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Growth hormone-related genes from baboon (*Papio hamadryas*): characterization, placental expression and evolutionary aspects

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Abstract

Pregnancy is a complex physiological condition, and the growth hormone (GH)-related hormones produced in the placenta, which emerged during the evolution of primates, are thought to play an important metabolic role in pregnancy that is not yet fully understood. The aim of this study was to identify the genes and transcription products of the GH family in baboon (*Papio hamadryas*) and to assess these in relation to the evolution of this gene family. *GH*-related transcripts were amplified using total RNA from placental tissue, by reverse transcription coupled to polymerase chain reaction (RT-PCR). Three different *GH*-related transcripts were identified in baboon placental tissue, with two encoding chorionic somatomammotropins (CSH) and one the placental variant of GH (*GH-2*). The *CSH* transcripts showed some minor allelic variation, and a splice variant of *CSH-C* that retains its in-frame third intron. Gene sequences for *GH-1* (probably representing the *GH* gene expressed primarily in the pituitary gland), *GH-2* and the two *CSH*s were identified in the baboon genomic database, together with a *CSH*-related pseudogene. Phylogenetic analysis of the baboon *GH*-related sequences, together with those of a related Old World monkey, macaque, and ape outgroup (human), showed the equivalence of the genes in baboon and macaque, and revealed evidence for several episodes of rapid adaptive evolution. Many of the substitutions seen during the evolution of these placental proteins have occurred in the receptor-binding sites, especially site 2, contrasting with the strong conservation of the hydrophobic core.

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Keywords

Primates; pregnancy; GH locus; chorionic somatomammotropin hormone; molecular evolution; alternative splicing

1. Introduction

The placenta is a very active metabolic organ, derived from the early embryo, which attaches the fetus to the maternal endometrium, thereby modulating fetal development and allowing interaction of fetal and maternal metabolisms. It is also an active endocrine organ producing a wide variety of hormones, growth factors and other bioactive molecules, which in higher primates include members of the growth hormone (GH) family, including chorionic somatomammotropin hormones (CSH, also referred to as placental lactogens) and GH variant (GH-2, placental GH). The placental GH/CSH family is well characterized in human, where it is encoded by a cluster of genes on chromosome 17 (Harper *et al*., 1982), and has evolved by duplications of the *GH* gene itself. The cluster comprises five genes (Chen *et al*., 1989), which encode (from the 5' end) pituitary GH (*GH-1*), a CSH-like protein (*CSH-L*, probably in effect a pseudogene - Reséndez-Pérez *et al*., 1990), placental lactogen (*CSH-A*), *GH-2* and placental lactogen (*CSH-B*, sequence of mature protein identical to that of CSH-A - Barrera-Saldaña *et al*., 1983).

During human pregnancy, the fetal *GH*-1 gene remains practically inactive until birth, while the expression of maternal *GH*-1 declines to undetectable levels during the second half of pregnancy (Frankenne *et al*., 1988). This inactivation is preceded by up regulation of the placental *CSH* and *GH*-2 genes and release of their translation products, primarily into the maternal circulation (Frankenne *et al*., 1988; MacLeod *et al*., 1992). The physiological role of CSH is not fully understood, but it appears to regulate fetal growth both directly and by indirect effects on maternal metabolism, including induction of insulin resistance and glucose intolerance, stimulation of nitrogen retention, and regulation of lipolysis (Walker *et al*., 1991). The actions of GH-2 are also not fully understood. It appears to take over as the main GH in the maternal circulation in the second half of pregnancy where it has growth-promoting activity equivalent to that of GH-1 but no lactogenic activity (Lacroix *et al*., 2002, Solomon *et al*., 2006). It has been associated with increased levels of insulin-like growth factors (IGF) 1 and 2 in maternal serum, as well as stimulation of trophoblast invasion (Lacroix *et al*., 2005).

In humans, low circulating CSH levels have been used as an indicator of fetal distress (Handwerger 1991). In some cases absence of CSH-A, CSH-B and GH-2, due to genetic deletion, has been associated with very low birth weight (e.g. Rygaard *et al*., 1998), and potentially subsequent metabolic disorders including type 2 diabetes, hypertension and cardiovascular disease but, remarkably, in other cases deletion of these genes did not lead to low birth weight or poor fetal health (e.g. Wurzel *et al*., 1982).

The human *GH*-related genes can give rise to alternative mRNAs by differential splicing. This is well established for *GH*-1, where an alternative transcript encodes a shorter (20kDa) form of GH in the pituitary gland (Cooke *et al*., 1988, Wallis, 1980). Alternative splicing of the *GH*-2 and *CSH* genes is seen in the placenta, and also in the testis, though the detailed splice patterns are different from that of *GH*-1 in the pituitary (Cooke *et al*., 1988; Macleod *et al*., 1992; Untergasser *et al*., 1998). There are additional reports about lower and higher molecular weight isoforms of these hormones, which can in part be explained by partial proteolytic digestion, glycosylation or aggregation. At least some of these variants appear to

have interesting physiological properties, distinct from those of the intact hormone (Clapp *et al*., 2006).

The role of these placental hormones, including the isoforms, is still not well understood in humans, and even less so in other primates. Prosimians, such as slow loris (*Nycticebus pygmaeus*) and bush baby (*Galago senegalensis*), like most other mammals, have a single *GH* gene, with no associated gene cluster (Adkins *et al*., 2001; Wallis *et al*., 2001). New World monkeys (NWM) have complex clusters of *GH*-like genes, which appear to have arisen independently of those seen in Old World monkeys (OWM) and apes (González Alvarez *et al*., 2006; Li *et al*., 2005; Liu *et al*., 2001; Revol de Mendoza *et al*. 2001, 2004; Wallis and Wallis 2001, 2002; Wallis *et al*. 2006; Yi *et al*., 2002). An OWM, the rhesus macaque (*Macaca mulatta*), has a cluster of six *GH*-like genes, at least 4 of which are expressed in placenta (Golos *et al*. 1993; González Alvarez *et al*. 2006). *GH*-like genes of three other OWMs have been investigated; each of these species has at least 4 *GH*-like genes, but the overall organization and expression of these has not been investigated (Li *et al*., 2005, Ye *et al*., 2005). Even among apes there appear to be substantial differences between the *GH* gene clusters (Revol de Mendoza *et al*., 2004; Wallis, M. unpublished). It is notable that higher primates differ from prosimians and other mammals not only in the presence of a cluster of *GH*-like genes, but also in the sequence of GH itself, which appears to have undergone an episode of rapid evolution after divergence of prosimians but before divergence of NWM and OWM (Wallis, 1981, 1994; Wallis *et al*., 2001). Marked evolutionary rate variation also occurred during the subsequent divergence of the various members of the *GH/CSH* gene family (González Alvarez *et al*. 2006; Wallis 1996; Ye *et al*. 2005).

An enhanced understanding of the physiological role of the placental GH-like proteins in humans and higher primates is still needed. Of particular interest is the possibility that fetal-maternal conflict has contributed to the rapid evolution in this gene family (Haig, 2008). Progress has been limited by the lack of a well characterized animal model, and the difficulty of recovering placentas followings birth in monkeys and apes. To address this need we have initiated studies in the baboon, *Papio hamadryas*, which is similar to human in its genetic make up, physiology, gestational process and fetal development (Chai *et al*., 2007; Santolaya-Forgas *et al*., 2007). Although little detailed information is available about the nature and organization of *GH*-like genes in baboon, there have been a number of previous studies on the secretion and actions of these hormones in this species (e.g. Musicki *et al*., 2003), and the baboon placenta has been reported to produce a placental lactogen-like protein (Josimovich *et al*., 1973). Here we describe studies on the characterization of the mRNAs of *GH*-related genes expressed in baboon placental tissue, relate the results to information currently available for the baboon genome sequence, and explore the implications for the evolution of the *GH* gene family in higher primates.

2. Material and methods

2.1. Animals

The animals utilized in this study are maintained at the Southwest Foundation for Biomedical Research in San Antonio, Texas. All animals shared the same diet and environmental conditions before and during pregnancy. All baboons are gang-housed and fed *ad libitum* on a standard low-fat chow diet (Harlan Tecklad 15% Monkey Diet, 8715). All animals underwent a Caesarean section at between days 136–139 of gestation, considered the term period of pregnancy for this species. Animal procedures were performed according to established ethical guidelines and reviewed by the Institutional Animal Care and Use Committee of the Southwest Foundation for Biomedical Research.

2.2. RNA isolation from placental tissue

Placental tissue was collected at the time of birth and stored in liquid nitrogen. RNA was isolated from six placentas using TRIZOL reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). RNA was treated with RQ1 DNAse (Promega, Madison, WI) for 15 min. at 37°C to remove traces of DNA. Purity and integrity of RNA were assessed by standard spectrophotometric and electrophoretic methods, respectively.

2.3. Reverse transcription and Polymerase Chain Reaction

Reverse transcription (RT) was conducted using the Thermoscript kit from Gibco (Gibco-BRL Life Technologies, Gaithersburg, MD) using one μg of RNA and random primers (Invitrogen). For amplification of *GH*-related cDNA, primers were designed using reported primate sequences for *GH*s and *CSH*s, including the human sequence. The forward or sense consensus primer (5′-CTCAGGATCCTGTGGACAGCTCACCTA-3′) was designed to initiate amplification 33 bases upstream from the translation initiation codon and the anti-sense primer (5′-TCGAATTCCAGGAGAGGCACTGGG-3′) to hybridize 28 bases after the termination codon. A polymerase chain reaction (PCR) was carried out using 5 μl of the RT reaction with the PCR kit of Qiagen (Valencia, CA). The program for amplification consisted of an initial hold at 94°C for 4.5 min, followed by 35 cycles, each including incubations at 94°C, 60°C and 72°C for 30s each, and finally an elongation step at 72°C for 6 min.

To achieve the amplification of the *GH-2* cDNA, the same sense primer was used, but a new, more-specific antisense primer (5′- GACACTGGAGTGGCACCTTCCAT-3′) was designed to hybridize bases after the termination codon for transcript specific for *GH-2*. This primer was designed using the *GH-2* sequences from human and rhesus macaque.

2.4. Molecular cloning and sequencing

Molecular cloning of PCR products was conducted using the TOPO-XL cloning vector with the pCR-XL-TOPO 3.5 kb kit (Invitrogen). Competent Top 10 *Escherichia coli* bacteria were transformed with the cloning products according to the manufacturer's instructions (Invitrogen). Cloning products and amplicons were sequenced using Big Dye terminator (Applied Biosystems, Foster City, CA) and universal M13 or specific primers and a DNA analyzer ABI PRISM® 3100 Genetic Analyzer and software (Applied Biosystems). Novel sequences have been deposited in the GenBank database (Accession numbers FJ916063-FJ916070).

2.5. Sequence analysis

Genomic sequences for baboon *GH*-like genes were established using the approach used previously for non-primate *GH* genes (Wallis 2008), by identifying appropriate sequences in the WGS Traces database for *Papio hamadryas* (http://www.ncbi.nlm.nih.gov/Traces) using the BLAST search method (Altschul *et al*. 1990) and the human *GH* coding sequence (CDS) as Query sequence. The Traces originated at the Baylor College of Medicine Human Genome Sequencing Center. Sequences from Traces were assembled using the Staden Package (https://sourceforge.net/projects/staden/), and evaluated by comparison with known sequences of human and macaque *GH*-like genes. The full sequences are given on the following website:
http://www.lifesci.sussex.ac.uk/Home/Mike_Wallis/GHAlign/baboonGHlikeGenes.

Sequences were aligned using the CLUSTAL W program (Higgins and Sharp 1988), followed by manual adjustment where appropriate. Nucleic acid open-reading frames (ORFs) were translated conceptually for analysis of their encoded polypeptides.

Phylogenetic analysis was carried out with the programme PAUP* (Swofford, 1998). Rates of evolution were analysed using the codeml programme in the paml package (Yang, 2007).

Investigation of the distribution of substitutions within the 3D structure was carried out using the molecular modelling programme Rasmol, after identifying unambiguous substitutions on branches of the phylogenetic tree with the parsimony-based programme MacClade (Maddison and Maddison 1992)

3. Results

The weights of the newborns showed no significant variation.

3.1. GH-related cDNAs from baboon placenta

A band of the expected amplicon size for the *GH*-related cDNA was seen in the agarose gel as an abundant PCR product. No other clearly-visible bands were seen that could reveal the existence of differentially spliced mRNA variants from the genes of the *GH* locus in the baboon placenta, although a splice variant was subsequently identified, as a minor component (see below). The *GH*-related PCR products were cloned, and sequencing revealed several showing the characteristic organization of *GH* family cDNAs: a short 5'-UTR, a CDS encoding a 26 residue signal peptide and 190 residue mature hormone, and a short 3'-UTR. Initial analyses revealed only transcripts from the 2–3 *CSH* genes thought to occur in the *GH* locus in this species and to be expressed in the baboon placenta (Rodríguez-Sánchez IP and Barrera-Saldaña HA, unpublished results). These were named *CSH-A* and *CSH-B*, differing at only a single nucleotide (nt), and *CSH-C*, differing from these at about 34 nt (Table 1). The amount of 3' and 5' UTR sequence obtained was limited by the nature of the RT-PCR strategy.

Initially no cDNA corresponding to the *GH-2* gene found in rhesus macaque (Golos *et al*., 1993) was found, and a second antisense primer more specific for this gene was therefore designed (see Section 2.3). This alternative strategy led to isolation of a *GH-2* related PCR product, which was sequenced directly. None of the approaches used revealed cDNA corresponding to the *GH*-1 gene in baboon placenta.

3.2. Features of the GH-related transcripts

The *CSH-A* and *CSH-B* transcripts differ at a single nucleotide (nt), leading to an amino acid (aa) difference (Arg -> Gln) at position 6 of the signal peptide. Whether they derive from separate non-allelic genes or allelic variation of a single gene is not clear, but allelic variation is suggested by the fact that *CSH-B* transcripts were only detected in two of the six placentas (Table 2), and only the *CSH-A* sequence was found in the genome (see below). A silent SNP (C/A at nt 627, initiating ATG being 1–3) was also found in the *CSH-A* sequence, with 5 animals homozygous for C and 1 homozygous for A. *CSH-C* and *CSH-A* transcripts differ at 34 nt. Allelic variation was found in *CSH-C* at two synonymous sites: 372 (four animals C/T heterozygous, two C/C homozygous) and 387 (four T/T homozygous, two C/C homozygous). The allelic variation seen in the sequences described here is summarized in Table 2. In addition, three clones corresponding to a differentially spliced *CSH-C* transcript were identified from two different animals (1–2% of all *CSH-C* clones).

This alternative transcript harbors an insertion of 93 nt, which appears to represent the retained third intron. It is potentially translated to a functional predicted protein 31 amino acids longer than the main CSH-C isoform.

Partial sequence was obtained from the amplicon of the *GH-2* cDNA. The missing portion corresponds to the first 171 nucleotides.

3.3 Baboon GH-related genes

Substantial information is now available about the baboon genome sequence, via the ncbi Traces database. A Blast search using the human *GH* CDS gave about 60 hits to *GH*-like sequences. These were resolved into 5 CDS consensus sequences, corresponding to *GH-1* (8 traces), *GH-2* (15 traces), *CSH-A* (9 traces), *CSH-C* (16 traces), and a *GH*-like sequence, apparently a pseudogene (9 traces). The first 4 of these are complete, the last is incomplete. The *GH-1* gene sequence is similar to that of macaque and human *GH-1* and presumably corresponds to the gene encoding baboon pituitary GH. The *GH-2*, *CSH-A* and *CSH-C* sequences correspond closely to the equivalent sequences derived from placental transcripts, as follows. The *GH-2* gene sequence encodes the complete baboon *GH-2* CDS. The CDS derived from the genome for *CSH-A* is identical to the predominant *CSH-A* transcript allele. The *CSH-B* sequence was not found in the genomic sequences, confirming that it is an allelic variant of *CSH-A*. The CDS derived from the genome for *CSH-C* is identical to the predominant *CSH-C* transcript allele. The pseudogene closely resembles the macaque *CSH-3* gene reported by González Alvarez *et al*. (2006), expression of which has not been detected (note that this *CSH-3* gene is not orthologous with the *CSH-3* transcript of Golos *et al*., 1993 - see Table 1). The results derived from the genome are thus in good agreement with those obtained on *GH*-related gene transcripts. The baboon *GH*-gene family appears to comprise 5 genes, *GH-1*, *GH-2*, *CSHA*, *CSH-C* and a pseudogene.

3.4. The predicted proteins encoded by baboon GH and CSH mRNAs

The aa sequence predicted for baboon GH-1 differs from that of the corresponding macaque protein at 3 aas (Fig. 1), none of which is located near a receptor-binding site. Baboon GH-2 (predicted mature protein), however, differs from macaque GH-2 at 25 aa, indicating rapid evolution for these proteins (see below). Nevertheless, it is clear from the sequence alignment (Fig. 1) that these proteins are closely related, presumably orthologous, and this is confirmed by the evolutionary analysis given below. On the other hand there is little similarity, in terms of shared derived characters, between human GH-2 and the macaque/baboon GH-2 sequences.

Baboon CSH-A is similar to macaque CSH-1/2, differing at only 8 residues, and baboon CSH-C and macaque CSH-3 are also very similar (9 differences). The monkey CSHs both differ substantially from human CSH-1 and PL-L; the alignment suggests a common origin for the CSHs of higher primates, followed by separate divergence in monkeys and apes.

3.5. Evolutionary analysis

Phylogenetic analysis using the Neighbor-Joining (NJ), parsimony and maximum likelihood methods in PAUP* clearly established that the monkey and human *CSH/PL* sequences form a grouping, with the exclusion of all *GH* sequences. However, resolution within the *GH/GH-2* group was less clear cut (Fig. 2). In particular, it is not established whether human *GH-2* is more closely related to the monkey *GH-2s* or to human *GH*, although the baboon and macaque *GH-2s* are clearly closely related.

To investigate the nature of the evolutionary processes driving divergent evolution of the various GHs and CSHs, rates of evolution for nonsynonymous and synonymous sites in the coding sequences for the mature proteins (dN and dS) were determined using codeml, branch model 1 (Yang, 2007), with a defined tree (Fig. 2). Evolutionary rates for the dN tree are very variable; for example the rates (dN) for the branches leading to baboon and macaque GH-2 are at least 10 fold greater than for branches leading to GH-1 for these species. An indication of the nature of the evolutionary forces involved can be obtained by determining dN/dS ratios (Yang and Bielawski, 2000). A dN/dS ratio << 1.0 is indicative of a conserved sequence maintained by purifying selection. A dN/dS ratio of 1.0 may indicate

evolution of a non-functional sequence undergoing neutral evolution. A dN/dS ratio significantly greater than 1.0 indicates positive selection, although failure to reach this level does not necessarily rule out selection. Eight branches in the tree shown in Fig.2b have dN/dS > 1.0 (as indicated by thick branches). Whether these ratios were significantly greater than 1 was tested using the branch-sites model in codeml (Yang and Nielsen, 2002; Zhang *et al*., 2005), but after correction for multiple hypothesis testing (Anisimova and Yang, 2007) on no individual branch was the rate shown to be significantly greater than one. However, use of the branch-sites model with all branches leading to genes expressed in the placenta as foreground (*GH-2* or *CSH*; branches on groups labelled a, b and c in Fig. 2) gave a foreground dN/dS value of 2.00, which was significantly greater than 1 (Table 3), establishing that evolution of at least some of the placental genes was driven by positive selection. The phylogenetic tree (Fig 2) establishes three clearly defined groups within the genes expressed in the placenta, (a) human *GH-2*, (b) OWM *GH-2* and (c) all *CSH*s. Analysis with each of these (separately) as foreground in the branch-sites method showed that for both (b) OWM *GH-2* and (c) all *CSH*s, dN/dS is significantly greater than one (Table 3), establishing that within each of these groups at least some evolution has occurred by positive selection.

3.6. Distribution of substitutions in the 3D structure

An indication of the potential significance of the aa substitutions accepted in a sequence during the course of evolution can be obtained by examining their distribution in the 3D structure of the protein. This was done for each of the branches on the evolutionary tree showing accelerated change (Fig. 2, branches i–viii), using only those substitutions that were unambiguously assigned to these branches, based on parsimony analysis with MacClade. Substitutions were assigned to hydrophobic core, receptor-binding sites 1 or 2, or elsewhere in the structure, on the basis of the 3D structure of human GH bound to 2 molecules of the extracellular domain of its receptor (de Vos *et al*., 1992). Results are shown in Table 4. Few substitutions occur in the hydrophobic core of the molecule, suggesting that overall structural integrity is strongly conserved. On the other hand there are many substitutions into the binding sites, especially site 2, presumably reflecting changes in biological specificity and activity. For site 2 (comprising 18 residues within 5Å of receptor 2) a total of 16 residues were substituted on branches i–viii. This is significantly greater than the proportion of substitutions (43/144) in the regions of the protein outside the binding sites (P=0.0039 after Bonferroni correction; Fisher's Exact Test). Remarkably there is very little parallelism for the site 2 substitutions, with most of the 18 residues involved in this site being substituted along at least one of branches i–viii.

4. Discussion

4.1. Gene equivalences between species, and placental expression

Placental hormones closely related to GH (CSHs and GH-2) occur only in primates. Superficially equivalent placental hormones in rodents and ruminants arose from independent duplications of the prolactin gene. Since the GH-related placental hormones of NWM and OWM/apes arose independently (Li *et al*., 2005; Revol de Mendoza *et al*., 2004; Wallis and Wallis, 2002, 2006), genes truly orthologous to human *CSH*s and *GH-2* occur only in the latter group. *CSH*s appear to occur in all OWMs and apes but *GH-2* is not found in all OWM species - for example green monkey (*Cercopithecus aethiops*) lacks it (Rodríguez-Sánchez IP and Barrera-Saldaña HA, unpublished results). In baboon our studies on genes expressed in the placenta and occurring in the baboon genome indicate that there are five *GH*-related genes: *GH-1*, identified in the genome and presumably expressed predominantly in the pituitary gland (Luque *et al*., 2006, showed expression of *GH* in the pituitary of the olive baboon, *Papio anubis*), *GH-2* expressed in the placenta, *CSH-A* and

CSH-C expressed in the placenta, and a non-functional pseudogene. *GH-2*, *CSH-A* and *CSH-C* are clearly equivalent to corresponding genes in macaque, detected as cDNAs, *GH-2*, *CSH1/2*, and *CSH3* respectively (Golos *et al*., 1993; González Alvarez *et al*., 2006) (Table 1). Equivalent genes have also been detected in other OWM species (Li *et al*., 2005; Ye *et al*., 2005), but there is clearly considerable variation in the organization of the *GH* gene cluster within OWM. For baboon there is good agreement between the genes detected in the genome and those expressed in the placenta.

The available data cannot rule out completely expression of *GH-1*, in the placenta, though specific attempts to detect this were unsuccessful. It is possible, of course, that the *GH*-related genes are also expressed at low levels in other tissues, as is the case for human (e.g. Untergasser *et al*. 1998).

4.2. Polymorphisms in the CSH genes

Analysis of placentas from 6 individual baboons has revealed some clear polymorphism. *CSH*-A and *CSH*-B mRNAs are identical except for a single substitution in the sequence encoding the signal peptide. Although this substitution is expressed as an amino acid change, it seems likely that it reflects an allelic polymorphism rather than a separate gene, given that it was not found in all individual baboons examined, it was not found in the genomic sequences, and the total number of hits (traces) obtained for *CSH-A* in the genomic data was not greater than that for the other genes. A second, synonymous, allelic polymorphism was also found in *CSH-A* (Table 2). CSH-C differs from CSH-A at about 34 amino acids, and also shows allelic polymorphisms at two sites, both synonymous. In addition, a rare splice variant was found for *CSH-C* in two animals. This alternatively-spliced transcript is potentially translatable, given the absence of stop codons within the retained in-frame third intron, and would give a protein with an additional 31 residues at the end of the first long loop of the 3D structure. Whether this alternative transcript, which represented 1–2% of all *CSH-C* transcripts observed, gives rise to a novel CSH-like protein remains to be seen; it may alternatively correspond to an immature transcript.

4.3. Evolutionary relationships among GH-related primate sequences

Evolutionary analysis of the baboon CSH- and GH-like proteins, along with those of other higher primates, confirms the rapid evolution of these proteins that has been observed previously (Forsyth and Wallis, 2002; González Alvarez *et al*., 2006; Liu *et al*., 2001; Wallis 1996; Wallis *et al*., 2001; Ye *et al*., 2005) (Fig. 2). The CSHs of OWM and man clearly form a separate clade from the GH-like proteins, indicating that CSHs arose as the result of gene duplication early in the diversification of the family (probably the first duplication after separation of OWM and NWM). On the other hand, the origin of the *GH-2* genes is much less clear cut. Among the *GH-1* and *GH-2* gene sequences the phylogenetic resolution is weak. There is no clear evidence that the *GH-2* genes of OWM and apes are orthologous, but neither is there evidence that they are not. Rapid evolution on several branches of the evolutionary tree shown in Fig. 2 is clear, and the significantly elevated dN/ dS ratios confirm that this is adaptive in nature (Table 3). This may well reflect the evolution of new functions following gene duplications.

4.4. Significance of changes in baboon GH-related hormones

The distribution of the amino acid substitutions seen in the various baboon CSH and GH-2 proteins has been examined on the basis of the 3D structure of human GH bound to its receptor, as described by de Vos *et al*. (1992). Notable are the strong conservation of the hydrophobic core of these proteins, presumably indicating conservation of the main fold, and the large variation of the binding sites, especially binding site 2, presumably reflecting changes in biological activity/specificity. Differences between the biological activities of the

human hormones are quite well characterized, although their physiological significance is not clear. Thus, human GH-2 retains the growth-promoting activity of GH-1, but lacks its lactogenic activity (Solomon *et al*., 2006), while human CSH-1 shows high lactogenic activity in many assays, but rather low growth-promoting activity (Florini *et al*., 1966; Josimovich and MacLaren, 1962). The biological activities and physiological roles of CSHs and GH-2 in OWM are currently poorly understood. The baboon provides a useful model for investigating these hormones, and the information provided here should give a substantial basis for such investigation.

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References

Adkins RM, Nekrutenko A, Li W-H. Bushbaby growth hormone is much more similar to nonprimate growth hormones than to rhesus monkey and human growth hormones. Mol. Biol. Evo. 2001; 18:55–60.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic Local Alignment Search Tool. J. Mol. Biol. 1990; 215:403–410. [PubMed: 2231712]

Anisimova M, Yang Z. Multiple hypothesis testing to detect lineages under positive selection that affects only a few sites. Mol. Biol. Evo. 2007; 24:1219–1228.

Barrera-Saldaña HA, Seeburg PH, Saunders GF. Two structurally different genes produce the same secreted human placental lactogen hormone. J. Biol. Chem. 1983; 258:3787–3793. [PubMed: 6300056]

Chai D, Cuneo S, Falconer H, Mwenda JM, D' Hooghe T. Olive baboon (Papio anubis anubis) as a model for intrauterine research. J. Med. Primatol. 2007; 36:365–369. [PubMed: 17976041]

Chen EY, Liao Y-C, Smith DH, Barrera-Saldaña HA, Gelinas RE, Seeburg PH. The human growth hormone locus: Nucleotide sequence, biology, and evolution. Genomics. 1989; 4:479–497. [PubMed: 2744760]

Clapp C, Aranda J, González C, Jeziorski MC, Martínez de la Escalera G. Vasoinhibins: endogenous regulators of angiogenesis and vascular function. Trends Endocrinol. Metab. 2006; 17:301–307. [PubMed: 16934485]

Cooke NE, Ray J, Watson MA, Estes PA, Kuo BA, Liebhaber SA. Human growth hormone gene and the highly homologous growth hormone variant gene display different splicing patterns. J. Clin. Inv. 1988; 82:270–275.

De Vos AM, Ultsch M, Kossiakoff AA. Human growth hormone and extracellular domain of its receptor: crystal structure of the complex. Science. 1992; 255:306–312. [PubMed: 1549776]

Florini JR, Tonelli G, Breuer CB, Coppola J, Ringler I, Bell PH. Characterization and biological effects of purified placental protein (human). Endocrinology. 1966; 79:692–708. [PubMed: 4959588]

Forsyth IA, Wallis M. Growth hormone and prolactin-molecular and functional evolution. J. Mamm. Gland Biol. Neoplasia. 2002; 7:291–312.

Frankenne F, Closset J, Gomez F, Scippo ML, Smal J, Hennen G. The physiology of growth hormones (GHs) in pregnant women and partial characterization of the placental GH variant. J. Clin. Endocrinol. Metab. 1988; 66:1171–1180. [PubMed: 3372680]

Golos TG, Durning M, Fisher JM, Fowler PD. Cloning of four GH/chorionic somatomammotropin-related complementary deoxyribonucleic acids differentially expressed during pregnancy in the rhesus monkey placenta. Endocrinology. 1993; 133:1744–1752. [PubMed: 8404617]

González Alvarez R, Revol de Mendoza A, Esquivel Escobedo D, Corrales Félix G, Rodríguez Sánchez I, González V, Dávila G, Cao Q, de Jong P, Fu YX, Barrera Saldaña HA. Growth hormone locus expands and diverges after the separation of New and Old World Monkeys. Gene. 2006; 380:38–45. [PubMed: 16872758]

Haig D. Placental growth hormone-related proteins and prolactin-related proteins. Placenta. 2008; 29 Supplement 1:S36–S41. [PubMed: 17981323]

Handwerger S. Clinical counterpoint: the physiology of placental lactogen in human pregnancy. Endocrine Rev. 1991; 12:329–336. [PubMed: 1662129]

Harper ME, Barrera-Saldaña HA, Saunders GF. Chromosomal localization of the human placental lactogen-growth hormone gene cluster to 17q22–24. Am J Hum Genet. 1982; 34:227–234. [PubMed: 7072716]

Higgins DG, Sharp PM. CLUSTAL: a package for performing multiple sequence alignment on a microcomputer. Gene. 1988; 73:237–244. [PubMed: 3243435]

Josimovich JB, Levitt MJ, Stevens VC. Comparison of baboon and human placental lactogens. Endocrinology. 1973; 93:242–244. [PubMed: 4197195]

Josimovich JB, MacLaren JA. Presence in human placenta and term serum of a highly lactogenic substance immunologically related to pituitary growth hormone. Endocrinology. 1962; 71:209–220. [PubMed: 14452554]

Lacroix MC, Guibourdenche J, Frendo JL, Muller F, Evain-Brion D. Human placental growth hormone - a review. Placenta. 2002; 23:S87–S94. (Suppl. A, Trophoblast Research). [PubMed: 11978064]

Lacroix MC, Guibourdenche J, Fournier T, Laurendeau I, Igout A, Goffin V, Pantel J, Tsatsaris V, Evain-Brion D. Stimulation of human trophoblast invasion by placental growth hormone. Endocrinology. 2005; 146:2434–2444. [PubMed: 15718272]

Li Y, Ye C, Shi P, Zou X-J, Xiao R, Gong Y-Y, Zhang Y-P. Independent origin of the growth hormone gene family in New World monkeys and Old World monkeys/hominoids. J. Mol. Endocrinol. 2005; 35:399–409. [PubMed: 16216919]

Liu J-C, Makova KD, Adkins RM, Gibson S, Li W-H. Episodic evolution of growth hormone in primates and emergence of the species specificity of human growth hormone receptor. Mol. Biol. Evo. 2001; 18:945–953.

Luque RM, Gahete MD, Valentine RJ, Kineman RD. Examination of the direct effects of metabolic factors on somatotrope function in a non-human primate model, *Papio anubis*. J. Mol. Endocrinol. 2006; 37:25–38. [PubMed: 16901921]

MacLeod JN, Lee AK, Liebhaber SA, Cooke NE. Developmental control and alternative splicing of the placentally expressed transcripts from the human growth hormone gene cluster. J. Biol. Chem. 1992; 267:14219–14226. [PubMed: 1378436]

Maddison, WP.; Maddison, DR. MacClade: analysis of phylogeny and character evolution, version 3.0. Sunderland, MA: Sinauer; 1992.

Musicki B, Pepe GJ, Albrecht ED. Functional differentiation of the placental syncytiotrophoblast: effect of estrogen on chorionic somatomammotropin expression during early primate pregnancy. J. Clin. Endocrinol. Metab. 2003; 88:4316–4323. [PubMed: 12970304]

Reséndez-Pérez D, Ramirez-Solís R, Varela-Echavarría A, Martínez-Rodríguez HG, Barrera-Saldaña HA. Coding potential of transfected human placental lactogen genes. Nucl. Acids Res. 1990; 18:4665–4670. [PubMed: 2395633]

Revol de Mendoza A, Esquivel Escobedo D, Santiago Alarcón D, Barrera Saldaña H. Independent duplication of the growth hormone gene in three anthropoidean lineages. J. Endocr. Gen. 2001; 2:151–159.

Revol de Mendoza A, Esquivel Escobedo D, Martínez Dávila I, Barrera Saldaña H. Expansion and divergence of the GH locus between spider monkey and chimpanzee. Gene. 2004; 336:185–193. [PubMed: 15246530]

Rygaard K, Revol A, Esquivel-Escobedo D, Beck BL, Barrera-Saldaña HA. Absence of human placental lactogen and placental growth hormone (hGH-V) during pregnancy: PCR analysis of the deletion. Human Genet. 1998; 102:87–92. [PubMed: 9490304]

Santolaya-Forgas J, Edwin S, Zeiter K, Pitt A, Pineles B, Tarca AL, Espinoza J, Kusanovic JP, Erez O, Wolf R, Romero R. The timed-pregnant baboon animal model can be used for determining the role of soluble vascular endothelial growth factor receptors 1 and 2 during development. J. Med. Primatol. 2007; 36:370–374. [PubMed: 17976042]

Solomon G, Reicher S, Gussakovsky EE, Jomain J-B, Gertler A. Large-scale preparation and *in vitro* characterization of biologically active human placental (20 and 22K) and pituitary (20K) growth hormones: Placental growth hormones have no lactogenic activity in humans. GH & IGF Res. 2006; 16:297–307.

Swofford, DL. Phylogenetic Analysis using Parsimony (* and Other Methods). Version 4. Sunderland, MA: Sinauer; 1998. PAUP*.

Untergasser G, Hermann M, Rumpold H, Berger P. Complex alternative splicing of the GH-V gene in the human testis. Eur. J. Endocrinol. 1998; 139:424–427. [PubMed: 9820620]

Walker WH, Fitzpatrick SL, Barrera-Saldaña HA, Reséndez-Pérez D, Saunders GF. The human placental lactogen genes: structure, function, evolution and transcriptional regulation. Endocrine Rev. 1991; 12:316–328. [PubMed: 1760991]

Wallis M. Growth hormone: deletions in the protein and introns in the gene. Nature. 1980; 284:512. [PubMed: 7366719]

Wallis M. The molecular evolution of pituitary growth hormone, prolactin and placental lactogen: a protein family showing variable rates of evolution. J. Mol. Evol. 1981; 17:10–18.

Wallis M. Variable evolutionary rates in the molecular evolution of mammalian growth hormones. J. Mol. Evol. 1994; 38:619–627. [PubMed: 8083887]

Wallis M. The molecular evolution of vertebrate growth hormones: a pattern of near-stasis interrupted by sustained bursts of rapid change. J. Mol. Evol. 1996; 43:93–100. [PubMed: 8660433]

Wallis M. Mammalian genome projects reveal new growth hormone (GH) sequences. Characterization of the GH-encoding genes of armadillo (*Dasypus novemcinctus*), hedgehog (*Erinaceus europaeus*), bat (*Myotis lucifugus*), hyrax (*Procavia capensis*), shrew (*Sorex araneus*), ground squirrel (*Spermophilus tridecemlineatus*), elephant (*Loxodonta africana*), cat (*Felis catus*) and opossum (*Monodelphis domestica*). Gen. Comp. Endocrinol. 2008; 155:271–279. [PubMed: 17574247]

Wallis OC, Wallis M. Characterisation of the GH gene cluster in a new-world monkey, the marmoset (*Callithrix jacchus*). J. Mol. Endocrinol. 2002; 29:89–97. [PubMed: 12200231]

Wallis OC, Wallis M. 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. Evo. 2006; 63:591–601.

Wallis OC, Zhang Y-P, Wallis M. Molecular evolution of GH in primates: characterization of the GH genes from slow loris and marmoset defines an episode of rapid evolutionary change. J. Mol. Endocrinol. 2001; 26:249–258. [PubMed: 11357061]

Wurzel JM, Parks JS, Herd JE, Nielsen PV. A gene deletion is responsible for absence of human chorionic somatomammotropin. DNA. 1982; 1:251–257. [PubMed: 7169010]

Yang Z. PAML 4: Phylogenetic analysis by maximum likelihood. Mol. Biol. Evol. 2007; 24:1586–1591. [PubMed: 17483113]

Yang Z, Bielawski JP. Statistical methods for detecting molecular adaptation. TRENDS Ecol. Evol. 2000; 15:496–503. [PubMed: 11114436]

Yang Z, Nielsen R. Codon-substitution models for detecting molecular adaptation at individual sites along specific lineages. Mol. Biol. Evol. 2002; 19:908–917. [PubMed: 12032247]

Ye C, Li Y, Shi P, Zhang YP. Molecular evolution of growth hormone gene family in old world monkeys and hominoids. Gene. 2005; 350:183–192. [PubMed: 15848116]

Yi S, Bernat B, Pal G, Kossiakoff A, Li W-H. Functional promiscuity of squirrel monkey growth hormone receptor toward both primate and nonprimate growth hormones. Mol. Biol. Evo. 2002; 19:1083–1092.

Zhang J, Nielsen R, Yang Z. Evaluation of an improved branch-site likelihood method for detecting positive selection at the molecular level. Mol. Biol. Evo. 2005; 22:2472–2479.

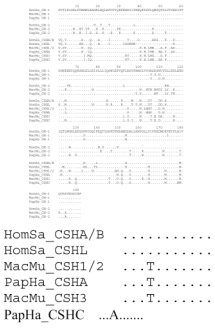

Fig. 1. Alignment of GH-related sequences (mature protein) from baboon, macaque and human

The human GH sequence is shown in full, others are shown in relation to this, with indicating identity, - a gap. Species names are abbreviated as follows: HomSa, *Homo sapiens*; MacMu, *Macaca mulatta*; PapHa, *Papio hamadryas*.

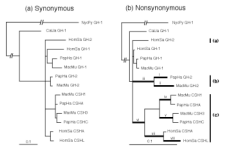

Fig. 2. Phylogenetic trees for *GH*-related sequences (CDS) encoding mature protein, based on (a) Synonymous substitutions and (b) Nonsynonymous substitutions

Constructed using codeml, model 1, and a defined tree. Thick branches are those with dN/dS >1. Branch labels i–viii are as referred to in Table 4. a–c are the groups analysed in Table 3. Species names are abbreviated as given in the legend to Fig.1, plus NycPy, *Nycticebus pygmaeus* (slow loris); CalJa, *Callithrix jacchus* (marmoset).

Table 1

GH-family genes in man, macaque and baboon

man (transcript and genome)[a]	**macaque (placental transcripts)**[b]	**macaque (genome)**[c]	**baboon (placental transcripts)**[d]	**baboon (genome)**[d]
GH-1		*GH-1*		*GH-1*
GH-2				
	GH-2	*GH-2*	*GH-2*	*GH-2*
CSH-A				
CSH-B				
CSH-L				
	CSH-1	*CSH-1*	*CSH-A/B*	*CSH-A*
	CSH-2			
	CSH-3	*CSH-2*	*CSH-C*, *CSH* isoform	*CSH-C*
		CSH-3		*CSH-pseudogene*
		CSH-4		

Transcripts/genes shown on the same line appear to be true orthologs.

[a] Chen *et al* (1989),

[b] Golos *et al* (1993),

[c] Gonzalez Alvarez *et al*. (2006),

[d] this study.

Table 2

Polymorphism of baboon *GH*-related genes

Placenta/genome	***CSH-A/B*** **nt 17**	***CSH-A*** **nt 627**	***CSH-C*** **nt 372**	***CSH-C*** **nt 387**
1	G/A	C/C	C/C	C/C
2	G/A	C/C	C/T	T/T
3–5	G/G	C/C	C/T	T/T
6	G/G	A/A	C/C	C/C
genome	G/G	C/C	C/C	T/T

Residue numbers are based on the initiating ATG being nt 1–3.

Table 3

Analysis of rate variation during the evolution of baboon, macaque and human *GH*-related genes

Branches tested	dN/dS	2×Δlnlikelihood	P value
Branches to all genes expressed in placenta (a + b + c in Fig.2b)	2.00	12.68	0.0003
Branch to human *GH-2* (a in Fig.2b)	1.00	0	1
Branches to OWM *GH*-2 (b in Fig.2b)	5.46	9.99	0.0045[a]
Branches to all *CSH*s (c in Fig.2b)	2.29	7.21	0.022 [a]

dN/dS values are for branches as indicated in Fig.2b. 2×Δlnlikelihood and significance are from branch-sites analysis and chi-square test.

[a]P-values corrected using Bonferroni method.

Table 4

Distribution of substitutions in 3D structure during the evolution of GH-related proteins in primates

branch (Fig.2)	total substitutions (total res = 191)[a]	hydrophobic core (core res = 39)	binding site 1 (site 1 res = 29)	binding site 2 (site 2 res = 18)	remainder (105)
i	8	0	0	1	7
ii	11	1	1	3	6
iii	11	0	2	3	6
iv	6	0	1	1	4
v	8	0	0	2	6
vi	3	0	0	3	0
vii	12	2	2	1	7
viii[b]	8	1	1	2	4
total	67	4	7	16	34

[a] substitutions were unambiguous changes on branches, identified using MacClade.

[b] for viii the deletion in hCSH-L, and adjacent residues, are excluded.