Chorionic gonadotrophin β subunit mRNA but not luteinising hormone β subunit mRNA is expressed in the pituitary of the common marmoset (*Callithrix jacchus*)

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Abstract

The pituitary gonadotrophins LH and FSH are responsible for regulation of gametogenesis in the testis and ovary. Chorionic gonadotrophin (CG), a third closely related glycoprotein hormone derived by gene duplication of the *LHβ* gene and secreted by the placenta in primates, is essential for the rescue of the corpus luteum and maintenance of pregnancy. We have recently shown that marmoset (m) CGβ mRNA is highly expressed in the pituitary of the common marmoset (*Callithrix jacchus*) and that LH is less active than human CG in activating the human LH receptor lacking exon 10. To investigate further which gonadotrophin is the actual ligand of the LH receptor (LHR) of the marmoset monkey that naturally lacks exon 10, we identified and characterised the genomic organisation of the *mLHβ* gene and its expression. Intergenic PCR amplification of the region encompassing the *mLHβ* and the *mCGβ* genes revealed that, surprisingly, *mCGβ* is located 20 kbp upstream of the *LHβ* gene, whereas in other species the intergenic distance is approximately 2–3 kbp. Sequence analysis of the mLHβ coding region showed 70% identity to mCGβ and 90% identity to human LHβ at the amino acid level. Both gonadotrophin β subunits are present at the genomic level, but RT-PCR of pituitary and placental total RNA using specific oligonucleotides for mCGβ and mLHβ showed high expression of mCGβ mRNA in both tissues, whereas LHβ was expressed neither in the pituitary nor in the placenta. Thus mLHβ mRNA is lacking in the marmoset pituitary. Immunohistochemistry of marmoset pituitaries showed that mCG was confined to the gonadotrophes, and partly co-localised in cells stained positively for FSH. Western blot analysis confirmed the presence of mCG in the pituitary. Northern blot analysis using mCGβ as a probe displayed one transcript of 0·7 kb in the pituitary and detected two transcripts of 1·1 kb and 2 kb in the marmoset placenta. Our results suggest that, in the common marmoset, CG is the only gonadotrophin with luteinising function that is present in the pituitary. We postulate that, owing to an unknown mutational event in evolution, expression of mLH was completely abolished, and CG – which, unlike LH, acts normally even when exon 10 is missing from the LHR – took over its function.

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Introduction

In primates there are two pituitary gonadotrophins, luteinising hormone (LH) and follicle-stimulating hormone (FSH), and one gonadotrophin of placental origin, the chorionic gonadotrophin (CG) (Hobson & Wide 1981, Dufau 1998, Simoni *et al.* 1999, Weinbauer *et al.* 2000). The general functions of LH and FSH are the regulation of gametogenesis in the testis and ovary, whereas CG rescues the corpus luteum and maintains pregnancy. Glycoprotein hormones are heterodimers of two

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non-covalently linked subunits, of which the α subunit is the same for all glycoprotein hormones of a species, whereas the β subunit determines the biological specificity for the respective receptor (Pierce & Parsons 1981).

The genomic organisation of the LH and CGβ subunits comprises three exons separated by two introns. In the human, there are seven copies of gonadotrophin β subunits present in a complex gene cluster, six of which represent *CGβ* and one *LHβ*. Four of the *CGβ* copies are located in inverted pairs and are not translated (Policastro *et al.* 1983). The genomic structure of the *LHβ*, in addition to the different *CGβ* gene copies, represents nearly accurate copies with respect to exon and intron size. *CGβ* evolved from a duplication of the *LHβ* gene, followed by a frameshift mutation in exon 3 leading to a readthrough into the 3′-untranslated region, thereby elongating the protein by 24 amino acids, an extension known as the carboxy-terminal peptide (CTP) (Fiddes & Goodman 1980, Talmadge *et al.* 1984).

The exact time of appearance of CG in primates is unknown, but the duplication of the *LHβ* gene should be approximately 30–40 million years ago (Kumar & Hedges 1998, Maston & Ruvolo 2002). Although trace amounts of genuine human (h) CG have been demonstrated in the human pituitary (Birken *et al.* 1996), LH is the only luteinising gonadotrophin present in physiologically relevant amounts in the pituitary of mammals, with the exception of horses and guinea pigs, which have a single copy *LH/CGβ* gene expressed in the pituitary and placenta (Sherman *et al.* 1992, 2001). Furthermore, the primate genome usually bears only one copy of *LHβ*, but one or more copies of *CGβ* (Sherman *et al.* 2001, Maston & Ruvolo 2002). Sequence information for many primate gonadotrophins is available today (Maston & Ruvolo 2002), mostly from Old World monkeys (Schmidt *et al.* 1999). In some species, such as the marmoset monkey (*Callithrix jacchus*), an important animal model for human reproduction, CG has already been characterised and cloned (Hobson & Wide 1981, Simula *et al.* 1995) from the trophoblast cells of the placenta, whereas the sequence and pattern of expression of the *LHβ* subunit remain unknown.

Findings from our group raised interest in marmoset (m) LH. In the human, the LH receptor (LHR), mediating the action of both LH and CG, shows considerable differences in signal transduction *in vivo* and *in vitro* if exon 10, encoding part of the extracellular hinge region, is deleted (Gromoll *et al.* 2000, Müller *et al.* 2003). We showed that the hLHR lacking exon 10 could bind normally both LH and CG, but a 30-fold greater dose of LH was needed to induce cAMP formation comparable to that obtained by hCG (Müller *et al.* 2003). As this was not the result of receptor desensitisation or permanent activation of the G_i protein, one possible explanation could be the different structure of the gonadotrophins, e.g. the CTP.

The LHR of the marmoset monkey naturally lacks exon 10 (Zhang *et al.* 1997), as is the case for other members of the *Platyrrhini* lineage (Gromoll *et al.* 2003). This peculiar, naturally occurring LHR which we named LHR type II, encompasses exon 10 at the genomic level, but is absent at the transcriptional level. Because of the high homology (94% identity at the nucleotide level) between hLHR and mLHR (Zhang *et al.* 1998), the question emerges as to how the marmoset monkey could maintain normal gonadal function if LH is not working correctly on an LHR lacking exon 10. In previous experiments we detected a surprisingly high expression of mCGβ mRNA by RT-PCR in the marmoset pituitary (Gromoll *et al.* 2003). This unexpected finding suggested that mCG, and not mLH, could be the relevant pituitary gonadotrophin in the marmoset. In the present study we characterised both genes and demonstrate expression of mCG only in the pituitary.

Materials and methods

PCR, RT-PCR and long template intergenic PCR

PCR generally followed 30 cycles with annealing temperatures from 55 °C to 78 °C for 40 s (depending on the primers used) and elongation time of 72 °C for 2 min. PCR products were resolved on a 1·5% agarose gel and the DNA bands were isolated, purified by a DNA purification kit (Roche) and cloned into pGEM T-easy vector (Stratagene, La Jolla, CA, USA). Both strands of denatured double-stranded DNA were sequenced with a commercial kit (Amersham) using T7 and T3 primers and the dideoxynucleotide termination method.

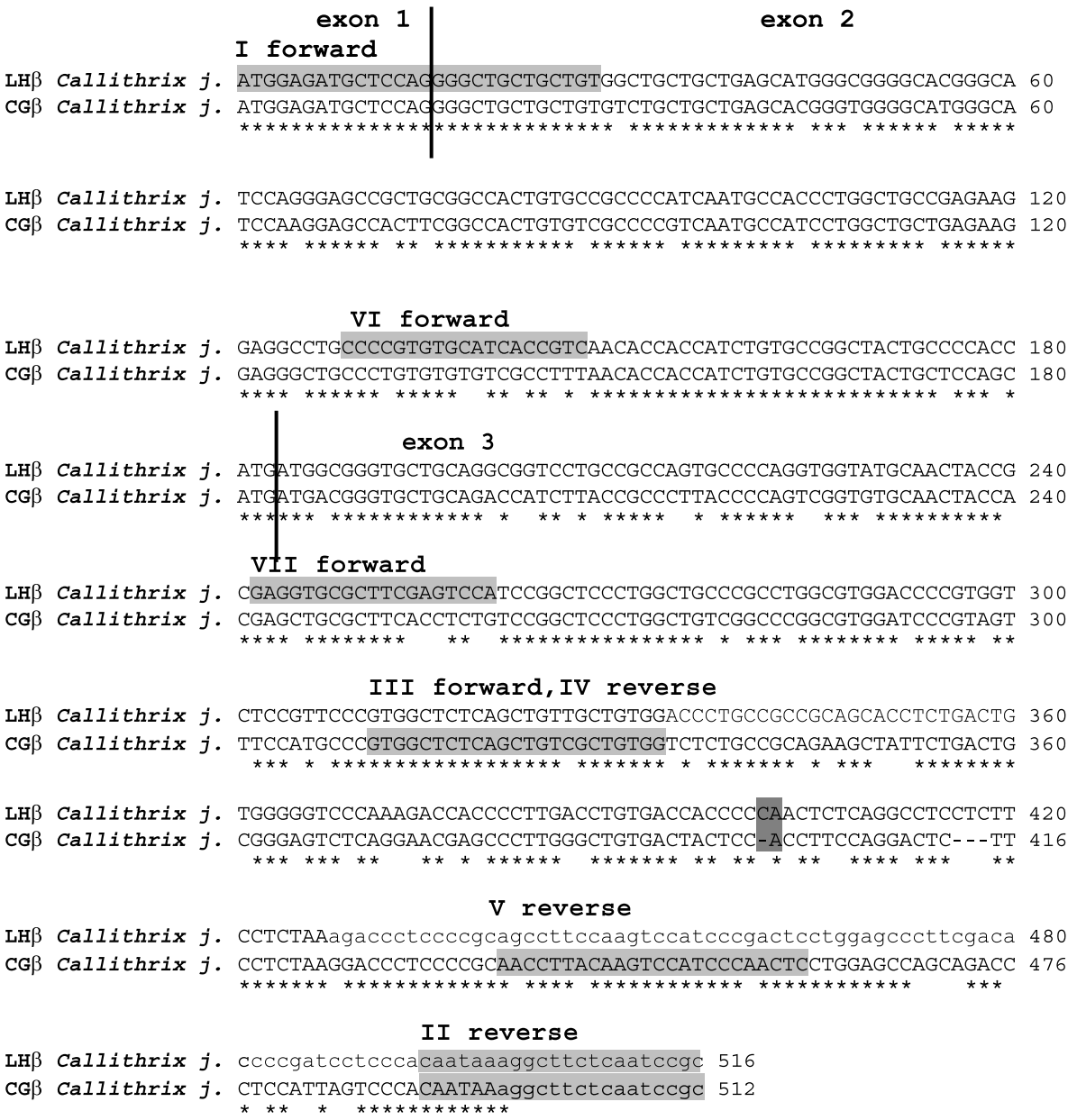


Figure 1 Nucleotide sequence comparison of *mLHβ* and *mCGβ* of the common marmoset *Callithrix jacchus* (non-translated region of *mLHβ* is given in small letters). Oligonucleotide pairs indicated by grey boxes used in the different amplifications: I forward/II reverse, amplification of *mCGβ* in RT-PCR and DNA; III forward/IV reverse, bidirectional long template PCR from DNA; III forward/II reverse, amplification of endpiece of *mCGβ* from bidirectional long template PCR; III forward/V reverse, amplification of endpiece of *mLHβ* from bidirectional long template PCR; I forward/IV reverse, amplification of *mLHβ* from bidirectional long template PCR; VI/VII forward/IV reverse, specific amplification of *mLHβ* from RNA and DNA. Vertical lines give the exon boundaries.

Long template, intergenic PCR was performed using an Expand Long Template PCR System kit (Roche) with oligonucleotides located in the middle of exon 3 designed from a interspecies comparison (Fig. 1). The reactions were carried out according to the manufacturer's recommendations

and were run in a Robocycler 96 PCR machine (Stratagene).

The reverse transcriptase (RT) reaction was carried out with 1 μg RNA as template for murine myeloma virus RT (Promega) primed with 1 ng reverse primer. PCR using different primer combinations (indicated in Fig. 1) was performed with 5 μl RT product using 0·2 U *Thermophilus aquaticus* DNA polymerase (Qiagen) in a Hybaid PCR Express (Hybaid, Heidelberg, Germany) or Stratagene Robocycler 96 (Stratagene).

Marmoset monkey DNA was obtained from blood samples of one male and two female animals, using a commercial kit (FlexiGene DNA 50, Qiagen), and stored at 4 °C. Total RNA was isolated from five (two males and three females) healthy monkey pituitaries and two placentas, using a commercial kit (Ultraspec, Biotex Laboratories, Houston, TX, USA).

The design of the oligonucleotides was based on preliminary results from our group (Gromoll *et al.* 2003) and others (Sherman *et al.* 2001, Maston & Ruvolo 2002) assuming high sequence homology with, or diversity from, other monkey gonadotrophin subunits respectively. The oligonucleotides covered a variety of positions between the 5′- and the 3′- ends of the monkey *LHβ* and *CGβ* coding sequences and genomic DNA; they are listed in Fig. 1.

Northern blot hybridisation

Fifteen micrograms total RNA from pituitary, liver, ovary and placenta of a marmoset monkey were run on a 1% agarose–formaldehyde gel and then transferred overnight to a nylon membrane (Hybond-N, Amersham) for northern hybridisation. RNA was fixed on the membrane by irradiation with UV light at 302 nm for 1 min. ^{32}P-Labelled cDNA probes were prepared from an EcoRI-digested plasmid containing hCGβ cDNA. Hybridisation was performed in glass tubes containing the hybridisation solution (5 × SSPE, 2 × Denhardts, 50% formaldehyde, 100 μg/ml denatured salmon sperm DNA, 1% SDS, 5×10^6 c.p.m./μl labelled probe) at 68 °C overnight. The membrane was washed three times with 0·1 × SSC containing 1% SDS at 68 °C for 30 min, followed by exposure on X-ray film (Kodak) at −80 °C using an intensifying screen (Amersham).

Immunohistochemistry

Sections 4 μm thick were cut from Bouin-fixed, paraffin-embedded specimens of pituitaries of three male marmoset monkeys. After the sections had been deparaffinised and rehydrated, primary antibody against hCG (polyclonal anti-human CG (AHP 536, Serotec, Düsseldorf, Germany) with crossreactivity $<1\%$ with LH and $<0{\cdot}3\%$ with FSH) was applied for 30 min at room temperature in blocking buffer. Controls were performed by omitting the primary antibody. The available antibody against mCG (R64–050396) showed binding only in the western blots and did not work on tissue sections, presumably because of the paraffin embedding procedure; for histological sections we therefore used the human hCG antibody. To visualise FSH in the pituitary gland, a primary antibody against human FSH (0373, Immunotech, Marseille, France) was utilized. After washing, slides were incubated with biotin-labelled goat anti-rabbit immunoglobulins and then with streptavidin conjugated to horseradish peroxidase for 15 min at room temperature, followed by incubation with diaminobenzidine substrate solution for 15–30 min. The staining reaction was stopped by washing in 1 × tris-buffered-saline (TBS). The slides were counterstained with haematoxylin for 10 s and mounted under coverslips with Dako Faramount medium (Dako Diagnostika, Hamburg, Germany) before observation using an Axioskop microscope (Carl Zeiss, Jena, Germany) at different magnifications (10 × and 20 × objectives). Digital images from equal exposure times were obtained with a CCD camera (Axiocam, Zeiss) controlled by Axiovision image software (Carl Zeiss).

Immunofluorescence staining

Immunofluorescence staining for mCG was performed with either the specific mCG (R64) primary antibody or the hCG antibody on CHO cells stably expressing recombinant mCG (Amato *et al.* 1998). The cells were grown on Falcon culture slides (Becton Dickinson Labware, Le Pont de Claix, France) overnight and fixed for 30 min with 4% paraformaldehyde. After a blocking step with 5% BSA and 0·1% Triton X-100, the mCG antibody together with 1% BSA and 0·1% Triton X-100 was applied. The rabbit polyclonal mCG antibody

was detected with an anti-rabbit antibody conjugated with TRITC (T-5268, Sigma). All incubations were performed at room temperature, for 2 h with the primary antibody and 1 h with the secondary antibody. The cells were counterstained with 1 μg/ml 4′-6-diamidino-2-phenylindole (DAPI, Sigma) for 5 min. Both fluorescent dyes were excited with the appropriate filter set on an Axioskop (Carl Zeiss, Jena, Germany) at a magnification of 200 × . Control cells, without the primary antibody, were negative for the TRITC fluorescent signal. Images were taken with the Axiocam controlled by Axiovision software.

Western blot

Proteins resolved by 12% SDS-PAGE, under reducing conditions, were transferred to a nitrocellulose membrane (0·4 μm) at 100 mA for 1 h. The membrane was incubated with 5% low-fat dried milk in PBS (pH 7·4) with 150 mM NaCl and 0·1% Tween 20 for 1 h. A further incubation was performed with the polyclonal mCG antibody R64 (diluted 1:2000) (Amato *et al.* 1998) for 1 h at room temperature, followed by incubation with anti-rabbit IgG horseradish peroxidase conjugate (Sigma; diluted 1:20 000) for a further 1 h. After each incubation, membranes were washed three times with the PBS–Tween 20 solution. Detection was achieved with enhanced chemiluminescence using the SuperSignal West Pico Chemiluminescent Substrate Kit from Pierce (Bonn, Germany).

Results

Search for LHβ mRNA expression in the pituitary

Using oligonucleotides published previously by Simula *et al.* (1995) and suitable for amplification of mCGβ mRNA from placental tissue (Fig. 1, I forward/II reverse), we were able to amplify mCGβ mRNA from the pituitary of the common marmoset (Gromoll *et al.* 2003). However, using this primer combination, no clone containing nucleotide sequence reminiscent of *LHβ* was found. In an additional approach we used 12 oligonucleotides (not shown) directed to consensus sequences derived from sequence comparisons of the known *LHβ* genes from other primates to amplify the mLHβ mRNA from the pituitary (Maston & Ruvolo 2002). The amplicons obtained were further analysed by either single-stranded conformation polymorphism or direct sequencing to distinguish *LHβ* from *CGβ*. Again, no mLHβ mRNA could be detected. Thus the following two options emerged: i) the sequence of the *LHβ* gene is very different from that of the *CGβ* gene in the marmoset, thereby hindering correct PCR amplification, or ii) the *LHβ* gene is not expressed.

Identification and characterization of the marmoset *LHβ* gene

The organization of *mLHβ* gene was analysed using genomic DNA. With two oligonucleotides (Fig. 1, III forward/IV reverse) directed to one *LHβ/CGβ* consensus sequence from different species, we attempted to amplify the genomic region between the *LHβ* and *CGβ* genes. Such a strategy would result in amplification only when two (*LHβ* and *CGβ*) or more highly homologous genes are present. Furthermore, it could give important information on the orientation of the genes. If more copies of the *mCGβ* gene were present, it is likely that differences in the intronic sizes between the genes would produce more than one amplification product. A similar amplification of several bands representing different gene copies has previously been shown for the *hCGβ* and *hLHβ* gene cluster, where the intergenic distance is approximately 3 kbp (Maston & Ruvolo 2002). With long template PCR techniques using marmoset genomic DNA, we amplified one large amplicon of approximately 20 kbp (Fig. 2*b*, R1). The amplicon was purified and used as a template for the subsequent PCR reactions to confirm whether it contained the *mCGβ* and *mLHβ* genes.

By using primer combinations capable of amplifying either the 3′ or the 5′ part of the presumed genes and subsequent cloning and sequencing of the amplicons, we gathered insights into the organisation and the sequence of the genes. With the primer combination III forward/II reverse we were able to amplify the 3′ portion of the *CGβ* gene with its characteristic mutation giving rise to the translation of the CTP. With the primer combination I forward/IV reverse, we obtained a 900 bp product with sequences significantly different from those of the mCGβ cDNA (Fig. 1). This part contains exons 1, 2 and part of exon 3 of the *LHβ* gene (Fig. 2b, R2).

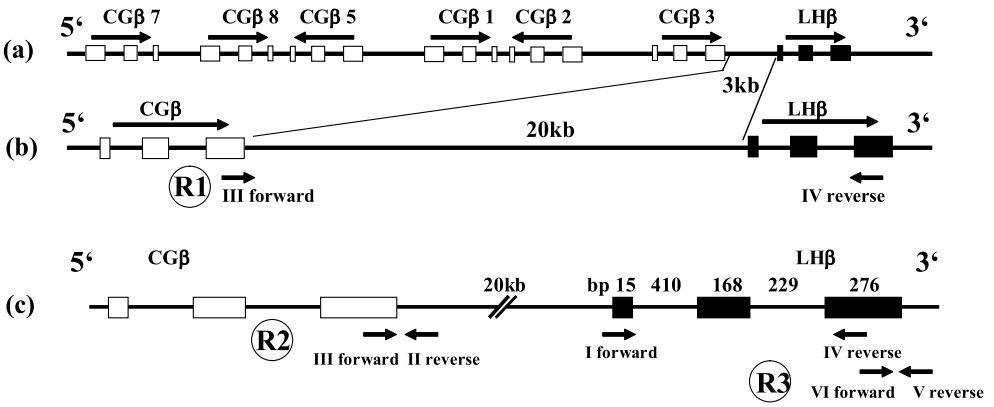


Figure 2 Strategy to detect marmoset LHβ at the genomic level. (a) Schematic organisation of *hLHβ*/*hCGβ* cluster. (b) Situation in the marmoset: *mCGβ* is separated from *mLHβ* by a 20 kb insertion, as revealed by intergenic PCR reaction (R1) (oligonucleotide pair: III forward/IV reverse). (c) Magnification of (b): fragment 1 located at the 5′ end with 163 bases matched 100% of the *mCG* sequence, whereas a 900 base fragment at the 3′ end showed just 81% identity to *mCG* but 93% to *hLH* (R2). Amplification of the 3′ end of *mLHβ* was performed with specific oligonucleotide (VI forward/V reverse) designed from sequence analysis of fragment 2 (R3). The corresponding exon and intron sizes for the *mLHβ* gene are indicated in bp numbers.

Unilateral specific PCR

The genomic approach using one nucleotide sequence to amplify two genes left the 3′ part of the *LHβ* gene unidentified (Fig. 2b). The missing 3′ part of the *LHβ* gene was obtained by unilateral specific PCR. For this we designed specific oligonucleotides (VI forward, VII forward) based on the obtained *LHβ* sequences, which were used in conjunction with the less specific V reverse primer to amplify the missing 3′ region (Fig. 1). Two amplicons of 900 and 350 bp respectively were obtained from genomic DNA, of which the last 160 bases contained the remaining portion of the *LHβ* gene (Fig. 2c, R3). Upon cloning and sequence analysis, we could identify sequences completing the *LHβ* gene of the common marmoset.

Sequence analysis

The *LHβ* gene of the common marmoset consists of three exons and two introns (Fig. 2). Exon 1 encodes 15 bp, exon 2 168 bp and exon 3 276 bp; the two corresponding introns cover 410 bp and 229 bp respectively. The exon and intron sizes are in good agreement with other *LHβ* genes (Jameson *et al.* 1984, Talmadge *et al.* 1984). The typical splice sites are preserved in the *LHβ* gene (not shown). The deduced open reading frame consists of 426 bp.

Interestingly, a CTC triplet coding for leucine is lacking in the mCGβ subunit cDNA, but is present in the *LHβ* gene of the marmoset and other species. The hallmark of all *LHβ* genes, the presence of an adenosine at position 400 leading to a translational stop codon at position 426, could be shown. *N*-Linked glycosylation at position Asn 30 is possible, and it is likely that mLH would also be glycosylated at position Asn 13, where mCG has lost its glycosylation recognition motif (N-A-T) (Fig. 3). All conservative cysteines were present, similar to human gonadotrophins and mCG (Fig. 3). At the amino acid level, LHβ has an identity of 89% to hLHβ and 78% to hCG, whereas identity to mCGβ reached only 70%. The signal peptide region of mLH corresponds to that of hLH.

Dendrogram

Using CLUSTAL W software (European Bioinformatics Institute, Cambridge, UK) a dendrogram was deduced showing the homologies of *LHβ* and *CGβ* genes from several primate species, including humans, covering all different important

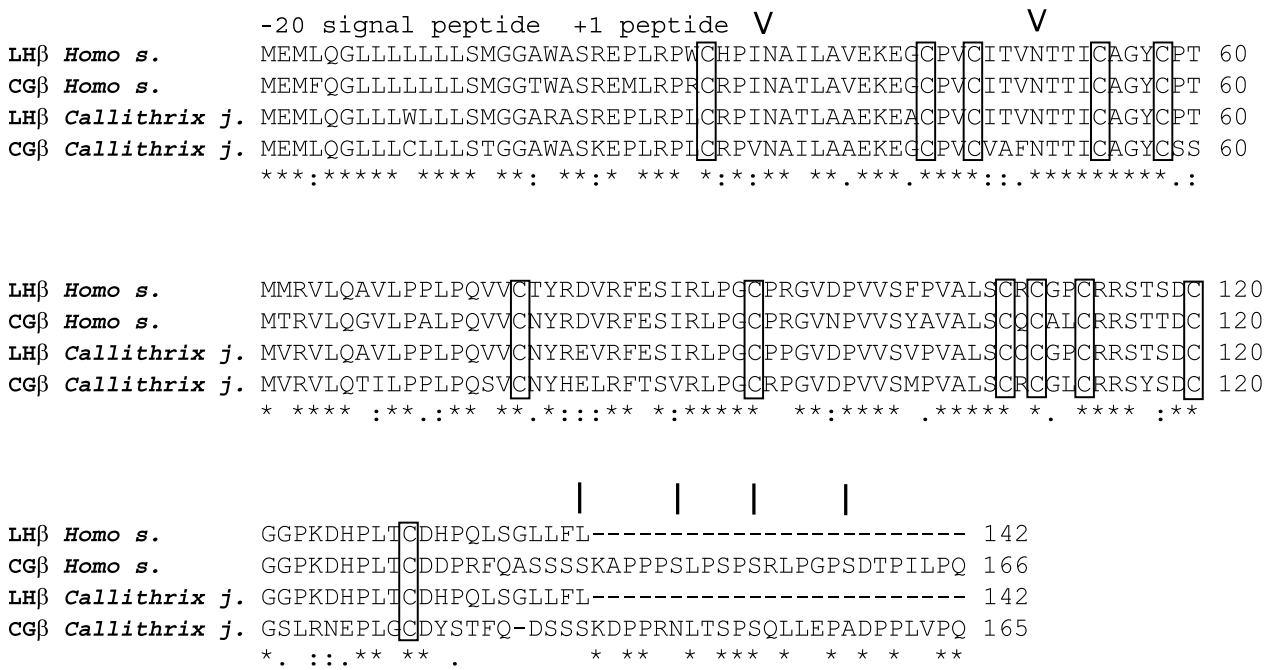


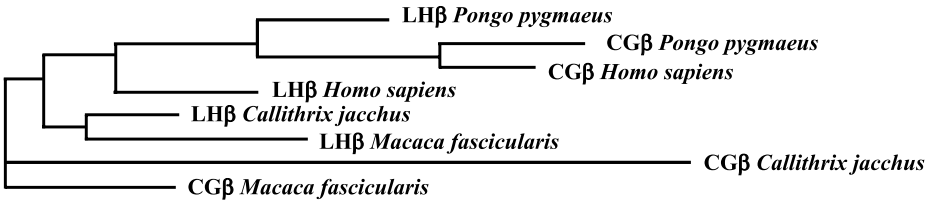
Figure 3 Multiple sequence analysis of amino acids of marmoset monkey *LHβ* (based on genomic information), *mCGβ*, *hLHβ* and *hCGβ* subunits. *mLHβ* shows 89% identity to *hLHβ* and 78% to *hCGβ*, but only 70% to *mCGβ* at the amino acid level. *N*-Linked glycosylation sites are represented by ∨, | symbols represent putative *O*-linked carbohydrates, not present in LH. The 12 typically conserved cysteines in gonadotrophins are boxed.

lineages (Fig. 4). We analysed in detail the *LHβ* and *CGβ* sequences from the New World monkey *Callithrix jacchus* (*m*), the Old World monkey *Macaca fascicularis* (*cyn*), the great ape *Pongo pygmaeus* and the human. The *LHβ* genes and the *CGβ* genes from human and the chimpanzee were closely related, whereas the New World and Old World monkeys seemed to form a separate group with respect to the *LHβ* genes (Fig. 4). Interestingly, both *CGβ* genes showed markedly reduced homology to the *hLHβ* or *hCGβ* genes: *hLH* compared with *cynCG* 84% and *hCG* compared with *cynCG* 81% identity for *M. fascicularis*; *hLH* compared with *mCG* 69% and *hCG* compared with *mCG* 66% for *C. jacchus* (Fig. 4). Thus the *mCGβ* gene is the most divergent nucleotide sequence among the *LHβ*/*CGβ* gene family (Fig. 4).

Expression analysis of the mLHβ and CGβ mRNA in pituitary and placenta

Using the new sequence information obtained from the genomic cloning of the *mLHβ* gene, we investigated the expression of the *LHβ* gene in the pituitary. Using primer combinations specific either to LHβ or mCGβ, we performed RT-PCR of total RNA preparations from the pituitary or the placenta (Fig. 5). According to the sequence data obtained and the genomic organization of the genes, a 333 bp fragment is to be expected for LHβ/CGβ RNA in the pituitary and placenta when using a primer combination capable of amplifying both genes (I forward/IV reverse) and an *LHβ* gene-specific band of 205 bases (VI forward/IV reverse) is expected when using the primer combination VI forward/V reverse (Fig. 1). Genomic DNA served as template to confirm PCR fidelity of the primer combinations used. As expected, we observed an amplicon of approximately 333 bp for the potentially simultaneous amplification of *CGβ*/*LHβ* from pituitary RNA; however, no product was obtained when the *LHβ*-specific primer was used (Fig. 5). Thus *LHβ* is not transcribed in the pituitary. The amplicon from the first RT-PCR (lane 1) corresponded completely to mCGβ cDNA revealed by DNA sequencing (data not shown). A similar picture was obtained in the placenta, with *mCGβ* being the only gene expressed. The fragments obtained from genomic DNA correspond to the fragment length expected

(a)



(b)

Homology AA's (%)	*Pongo p.* LHβ	*Pongo p.* CGβ	*Callithrix j.* LHβ	*Callithrix j.* CGβ	*Homo s.* CGβ	*Homo s.* LHβ	*Macaca f.* CGβ
Macaca f. LHβ	81	68	90	68	73	87	85
Macaca f. CGβ	79	79	91	73	81	84	
Homo s. LHβ	88	77	90	69	81		
Homo s. CGβ	84	92	78	66			
Callithrix j. CGβ	66	67	70				
Pongo p. CGβ	88						

Figure 4 (a) Dendrogram of LHβ and CGβ subunit genes from different primates, derived using CLUSTAL W software. (b) Percentage of amino acid (AA) identities between the different species and subunits.

for the amplification of portions from the *LHβ* or *CGβ* gene. The larger size observed is attributable to intronic sequences, as the primers were designed to cover exons 2 and 3.

Northern blot hybridisation

The expression of mCGβ mRNA in different tissues was analysed by Northern blot hybridisation using the complete mCGβ cDNA as a probe (Fig. 6). mCGβ mRNA was specifically expressed in the pituitary and placenta of the marmoset monkey, whereas other tissues, such as liver or ovary, were negative. The pituitary transcript size was approximately 0·7 kb, whereas two transcripts of 1·1 kb and 2 kb were detected in the placenta (Fig. 6).

Immunohistochemistry

Using an antibody against hCG, specific staining of CG in the marmoset pituitary could be demonstrated (Fig. 7A). The CG-positive cells were distributed randomly throughout the adult pituitary gland, showing that approximately 25% of the cells produce CG. The concentration of CG in the cytoplasm of these cells showed equal staining, giving the impression that all CG-producing cells could release a similar amount of CG. The polyclonal mCG antibody used did not work in paraffin-embedded sections of marmoset tissues; however, we obtained specific signals in cell cultures stably expressing mCG (Fig. 7D, E). Similar signals were obtained in these cell cultures using the hCG antibody (data not shown). Thus detection of mCG using the mCG antibody is specific. Some CG-producing pituitary cells were also positive for expression of FSH protein (not shown). This FSH expression showed that more cells produce FSH (Fig. 7B) than produce CG; three types of cells were found, some producing only CG, a low number producing both CG and FSH, and a larger group of cells producing solely FSH.

Western blot

A western blot using the polyclonal mCG antibody was performed using protein extracts from CHO cells stably expressing the recombinant mCG (see above) and from rat, cynomolgus and marmoset

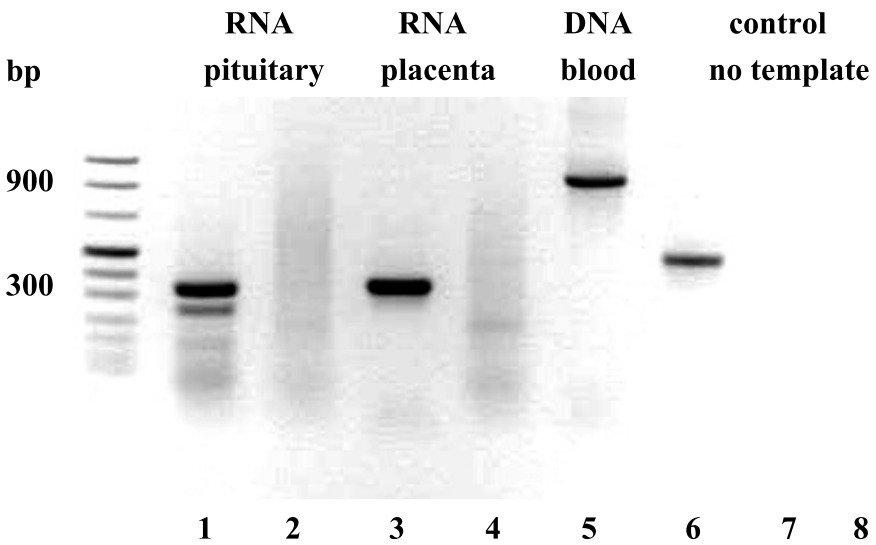


Figure 5 RT-PCR from marmoset pituitary and placenta, demonstrating that only mCGβ is expressed. Amplification of marmoset CGβ obtained from placenta, pituitary and genomic DNA isolated from blood samples. Lanes 1, 3, 5: I forward/IV reverse amplifies both CGβ and LHβ. Lanes 2, 4, 6: VI forward/IV reverse amplifies LHβ exclusively. Lanes 7, 8: Negative controls. A 333 base fragment was found for both LH and CG at the mRNA level (lane 1 and 3). LHβ mRNA would yield a 205 bp fragment (lane 2 and 4), but was not amplified. At the genomic DNA level, mCGβ and mLHβ produced a fragment of 900 bp sequences (lane 5); LHβ-specific amplification gave rise to a 550 bp fragment (lane 6); both fragments are considerably larger than RT-PCR fragments, which is the result of intronic sequences. For the genomic amplification, a shorter forward primer I, corresponding to the boundaries of exon 1, was used (Fig. 1). RT-PCR was independently repeated with RNA from three pituitaries with different PCR conditions.

monkey pituitaries (Fig. 8). Double bands, presumably corresponding to the β subunit and probably exhibiting different glycosylation patterns, were detectable in both the cell extracts from the CHO cells (Fig. 8, lanes 1 and 5) and the pituitary from the marmoset monkey (Fig. 8, lane 3). The bands in the pituitary were shifted towards a greater molecular weight, which might be a reflection of different glycosylation patterns. No bands were visible in the pituitaries of rat and cynomolgus monkey (Fig. 8, lanes 2 and 4), indicating the lack of expression of CG and also that this antibody did not specifically bind to LH in those animals.

Discussion

The marmoset monkey belonging to the *Platyrrhini* lineage is one of the most important animal models in clinical and toxicological research around the world. Its small size and easy reproduction in captivity are considered an advantage for studies in non-human primates (Smith *et al.* 2001, Zuehlke & Weinbauer 2003). Marmoset placental CGβ has been the subject of several studies in the past (Hobson & Wide 1981, Saunders *et al.* 1987, Rosenbusch *et al.* 1994, Simula *et al.* 1995), whereas the genomic sequence of mLH was unknown. In the present work we used intergenic PCR to demonstrate the existence of both *mCGβ* and *mLHβ* subunit genes, separated by an intronic sequence of approximately 20 kb, with *CGβ* placed 5′ of the *LHβ* gene. Sequence analysis of the coding region and deduced amino acids of *mLHβ* compared with *mCGβ* showed 70% identity, compared with 81% in the human. *mLHβ* showed 90% identity to *hLHβ* at the amino acid level.

We were able to confirm that mCGβ is highly expressed in the pituitary (Gromoll *et al.* 2003), but were unable to detect the expression of LHβ mRNA. Pituitary expression of trace amounts of

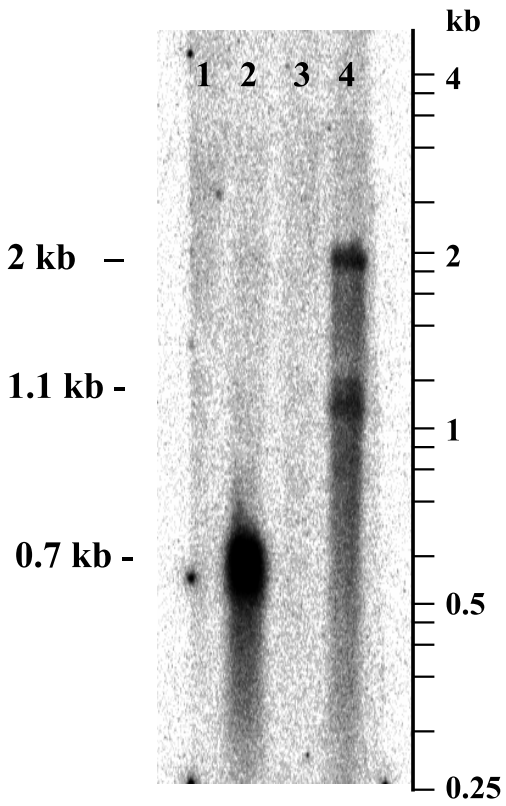


Figure 6 Northern hybridisation of marmoset CGβ mRNA. Lane 1: ovary; lane 2: pituitary; lane 3: liver; lane 4: placenta. In the pituitary, one specific band of 0·7 kb is visible, whereas in the placenta two bands of 1·1 kb and 2 kb are detectable.

hCG is also found in the human. The isolation and characterisation of hCG from the human pituitary showed that it bears a glycosylation pattern that makes it more similar to LH than to placental hCG (Birken *et al.* 1996). However, no physiological role for pituitary hCG is known. In the marmoset, we observed the opposite phenomenon: CG is the major luteinising gonadotropin in the pituitary gland. The fact that expression of LHβ mRNA is lacking in the marmoset pituitary could be the result either of a lack of transcription factors involved in the regulation of *mLHβ*, or of genetic alterations. We did not find any unusual genetic changes within the exon–intron structure of the *LHβ* gene that prevented gene expression; however, we noticed an unusual extension of the intergenic region between the *mCGβ* and *mLHβ* genes. In other primates, including the human, the intergenic distance is approximately 2–3 kbp, but the marmoset displays a 20 kbp region. Such an expansion might be the result of an insertion – for example retrotransposition of long interspersed nuclear element (LINE) elements, which are known to be responsible for gene silencing. In the cotton tamarin, such a retrotransposon has led to the silencing of the semenogelin II gene and subsequently to its elimination from the genome (Lundwall & Olsson 2001). Future studies are needed to reveal the structure and sequences of the large intergenic region and the mechanism underlying the inactivation of LHβ in the marmoset.

The transcription of the *mCGβ* gene is highly tissue specific and yielded two different transcript patterns for the pituitary and the placenta. The 0·7 kb transcript in the pituitary and the 1·1 kb in the placenta are in good agreement with transcript sizes described in other species (Fiddes & Goodman 1980, Gromoll *et al.* 1993), whereas the existence of the 2 kb transcript might be attributable to a hitherto unknown new transcriptional start site in the *mCGβ* gene. In the horse, the *LHβ* gene is expressed both in the pituitary and in the placenta, using the same transcriptional start site, which is homologous to that of the human *LHβ* gene (Sherman *et al.* 1992), giving rise to identical transcript sizes. In a functioning CGβ/LHβ system it is assumed that, during evolution, changes within the promoter regions of *CGβ* genes have led to the placenta-specific expression, and the usage of a novel transcriptional start site for the *CGβ* gene yields transcripts of 1·1 kb (Jameson *et al.* 1984, Hollenberg *et al.* 1994). In the marmoset, the situation is very different. The various transcripts observed must be derived from the same gene. This implies that different transcriptional start sites are being used in a cell-specific manner. Thus cell-specific transcription factors must be involved in silencing or activating the corresponding transcription of *mCGβ* in the pituitary and placenta. Analysing the promoter structure of the *mCGβ* gene should give insight into a promoter version that resembles an ambiguous form in terms of cell specificity when compared with other *CGβ* promoters, in which expression of CGβ mRNA is restricted to the trophoblasts.

The Western blot experiments proved that mCG is expressed in the pituitary of the marmoset monkey. Detection of the mCG β subunit is specific because a positive control, CHO cells stably expressing the recombinant mCG, gave

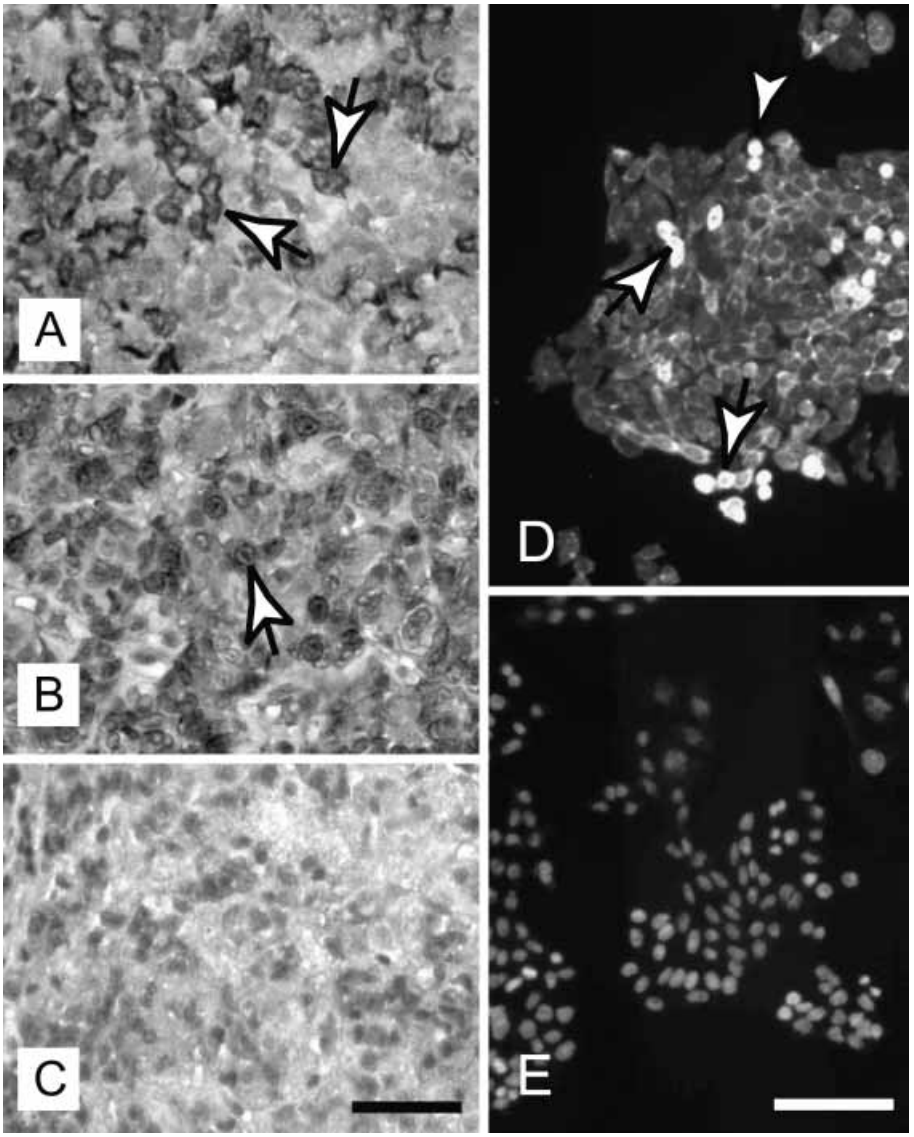


Figure 7 Immunocytochemistry of mCG in the marmoset pituitary. CG-positive cells and FSH-positive cells were stained with diaminobenzidine using an anti-human chorionic gonadotrophin antibody (A) or an anti-human FSH antibody (B) in marmoset pituitary. Negative controls (C) were generated by omitting the primary antibodies. (D) CHO cells stained positive for mCG; the nuclei are counterstained with DAPI. (E) Negative control: staining was absent when the first antibody was omitted. Scale bar represents 50 μm.

comparable signals whereas pituitaries from rat and cynomolgus monkey, only expressing LH in the pituitary, remained without any detectable signal (Amato *et al.* 1998). The observed slight shift in size in the marmoset pituitary could be the result of a different glycosylation pattern, known from other recombinant gonadotrophins. The molecular mass obtained is in agreement with the findings of previous studies (Simula *et al.* 1995).

Immunocytochemical data showed that both CG and FSH were expressed in gonadotrophic cells of the marmoset pituitary and that, as in other species, both gonadotrophins are partly co-localized in the same cells (Wittkowski 1971, Watanabe *et al.* 1998,

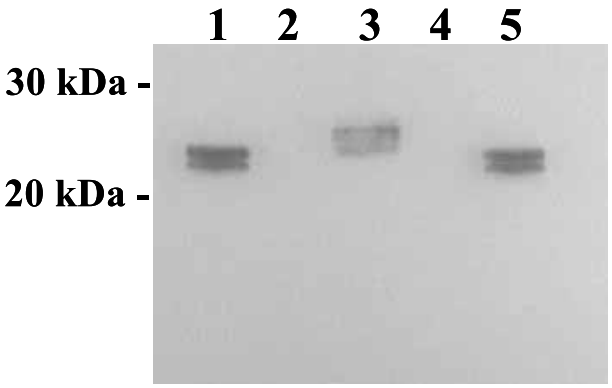


Figure 8 Western blot for mCG. Protein extracts from a CHO cell line stably expressing recombinant mCG (lanes 1 and 5) or pituitaries from rat (lane 2), marmoset monkey (lane 3) and cynomolgus monkey (lane 4) were subjected to SDS-PAGE and blotted. Using a specific mCG polyclonal antibody, duplicate bands were detectable in the protein extracts of the CHO cells expressing recombinant mCG (approx. 25 kDa) and the marmoset pituitary (approx. 27 kDa). No such bands could be detected in the pituitaries of the cynomolgus monkey or rat.

Crawford & McNeilly 2002, Meeran *et al.* 2003). The pattern of localisation of mCG and FSH and the numbers of positive cells resembled previously published distributions for LH and FSH (Tixier *et al.* 1975, Newman *et al.* 1989, Sharpe *et al.* 2002). This suggests that gonadotropin-releasing hormone also regulates synthesis and secretion of both CG and FSH from the pituitary gland in the marmoset monkey. Normally, pulsatile LH secretion is important for normal steroidogenesis, which can be supported optimally with a short gonadotrophin half-life. Because of the presence of the CTP and a different pattern of glycosylation, placental CG has a longer half-life, which is optimal for maintaining pregnancy by rescuing progesterone production by the corpus luteum. In most species, CGβ shows four *O*-glycosylations in its CTP that are responsible for the extension of the half-life of this gonadotrophin (Pierce & Parsons 1981, Chen *et al.* 1982, Matzuk *et al.* 1990, Wilson *et al.* 1990). mCGβ lacks one *N*-glycosylation at Asn 13 and two *O*-glycosylations in its terminal region, but has gained an additional *N*-glycosylation site at Asn 126 (Fig. 3) (Simula *et al.* 1995). Subsequent biochemical studies indicated two *N*-linked and one *O*-linked oligosaccharides in the secreted protein (Amato *et al.* 1998). In addition, the pattern of glycosylation of pituitary hCG is different from that of placenta hCG, because of the presence of sulphate and sialic acid residues, which renders the molecule more similar to LH (Birken *et al.* 1996). The pituitary gland is known to express the relevant glycosylation enzymes, which are not expressed in the placenta. In addition, the terminal sulphate residues on LH are recognized by the mannose/GalNAc-4-SO(4) receptor located in hepatic endothelial cells, which sequester and metabolise the hormone, contributing to the pulsatile pattern of the presence of LH in blood (Baenziger *et al.* 2003). If this also occurs in the marmoset, this could result in a shorter half-life of pituitary mCG, resulting in pulsatility. We suggest that the data reported by Hodges (1978) and O'Byrne *et al.* (1988), showing that mLH secretion is pulsatile with a peak every hour, should be reinterpreted as showing that CG is secreted in a pulsatile fashion from the marmoset pituitary. These authors measured LH by Leydig cell bioassay, which cannot distinguish between LH and CG, and by RIA using an anti-ovine rabbit LH 610 V antiserum, which is not specific for mLH. As the pattern of glycosylation of LH is essential to maintain pulsatile concentrations in serum (Baenziger 2003), these data suggest that glycosylation of pituitary CG is compatible with a short-lived gonadotrophin. Further studies are required to investigate whether the pattern of glycosylation of placental CG is different from that of pituitary CG.

Both hLH and hCG can activate the marmoset LHR, as demonstrated by *in vitro* experiments in transiently transfected COS7-cells (unpublished data) and by isolated and perfused corpora lutea (Einspanier & Hodges 1994). It is unclear whether genuine mLH would act on its own receptor, but most probably it would, even if it is expected that mLH would show lower bioactivity than CG. In fact, we have shown that mCG lacking CTP is less bioactive than complete mCG, suggesting that the CTP of *CGβ* can compensate for the loss of exon 10 of the LHR in receptor activation (T Müller, J Gromoll, AP Simula, R Norman, R Sandhowe-Klaverkamp and M Simoni, Unpublished Observation).

In summary, the surprising fact that *LHβ* is a pseudogene in the marmoset monkey is a peculiar finding in the LH–CG–LHR system of this species and may also be true in other New World monkeys that also possess the LH receptor type II lacking

exon 10 (Gromoll *et al.* 2003). In view of recent data reported by Maston & Ruvolo (2002), there are two possible explanations for the loss of expression of *LHβ*. For both hypotheses, the first steps are identical: the *LHβ* gene duplicates and mutates, giving rise to the *CGβ* gene originally expressed in pituitary and placenta, and *CGβ* acquires expression in the placenta of species with a hemochoral placenta (Tarsiers). At this point, two evolutionary pathways are possible. The first is that a mutation or another genomic event (insertion of a retrotransposon, indicated by the unusually large DNA fragment inserted between the *mLHβ* and *mCGβ* genes) disrupts the regulatory sequence of the *LHβ* gene in *Platyrrhini*; *CGβ*, however, compensates for loss of *LHβ* expression. Then the expression of exon 10 of the LHR, redundant for CGβ action, is lost as a result of mutation(s) in the New World monkeys; the mutation is irrelevant for receptor function, driven by CGβ. Alternatively, a mutation or another genomic event disrupts exon 10 of the LHR in the *Platyrrhini* lineage and, because of the pituitary expression of CG, which is more bioactive on the LHR lacking exon 10, expression of *LHβ* is lost. As a consequence the 'luteinising' function is maintained by CG. Finally, the glycosylation machinery of the pituitary gland, different from that of the placenta, could result in a pattern of glycosylation compatible with short hormone half-life and pulsatility.

Our findings strongly suggest that *LHβ* is a pseudogene in the marmoset monkey, and that all previous studies dealing with LH secretion and serum concentration in this species (Rosenbusch *et al.* 1994, O'Byrne *et al.* 1988) should be reconsidered from the viewpoint that CG, and not LH, is produced by the pituitary gland.

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