A novel two-promoter-one-gene system of the chorionic gonadotropin β gene enables tissue-specific expression

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Abstract

The New World monkey (NWM), Callithrix jacchus, a preferred model in medical research, displays an interesting endocrine regulation of reproduction: LH, the heterodimeric glycoprotein hormone, is functionally replaced by the chorionic gonadotropin (CG), a hormone indispensable for establishment of pregnancy in humans and normally expressed in the placenta. In the marmoset pituitary, the expression of the β-subunit (CGB) gene is regulated similar to human LH β-subunit, but its placental regulation is unknown. This study intended to decipher the underlying mechanism of tissue-specific expression of CGB in the marmoset placenta. We identified a new placental transcriptional start site, described a new, previously undiscovered exon, and define a novel placental core promoter in the marmoset CGB gene. This promoter contains a TATA box and binding sites for activating protein 2 and selective promoter factor 1, the latter acting synergistically by forming a regulation cassette. Differential first exon usage directed the tissue-specific expression. Methylation analyses revealed a tissue-specific pattern in the placental promoter indicating additional epigenetic regulation of gene expression. Our findings point toward a hitherto unknown evolutionary plasticity in the LH/CG hormonal system in NWM, which could be used as a model to study human CGB regulation in clinical pathologies.

Journal of Molecular Endocrinology (2011) 47, 285–298

Introduction

Mammalian reproduction is controlled by the hypothalamic GnRH that stimulates the pituitary to release FSH, which triggers folliculogenesis in the female and spermatogenesis in the male, and LH, which directs ovulation in the female and testosterone production in the male. A third glycoprotein hormone, the chorionic gonadotropin (CG), is essential for establishing pregnancy as it is the first biochemical signal from the embryo and ‘rescues’ the corpus luteum (Bousfield & Ward 2006). Moreover, in humans, CG triggers fetal testosterone production and is hence crucial for male sexual development (Gromoll et al. 2000). The gonadotropins are heterodimeric with identical α-subunits but hormone-specific β-subunits that confer specificity (Bousfield & Ward 2006). While LHB and FSHB genes are present in nearly all mammals, the CGB gene is exclusively present in Anthropoidea, such as New World monkeys (NWM), Old World monkeys (OWM), great apes, and humans. Produced mainly by placental trophoblasts (Maston & Ruvolo 2002), CG is derived from the LHB gene by gene duplication and a frameshift mutation. Thus, this leads to an extension of the protein, named the carboxy-terminal peptide (Talmadge et al. 1984), followed by several duplications in OWM and humans. These result in the presence of six CGB genes and one LHB gene in humans, all expressed at different levels, with CGB5 being the most abundant (Talmadge et al. 1983, Jameson et al. 1984, Bo & Boime 1992, Miller-Lindholm et al. 1997). Notably, the CGB variants are arranged in a gene cluster together with the single-copy LHB gene (Maston & Ruvolo 2002, Hallast et al. 2005), suggesting rapid evolution in parallel to the LHB gene itself (Wallis 2001). However, the ongoing evolution of the CGB/LHB genes in the NWM lineage is underlined by the absence of the LHB gene, while CGB is a single-copy gene in this taxon (Maston & Ruvolo 2002, Muller et al. 2004a, Scammell et al. 2008). It is noteworthy that equine species also possess a gonadotropin with CG-like properties that has evolved differently from primate CG via convergent evolution (Sherman et al. 1992, Chopineau et al. 1999). Understanding the mechanism of CGB gene regulation is relevant for clinical pathologies, e.g. reduced serum levels of CG are a risk factor for miscarriage, and association of specific CGB polymorphisms and recurrent miscarriage (Rull et al. 2008b). Furthermore, increased serum CGB can serve as a marker for trisomy 21 and malignancies of the reproductive system (Iles et al. 1987, Cuckle et al. 1994, Hotakainen et al. 2007).
The CGB gene is also important from an evolutionary point of view for primates and humans, as it is only present in that particular taxa (Fiddes & Talmadge 1984, Henke & Gromoll 2008). The marmoset is the most common non-human primate animal model in reproductive sciences and preclinical toxicology (Zuhlke & Weinbauer 2003). Our previous study showed that the regulation of human LHB and marmoset CGB expression shows similarities in the pituitary. Both have a very short 5′-untranslated region (5′-UTR) of only 9 and 7 bp respectively. Furthermore, human LHB and marmoset CGB promoters show similar GNRH responsiveness and identical transcription factor binding sites (TFBS) in the core promoter of the pituitary (Henke et al. 2007). Findings were similar to that of other NWM species as well (Scammell et al. 2008, Vasauskas et al. 2011). In contrast, human placental CGB transcription starts 365 bp upstream of exon 1 so that the human CGB 5′-UTR encompasses the region that would serve as a regulatory region in paralogous human LHB and orthologous marmoset CGB. Thus, the regulation of CGB gene expression in the placenta of NWM deserves to be explored in detail. The fact that the marmoset has only one CGB gene expressed in two different tissues (Muller et al. 2004b), as well as other distinct biochemical properties, hints at a mechanism for tissue-specific regulation. In the last years, the role of epigenetic processes got more focus in research, which is also true for CGB. Very recently, Uuskula et al. (2010) demonstrated epigenetic mechanisms regulating the human CGB genes during pregnancy. Meanwhile, the need to consider protein–DNA interaction as well as epigenetic aspects of gene regulation was highlighted for placental trophoblast differentiation and function in a recent review (Maltepe et al. 2010). Therefore, this study aimed to determine the placental regulation of the CGB gene in the marmoset, taking into account classical transcriptional regulation as well as additional epigenetic regulation.

**Materials and methods**

**Animals and tissues**

Animals were maintained in the center’s animal house and kept under constant conditions. All animal procedures were approved by an ethics committee (G64/01) and were carried out in accordance with the German Animal Protection Law. Pituitaries and placental tissues of the common marmoset (Calithrix jacchus) for RNA and DNA isolation were collected in a previous study (Henke et al. 2007), while genomic DNA of the brown capuchin monkey (Cebus apella), the cotton-top tamarin (Saguinus oedipus), the brown-headed tamarin (Saguinus fuscicollis), and the common squirrel monkey (Saimiri sciureus) were obtained from our center’s own tissue bank.

**DNA isolation**

Genomic DNA was isolated using the Qiagen ‘Whole DNA Tissue Kit’ (Qiagen) following the manufacturer’s instructions.

**Sequences and in silico tools**

Marmoset DNA sequence data were obtained from the Ensembl website (www.ensembl.org). Sequence alignments were performed with ClustalW (Chenna et al. 2003) and translation of DNA sequences was conducted by EMBOSS transeq (Rice et al. 2000). Prediction of potential TFBS in the CGB promoter was performed by MatInspector (Cartharius et al. 2005) and protein cleavage sites were predicted by SignalIP (Emanuelsson et al. 2007).

**Cloning experiments**

Primers (outlined in Supplementary Table 1, see section on supplementary data given at the end of this article) were synthesized by Eurofins (Ebersburg, Germany). Segments of the marmoset placental CGB gene promoter were amplified by PCR, cloned into pCR2.1 (Invitrogen, Karlsruhe, Germany) and verified by sequencing. Constructs were then subcloned into the luciferase vector pGL3-basic via XhoI and KpnI restriction sites (Promega, Mannheim, Germany).

**RNA isolation, rapid amplification of cDNA ends, and reverse transcriptase PCR**

Total RNA was isolated with Ultraspec (AMS Biotechnology, Frankfurt, Germany) according to the manufacturer’s instructions. Reverse transcription of RNA was performed using Superscript II Reverse Transcriptase (Invitrogen), random hexamer primers (Promega), and RNAsin (Promega) according to the manufacturer’s instructions. 5′-Rapid amplification of cDNA ends (RACE) was performed using the GeneRacer kit with Superscript III (both Invitrogen) according to the product instructions. Reverse transcriptase PCR (RT-PCR) and RACE primer sequences are given in Supplementary Table 1, see section on supplementary data given at the end of this article. The first round of nested RACE PCR was performed with 1 μl cDNA. The mix was run on a Robocycler (Stratagene, La Jolla, CA, USA). For the second round of nested RACE-PCR, 500 ng DNA from round 1 was used.

The products were purified and cloned into the pCR4TOPO vector (Invitrogen) according to the
These cells were cultured at 37°C and analyzed using the accompanying software. The expression values of both transcript variants were each normalized to β-actin expression and set as 100%.

**Cell culture**

The monkey kidney cell line COS7 was kept at 37°C and 5% CO₂ (v/v). Cells were cultured in DMEM/F12 1:1 containing 10% (v/v) FCS and 1% (w/v) antibiotics. The human placental choriocarcinoma cell line BeWo was purchased from Cell Line Service (CLS, Eppelheim, Germany). BeWo was established in 1968 and is an accepted model cell line for cytotrophoblasts (Pattillo et al., 1968, Sullivan 2004). These cells were cultured at 37°C, 5% (v/v) CO₂ in Ham’s F12 medium with 10% (v/v) FCS.

**Transient transfections**

BeWo (1·5×10⁵) and COS7 cells (2·5×10⁵) per well were seeded in 12-well plates. After 24 h, cells were covered with 400 μl serum-free medium (Ham’s F12 for BeWo and DMEM/F12 for COS7, Gibco), which contained 1 μg promoter construct DNA, 0·05 μg pRLSV40 Renilla vector (for normalization of transfection efficiency), and 2 μl Lipofectamine (Invitrogen). Each DNA construct was transfected in duplicates. After 18 h, the medium was replaced with a medium containing 10% (v/v) FCS (heat inactivated and charcoal stripped). After 24 h, the cells were washed with PBS, then lysed with 300 μl PLB, and assayed by luciferase assay. Overexpression experiments were similarly performed, using 3 μg transfected DNA (promoter, expression plasmid, and vehicle). Expression plasmids used were activating protein 2 (AP2), human AP2γ isomorph cloned in pBKCMV, and selective promoter factor 1 (SP1), human isomorph cloned in pFCMV. Expression vectors were provided by P Mellon, UCSD, La Jolla, USA.

Luciferase assay

Luciferase activity was measured with the Dual-Luciferase Reporter Assay (Promega) according to the manufacturer’s protocol, using 20 μl lysate and 50 μl reaction buffers I and II. Firefly and Renilla luciferase activity was measured using the 96-well plate reader MicroLumat Plus LB96 (Berthold Technologies, Bad Wildbad, Germany). Each DNA construct was measured in duplicates with two to five repetitions. Experiments were normalized to the negative control (empty vector, pGL3 basic), which was set arbitrarily as 1 relative light unit (RLU).

**In vitro mutagenesis**

Mutagenesis of promoter sequences was performed using the QuickChangeII Site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions with an annealing temperature of 55°C for 30 s. The primers used for mutagenesis are given in Supplementary Table 1, see section on supplementary data given at the end of this article. Correct mutagenesis was verified by sequencing, the correct clone amplified; DNA was isolated and stored at -20°C.

**Immunohistochemistry**

We obtained tissue sections of formalin-fixed and paraffin-embedded human full-term placenta from two individuals from the Edinburgh Reproductive Tissue Bio Bank (ethical approval no. 09/S0704/3). These sections served as positive controls for the immunohistochemistry detection of SP1 and AP2γ. Tissue sections of marmoset placenta from day 80 of gestation were retrieved from the tissue bank of the Medical Research Council, Centre for Reproductive Health. Monoclonal mouse anti-SP1 antibody (Abcam, Cambridge, UK, cat. no. Ab58199) was used at a concentration of ~1 μg/ml, while the monoclonal mouse anti-AP2γ antibody (sc-12762) and the mouse anti-hCGB (sc-51605; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used at ~3 and 2 μg/ml respectively. The peptides against which SP1 and AP2γ antibodies were raised showed ~95% homology between human and marmoset. Immunohistochemistry (IHC) was performed as follows: tissue sections were dewaxed and rehydrated in a xylene/ethanol/water series, and antigens were retrieved by pressure-cooking in 0·01 M citrate buffer (pH 6). The antigen detection procedure for AP2γ and CGB was automated on a Bond-Max machine (Leica Microsystems, Milton Keynes, UK) with the Bond Polymer Refine Detection Kit (cat. no. DS9800; Vision BioSystems bond, Milton Keynes, UK) according to the manufacturer’s instructions and slides were mounted in Pertex (Histolab, Göteborg, Sweden) and coverslips. Antigen
detection for SP1 was carried out manually: dewaxin-
g/rehydration in xylene/ethanol/water series, antigen
retrieval via pressure-cooking, blocking in methanol
with 3% (v/v) H2O2, blocking with streptavidin and
biotin blocking solutions (Vector Laboratories, Burlingame, CA, USA, cat. no. SP-202), and blocking
with a mixture of 20% normal rabbit serum (NRS),
TBS, and 5% (w/v) BSA (NRS/TBS/BSA). Slides were
incubated with the primary antibody in NRS/TBS/BSA
overnight in a humidity chamber at 4 °C. The following
day, the slides were incubated at room temperature with
first 1:500 dilutions of a secondary rabbit anti-mouse
biotinylated antibody (Dako, Glostrup, Denmark; cat.
no. E464) for 2 h in NRS/TBS/BSA and then 1:1000
dilutions of tertiary streptavidin–HRP conjugate in TBS
(Vector Laboratories) for 2 h. ImmPACT DAB kit
(Vector Laboratories, SK-4105) was used to visualize
antibody binding. Afterwards slides were mounted and
tissue sections were photographed with an Axiocam
HRC (Zeiss, Welwyn Garden City, UK) mounted to a Provis
AX70 microscope from Olympus (Southend-on-Sea, UK).
Images were white balanced, composed, and annotated
with Photoshop. Omission of the primary antibody was
considered to be a sufficient negative control.

Chromatin immunoprecipitation with transiently
transfected cells
BeWo cells were seeded in 10 cm dishes at 80% 
confluence for 24 h. The next day, the cells were
transfected with 15 μg of the promoter construct with
or without mutation using 66 μl Lipofectamine trans-
fection reagent (Invitrogen). Plates were incubated for
24 h at 37 °C after which cells were cross-linked with
formaldehyde, harvested, and chromatin immuno-
precipitation (ChIP) was performed. Cells (5×10⁶)
were used per immunoprecipitation reaction, using the
ChampionChIP One-Day Kit (SABiosciences, Frederick,
MD, USA) according to the previously published
protocols (Wells & Farnham 2002) for AP2. For SP1,
the Cell Signalling Simple ChIP Kit (Qiagen, Hilden,
Germany) was used, according to the manufacturer’s
recommendations. The resulting DNA was analyzed by
PCR with primers flanking the AP2/SP1 binding
cassette (see Supplementary Table 1, see section on
supplementary data given at the end of this article).
Antibodies used in the ChIP procedure included AP2
(sc-12762X), SP1 (sc-17824X), Pol II (sc-9001X; all
Santa Cruz Biotechnology), and anti-mouse IgG
(included in the ChampionChIP One-Day Kit).

Bisulfite sequencing
MethPrimer software (Li & Dahiya 2002) was used to predict in silico CpG islands in the marmoset CGB
promoter, and primers designed accordingly to amplify
a region assumed to regulate placental CGB expression.
A total of 2 μg DNA were bisulfite converted in a volume
of 50 μl, according to the protocol of Hayatsu et al.
(1970). Converted DNA was desalted and purified using
the DNA Purification Kit (Promega). PCR was
performed using the primer listed in Supplementary
Table 1, see section on supplementary data given at
the end of this article. 10× Buffer, 10 mM dNTPs,
forward primer (20–100 pmol/μl), reverse primer
(20–100 pmol/μl), 0-3 μl Taq Polymerase (Qiagen),
dH2O2, and 2 μg bisulfite DNA were added to a total
volume of 25 μl. Thermocycling program used was
an initial denaturation at 95 °C for 120 s, 95 °C for 30 s,
48–54 °C for 30 s, and 72 °C for 30 s, final extension
72 °C for 10 min respectively, PCR product was checked
via gel electrophoresis, purified, and cloned into the
pGemT-easy vector (Promega) in E. coli XL-1Blue. Ten
resulting clones were sequenced.

CGB promoter fragments were cloned into a CpG-
free backbone luciferase vector (Klug & Rehli 2006)
followed by in vitro methylation by SssI Methylase
(New England Biolabs, Frankfurt, Germany) according to the
manufacturer’s instructions. Briefly, 1 μg vector DNA,
2 μl 10× NEB-Buffer, 2 μl 1× Sadenosylmethionine,
and 1 μl SssI methylase (4 U/μl) were incubated for 1 h
at 37 °C before the reaction was stopped by heating at
65 °C for 20 min and vectors subjected to the
reporter assay.

Statistical analysis
Statistical analysis was performed using GraphPad
Prism (GraphPad Software, La Jolla, CA, USA), paired
t-test, one-way ANOVA with the Bonferroni post-test,
and the Pearson test where appropriate.

Results
CGB mRNA from marmoset placenta contained
a novel exon
We initially determined the transcriptional start site
(TSS) for placental CGB expression in the marmoset via
5’-RACE and sequenced the resulting clones. From
these, we identified a previously unknown TSS of
CGB and a novel exon 1 (Fig. 1A). The conventional exon 1,
present in all known LHB and CGB transcripts from
OWM, is absent with the novel exon 1 directly spliced to
exon 2. This novel exon contains 212±11 bp 5’-UTR
and a 15 bp protein-coding region after the first ATG
codon (Fig. 1A). Thus, both exon 1 sequences, the
novel as well as the conventional, encode the first five
amino acids (aa) of a signal peptide, while exon 2
encodes the remaining 15 aa of the signal peptide and
41 aa of the mature peptide. The in silico translated
peptide sequence of the marmoset CGB new exon 1 was changed from the previously known pituitary CGB protein sequence MEMLQ – which is also conserved in human CGB peptide sequences – to MALVE. This 4 aa substitution alters the polarity and charge of the signal peptide. A negatively charged glutamic acid is exchanged for an uncharged alanine, positively charged methionine for an uncharged leucine, while in position –17 a lipophilic character is maintained and in position –16 a polar glutamine is changed to anionic glutamate. Analysis of the peptide sequence via SignalP showed no altered cleavage site of the signal peptide from the mature peptide.

**Presence of the novel exon 1 in NWM**

The surprising findings of a novel exon 1 in the marmoset raised the question of whether other primate species, especially the NWM, also possess the novel exon 1. To address this, we retrieved CGB gene and
upstream sequences from public databases. In addition, the marmoset CGB gene and its upstream sequence were built in silico from raw data provided from the marmoset genome project. This sequence served for designing primers for cloning of the CGB region from genomic DNA. We were able to clone the CGB genes and their upstream promoter sequences from the common marmoset as well as four other NWM species. The sequences were deposited in public databases (see Supplementary Table 2, see section on supplementary data given at the end of this article, for accession numbers) and used for sequence alignments.

Prerequisites for a functional exon 1 are generally a start codon and a donor splice site. These two conditions for a potential novel exon 1 were only fulfilled in the marmoset and three other NWM (S. oedipus, S. fuscicolis, and C. apella). All three CGB genes of the rhesus macaque, chimpanzee, and human CGB7 showed an ATG codon for a potential novel exon 1 but not the consensus sequence for donor splice sites. The other human and chimpanzee sequences fulfilled neither criterion, suggesting that the presence and use of the novel exon 1 of CGB is limited to the NWM (Fig. 1B). The signal peptide sequences were also aligned with known LHB/CGB signal peptide sequences from OWM (Macaca mulatta, Macaca fascicularis and Pan trichyderes), human, mouse (Mus musculus), and horse (Equus caballus) as outliers (Fig. 1C). The conventional consensus sequence MEM(L/F)Q was found in all primates but not in mouse or horse. Different amino acids were restricted to the third or fourth position in the human, chimpanzee, and olive baboon sequences (Fig. 1B and Supplementary Figure 2, see section on supplementary data given at the end of this article). The horse and mouse showed a single aa difference as well (Fig. 1C).

The cladogram (Supplementary Figure 2, see section on supplementary data given at the end of this article), showing the relationship between the different primates as well as horse and mouse, revealed similarities in the NWM group. The protein consensus sequence for the novel exon 1 appeared to be MALVE and was found to be exclusively present only in the NWM. Only the brown capuchin C. apella showed one different aa, with a lysine residue replacing glutamine at the fifth position.

**Preferential use of novel exon 1 in the placenta**

Having identified a novel exon 1, we next addressed the question of whether the marmoset uses the two different exons in a tissue-specific manner. We examined the distribution of the two CGB transcript forms in pituitary and placenta by semi-quantitative RT-PCR from cDNA samples generated in a previous study from marmoset pituitaries and placentas of pregnant (8–12 weeks of gestation) and non-pregnant female marmosets (Henke et al. 2007). In the pituitaries, a significantly elevated expression of the CGB isoform containing the conventional exon 1 was evident in both pregnant (n = 4) and non-pregnant females (n = 4) (Supplementary Table 3, see section on supplementary data given at the end of this article). As there was no statistically significant difference between these two groups (P = 0.678), they were combined (n = 8) for subsequent comparison with the prevalence of the isoforms in the placenta. In the placenta (n = 6), the novel exon 1 was the predominant transcript isoform. A significant difference of exon 1 usage between pituitary and placenta was detected (P < 0.0001; Fig. 2, Supplementary Tables 3 and 4, see section on supplementary data given at the end of this article).

**Identification of the placental core promoter region for marmoset CGB**

Due to the findings of a new TSS, a novel exon 1, and a tissue-specific expression of exon 1, we concluded that the preferential use of the novel exon 1 in the placenta must be accompanied by a new promoter region, which we wanted to identify. In the following, the positions are given in relation to the ATG start codon of conventional exon 1. In the human, the TSS for CGB3/5/7/8 is located 365 bp upstream of the start codon of the conventional exon 1 and the TSS for CGB1/2 is 175 bp upstream (Henke & Gromoll 2008). However, this region is part of the 5'-UTR in the placental transcript of the orthologous marmoset CGB gene. Promoter regions of different lengths were used in a reporter assay. Luciferase activity was measured after transient transfection into the human choriocarcinoma cell line BeWo. The marmoset CGB promoter constructs were successively truncated, leading to a subsequent loss of TFBS (see Supplementary Figure 1, see section on...
subjected this sequence to TFBS analysis. Promoter activity after removal of this 200 bp and K essential placental CGB decrease in promoter activity. Thus, we identified the further than 1176 nucleotide (nt) upstream led to a of all constructs generated (Fig. 3). A construct located minimal activity in BeWo cells. In contrast, distinct expression in the pituitary (CGB construct containing the core promoter for marmoset CGB specific transcription factor GCMa (Johnson & Jameson 1999) and by AP2 in the squirrel monkey (Vasauskas et al. 2011). The GCMa factor was also predicted, which is absent in the human CGB promoter. We mapped the AP2 and SP1 sites on the promoter constructs. Hence, only two EGR1, two SP1 sites, and both AP2 sites are located within the core promoter sequence (Fig. 3). Promoter sequences with both AP2 and SP1 trans elements displayed higher activity (up to 50-fold), compared with constructs without AP2 or SP1 sites (120 RLU versus 2 RLU; Fig. 3). This correlates with studies showing placental transcriptional regulation by AP2 and SP1 in the human hCGB5 gene (Johnson & Jameson 1999) and by AP2 in the squirrel monkey (Vasauskas et al. 2011). The GCMa factor was excluded from further studies due to the minimal activity of the promoter constructs containing only the GCMa variant (−645/−381 vs −624/−381).

Localization of AP2γ, SP1, and CGB in the placenta

Having excluded several possible candidate factors, we examined whether the two remaining factors AP2 and SP1 are expressed together with CGB. We found AP2γ expression exclusively in the nuclei of supplementary data given at the end of this article, for 5'-ends of truncated promoter constructs). A reporter construct containing the core promoter for marmoset CGB expression in the pituitary (−264/−1) showed minimal activity in BeWo cells. In contrast, distinct activity was detected from the constructs containing regions suspected of involvement in placental CGB regulation. The shortest construct (−624/−381) stretched to the beginning of the novel exon 1 (at position −381) and had a very low promoter activity, while longer constructs (−1167/−381 and −1024/−381) displayed the highest promoter activity of all constructs generated (Fig. 3). A construct located further than 1176 nucleotide (nt) upstream led to a decrease in promoter activity. Thus, we identified the essential placental CGB core promoter sequence (between −837 and −624) due to a significant loss of promoter activity after removal of this 200 bp and subjected this sequence to TFBS analysis.

In silico prediction of TFBS within the placenta-specific CGB core promoter

After identification of the placental core promoter, we wanted to address its regulation via transcription. Within the placental CGB core promoter, several TFBS were predicted in silico by MatInspector, including three trans binding sites for early growth response protein 1 (EGR1; −875/−883, −744/−735, and −645/−639), five binding sites for SP1 (−885/−879, −856/−849, −845/−838, −805/−798, and −655/−649), and two binding sites for AP2 (aliases TFAP2C, ERF1, and hAP2-g) (−659/−650 and −641/−631) (see Supplementary Figure 1, see section on supplementary data given at the end of this article, for position). In addition, within an AT-rich region, a TATA box was predicted, whose position is in accordance with the average distance between a TATA box and a TSS. Finally, a binding site for the chorion-specific transcription factor GCMa (−639/−633) was also predicted, which is absent in the human CGB promoter.

We mapped the AP2 and SP1 sites on the promoter constructs. Hence, only two EGR1, two SP1 sites, and both AP2 sites are located within the core promoter sequence (Fig. 3). Promoter sequences with both AP2 and SP1 trans elements displayed higher activity (up to 50-fold), compared with constructs without AP2 or SP1 sites (120 RLU versus 2 RLU; Fig. 3). This correlates with studies showing placental transcriptional regulation by AP2 and SP1 in the human hCGB5 gene (Johnson & Jameson 1999) and by AP2 in the squirrel monkey (Vasauskas et al. 2011). The GCMa factor was excluded from further studies due to the minimal activity of the promoter constructs containing only the GCMa variant (−645/−381 vs −624/−381).

Figure 3 Identification of the placental CGB core promoter of marmoset. The placental core promoter resides within −837 to −624 bp upstream, displaying significant differences between these two promoter constructs. Empty vector pGL3 basic served as negative control whereas the human CGB5 promoter served as positive control. The construct (−264/−1) represents the CGB pituitary promoter. All activities are relative to pGL3 basic, for which the activity was set to 1. The different symbols indicate binding sites for transcription factors AP2, EGR1, GCMa, and SP1 on the left side. RLU, relative light unit. Given are the means ± s.e.m. of three independent transfections of BeWo cells per experiment. One-way ANOVA; *P < 0.001.

![Image](https://via free access)
syncytiotrophoblasts and cytotrophoblasts of both marmoset and human placenta (Fig. 4A, a–c). SP1 expression in the marmoset placenta was detected in varying levels in the nuclei of nearly all syncytiotrophoblasts, most cytotrophoblasts, but also in endothelial and stromal cells. In the human placenta, SP1 expression was similarly detectable in most cell types but to a smaller extent than in the marmoset, particularly in syncytiotrophoblasts (Fig. 4A, d–f). CGB is expressed in syncytiotrophoblasts in the marmoset placenta. However, the strength of the signal is weak compared with human tissue (Fig. 4A, g–i).

Synergistic effects of AP2 and SP1 on promoter activity

Since it was previously demonstrated for human CGB that AP2 binding stimulates CGB expression, and a

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Figure 4 Immunohistochemistry, mutation analyses, and chromatin immunoprecipitation (ChIP) indicating importance of AP2 and SP1 for promoter activation. (A) Immunohistochemical detection of AP2γ, SP1 and CGB in syncytiotrophoblasts of the marmoset placenta. (a–c) Staining for AP2γ of marmoset placenta showed exclusive AP2γ expression in syncytiotrophoblasts and cytotrophoblasts (small inset: negative control). Human term placenta showed a staining pattern identical to the marmoset. (d–f) Identical staining was also seen for marmoset and human SP1, with SP1 expression mainly in trophoblastic cells but also in endothelial cells and stromal cells. Human placenta has a similar SP1 pattern. (g–i) Marmoset placenta showing positive trophoblasts after staining for CGB. As control human placenta has a similar SP1 pattern. (A) Immunohistochemical detection of AP2γ, SP1 and CGB in syncytiotrophoblasts of the marmoset placenta. (a–c) Staining for AP2γ of marmoset placenta showed exclusive AP2γ expression in syncytiotrophoblasts and cytotrophoblasts (small inset: negative control). Human term placenta showed a staining pattern identical to the marmoset. (d–f) Identical staining was also seen for marmoset and human SP1, with SP1 expression mainly in trophoblastic cells but also in endothelial cells and stromal cells. Human placenta has a similar SP1 pattern. (g–i) Marmoset placenta showing positive trophoblasts after staining for CGB. As control human placenta has a similar SP1 pattern. (A) Immunohistochemical detection of AP2γ, SP1 and CGB in syncytiotrophoblasts of the marmoset placenta. (a–c) Staining for AP2γ of marmoset placenta showed exclusive AP2γ expression in syncytiotrophoblasts and cytotrophoblasts (small inset: negative control). Human term placenta showed a staining pattern identical to the marmoset. (d–f) Identical staining was also seen for marmoset and human SP1, with SP1 expression mainly in trophoblastic cells but also in endothelial cells and stromal cells. Human placenta has a similar SP1 pattern. (g–i) Marmoset placenta showing positive trophoblasts after staining for CGB. As control human placenta has a similar SP1 pattern.
mutation of AP2 binding sites that eliminates AP2 binding leads to decreased CGB activity (LiCalsi et al. 2000). We mutated the two predicted AP2 binding sites (−659/−650 and −641/−631) within the core promoter sequence, according to LiCalsi et al. (2000). The constructs bearing both AP2 sites (−837 to −381 bp) displayed higher activity, up to fivefold in the reporter assay, than constructs without AP2 sites (120 RLU versus 25 RLU; Fig. 3). Mutating each AP2 site alone decreased the activity by 50% (60 RLU; M2) or 33% (80 RLU; M1) compared with the wild-type construct (WT, construct −837/−381) in transfected BeWo cells (Fig. 4B). LiCalsi et al. (2000) hypothesized a possible interaction of SP1 with AP2 activation. Mutations of the two SP1 core promoter binding sites (−805/−798 and −655/−649) according to LiCalsi et al. (2000) showed similar effects, which SP1 M1 decreased activity to 25% (35 RLU), whereas SP1 M2 mutation showed 20% (23 RLU) remaining activity. The SP1 M1 + 2 double mutant showed 15% activity (20 RLU), which was similar to the activity of the construct bearing no AP2 and SP1 sites (−645/−381, Fig. 4B). Additional mutations of AP2 and SP1 sites (AP2 M1 + 2 and SP1 M1 or AP2 M1 + 2 and SP1 M2) as well as the mutation of all four binding sites further decreased promoter activity to 15% (15–20 RLU). Mutation of the distal EGR1 binding site, however, showed no effect on placental promoter activity (data not shown). In COS7 cells, these effects were absent, indicating a cell-specific regulatory mechanism only present in the placenta and displayed by the trophoblastic cell line BeWo (data not shown). Co-transfection of the placental CGB wild-type core promoter construct with either AP2 or SP1 overexpression constructs alone displayed a moderate stimulative effect or no significant upregulation of the reporter respectively. However, simultaneous addition of both factors even at lower concentrations resulted in an increase yet a decrease at high concentrations. Transfecting the promoter constructs that were mutated either at the AP2 or at the SP1 binding site, in the presence of a vehicle only, led to a slight non-significant decrease in activity (Fig. 4C). Interestingly, transfecting cells with the wild-type promoter construct and 0.25 µg AP2 with increasing levels of SP1 showed an inhibitory effect after activation with AP2 alone.

In order to confirm AP2 and SP1 binding to the CGB promoter, we performed ChIP analyses. Enrichment of AP2 and SP1 at the promoter could be demonstrated by PCR (Fig. 4D). Mutation of the respective binding sequences resulted in signal loss for AP2 and a signal reduction for SP1. For SP1, these conclusions were drawn from the SP1 band intensities being above or, in case of the mutated form, below the surprisingly high IgG signal.
We first determined the CG base content within the placental promoter sequence, as high CG content is indicative of possible regions for methylation. The high CG bases number in the placental CGB promoter region (75% compared with 60% in the pituitary and gene) points to the potential of CGB regulation via epigenetic mechanisms. MethPrimer software (Li & Dahiya 2002) was used to predict four CpG islands in silico in the marmoset CGB. Two are located in the placental promoter (−1434/−1307 and −1208/−661), one in the novel exon 1 (−512/−411), and one in exon 3 (+773/+955), indicated in Fig. 5A. As the second CpG-island within the placental promoter is the longest, spanning nearly the complete promoter, it was chosen for further analysis by bisulfite sequencing. Interestingly, no CpG islands were identified in the pituitary promoter (Fig. 5A).

A genomic DNA fragment containing the placental promoter region (−964/−381) was isolated, bisulfite treated, and sequenced (n=3 for placenta, n=4 for blood and pituitary). The analysis showed that the mean CGB methylation level is drastically lowered in the placenta compared with the other tissues (Fig. 5B). In the placenta, the mean methylation level in the promoter (−961 to −624) throughout the analyzed sequence was 10.8±3.7%, significantly lower than pituitary tissue (27.5±6.1%) and blood leukocytes (45±4.5%). Interestingly, the placental core promoter (−837 to −624) showed a significant hypermethylation, which was much stricter (28.1±0.2% vs 65.4±9.8% vs 62.5±5.4%). However, mean methylation in the placental CpG island increased toward the novel exon 1 in placental tissue.

We next examined whether the methylation does indeed downregulate CGB transcription. The CGB core promoter sequences were cloned into the methylation insensitive vector pCpG (Klug & Rehli 2006) and in vitro DNA methylation was performed. Promoter activity after in vitro methylation was dramatically decreased compared with the activity of non-methylated plasmids (Fig. 5C). Compared to the results of unmethylated plasmid constructs, activity declined significantly to 5–10% in all methylated plasmids. As shown in Fig. 5C, CMV promoter activity was not affected by in vitro methylation, proving that the vector backbone was insensible to the in vitro methylation procedure.

Discussion

In anthropoid primates, including humans, CGB expression has been limited to the trophoblast and placental syncytiotrophoblast, with minimal amounts in the pituitary (Dinnofer et al. 1996). For the common marmoset, which serves as a preferred primate model in biomedicine in general and in reproductive research specifically (Moore et al. 1985, Hearn 1986, Saunders et al. 1987, Smith et al. 1987, Summers et al. 1987, Hearn et al. 1988), we previously found that CGB is strongly expressed in the marmoset pituitary instead of LH (Muller et al. 2004b). This finding was confirmed when only CGB but not LHB gene expression was found in the NWM Ma’s night monkey (Aotus nancymaeae) and the brown squirrel monkey (Saimiri boliviensis; Scammell et al. 2008). An investigation of CGB gene copy numbers in different primate taxa revealed that CGB is a single-copy gene in NWM (Maston & Ruvolo 2002), which raises the question ‘how is the marmoset able to differentially regulate CGB expression in the pituitary and placenta?’ We have previously shown that the regulation of CGB expression is similar to that of human LHB in the marmoset pituitary (Henke et al. 2007). Both genes have a similar short 5′-UTR of the mRNA, are GNRH responsive, and have identical binding sites for the transcription factor pituitary-induced factor (PITX1), stimulating factor (SF1), and early growth receptor (EGRI) in the pituitary-specific core promoter region (Henke et al. 2007). In the current study, we investigated placental CGB regulation in the marmoset and identified a new exon 1, termed ‘novel exon 1 (nExon 1)’, to distinguish it from the conventional exon 1, which is present only in OWM CGB genes. It has its own TSS, located more than 200 bp upstream of the human CGB TSS, with the region involved in transcriptional regulation in orthologous human CGB5 being now part of the marmoset’s 5′-UTR of placental CGB. Thus, we discovered tissue-specific CGB isoforms between the marmoset monkey pituitary and the placenta. The detection of tissue-specific transcripts is not exclusive to the marmoset. Several genes share the phenomenon of multiple variable first exons, e.g. the novel spermatogenesis regulator in Drosophila (Ding et al. 2010). Typically, tissue-specific expression is predominantly found in 70–80% of mammalian regulatory genes (Lareau et al. 2004, Ben-Dov et al. 2008).

Very recently, the CGB1/2 gene expression was confirmed in the human testis in alternative splice forms, one of which could potentially give rise to a normal CGB protein. So far, no evidence exists for the functionality of CGB1 or CGB2 (Parrott et al. 2011). In contrast, the marmoset CGB is actually the only known glycoprotein hormone gene that encodes two different peptides due to alternative promoter/exon 1 usage. This situation could potentially affect other processes, e.g. mRNA nuclear export, cytoplasmic localization, translational efficiency, and stability and regulatory motifs (Hughes 2006).

We also investigated the prevalence of the novel exon 1 within the primate taxon. Interestingly, the putative novel exon 1 was found in all NWM analyzed but not
other primate species. Despite the high-sequence homology among LHB/CGB genes (85–99%), there are signs of functional differentiation among the gene copies (Hallast et al. 2008). It seems that in the NWM, the exon gains function during evolution, whereas in the OWM and humans no such function evolves. The differential use of alternative first exons often indicates a two-promoter system. In a previous study, we demonstrated that marmoset CGB expression in the pituitary is GNRH inducible and driven by SF1, EGR1, and PITX1 (Henke et al. 2007). Conversely, the placental core promoter seemed to be regulated differently. In silico predictions underlined the importance of two transcription factors, SP1 and AP2, in the activation of placental CGB expression. We showed the presence of SP1 and AP2γ in the nuclei of syncytiotrophoblasts in marmoset placenta, which also express CGB. Previous investigations showed that CGB is solely expressed in syncytiotrophoblasts in placentas (Gauster & Huppertz 2008), leading us to conclude that AP2γ and SP1 protein is present in CGB-expressing cells. This premise was confirmed by the detection of CGB in syncytiotrophoblasts.

In addition, our promoter mutagenesis and ChIP experiments confirmed AP2 and SP1 as transcription factors binding to the CGB promoter. Compared to IgG and polymerase, which display similar signal strength, the signal of the SP1 fraction is enhanced in the wild type, suggesting an enrichment of SP1. The weaker signal in the SP1 fraction with mutated DNA sequence is due to a loss of SP1 binding to the SP1 binding site. In order to successfully perform the SP1 ChIP, the method has been slightly modified. This could be the reason why we observed a higher background in the IgG fraction. Nevertheless, the loss of SP1 binding could be clearly identified by the signal strength above background in the wild type and the weaker signal in the mutated fraction.

The presence of AP2 and SP1 binding sites within the placental core promoter correlates with previous human CGB studies that show the existence of two clusters of TFBS in the human CGB5 promoter (Bo & Boime 1992, Johnson et al. 1997). Within these clusters, AP2 and SP1 were the most prominent factors (Steger et al. 1993, 1994, LiCalsi et al. 2000, Thackray et al. 2010). Johnson et al. (1997) also showed that AP2 and SP1 cooperate to induce gene activation, a finding confirmed in our overexpression experiments. It appears that the model postulated by Johnson et al. (1997) and LiCalsi et al. (2000) for the activation of human CGB expression in general also holds true for the marmoset, albeit with some differences. First is the enhancer function of the SP1 site (−805/−796), which had been postulated (Johnson et al. 1997). Additionally, our results indicate that the proximal SP1 site functions as a linker between the AP2 sites, forming a cassette, akin to that described for human CGB (Johnson et al. 1997): hereby, SP1 binding is the prerequisite for AP2 binding, confirmed by Vasauskas et al. (2011), who independently identified a functional AP2 binding site in the CGB promoter of the NWM squirrel monkey (Vasauskas et al. 2011). Therefore, we propose a concurring model for AP2 and SP1 regulation of placental CGB (Fig. 6A) in which one SP1 site acts as a linker between AP2 and the other as an enhancer of the AP2 and SP1 cassette. This model combines our findings with those of Johnson et al. (1997) and LiCalsi et al. (2000), for control of the human CGB promoter by SP1, SP3, and AP2 (Fig. 6B). In this case, two clusters exist. At the first site, SP1 and AP2 binding sites overlap, and binding is mutually exclusive. The second site shows similarity to our findings in the marmoset. At this site, comparative SP1 and AP2 binding exists. The

Figure 6 Current model of placental CGB activation by SP1 and AP2. (A) Model for CGB regulation in the marmoset placenta and pituitary. Placental expression is controlled by SP1, in combination with AP2 binding to specific regions within the minimal CGB core promoter (binding cassette, white box). The distal SP1 acts as an enhancer on the proximal SP1/AP2 binding cassette. SP1 serves as a linker between the two AP2 sites (black circles). The loss of one of these components leads to a decrease in CG expression. The promoter sequence in the placental tissue is activated by hypomethylation (small open circles). This leads to expression of the placental CGB variant. In the pituitary, the binding of AP2 and SP1 is disturbed, because of methylated sequences (small filled black circles) within the binding cassette. In the pituitary, the activation is triggered by EGR1 and SF1 (black hexagonal boxes). Therefore, stimulation of placental CGB expression is not that strong. The novel exon 1 is represented by the black box between the two TSS (angled black arrows) sites and the conventional exon is given by the gray box. (B) Model for human placental CGB regulation. In the human, placental CGB regulation is controlled by a similar binding cassette, an SP1 site is flanked by two AP2 sites. The AP2 binding is stimulated by cAMP and SP1 itself interacts with SP3. Adapted from Johnson et al. (1997).
different effects of AP2 and SP1 (activation versus inhibition) seem to be present in the marmoset as well, as indicated by our overexpression experiments. Adding low levels of equimolar concentrations of AP2 and SP1 led to an increase in promoter activity but this decreased when high concentrations were added. This might be due to the action of SP1 that at high levels may reverse the activating effect of AP2, but more likely the high concentrations of these factors are far beyond their normal physiological range. This proposition is in agreement with the finding that AP2 alone increases promoter activity while SP1 alone neither stimulates nor inhibits promoter activity. Our findings that the addition of increasing amounts of SP1 to constant AP2 levels, however, do display an inhibitory effect, add evidence to the concept of a balanced interaction of AP2 and SP1. Hence, more AP2 leads to activation, whereas more SP1 leads to inhibition or repression (Nowak-Markwitz et al. 2004a,b, Rull et al. 2008a). However, further studies addressing the influence of methylation on CGB promoter regulation are necessary.

In summary, this study gives new insights into the placental regulation of marmoset CGB via a novel two-promoter, one-gene system. Thus, our findings represent a hitherto unknown scenario of evolutionary plasticity in the CG hormonal system in NWM, which involves regulation by transcription factors as well as epigenetic mechanisms to address tissue-specific expression.

**Supplementary data**

This is linked to the online version of the paper at http://dx.doi.org/10.1530/JME-11-0026.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

**Funding**

This study was supported by the Deutsche Forschungsgemeinschaft (DFG, grant nos GR15746/-8/1), the funding of Innovative Medizinische Forschung (IMF grant SI520601) of the University of Münster, and the Alexander-von-Humboldt Foundation.

**Acknowledgements**

The authors thank Prof. Pamela Mellon (UCSD, La Jolla, USA) for donating the AP2 expression plasmid, Reinhild Sandhowe-Klaver-kamp for technical assistance in cell culture, Elisabeth Lahrmann for assistance in molecular biology, Dr Simon Riley for advice, and Dr James L. Hutchinson and Dr Con Mallidis for language editing and comments.

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Received in final form 22 July 2011

Accepted 5 August 2011

Made available online as an Accepted Preprint 5 August 2011