Published in final edited form as:

Gen Comp Endocrinol. 2011 February 1; 170(3): 514–521. doi:10.1016/j.ygcen.2010.11.023.

Tissue-specific expression of squirrel monkey chorionic gonadotropin

Audrey A. Vasauskas^{a,b}, Tina R. Hubler^c, Lori Boston^c, and Jonathan G. Scammell^{a,b,*}

^a Department of Comparative Medicine, University of South Alabama College of Medicine, Mobile, Alabama 36688

^b Department of Pharmacology, University of South Alabama College of Medicine, Mobile, Alabama 36688

^c Department of Biology, University of North Alabama, Florence, Alabama 35632

Abstract

Pituitary gonadotropins LH and FSH play central roles in reproductive function. In Old World primates, LH stimulates ovulation in females and testosterone production in males. Recent studies have found that squirrel monkeys and other New World primates lack expression of LH in the pituitary. Instead, chorionic gonadotropin (CG), which is normally only expressed in the placenta of Old World primates, is the active luteotropic pituitary hormone in these animals. The goal of this study was to investigate the tissue-specific regulation of squirrel monkey CG. We isolated the squirrel monkey CG β gene and promoter from genomic DNA from squirrel monkey B-lymphoblasts and compared the promoter sequence to that of the common marmoset, another New World primate, and human CG β and LH β . Using reporter gene assays, we found that a squirrel monkey CG β promoter fragment (–1898/+9) is active in both mouse pituitary L β T2 and human placenta JEG3 cells, but not in rat adrenal PC12 cells. Furthermore, within this construct separate *cis*-elements are responsible for pituitary- and placenta-specific expression. Pituitary-specific expression is governed by Egr-1 binding sites in the proximal 250 bp of the promoter, whereas placenta-specific expression is controlled by AP-2 sites further upstream. Thus, selective expression of the squirrel monkey CG β promoter in pituitary and placental cells is governed by distinct *cis*-elements that exhibit homology with human LH β and marmoset CG β promoters, respectively.

Keywords

New World primate; luteinizing hormone; marmoset; placenta

1 Introduction

LH and chorionic gonadotropin (CG) are glycopeptide hormones made up of an identical α -subunit and different β -subunits. The β -subunits of these heterodimeric glycopeptides confer their biological specificities [20,23]. Although LH and CG activate the same LH/CG

*Corresponding author: Jonathan G. Scammell, Ph.D., Department of Comparative Medicine, MSB 992, University of South Alabama, Mobile, Alabama 36688. Telephone: +1 251-460-6239, jscammel@jaguar1.usouthal.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

receptor (LHR), they are differentially regulated in pituitary gland and placenta of humans and other Old World primates, consistent with their respective roles during the reproductive cycle and during pregnancy. Reproductive function is dependent on the proper expression and release of LH from gonadotrophs of the anterior pituitary gland. The upregulation and secretion of LH is stimulated by the pulsatile release of GnRH from the hypothalamus. In females, LH stimulates granulosa cell differentiation, cumulus expansion, and ovulation, as a mid-cycle surge of LH causes rupture of the mature follicle [17]. In males, LH activates testosterone biosynthesis in Leydig cells of the testes [11]. On the other hand, CG is expressed by syncytiotrophoblasts of the placenta of humans and other Old World primates during the first ten weeks of pregnancy and supports implantation and placental development by maintaining progesterone production by the corpus luteum [8].

Recent work from this laboratory and others has shown that New World primates, such as marmoset and squirrel monkey, do not express LH in the pituitary gland, but rather CG is the relevant luteotropic hormone [19,22]. Müller et al. showed that the LH β gene is present in marmoset. However, LH β mRNA is absent from both pituitary and placental tissue, whereas CG β mRNA is expressed in both tissues [19]. It is likely that pituitary expression of CG in New World primates arose secondary to expression of LHR that lack exon 10. The absence of amino acids encoded by exon 10 in LHR impairs LH, but not CG activity [5,18,30]. Therefore, unlike Old World primates, New World primates express CG in both pituitary and placenta, a finding that raises several questions. For example, what is the molecular organization of the CG gene in New World primates? Second, how is CG expressed in different tissues, specifically the pituitary gland and placenta? Answers to these questions are emerging.

Work in the common marmoset suggests that the CG β gene is a single copy gene in New World primates [19]. Furthermore, differently sized mRNA transcripts of CG β were detected in pituitary and placenta of the marmoset. These transcripts most likely arise from the tissue-specific utilization of different promoters in the CG β gene. More recently, Henke and colleagues identified sites in the putative pituitary-specific promoter of the marmoset CG β gene to which transcription factors steroidogenic factor 1 (SF-1) and early growth response protein 1 (Egr-1) bind [10]. These factors are important for the GnRH-responsiveness of the rat and bovine LH β genes [3,6,27]. Here, in studying regulation of CG β expression in squirrel monkey, another species of New World primate, we confirm and expand these previous studies in marmoset. We identified independent promoter regions in the squirrel monkey CG β promoter that mediate pituitary- and placenta-specific expression of the gene. Comparisons are made to these regulatory regions in the marmoset and human CG β promoters and the human LH β promoter. Within these regions, we showed that specific *cis*-elements contribute to the tissue-specific expression of squirrel monkey CG β .

2 Materials and Methods

2.1 Cell Culture

L β T2 mouse pituitary gonadotroph cells [1] were kindly provided by Dr. Pamela Mellon (University of California at San Diego, La Jolla, CA). JEG-3 human choriocarcinoma cells were obtained from American Type Culture Collection (Manassas, VA). PC12 rat pheochromocytoma cells were kindly provided by Dr. Edward Hawrot (Brown University, Providence, RI). Monolayers of L β T2, JEG-3, and PC12 were grown in DMEM (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS, Hyclone Logan, UT), 50 U/mL penicillin G, and 0.05 mg/mL streptomycin. Epstein-Barr virus-transformed squirrel monkey lymphoblast cells (SML) [21] were grown in RPMI 1640, 10% FBS, 4 mM L-glutamine, and antibiotics as above. Cells were grown at 37°C in a humidified atmosphere of 95% air-5% CO₂.

2.2 Isolation of the squirrel monkey CG β gene and promoter

Total genomic DNA was isolated from SML using the QIAGEN DNeasy Blood and Tissue Kit or the Flexigene Kit (QIAGEN, Valencia, CA). A DNA fragment containing the squirrel monkey CG β gene was obtained by PCR using the Taq PCR Core Kit (QIAGEN) and upstream primer 5'-GAC GCA CCA AGG ATG GAG-3' and downstream primer 5'-GCG GAT TGA GAA GCC TTT A-3' corresponding to the human CG β cDNA sequences 354–371 and 879–861 (NM_000737). The squirrel monkey CG β promoter was obtained with the GenomeWalker Universal and Advantage-GC 2 PCR Kits (Clontech, Mountain View, CA), using an outer adaptor primer included in the kit and a reverse primer, 5'-TTG GAT CCC CAT GGC CCA CCT GTG CTC A-3', corresponding to sequences 403–376 in the squirrel monkey CG β gene. The squirrel monkey CG β promoter and gene sequences were submitted to GenBank with accession number GU117708. Sequences were confirmed in several, independently generated DNA fragments.

2.3 Construction of luciferase plasmids and mutagenesis

A DNA fragment containing the squirrel monkey CG β promoter (–1898 to +9) was amplified using a forward primer containing a linker sequence for *Mlu*I, TGT ACG CGT TCT AGC GAT TCT CTT GCC TCA ACC TC, corresponding to position –1898 to –1872 and a reverse primer containing a *Bgl*II site, CAC AGA TCT CCT TGG TGC CTC CCC TGC CTC GT, corresponding to position –16 to +9, (*Mlu*I and *Bgl*II sites underlined). After digestion with *Mlu*I and *Bgl*II, the amplified DNA fragment was subcloned into the *Mlu*I- and *Bgl*II-cut vector pGL3-Basic (Promega Corp, Madison, WI).

To study *cis*-elements responsible for cell type-specific activity of the squirrel monkey CG β promoter, deletion constructs of the promoter were made. The 5' deletion construct (–253 to +9) was generated by amplifying the region of interest using a forward primer containing a *Mlu*I site (underlined); TGT ACG CGT GGC GAG GCC TTT CTG TAC CCT ACT T, corresponding to position –253 to –229 and the reverse primer containing a *Bgl*II site described above. The product was cut with *Mlu*I and *Bgl*II and inserted into pGL3-Basic. To generate a construct in which 248 bp were deleted from the 3' end of the squirrel monkey CG β promoter, a *Hind*III restriction site was inserted at position –249 using site-directed mutagenesis. The mutagenesis was performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), using forward and reverse primers as follows: (*Hind*III site underlined); 5'-CAG GAA GAG ACA GGG CGA AGC TTT TCT GTA CCC TAC TTC-3' and 5'-GAA GTA GGG TAC AGA AAA GCT TCG CCC TGT CTC TTC CTG-3', corresponding to position –227 to –266. The promoter fragment was digested with *Mlu*I and *Hind*III and subcloned into pGL3-Basic.

Mutations were made in the proximal and/or distal Egr-1, SF-1, or AP-2 binding sites of the squirrel monkey CG β promoter (–1898 to +9) using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) (Table 1). PCR was performed according to the manufacturer's instructions except that DMSO (5%) was added to the reactions. PCR products were digested to remove parental DNA according to the kit protocol, grown up in One Shot® Top 10 competent cells (Invitrogen), isolated, and sequenced.

2.4 Luciferase reporter gene assays

Cells were plated at a density of 3×10^5 cells/well in 6-well dishes. The next day, cells were transfected with 2 μ g DNA/well of either promoterless luciferase plasmid (pGL3-Basic or pLuc-Link) or luciferase plasmid containing various promoter fragments using Superfect Transfection Reagent (QIAGEN) in complete medium for 3 h. Cells were washed, and fresh medium was added. The following day, after treatment with 100 nM GnRH (Sigma, St. Louis, MO) for 6 h, cells were harvested in 300 μ L ice-cold Monolight lysis buffer (BD

Biosciences, San Jose, CA), and luciferase activity measured in a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). Results were normalized to the promoterless luciferase plasmid control. Experiments were performed at least three times.

2.5 Statistical analysis

Statistical analysis was performed by using GraphPad Prism version 4.0 software (San Diego, CA). Comparisons between multiple groups were performed by using a one-way ANOVA and the Newman-Keuls posthoc test. Values were considered significantly different when the *P* value was less than 0.05.

3 Results

3.1 Isolation and analysis of the squirrel monkey CG β promoter

A DNA fragment containing the squirrel monkey CG β gene was amplified by PCR from genomic DNA and found to contain three exons and two introns with a similar organization to the human CG β 5 gene. Using a genome walking strategy, additional 5' upstream DNA was isolated and approximately 1.9 kb were sequenced. This sequence was compared to the proximal marmoset CG β promoter (*Callithrix jacchus* trace archive ti 1063492599) and rhesus macaque CG β promoter (*Macaca mulatta* trace archive ti 542139165) and found to be highly similar (88% identity and 76% identity, respectively) (Figure 1). Sequences shown to bind the distal SF-1 and distal Egr-1 *cis*-elements in the marmoset promoter are conserved in the squirrel monkey CG β promoter (Figure 2). Additionally, the squirrel monkey CG β promoter was compared to the human CG β , human LH β , and rhesus macaque LH β promoters (*Macaca mulatta* trace archive ti 349792889) and was found to contain regions of similarity with these promoters (79% and 86% identity with the human CG β and LH β promoters, respectively, and 74% identity with the rhesus macaque promoter) (Figures 1 and 2, respectively). *Cis*-elements known to be important for expression of human LH β and CG β genes are largely conserved in the squirrel monkey CG β promoter. Taken together, these data suggest that the squirrel monkey CG β promoter contains likely sequence important for both pituitary- and placenta-specific expression.

Thus, based on the sequence comparison of squirrel monkey CG β promoter with marmoset CG β , human CG β and human LH β promoters, we propose a model of the squirrel monkey CG β gene and promoter wherein the expression of the gene is governed by two promoters: expression of the squirrel monkey CG β gene in the pituitary is driven by a promoter that is found immediately upstream of exon 1, and contains a TATA-box, and *cis*-elements important for pituitary-specific expression; on the other hand, expression of the gene in the placenta is governed by an upstream TATA-less promoter that contains *cis*-elements similar to regions in the human CG β promoter shown to be important for placenta-specific expression (Figure 3A).

3.2 Pituitary-specific regulation of the squirrel monkey CG β promoter

To determine whether the squirrel monkey CG β promoter confers pituitary- and placenta-specific expression, we generated a construct in which 1907 bp (−1898/+9) of 5'-flanking sequence of the squirrel monkey CG β gene was placed upstream of the luciferase reporter gene in pGL3-Basic (Figure 3A). This construct was transiently transfected into either mouse pituitary gonadotroph L β T2 cells, human placental JEG-3 cells, or rat pheochromocytoma PC12 cells that do not express CG β . For each experiment, a separate set of cells was transfected with empty pGL3-Basic vector to account for background and experiment-to-experiment variability. The squirrel monkey CG β promoter fragment exhibited cell-type specific expression in both pituitary and placental cells (Figure 3B). Less

than three-fold activity over the promoterless control was observed when the promoter fragment was transfected into PC12 cells. These data suggest that the $-1898/+9$ promoter fragment contains sequences that are sufficient for pituitary- and placenta-specific expression.

The LH β gene promoter exhibits GnRH-responsiveness in pituitary cells [3,27]. Additionally, Henke et al. showed that the marmoset CG β promoter is also GnRH-responsive [10]. To examine whether the squirrel monkey CG β promoter behaves similarly, we tested the GnRH-responsiveness of the squirrel monkey CG β promoter in L β T2 gonadotroph cells. The $-1898/+9$ construct, and two deletion constructs, in which 5' upstream ($-253/+9$) or 3' downstream sequences ($-1898/-249$) were deleted (Figure 3A), were tested for GnRH-responsiveness. Treatment with 100 nM GnRH resulted in stimulation of reporter gene activity in cells transfected with both the $-1898/+9$ and $-253/+9$ constructs, whereas the $-1898/-249$ construct showed no GnRH-responsiveness (Figure 4A). These results suggest that the squirrel monkey CG β promoter is regulated by GnRH in pituitary cells and expression is conferred by sequences within the most proximal ~250 bp of the promoter.

Within this ~250 bp region of the squirrel monkey CG β promoter are sequences homologous to known *cis*-elements in the LH β promoter to which Egr-1, SF-1 and Pitx-1 bind (Figure 2). These factors are conserved among several species, and have been shown in the rat, mouse, and bovine LH β promoters to act in concert to regulate basal and GnRH-induced expression in pituitary gonadotropes [3,6,27,29]. To determine whether these sites are similarly involved in pituitary-specific expression and GnRH-responsiveness of the squirrel monkey CG β promoter, we employed site-directed mutagenesis to mutate both the proximal and distal Egr-1 or SF-1 sites (Figure 4B). L β T2 pituitary gonadotroph cells were transiently transfected with the wild type (WT) promoter fragment ($-1898/+9$) or constructs containing mutations of Egr-1 and SF-1 binding sites (Table 1) that are known to affect GnRH-responsiveness of the LH β promoter [3]. A mutation within the distal Egr-1 binding site (dEgr1m) resulted in a 60% reduction in GnRH-stimulated promoter activity, whereas a mutation within the proximal Egr-1 binding site (pEgr1m) reduced the GnRH response by 90% (Figure 4C). The construct containing mutations in both the proximal and distal Egr-1 (pdEgr1m) sites completely abrogated the effect of GnRH on the promoter. Thus, as reported for the LH β promoter in other species, Egr-1 binding sites play a critical role in the regulation by GnRH of the pituitary-specific squirrel monkey CG β promoter. On the other hand, mutation of either or both of the SF-1 binding sites within the squirrel monkey CG β promoter (Figure 4D) had no effect on GnRH-responsiveness (Figure 4E).

3.3 Placenta-specific expression of the squirrel monkey CG β promoter

Having identified regions in the squirrel monkey CG β promoter that mediate pituitary-specific regulation, placenta-specific expression of the squirrel monkey CG β gene was studied. Reporter constructs containing the squirrel monkey CG β promoter were transiently transfected into JEG-3 cells, a human choriocarcinoma cell line used to study placenta-specific expression of reporter genes [4,16]. The activity of the reporter construct containing $-1898/+9$ of the squirrel monkey CG β promoter was dramatically higher than that of the promoterless vector in JEG-3 (Figure 5A). When upstream elements were deleted ($-253/+9$), promoter activity was eliminated. On the other hand, the construct containing -1898 to -249 of the promoter had similar activity to the $-1898/+9$ construct. These results suggest placenta-specific expression is governed by *cis*-elements between -1898 and ~-250 bp of the squirrel monkey CG β promoter.

Previous studies have shown that three AP-2 binding sites within the human CG β promoter are important for placenta-specific expression of the gene [12,16]. These *cis*-elements are

well conserved within the squirrel monkey CG β promoter (Figure 1). We asked whether these binding sites play a role in the expression of the squirrel monkey CG β promoter in placental cells. Site-directed mutagenesis was employed to mutate the proximal, middle, and distal AP-2 sites within the $-1898/+9$ construct (Figure 5B), and these reporter constructs were transiently transfected into JEG-3 placental cells. These mutations (Table 1) have been shown to affect the expression of the human CG β promoter [12,13]. Mutations in each of the AP-2 sites reduced the activity of the promoter by at least two-fold (Figure 5C). Similar results were observed in the JAR choriocarcinoma cell line, another cell line used to study placenta-specific gene expression (data not shown). These results suggest that like the human CG β promoter, AP-2 sites play a role in placenta-specific expression of the squirrel monkey CG β promoter.

4 Discussion

Recently, it has been found that New World primates lack expression of LH, and instead express CG as the luteotropic hormone secreted from the pituitary gland. Therefore, New World primates express CG in both the pituitary gland and the placenta. In this study, we isolated the squirrel monkey CG β gene and demonstrated that its promoter is active in both pituitary and placental cells. Furthermore, distinct regions of the promoter appear to be important for this cell-type specific activity. Egr-1 sites play a critical role in pituitary-specific expression, whereas AP-2 sites are important for placenta-specific expression. This work confirms and expands work on the marmoset promoter by Gromoll and colleagues, who introduced the notion that New World primates express CG, and not LH, in the pituitary gland using *cis*-elements with homology to LH β [10,19].

Like the marmoset CG β promoter, the squirrel monkey CG β promoter shows high sequence identity to both the human CG β and human LH β promoters. The squirrel monkey CG β promoter contains sequences known to confer pituitary-specific LH β expression and GnRH-responsiveness. These include proximal and distal Egr-1 sites, which are paired with adjacent SF-1 sites [3,6,7]. A Pitx-1 site resides between the two SF-1/Egr-1 pairs [27]. Egr-1 appears to be a critical effector of GnRH stimulation, as Egr-1 $-/-$ mice exhibit female infertility and male fertility dysfunction caused by lack of LH β transcriptional activity in pituitary gonadotrophs [15,26]. Egr-1 also appears to be a critical factor in the regulation of the squirrel monkey CG β promoter, as mutations of the two Egr-1 sites eliminated GnRH-responsiveness. Henke *et al.* showed that basal and GnRH-responsive elements are located in the proximal 264 bp of the marmoset CG β promoter, and that Egr-1 and SF-1 bind to a region of the marmoset CG β promoter ($-130/-92$ in Figure 1) containing the distal Egr-1 and SF-1 sequences [10]. Additionally, specific point mutations in the distal Egr-1 binding site, which convert the marmoset CG β promoter to corresponding sequences in the human CG β promoter, reduced GnRH-responsiveness approximately two-fold [10]. Here, we conclude that both Egr-1 binding sequences in the squirrel monkey CG β promoter are sufficient for its GnRH-responsiveness in pituitary gonadotrophs.

On the other hand, we did not observe significant changes in GnRH-responsiveness of the CG β promoter when SF-1 elements were mutated. While the synergy of Egr-1 with SF-1 is necessary for GnRH-responsiveness of the LH β promoter [3], SF-1 may play a lesser role in the regulation of the squirrel monkey CG β promoter. Although SF-1 appears to bind to the distal SF-1 binding site of the marmoset CG β promoter, it is not known whether SF-1 contributes to GnRH-responsiveness of the marmoset CG β promoter [10]. SF-1 does not appear to be necessary for the GnRH-responsiveness of the squirrel monkey CG β promoter. Collectively, these data add to the larger evolutionary picture of pituitary CG β expression in New World primates.

Less is understood regarding the complex transcriptional regulation of the CG β promoter in placenta. There are two major DNA footprint regions of the human CG β 5 promoter which bind several transcription factors. The most important *cis*-elements within the CG β 5 promoter, the most active promoter in humans [2], appear to be three AP-2, two Sp1, and five Ets-2 binding sites [2,4,12,14,16]. Of these, binding of AP-2 family members to the three AP-2 *cis*-elements is responsible for basal expression of the CG β promoter, and Sp1 binding may contribute to this activity [12,14,16]. The three AP-2 sites are largely conserved within the squirrel monkey CG β promoter, suggesting that they also play a role in the basal expression of this promoter. We found that the squirrel monkey CG β promoter is very active in placental cells and that mutations in the AP-2 sites lead to a significant decrease in basal promoter activity. These data suggest that the squirrel monkey CG β promoter is regulated similarly to that of the human CG β promoter and highlights the role of AP-2-binding sites in basal expression of the gene in placenta.

Collectively, the work of a number of groups have highlighted the important role of CG in the regulation of reproductive function in New World primates [9,10,19,24,25]. The novel finding of pituitary expression of CG in New World primates raises a number of questions regarding its storage and secretion. In other species, such as humans and rodents, the luteotropic hormone LH is tightly regulated with the granin, secretogranin II (SgII). A question is whether a similar relationship is found between SgII and CG. This question is answered in a companion publication [28].

Research Highlights

- Squirrel monkey CG β promoter is regulated in a tissue-specific manner
- *Egr-1 cis*-elements necessary for GnRH-responsiveness of squirrel monkey CG β
- AP-2 *cis*-elements contribute to placenta-specific activity of CG β promoter

Acknowledgments

This study was supported by Grant number 13200 from the National Center for Research Resources (NCRR), a component of the National Institutes of Health (NIH). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of NCRR or NIH. Ms. Lori Boston and Dr. Tina Hubler were supported by REU: Structure and Function of Proteins National Science Foundation Research Experiences for Undergraduates (NSF #0751684) and an intramural grant from the University of North Alabama, respectively. We are grateful to Ms. Subha Pyakurel for her technical assistance.

References

1. Alarid ET, Windle JJ, Whyte DB, Mellon PL. Immortalization of pituitary cells at discrete stages of development by directed oncogenesis in transgenic mice. *Development* 1996;122:3319–3329. [PubMed: 8898243]
2. Bo M, Boime I. Identification of the transcriptionally active genes of the chorionic gonadotropin β gene cluster in vivo. *J Biol Chem* 1992;267:3179–3184. [PubMed: 1371113]
3. Dorn C, Ou Q, Svaren J, Crawford PA, Sadovsky Y. Activation of luteinizing hormone β gene by gonadotropin-releasing hormone requires the synergy of early growth response-1 and steroidogenic factor-1. *J Biol Chem* 1999;274:13870–13876. [PubMed: 10318795]
4. Ghosh D, Ezashi T, Ostrowski MC, Roberts RM. A central role for Ets-2 in the transcriptional regulation and cyclic adenosine 5'-monophosphate responsiveness of the human chorionic gonadotropin- β subunit gene. *Mol Endocrinol* 2003;17:11–26. [PubMed: 12511603]
5. Gromoll J, Wistuba J, Terwort N, Godmann M, Muller T, Simoni M. A new subclass of the luteinizing hormone/chorionic gonadotropin receptor lacking exon 10 messenger RNA in the New World monkey (*Platyrrhini*) lineage. *Biol Reprod* 2003;69:75–80. [PubMed: 12606382]

6. Halvorson LM, Ito M, Jameson JL, Chin WW. Steroidogenic factor-1 and early growth response protein 1 act through two composite DNA binding sites to regulate luteinizing hormone β -subunit gene expression. *J Biol Chem* 1998;273:14712–14720. [PubMed: 9614069]
7. Halvorson LM, Kaiser UB, Chin WW. Stimulation of luteinizing hormone β gene promoter activity by the orphan nuclear receptor, steroidogenic factor-1. *J Biol Chem* 1996;271:6645–6650. [PubMed: 8636081]
8. Hanson FW, Powell JE, Stevens VC. Effects of HCG and human pituitary LH on steroid secretion and functional life of the human corpus luteum. *J Clin Endocrinol Metab* 1971;32:211–215. [PubMed: 4923418]
9. Henke A, Gromoll J. New insights into the evolution of chorionic gonadotrophin. *Mol Cell Endocrinol* 2008;11–19. [PubMed: 18599193]
10. Henke A, Luetjens CM, Simoni M, Gromoll J. Chorionic gonadotropin b-subunit gene expression in the marmoset pituitary is controlled by steroidogenic factor 1, early growth response protein 1, and pituitary homeobox factor 1. *Endocrinology* 2007;148:6062–6072. [PubMed: 17872365]
11. Holdcraft RW, Braun RE. Hormonal regulation of spermatogenesis. *Int J Androl* 2004;27:335–342. [PubMed: 15595952]
12. Johnson W, Albanese C, Handwerger S, Williams T, Pestell RG, Jameson JL. Regulation of the human chorionic gonadotropin α - and β -subunit promoters by AP-2. *J Biol Chem* 1997;272:15405–15412. [PubMed: 9182571]
13. Johnson W, Jameson JL. AP-2 (activating protein 2) and Sp1 (selective promoter factor 1) regulatory elements play distinct roles in the control of basal activity and cyclic adenosine 3',5'-monophosphate responsiveness of the human chorionic gonadotropin- β promoter. *Mol Endocrinol* 1999;13:1963–1975. [PubMed: 10551788]
14. Johnson W, Jameson JL. Role of Ets2 in cyclic AMP regulation of the human chorionic gonadotropin β promoter. *Mol Cell Endocrinol* 2000;165:17–24. [PubMed: 10940479]
15. Lee SL, Sadovsky Y, Swirloff AH, Polish JA, Goda P, Gavrilina G, Milbrandt J. Luteinizing hormone deficiency and female infertility in mice lacking the transcription factor NGFI-A (Egr-1). *Science* 1996;273:1219–1221. [PubMed: 8703054]
16. LiCalsi C, Christophe S, Steger DJ, Buescher M, Fischer W, Mellon PL. AP-2 family members regulate basal and cAMP-induced expression of human chorionic gonadotropin. *Nucleic Acids Res* 2000;28:1036–1043. [PubMed: 10648798]
17. Matzuk MM, Burns KH, Viveiros MM, Eppig JJ. Intercellular communication in the mammalian ovary: oocytes carry the conversation. *Science* 2002;296:2178–2180. [PubMed: 12077402]
18. Muller T, Gromoll J, Simoni M. Absence of exon 10 of the human luteinizing hormone (LH) receptor impairs LH, but not human chorionic gonadotropin action. *J Clin Endocrinol Metab* 2003;88:2242–2249. [PubMed: 12727981]
19. Muller T, Simoni M, Pekel E, Luetjens CM, Chandolia R, Amato F, Norman RJ, Gromoll J. Chorionic gonadotrophin β subunit mRNA but not luteinising hormone β subunit mRNA is expressed in the pituitary of the common marmoset (*Callithrix jacchus*). *J Mol Endocrinol* 2004;32:115–128. [PubMed: 14765996]
20. Pierce JG, Parsons TF. Glycoprotein hormones: similar molecules with different functions. *UCLA Forum Med Sci* 1979;99–117. [PubMed: 400318]
21. Reynolds PD, Roveda KP, Tucker JA, Moore CM, Valentine DL, Scammell JG. Glucocorticoid-resistant B-lymphoblast cell line derived from the Bolivian squirrel monkey (*Saimiri boliviensis boliviensis*). *Lab Anim Sci* 1998;48:364–370. [PubMed: 10090044]
22. Scammell JG, Funkhouser JD, Moyer FS, Gibson SV, Willis DL. Molecular cloning of pituitary glycoprotein α -subunit and follicle stimulating hormone and chorionic gonadotropin β -subunits from New World squirrel monkey and owl monkey. *Gen Comp Endocrinol* 2008;155:534–541. [PubMed: 17897645]
23. Shupnik MA. Gonadotropin gene modulation by steroids and gonadotropin-releasing hormone. *Biol Reprod* 1996;54:279–286. [PubMed: 8788177]
24. Tannenbaum PL, Schultz-Darken NJ, Saltzman W, Terasawa E, Woller MJ, Abbott DH. Gonadotrophin-releasing hormone (GnRH) release in marmosets I: in vivo measurement in ovary-intact and ovariectomised females. *J Neuroendocrinol* 2007;19:342–353. [PubMed: 17425609]

25. Tannenbaum PL, Schultz-Darken NJ, Woller MJ, Abbott DH. Gonadotrophin-releasing hormone (GnRH) release in marmosets II: pulsatile release of GnRH and pituitary gonadotrophin in adult females. *J Neuroendocrinol* 2007;19:354–363. [PubMed: 17425610]
26. Topilko P, Schneider-Maunoury S, Levi G, Trembleau A, Gourdji D, Driancourt MA, Rao CV, Charnay P. Multiple pituitary and ovarian defects in Krox-24 (NGFI-A, Egr-1)-targeted mice. *Mol Endocrinol* 1998;12:107–122. [PubMed: 9440815]
27. Tremblay JJ, Drouin J. Egr-1 is a downstream effector of GnRH and synergizes by direct interaction with Ptx1 and SF-1 to enhance luteinizing hormone β gene transcription. *Mol Cell Biol* 1999;19:2567–2576. [PubMed: 10082522]
28. Vasauskas AA, Hubler TR, Mahanic C, Gibson SV, Kahn AG, Scammell JG. Distribution and regulation of squirrel monkey chorionic gonadotropin and secretogranin II in the pituitary. *Gen Comp Endocrinol* (in review).
29. Wolfe MW, Call GB. Early growth response protein 1 binds to the luteinizing hormone- β promoter and mediates gonadotropin-releasing hormone-stimulated gene expression. *Mol Endocrinol* 1999;13:752–763. [PubMed: 10319325]
30. Zhang FP, Rannikko AS, Manna PR, Fraser HM, Huhtaniemi IT. Cloning and functional expression of the luteinizing hormone receptor complementary deoxyribonucleic acid from the marmoset monkey testis: absence of sequences encoding exon 10 in other species. *Endocrinology* 1997;138:2481–2490. [PubMed: 9165039]

Figure 1.

Nucleotide sequence alignment of the squirrel monkey CG β promoter to the common marmoset CG β (cmCG β), human CG β (huCG β), and rhesus macaque CG β (rmCG β) promoters. *Cis*-elements known important for human CG β promoter activity are conserved in the squirrel monkey CG β promoter (underlined and in bold), the common marmoset CG β , and the rhesus macaque CG β promoters. The putative initiator element, based on the hCG β initiator element, is underlined and in bold. Dashes indicate nucleotides bases that are identical to the squirrel monkey sequences. Asterisks indicate nucleotide deletions. The sequence is numbered according to the putative transcription start site for pituitary expression of squirrel monkey CG β , labeled as +1, based on the transcription start site for human LH β .

Figure 2. Nucleotide sequence alignment of the squirrel monkey CGβ (smCGβ) promoter to the common marmoset (cmCGβ), human LHβ (huLHβ), and rhesus macaque LHβ (rmLHβ) promoters. *Cis*-elements known to be important for human LHβ promoter activity are conserved in the squirrel monkey CGβ promoter (underlined and in bold), the common marmoset CGβ and rhesus macaque LHβ promoters. Putative transcription start sites for pituitary-specific expression are indicated as +1, based on the start site for human LHβ. Dashes indicate nucleotides bases that are identical to the squirrel monkey sequences. Asterisks indicate nucleotide deletions.

Figure 3.

Squirrel monkey CG β is regulated in a tissue-specific manner by distinct promoter elements. A, Schematic depicting the putative structure of the squirrel monkey CG β gene and promoter (not drawn to scale). The squirrel monkey CG β gene is made up of three exons (solid bars) and two introns. The putative transcription start sites for pituitary- and placenta-specific expression are indicated by arrows. Pituitary-specific expression is governed by a promoter immediately upstream of the gene, whereas placenta-specific expression is governed by elements approximately 300 bp upstream of the pituitary start site. Luciferase promoter constructs are shown below the gene schematic, and sizes of each promoter fragment are indicated by position relative to the putative pituitary transcription start site: -1898/+9, -253/+9, and -1898/-249. B, L β T2, JEG-3, or PC12 cells were transiently transfected with pGL3-Basic promoter luciferase reporter plasmids driven by the -1898/+9 squirrel monkey CG β promoter. Cells were harvested and assayed for luciferase activity, and the data are shown as fold-induction over that achieved with pGL3-Basic vector. Each bar represents the mean $\pm$ SEM of at least three independent experiments. *, significantly different from promoter activity in L β T2 and JEG3 cells.

Figure 4.

GnRH-responsiveness of squirrel monkey CG β promoter in pituitary cells. A, L β T2 cells were transiently transfected with luciferase-reporter plasmids driven by either the -1898/+9, -253/+9, or -1898/-249 squirrel monkey CG β promoter constructs. After 24 h, cells were incubated in the absence or presence of 100 nM GnRH for 6 h. Cells were harvested and assayed for luciferase activity, and data are shown as percent change in luciferase activity over that achieved in unstimulated cells, set at 100 percent. Each bar represents the mean $\pm$ SEM of at least three independent experiments. *, significantly different than activity in control cells. B, Schematic depicting relative positions of Egr-1, SF-1, and Pitx-1 binding sites of the squirrel monkey CG β promoter. Mutated elements are denoted by "MUT." The luciferase reporter plasmid containing mutations in the proximal Egr-1 binding site is designated as pEgr1m, the distal Egr-1 site as dEgr1m, and the construct containing mutations of both sites as pdEgr1m. C, L β T2 cells were transiently transfected with either the luciferase-reporter plasmid driven by the -1898/+9 squirrel monkey CG β promoter (WT) or the same plasmid in which mutations were made in the proximal (pEgr1m), the distal (dEgr1m), or both Egr-1 binding sites (pdEgr1m). After 24 h, the cells were incubated in the absence or presence of 100 nM GnRH for 6 h. Cells were harvested and assayed for luciferase activity, and data are shown as percent change in luciferase activity over that achieved in unstimulated cells, set at 100 percent. Each bar represents the mean $\pm$ SEM of at least three independent experiments. *, significantly different from activity in control cells. †, significantly different from WT activity. D, Mutated SF-1 elements are denoted by "MUT." The luciferase reporter plasmid containing mutations in the proximal SF-1 binding site is designated as pSF1m, the distal SF-1 site as dSF1m, and the construct containing

mutations of both sites as pdSF1m. E, L β T2 cells were transiently transfected with either the luciferase-reporter plasmid driven by the -1898/+9 squirrel monkey CG β promoter (WT) or the same plasmid in which mutations were made in the proximal (pSF1m), the distal (dSF1m), or both SF-1 binding sites (pdSF1m). After 24 h, the cells were incubated in the absence or presence of 100 nM GnRH for 6 h. Cells were harvested and assayed for luciferase activity, and data are shown as percent change in luciferase activity over that achieved in unstimulated cells, set at 100 percent. Each bar represents the mean $\pm$ SEM of at least three independent experiments.

Figure 5.

Placenta-specific expression of the squirrel monkey promoter. A, JEG3 cells were transiently transfected with luciferase-reporter plasmids driven by either the -1898/+9, -253/+9 or -1898/-249 squirrel monkey CG β promoter constructs. After 24 h, cells were harvested and assayed for luciferase activity, and the data are shown as fold-induction over that achieved with pGL3-Basic vector. *, significantly different than activity in -1898/+9 and -1898/-249. B, Schematic depicting relative positions of AP-2 binding sites of the squirrel monkey CG β promoter. Mutated elements are denoted by "MUT." The luciferase reporter plasmid containing mutations in the proximal AP-2 binding site is designated as pAP2m, the middle AP-2 site as mAP2m, and the distal AP-2 site as dAP2m. C, JEG-3 cells

were transiently transfected with either the luciferase-reporter plasmid driven by the -1900/+7 squirrel monkey CG β promoter (WT) or the same plasmid in which mutations were made in either the proximal (pAP2m), middle (mAP2m), or distal (dAP2m) AP-2 binding sites. After 24 h, cells were harvested and assayed for luciferase activity, the data are shown as fold-induction over that achieved with pGL3-Basic vector. Each bar represents the mean $\pm$ SEM of at least three independent experiments. *, significantly different than activity in WT.

TABLE 1

Oligonucleotide primers used for mutagenesis studies

Site	Sequence and position	Primer sequence
pEgr-1	5'-CGC CCC CAC-3' (-48/-40)	5'-AAC ACC ACC TGG TGG CCT TGT GAT TCT TAT AAC CCC GAG GTA T-3'
dEgr-1	5'-CG CCC CCG G-3' (-110/-102)	5'-CTT GTC CGC CTC CTA GTA CTC AGG GGA TTA GTG TCC AGG TTA C-3'
pSF-1	5'-T GGC CTT G-3' (-57/-50)	5'-ATG CTA ACA CCA CCT GGT GGA ATT CTC GCC CCC A CAA CCC CGA-3'
dSF-1	5'-TG GCC TTG-3' (-127/-120)	5'-CTG AGC CCC TGC TGC TCC CTG ATC ATG ATG TCC GCC TCC CG-3'
pAP-2	5'-GG CTG AGA G-3' (-526/-518)	5'-ACT GCT CTG GGC TTC TTG ATG ATA GTG GTG TGG GAG AAG-3'
mAP-2	5'-CCT GCG GGT G-3' (-599/-591)	5'-GCA GGC ACG CCC CCT GGA GAT TTT TTA AAT AAT GAG TTA AAT-3'
dAP-2	5'-GCC CCG AGG GC-3' (-620/-610)	5'-ATG TTC ATG CAG GCT GTG GAT CCG AGA TCA GGC ACG CCC CCT G-3'

pEgr-1, pSF-1, pAP-2 denote proximal Egr-1, SF-1, AP2 binding sites, respectively. mAP-2 denotes middle AP-2 site. dEgr-1, dSF-1, dAP-2 denote distal Egr-1, SF-1, AP-2 binding sites, respectively