Messenger RNAs encoding the β subunits of guinea pig (Cavia porcellus) luteinizing hormone (gpLH) and putative choriionic gonadotropin (gpCG) are transcribed from a single-copy gpLH/CGβ gene

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ABSTRACT

Neither gene locus nor gene sequence characterizations have been reported for the β subunits of guinea pig (gp) LH and putative gp chorionic gonadotropin (CG). Descriptions of this locus would allow comparison with functionally relevant molecular genetic features of other species' homologous loci including the single-copy equid LH/CGβ gene and the primate LH–CGβ gene cluster locus. Contiguous cDNA and genomic DNA fragments spanning the entire mature coding sequence of gpLH mRNA, gpCG mRNA and a homologous gpLH/CGβ gene were amplified using PCR methodologies. With the exception of one silent mutation, the two cDNA and the genomic sequences were identical where they overlapped. Comparison of guinea pig coding sequence with LH, CG and LH/CG sequences of other vertebrate species revealed the following order of similarity expressed as percent coding sequence identity: rhinoceros LHβ (83.6%) > pig LHβ (81.8%) > donkey LH/CGβ > bovine LHβ (81.5%) > horse LH/CGβ (80.6%) > dog LHβ (79.7%) > human LHβ (78.2%) > rat LHβ (77.9%) > human CGβ (75.8%) > turkey LHβ (52.7%); values that are generally consistent with recently postulated phylogenetic relationships. Like the consensus mammalian LHβ gene, the 5’-flanking region of the gpLH/CGβ gene contains a single TATA sequence 37 bp upstream of the translation start codon. The first in-frame stop codon occurred at codon position +122 which is consistent with the 121 amino acid residue length of the consensus mammalian mature LHβ peptide. To estimate gene copy number, full-length gpLHβ cDNA was radiolabeled and hybridized to Southern blots of guinea pig genomic DNA digested with a panel of six restriction endonucleases. The resulting simple hybridization pattern strongly suggested that there is a single-copy gpLH/CGβ gene. Northern analysis of total pituitary RNA using the same probe indicated that gpLHβ transcript size is indistinguishable from that of consensus mammalian pituitary LHβ mRNAs (~750 nucleotides). Despite amplifying gpCGβ from placental RNA, positive signal was not detected in Northern blot lanes containing guinea pig total RNA prepared from placentae collected at three gestational ages (17.3 days, 24.3 days and 68 days (term)). Other data suggest that inability to detect Northern blot signal could have been due to low relative tissue concentrations of gpCGβ transcript and/or sampling at gestational time-points that missed peak periods of mRNA expression. We conclude that, with respect to gene copy number, coding sequence and pituitary mRNA size, the gpLH/CGβ gene locus reflects the CTP-less consensus mammalian LHβ condition. However, based on the capacity of this single-copy gene to express in both pituitary and placental tissues, gpLH/CGβ also exhibits functional similarities with the single-copy equine LH/CGβ locus.

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INTRODUCTION

There are two types of mammalian luteotropic glycoprotein hormones (LGH), luteinizing hormone (LH), produced by pituitary gonadotropes, and chorionic gonadotropin (CG), produced by placental trophoblasts. These two gonadotropins are biochemically related, each containing a common α subunit peptide (Fiddes & Goodman 1981) non-covalently bound to a hormone-specific β subunit peptide, a heterodimeric structure required for high-affinity receptor binding and induction of biological activity (Pierce & Parsons 1981, Bousfield et al. 1994).

Whereas LH is synthesized in the pituitary gland of all mammals, placental CG expression is well characterized only in equids and primates (Aschheim & Zondek 1927, Cole & Hart 1930). LH and CG bind the same gonadal LH/CG receptor and induce similar luteotropic responses. The analogous intraspecific biological activities of LH and CG reflect the fact that placenta-expressed CGβ peptide is encoded by either the same single-copy (LH/CGβ) gene that encodes pituitary LHβ (equids) (Sherman et al. 1992), or by CGβ genes that have evolved by recent duplication of the LHβ subunit (primates) (Policastro et al. 1983, Talmadge et al. 1984a, Graham et al. 1987).

In addition, there are distinctive 25 and 29 amino acid carboxyl terminal peptide extensions (CTP) that are encoded by functional human (h) CGβ and equid LH/CGβ genes respectively (Bousfield et al. 1987, 1994, Sugino et al. 1987). With the exception of the CTP-bearing equine LHβ subunit, however, all characterized mammalian LHβ subunit peptides lack such an extension (Bousfield et al. 1994).

The guinea pig (gp) placenta has been reported to produce considerable quantities of bioactive CG which, on purification, has physiochemical and immunological properties similar to those of hCG, including the presence of two non-covalently bound peptide subunits (Humphreys et al. 1982, Bambra et al. 1984). Immunohistochemical studies using cross-reacting antiserum raised against hCGβ revealed positive staining in the Golgi body of the guinea pig syncytiotrophoblast, which is homologous to the CG-producing cell types in equids and primates (Bambra 1981). In addition, elevated CG immunoactivity was detected in guinea pig blood at the end of the third week of gestation, as well as in extracts prepared from placentae collected from day 6 to day 46 of gestation (Bambra 1981). Finally, highly purified gpCG isolated from gestation day 26 placentae was reported to have greater biopotency in the ovarian ascorbic acid depletion assay than the highly purified contemporary hCG preparation (Bambra et al. 1984).

To begin to gain an understanding of structure–function relationships at the gpLGHβ locus, we undertook studies aimed at characterizing selected structural features of the gpLGHβ gene(s) and tissue-specific mRNAs that are functionally relevant at the corresponding loci in primates, equids and other mammalian species. Our specific goals were to determine gpLH/CGβ gene copy number, assay for pituitary and placental expression of the gpLH/CGβ gene, and establish whether gpLH/CGβ gene(s) and corresponding pituitary and placental tissue cDNA sequences predict the existence of a CTP.

MATERIALS AND METHODS

Animals and tissues

Placental tissues were collected from timed-pregnant guinea pigs (Cavia porcellus). A total of

FIGURE 1. Amplification strategy and nucleotide sequences for the gpLH/CGβ gene, and gpLHβ and gpCGβ cDNAs. Two aligned nucleotide sequences are shown. The upper line is gene sequence. The lower line, in italics, is a composite of pituitary and placental cDNA sequences. The ambiguous nucleotide, M, in codon +49 was ‘A’ (silent mutation) in gpLHβ cDNA and ‘C’ in gpCGβ cDNA. Deduced amino acid sequence is shown above the genomic sequence, with the putative first amino acid of the mature peptide as +1. Flanking and intervening gene sequences are in lowercase letters. Predicted exon sequence is in uppercase letters (note that the lengths of the 5’-UTR for gpLHβ and gpCGβ mRNAs have not been directly determined). Arrows, labeled as a P number, above and below the sequence correspond to PCR primers used to amplify gene and cDNA sequences respectively (see text for results of amplification reactions with specific primer pairs). Solid arrows indicate homologous primers designed after determining actual sequences of guinea pig genomic or cDNA fragments. Asterisks and nucleotides within arrows indicate sites of potential or known sequence mismatches (determined retrospectively) between a consensus primer and targeted guinea pig sequence. Reverse-type regions represent sequences of functional terminal consensus primers that may or may not contain sites of non-complementarity with true guinea pig sequence. Note that the 3’-terminal ‘G’ of primers P2a and P2b corresponds to the first nucleotide of exon 2. The consensus TATAA promoter and AATAAA polyadenylation sequences, and the 12 highly conserved half-cysteine residues, are in bold letters. Genbank accession numbers for gpLH/CGβ gene sequence, and gpLHβ and gpCGβ cDNA sequences are AF355775, AF356595 and AF356596 respectively.
five, four and two placentae were excised from three sows at estimated days 17·3, 24·3 and 68 of gestation. Gestational ages were determined retrospectively as a function of fetal weight (Draper 1920) and were in good agreement with estimates based on time of breeding post-estrus. Pituitary glands were excised from all sows for RNA isolation. Testes from two prepubertal males were collected for isolation of genomic DNA.

Amplification and cloning of gpLHβ cDNA

A guanidinium method (Kingston et al. 1995) for total RNA isolation was used to prepare pituitary RNA. Guinea pig pituitary total RNA served as template for reverse transcription-polymerase chain reaction (RT-PCR)-mediated amplification of gpLHβ cDNA. cDNA was reverse transcribed with a primer (AP) composed of polythymidine sequence and additional 5'-anchor' sequence (3' RACE System; Gibco BRL, Gaithersburg, MD, USA). Utilizing the resulting product as template, full-length gpLH cDNA was amplified (Fig. 1) by PCR employing an upstream primer corresponding to porcine LHβ first exon sequence (P2a) and a downstream primer corresponding to 5'-anchor sequence (UAP). Amplicons from two independent amplification reactions were cloned into plasmid and sequenced in both directions. Appropriate negative control reactions, in which reverse transcriptase was excluded from initial reactions, were performed.

Amplification and cloning of gpLH/CGβ gene

The gpLH/CGβ gene was amplified using a consensus upstream primer complementary to a highly conserved sequence immediately upstream of the mammalian LGHβ TATA box (P1), and a downstream primer (P7b) corresponding to homologous gpLHβ cDNA 3'-untranslated region (3'-UTR) sequence (Fig. 1). The downstream primer included the consensus AATAAA transcriptional termination signal. Amplicons from three independent amplification reactions were cloned into plasmid and sequenced in both directions.

Amplification of gpCGβ cDNA

Guinea pig placental total RNA, isolated as described previously (Kingston et al. 1995), served as template for PCR-mediated amplification of gpCGβ cDNAs. First strand cDNA was reverse transcribed (3' RACE System) with a primer (P7a) corresponding to homologous gpLHβ cDNA 3'-UTR sequence. First round PCR was performed using an upstream primer based on homologous exon 1 sequence (P2b; determined from gpLH/CGβ gene sequence) paired with downstream primer, P7a. Using this product as template, semi-nested PCRs were performed using various upstream primers (P3, P4, P5 and P6) paired with P7a (Fig. 1). Nested PCR approaches were employed because homologous guinea pig primer P2b was of poor quality due to: (1) high melting temperature values, (2) high end self complementarity and (3) excessive 3'-end stability (Rozen & Skaletsky 1998), parameters that could not be improved due to the short length of available target sequence (consensus LGHβ exon 1 is only 21–25 bp). Although P2b was inefficient as a first round PCR primer when paired with P7a, amplification of gpCGβ fragments by subsequent semi-nested PCR was routinely successful using 1 µl aliquots of the first round PCR mix as a source of template. Attempts were also made to amplify potentially 5'-extended gpCGβ transcripts using primers P1 and P8 located upstream of the consensus site for consensus LHβ transcriptional initiation, but within the corresponding 5'-UTR regions of the equine LH/CGβ or hCGβ genes (Fig. 1). Negative control reactions, in which reverse transcriptase was excluded, failed to produce gpCGβ amplification products in first round PCRs as well as subsequent semi-nested PCRs. Amplified gpCGβ fragments were sequenced directly.

PCR conditions

Amplification reactions were performed in a total volume of 50 µl using a Perkin Elmer 9700 DNA Thermal Cycler (Norwalk, CT, USA) and 1 µg genomic DNA as target. Final PCR reagent concentrations were: 200 µM deoxynucleotide triphosphates, 0·25 µM primers, 10 mM Tris, pH 8·3, 50 mM KCl, 1·5 mM MgCl2, 0·001% gelatin and 2·5 U/100 µl Taq polymerase (Gibco BRL). PCR was typically carried out for 30 cycles (denaturation at 95 °C for 1 min; annealing at 60 °C for 1 min; extension at 72 °C for 2 min). Annealing temperatures were varied between 55 °C and 65 °C to optimize PCR conditions for various primer pairs.

Nucleotide sequencing

Gel-purified amplification products and plasmid clone inserts were sequenced in both directions. Analysis of sequences longer than 750 bp were performed using a LI-COR model 4000 DNA sequencer (LI-COR, Inc., Lincoln, NE, USA). Sequences shorter than 750 bp were analyzed using an Applied Biosystems 373A Automated DNA Sequencer following standard protocols (Sequenase Version 2·0, US Biochemical Corporation, Cleveland, OH, USA).

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Genomic Southern blots

DNA was prepared by proteinase K digestion of testis tissue (Strauss 1995). Biochemicals and restriction enzymes were purchased from Gibco BRL. Ten micrograms of restriction enzyme-digested DNA were electrophoresed in a 0.7% agarose gel prepared using TBE buffer (89 mM Tris–89 mM borate–2.76 mM ethylenediaminetetraacetic acid, pH 8.3) at 2.5 V/cm for 8–11 h. Gels were prepared for blotting onto Duralon-UV membrane (Stratagene, La Jolla, CA, USA) according to the manufacturer’s specifications and DNA was transferred by standard capillary blotting (Brown 1995a). Blots were hybridized to [32P] labeled probe prepared by random-primed labeling (Multiprime DNA Labeling System; Amersham, Arlington Heights, IL, USA) for 2.5 h at 65 °C in QuikHyb solution (Stratagene). Template for probe synthesis was cloned full-length gpLHβ cDNA excised from plasmid and gel purified. Two washings in 2× SSC/0.1% sodium dodecyl sulfate at 25 °C for 15 min were followed by a final wash in 0.1× SSC/1% sodium dodecyl sulfate, 60 °C for 30 min. (Single-strength SSC is 0.15 M sodium chloride and 0.015 M sodium citrate.) Membranes were exposed to autoradiography film (X-OMAT AR; Eastman Kodak, Rochester, NY, USA) with an intensifying screen at −70 °C for 30–48 h.

Northern analysis

Pituitary, placental and control tissue RNA was prepared as previously described (Kingston et al. 1995). RNA (10 µg per lane) was electrophoresed in a 2.2 M formaldehyde/1.5% agarose gel for 14 h at 1.5 V/cm (Brown 1995b). Blotting, probe hybridization and film exposure were performed as described for genomic blots with the exception of an 11-h exposure of membrane to film.

RESULTS

To confirm pituitary expression of the gpLH/CGβ gene(s), and generate homologous primer and probe sequences for use in subsequent genomic PCR, Northern blot and Southern blot experiments, pituitary total RNA was used as template for RT-PCR amplification of gpLHβ cDNA. A single 523 bp fragment spanning the entire putative mature coding sequence of gpLHβ cDNA was amplified using P2a and UAP, cloned and sequenced (Fig. 1). The 3’-end of this fragment included a portion of the poly-A tail and the UAP primer.

Using a consensus upstream primer (P1) and a homologous downstream primer based on gpLHβ sequence (P7b), a single contiguous gpLH/CGβ genomic fragment routinely amplified (Fig. 1). The 950 bp fragment extended from immediately 5’ of the consensus mammalian LGHβ TATA box to the transcriptional termination signal (AATAAA), and thus spanned the entire putative coding region.

RT-PCR approaches were also used to amplify gpCGβ cDNA. Homologous primer P2b, which corresponds to the first exon sequence, did not produce a visible amplification product when used with downstream primer P7a. However, when aliquots of this reaction mix were used as template for PCR employing nested upstream primers P3, P4, P5 and P6 paired with homologous downstream primer P7a, corresponding CGβ fragments readily amplified. The longest fragment routinely isolated (450 bp) extended from the 5’-end of exon 2 to the 3’-UTR region and thus included three-quarters of the putative signal peptide coding region and all of the mature protein coding sequence (Fig. 1).

To evaluate the possibility that gpCGβ mRNA transcripts, like those of equine CGβ and hCGβ, bear an extended 5’-UTR, RT-PCRs alone and in combination with second round semi-nested PCR were performed. Following reverse transcription of placental RNA, homologous upstream primers P1 and P8, corresponding to known gpLH/CGβ gene sequence upstream of the consensus mammalian LHβ transcriptional initiation site, were used with downstream primer P7a to perform PCR (Fig. 1). No gpCGβ cDNA amplification products were generated from either first round or semi-nested PCRs (data not shown).

Sequence alignments revealed that, with the exception of one translationally silent nucleotide substitution in gpLHβ cDNA relative to gpCGβ cDNA and gpLH/CGβ gene sequences, all cDNA and genomic sequences were identical where they overlapped (Fig. 1). Comparison of genomic DNA to pituitary cDNA sequences revealed the presence of three exons and two introns, with exon–intron boundaries identical to those observed for other mammalian LHβ and CGβ genes (Bousfield et al. 1994). Intron b boundaries delineated by the gpCGβ cDNA were identical to those defined by the gpLHβ cDNA (Fig. 1).

Applying the reading frame defined by consensus mammalian LGHβ sequence, the first translation stop codon was located at amino acid position +122 (Fig. 1). Accordingly, our nucleotide sequences predict a mature peptide length of 121 amino acids which matches the length of the consensus
mammalian LHβ subunit, but not the consensus CGβ subunit (which bears a CTP). The deduced peptide structure also exhibits 12 cysteine residues in register with the highly conserved cysteines present in mammalian LGHβ subunits. A single potential consensus glycosylation site occurs at Asn13, which is the typical location of oligosaccharide attachment in other mammalian LHβ subunits (Bousfield et al. 1994). Because no gpLHβ or gpCGβ amino acid sequence data had previously been reported, it was not possible to directly compare known and deduced amino acid sequences to confirm that the fragments we isolated were bona fide guinea pig sequences. An alternative way to address this issue was to demonstrate that the characterized sequences were not identical to potentially contaminating species’ LHβ sequences and exhibited degrees of dissimilarity consistent with predicted phylogenetic distances. The distance matrix shown in Table 1 shows percent coding sequence differences between our gpLH/CGβ sequence and the LHβ coding sequences of nine other vertebrate species. The values presented confirm that the sequence we report is both unique and consistent with the evolutionary relationships recently postulated for the guinea pig (D’Erchia et al. 1996).

With the exception of a single silent mutation, all of the several genomic and cDNA sequences we isolated and sequenced were identical (Fig. 1). However, the possibility remained that we failed to amplify one or more additional gpLGHβ gene(s) which might encode peptides exhibiting divergent amino acid sequences, such as a CTP domain. To evaluate this possibility further, Southern blot analysis was performed (Fig. 2). Hybridization of genomic blots with gpLHβ cDNA probe resulted in single bands in each of six lanes containing genomic DNA digested with different restriction enzymes. The simple hybridization pattern strongly suggested that there is only one gpLH/CGβ gene per haploid genome.

**Table 1. Distance matrix**, expressed as number of substitutions per 100 bases, for mammalian LHβ and CGβ DNA sequences**

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<th>hCGβ</th>
<th>hLHβ</th>
<th>dkLH/CGβ</th>
<th>eLH/CGβ</th>
<th>pLHβ</th>
<th>rLHβ</th>
<th>gpLH/CGβ</th>
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<td>25</td>
</tr>
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<td>dkLH/CGβ</td>
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<td>9</td>
<td>10</td>
<td>9</td>
<td>12</td>
<td>10</td>
<td>11</td>
<td>12</td>
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<tr>
<td>eLH/CGβ</td>
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<td>10</td>
<td>12</td>
<td>12</td>
<td>18</td>
<td>12</td>
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<td>12</td>
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<tr>
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<td>10</td>
<td>11</td>
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<tr>
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<td>tLHβ</td>
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<td>15</td>
<td>16</td>
<td>11</td>
<td>15</td>
<td>11</td>
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<tr>
<td>Ranked distance relative to guinea pig:</td>
<td>Rhino &lt; Pig &lt; bovine ≡ donkey ≡ horse &lt; dog &lt; hLHβ &lt; rat &lt; hCGβ &lt; turkey</td>
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</table>

*Alignments and matrix construction performed with the assistance of Wisconsin Package software, version 8.1, September, 1995, Genetics Computer Group, Madison, WI, USA.

*dk=donkey, e=equine, p=porcine, rn=rhinoceros, d=dog, b=bovine, r=rat, t=turkey.

*Sequences retrieved from Genbank.

*Comparisons included encoding DNA sequence corresponding to codon positions +1 through +110.

**FIGURE 2.** Southern blot analysis of guinea pig genomic DNA. DNA was incubated with the indicated restriction enzymes, electrophoresed, blotted and hybridized with probe produced from full-length gpLHβ cDNA (see Materials and Methods). Kbp, kilobase pairs.
Northern blot analysis was performed to further characterize expression of the gpLH/CGβ gene (Fig. 3). RNA prepared from guinea pig pituitary gland produced a strong hybridization signal indicating the presence of a transcript of the predicted size. By contrast, there was insufficient signal intensity to detect gpCGβ transcript in RNA isolated from 17·3 day, 24·3 day and term gestation placentae.

DISCUSSION

A major objective of the present study was to determine whether the gpLH/CGβ gene locus bears structural similarity to any of the four primary structure–activity variants of LGHβ loci that have been characterized in mammals to date (Fig. 4A). The most prominent locus organization, thought to represent the ancestral, or consensus, condition is the simplest: a single-copy LHβ gene encoding a structurally conserved 121 amino acid mature β peptide that is expressed exclusively in the anterior pituitary gland (Bousfield et al. 1994). By contrast, the primate lineage has evolved a more complex LGHβ locus arrangement comprised of a single pituitary-expressed LHβ gene linked to a cluster of placentally expressed CGβ genes and pseudogenes (Talmadge et al. 1983, 1984a, Fiddes & Talmadge 1984). While the primate LHβ gene reflects the consensus condition, encoding a typical 121 amino acid peptide, expressed hCGβ genes encode a peptide that exhibits an extended carboxyl terminus (CTP) giving a 145 amino acid-long mature CGβ subunit. The third structure–activity variant is found in equids where there is a single-copy LH/CGβ gene that has acquired the capacity for both pituitary- and placenta-specific expression (Sherman et al. 1992). Like primate CGβ genes, the equid LH/CGβ gene encodes a CTP giving a mature peptide length of 149 amino acids for both pituitary LHβ and placental CGβ subunits (Bousfield et al. 1987, Sugino et al. 1987). A fourth LGHβ gene arrangement (not shown) is found in the white rhinoceros where two unlinked LHβ gene copies are present per haploid genome (Lund & Sherman 1998). The sequences of these two LHβ genes are identical and predict a mature peptide length of 121 amino acids. However, it remains to be determined which of these two genes are expressed, and in what tissues they may be transcriptionally active.

Our genomic blot analysis strongly suggests that the gpLH/CGβ gene is present as a single copy. In this regard, the guinea pig locus more closely resembles the consensus LHβ gene and equid
single-copy LH/CGβ gene. This contrasts with the primate scenario in which acquisition of the capacity for placenta-specific expression was temporally associated with a series of gene duplication events that converted the ancestral single-copy LHβ gene locus into an LHβ1–CGβn gene cluster locus (LHβ1–CGβn in humans). In the primate lineage, either during or some time after the first duplication event in which the ancestral primate LHβ gene gave rise to the first ancestral primate CGβ gene copy, the latter lost its ability to express in pituitary and gained the capacity for placenta-specific expression (Fiddes & Talmadge 1984). By contrast, evolution of the equid LH/CGβ single gene locus involved adaptations of a single-copy ancestral equid LHβ gene with a 5′-flanking region that ultimately enabled activation of placental expression, without disrupting functionality of promoter elements required for LHβ expression (Sherman et al. 1992). Thus, the equid and guinea pig single-gene LH/CGβ loci are similar to the extent that they have both evolved the capacity for dual pituitary/placental expression constrained by the limitations imposed by a single-gene sequence (Fig. 4).

Length of the gpLHβ mRNA (~750 nucleotides) as revealed by Northern analysis is indistinguishable from the lengths of other species' LHβ transcripts (Jameson et al. 1984, Ezashi et al. 1990, Sherman et al. 1997). Given that the lengths of gpLHβ and consensus LHβ coding sequences are identical, and that length of the poly-A tail (~200 bases) also appears to be conserved across species lines (Jameson et al. 1984, Ezashi et al. 1990, Sherman et al. 1997), the transcriptional start site in the guinea pig pituitary likely maps to the same general region as other mammalian LHβ genes. Furthermore, the gpLH/CGβ proximal 5′-flanking region contains a classical TATA sequence in the same position as the functional TATA element in the consensus mammalian LHβ gene (Fig. 4B). The implication of this juxtaposition is that, as in other species, pituitary expression in the guinea pig is likely to be TATA-mediated, with transcription initiating 28–32 bp downstream of the TATA element (Fig. 4B). This would produce a short (6–10 bp) gpLHβ 5′-UTR which is consistent with the 6–11 nucleotide-long 5′-UTR of other mammalian LHβ mRNAs (Jameson et al. 1984, Ezashi et al. 1990, Guthrie et al. 1991, Bousfield et al. 1994).

Acquisition of the capacity for placental expression in both equids and primates was associated with the development of novel proximal promoter and 5′-flanking structure–activity relationships (Fiddes & Talmadge 1984, Jameson et al. 1986, Sherman et al. 1992). Significant elongation of the 5′-UTR region is observed in both equid CGβ (51 bp) and primate CGβ (~350 bp) transcripts (Jameson et al. 1986, Sherman et al. 1992), as compared with the consensus LHβ gene (Fig. 4A and B). In equids, the lengthened 5′-UTR is a consequence of duplication of the TATA box (TATAα) and adjacent downstream sequences (Fig. 4A and B) (Sherman et al. 1992). In the horse, both TATA elements are functionally active in both pituitary and placental tissues (Sherman et al. 1992). By contrast, hCGβ genes adopted a novel TATA-less promoter approximately 350 bp upstream of the consensus mammalian LHβ TATA sequence (Jameson et al. 1986), thereby producing a transcript with a substantially elongated 5′-UTR containing an intact but non-functional TATA sequence (Fig. 4A and B).

Unfortunately, standard techniques for mapping the site(s) of placental transcriptional initiation of the gpLH/CGβ gene could not be employed in the present study because...
of the requirement for at least as much transcript as is necessary for Northern analysis. Thus, it could not be directly determined whether the promoter sequences regulating placental gpCGβ expression are TATA-associated, or the existing TATA sequence might be ignored in favor of an alternative upstream site, as with the primate CGβ gene locus. In an attempt to explore the latter possibility further, we used nucleotide sequence information gleaned from the 5'-end of the gpLH/CGβ gene to design PCR primers that would be expected to amplify putative gpCGβ reverse transcripts bearing 5'-extended UTRs similar to those observed in equid and primate CGβ mRNAs. Despite using these primers in both standard and nested PCR formats, no 5'-extended gpCGβ cDNA amplification products were produced. While these findings suggest that CGβ transcripts bearing elongated 5'-UTR sequences are not present in the guinea pig placenta, caution must be exercised when interpreting negative PCR outcomes.

If tandem replication of the equid TATA element and/or adjacent 5'-flanking sequences played a role in acquisition of the capacity for placental expression in equids, then the absence of any evidence for similar changes in the corresponding area of the gpLH/CGβ (Fig. 4B) gene would argue that a different combination of molecular mechanisms may be responsible for directing placental CGβ expression in the guinea pig. However, promoter elements mediating placental expression of equid CGβ, other than the TATA box, remain ill-defined and guinea pig 5'-flanking sequence upstream of the TATA element has yet to be determined. Accordingly, the possibility remains that the guinea pig locus may share some functional promoter features with equids and/or primates.

As expected, full-length gpLHβ cDNA was readily amplified from pituitary total RNA. Consistent with prior studies detailing isolation and immunodetection of biologically active gpCG (Bambra 1981, Humphreys et al. 1982, Bambra et al. 1984), both large and small fragments of gpCGβ cDNA were also amplified by RT-PCR from placental total RNA using a variety of homologous primer pairs. The largest placently derived cDNA amplicon spanned the entire mature coding sequence and exhibited consensus splicing of intron b (Fig. 1). Moreover, this fragment was amplified by nested PCR following initial amplification using P2b (first exon primer), thereby demonstrating that full-length CGβ cDNA was present after the first round of PCR. Nevertheless, in view of the absence of detectable Northern blot signal when probing placental RNA, there was concern that the positive placental RT-PCR assay result could be due to contamination with pituitary-derived amplicons. To protect against this possibility, negative control reactions were performed in which reverse transcriptase was excluded, prior to PCR. This control reaction failed to give amplification product (data not shown) in reactions run side-by-side with entire reactions which did amplify expected gpCGβ cDNA fragments. We conclude from these experiments that the gpLH/CGβ gene is transcriptionally active, at some level, in the placenta.

There are several possible explanations for the absence of detectable signal in Northern blot assays of placental RNA. Previous studies (Bambra 1981) revealed that the syncytiotrophoblast cells staining immunohistochemically positive for hCGβ represented only a small proportion of cells in the tissue sections examined. Moreover, positive cells were scattered individually amongst relatively large expanses of negative cells. Thus, there were no grossly dissectible placental regions containing concentrations of positive cells that could be preferentially excised during sample collection to enhance the yield of desired transcript. We surmise from this that the concentration of gpCGβ mRNA may have been too low to detect in our Northern blot assay because of tissue dilution effects.

Two other potential factors influencing our inability to detect CGβ transcripts in the Northern blot assay are that this mRNA species may be less stable, exhibiting a comparatively short in vitro or in vivo half-life, and that the timing of placental tissue collection missed the peak gestational periods of CGβ mRNA synthesis. In an attempt to address the later possibility, the two earlier gestational ages at which placental tissue was collected in the present study were selected based on the periods of greatest CG bioactivity and immunoactivity (gestational days 17–19 and days 21–26) reported in two previous studies (Humphreys et al. 1982, Bambra et al. 1984). Our original estimates of gestational age based on postpartum breeding dates agreed well with gestational age calculated using a formula based on fetal weight (Draper 1920). The calculated fetal ages were 17.5 days and 24.4 days, which were within the gestational windows targeted for sampling. Nevertheless, it is possible that period(s) of higher level gpLH/CGβ gene expression in placenta may be transient within these windows and, thus, easily missed. In view of these factors, further studies in which placental tissues are collected more frequently and assayed using cell-specific cytochemical methodologies such as in situ hybridization are warranted.

All genomic and cDNA clones and amplicons sequenced in this study predict an in-frame
translational stop site at codon position +122. Therefore, the absence of a CTP domain in the guinea pig constitutes a novel LGHβ structure–activity relationship as shown in Table 2. The consensus pituitary and placental conditions related to LGHβ length include the CTP-less pituitary LHβ subunit and the CTP-containing placental CGβ subunit (Bousfield et al. 1994). While the CTP-bearing equid LHβ subunit is the only known exception to the consensus pituitary state, the present study is the first to predict an expressed CGβ transcript that does not encode a CTP.

Nevertheless, existence of a CTP-less CGβ subunit (Fig. 4C) is consistent with a leading scenario for the temporal order of evolutionary events leading to placental CGβ subunit expression in equids and primates (Fiddes & Talmadge 1984, Talmadge et al. 1984b, Jameson et al. 1986). According to this model, promoter sequence mutation(s) first occurred which enabled transcriptional activation of a single LHβ gene (equids), or duplicated copies of the LHβ gene (primates), in placental trophoblasts. Some time following initial acquisition of the ability to express in placenta, additional selective pressure favored retention of a frame-shift deletion mutation(s) (Fig. 4C) leading to CTP expression. In such a scenario, the CTP-less gpLH/CGβ gene could reflect the intermediate evolutionary stage that would occur after the first promoter sequence mutation(s) enabling placental expression, and before the second mutation(s) leading to CTP expression.

Evidence for both luteotropic (Mwenda et al. 1989) and immunomodulatory (Bambra 1984) effects of highly purified gpCG have been reported in the guinea pig. However, the extent to which these or other physiologic mechanisms may or may not be under continued selective pressure remain unclear. The CTP domains of equid and primate LGHβ subunits have been shown to increase hormone half-life in vivo (Boime & Ben-Menahem 1999). Accordingly, both up-regulation of CGβ mRNA transcription in the placenta and acquisition of a CTP domain would address a common putative selective pressure, namely enhanced reproductive success consequent to elevated/prolonged luteotropic gonadotropin subunit presence during gestation. Whether or not mutations leading to creation of (1) functional placenta-specific promoter elements or (2) functional placental promoter elements plus expression of a CTP domain are selected for in any given species depends on whether all enabling chance mutations have yet occurred and, subsequently, on the magnitude of the selective pressure favoring elevated CG-like activity during pregnancy. It is possible that present levels of placenta-specific promoter activity of the LH/CGβ gene in the guinea pig may adequately meet selective pressure demands. Alternatively, selective pressure for greater gestational gpCGβ expression may persist, in which case retention of future mutations that further enhance promoter activity and/or generate a CTP domain may be favored.

Our discovery of a single-copy gpLH/CGβ gene leads to the prediction that gpLHβ and gpCGβ peptide sequences are identical, as in the case of equine LHβ and equine CGβ. Mammalian glycoprotein hormones also typically contain a common α subunit encoded by a single gene (Bousfield et al. 1994). Therefore, gpLH and putative gpCG would be predicted to have identical heterodimeric primary protein structure and similar, if not identical, three-dimensional structures. Consistent with this prediction is the report that anti-hCGβ antiserum used to monitor purification of large quantities (1·1 g) of highly purified heterodimeric gpCG from placental extracts exhibited similar cross-reactivities with hLH and hCG holo-hormone (Bambra 1981, Bambra et al. 1984). Thus, the majority of the principal epitopes recognized by this polyclonal antiserum must have been located within the highly homologous disulfide-bonded core regions of hLHβ and hCGβ, rather than in the CTP domain that is present in hCGβ but not hLHβ. Had epitope specificity of this antiserum been limited to the CTP domain of hCG, it would have exhibited little utility in the immunodetection of CTP-less gpCG.

An expanded model summarizing the postulated independent evolution of equid, primate and guinea pig LHβ loci is illustrated in Fig. 5. We have previously shown that evolution of expression of equid CGβ occurred through a mechanism independent and distinct from that of the primate CGβ cluster (Sherman et al. 1992). Recently revised phylogenetic relationships reveal that guinea pig radiation occurred after rodents and prior to divergence of primates, ungulates, carnivores and lagomorphs (Graur et al. 1991, D’Erchia et al. 1996). Given that the majority of mammalian

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**Table 2. Phylogenetic distribution of CTP expression among mammalian LHβ and CGβ subunits**

<table>
<thead>
<tr>
<th>β subunit type</th>
<th>No CTP</th>
<th>+CTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>LHβ</td>
<td>Most mammals</td>
<td>Equids</td>
</tr>
<tr>
<td>CGβ</td>
<td>Guinea pigs</td>
<td>Equids and Primates</td>
</tr>
</tbody>
</table>

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species in these clades are not known to express placental CG, it is likely that acquisition of the capacity for placental expression of CGβ is independently acquired in a limited subset of mammalian clades, an example of convergent evolution. Expression of a CTP domain in LHβ and CGβ subunits is variable. Subscripts indicate the number of LGHβ gene copies (n=variable gene number) per haploid genome. Asterisk indicates genes that are not physically linked.

Advancement of our understanding of the molecular mechanisms governing differential CGβ and LHβ gene expression in primates and equids has been hindered by the lack of access to a convenient laboratory animal model. It is anticipated the gpLH/CGβ gene structure–activity relationships described in this paper will provide fundamental information that will facilitate more comprehensive comparative analyses aimed at delineating the molecular mechanisms mediating tissue-specific expression of mammalian LHβ, CGβ and LH/CGβ subunits. Apart from the obvious importance of the roles played by LHβ and CGβ subunits in the regulation of gamete development and the maintenance of pregnancy, free CGβ subunit has recently been found to suppress tumor development associated with HIV infection (Lunardi-Iskandar et al. 1995, Gill et al.

**FIGURE 5.** Model for the evolution of LGHβ genes. The single-copy LHβ gene condition in the ancestral mammal is retained in individual lineages following mammalian radiation. The capacity for placental expression of CGβ is independently acquired in a limited subset of mammalian clades, an example of convergent evolution. Expression of a CTP domain in LHβ and CGβ subunits is variable. Subscripts indicate the number of LGHβ gene copies (n=variable gene number) per haploid genome. Asterisk indicates genes that are not physically linked.
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