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# Eutopic production of human chorionic gonadotropin β (hCGβ) and luteinizing hormone β (hLHβ) in the human testis

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**Abstract**

The classical pregnancy and tumor marker hCG has long been considered to be only accidentally expressed ectopically, e.g. by tumors. The biological functions of low levels of hCGβ, hCGα and holo-hCG in the sera of nonpregnant healthy individuals remained unclear. Immunological analyses by our ultrasensitive time-resolved fluoroimmunoassays revealed a concentration gradient from < 5 pg hCGβ/ml in cubital vein serum versus up to 480 pg hCGβ/ml in the corresponding benign testicular hydrocele fluids. Moreover, hCGβ and its cognate molecule luteinizing hormone β (LHβ) were present in cytosolic extracts of normal human testes. Both hCGβ and hLHβ are eutopically produced as proven by RT-PCR and subsequent Southern and dot blot analyses. Thus, the view of a purely systemic hormonal function of hLH, and of hCG during pregnancy needs a reassessement as hCGβ and hLHβ are synthesized in the human testis and autocrine/paracrine actions seem to be likely.

*Key words:* Human chorionic gonadotropin; Luteinizing hormone; Eutopic production; Human testis

## 1. Introduction

Physiologically, the most important pregnancy marker human chorionic gonadotropin (hCG) is considered to act systemically as a hormone on the human ovary in order to sustain progesterone secretion. In addition to its synthesis in the placenta, hCG is produced by a variety of both trophoblastic and nontrophoblastic malignancies and serves as a well established tumor marker for testicular cancer [1–3]. Moreover, hCG and its free α (hCGα) and β-subunits (hCGβ) have been demonstrated in various body fluids such as serum, urine and hydrocele fluid in normal healthy female and male populations [1,2,4], with levels correlated to age [5]. The pituitary which is the site of production for LH has been considered to be the main source of these ectopically synthesized hCG-derived molecules [6].

HCGβ is encoded by six genes linked physically to a single highly homologous gene for luteinizing hormone β-subunit (LHβ) all of which are arranged in a single cluster on chromosome 19 [7]. The six members of the hCGβ gene family are essentially transcriptionally active in the placenta and the choriocarcinoma cell line JAr [8]. More than a decade ago reports appeared that hCG-like substances are widespread in other normal tissues including normal testis (for review see [9]). After the advent of monoclonal antibodies and the introduction of two-site monoclonal antibody based immunoassays only few of these data could be verified [1,6]. The biological function of ectopically produced hCG which could be detected in minute concentrations in serum remained unclear. As there is increasing evidence for the importance of local operative autocrine/paracrine mechanisms in reproductive organs such as the testis [10–13] and as we previously demonstrated that irrespective of histology all testicular cancers secrete hCG-like molecules, we hypothesized that in addition to pituitary derived LH, which is definitely the primary support for Leydig cell function, locally produced LH- and hCG-derived molecules in the normal human testis might act as autocrine or paracrine modulators of testicular physiology.

## 2. Materials and methods

### 2.1. Tissues, cells and body fluids

Testes from 19 men undergoing orchiectomy for previously untreated prostatic cancer and a post mortem male pituitary (29 a) were snap frozen in liquid nitrogen and kept at −70° until used.

For immunological determinations of hCG- or LH-derived molecules one gram of each decapsulated testis was homogenized in 5 ml ice-cold phosphate-buffered saline (PBS, pH 7.2) with an Ultra turrax tissue homogenizer (5 × 20 s), followed by centrifugation at 19,000 × *g* for 20 min (4°C). The supernatant was stored at −20°C until tested. Serum and hydrocele fluids were obtained from 11 patients undergoing

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hydrocele operations for benign reasons and stored frozen. All the patients gave informed consent.

*2.2. Time-resolved fluoroimmunoassays (IFMA)*

Immunological determinations of holo-hCG, free hCG$\beta$, free hCG$\alpha$ and hLH$\beta$ were performed by highly specific and sensitive (2 pg/ml) time-resolved IFMAs [1,5,14] based on our monoclonal antibodies (MCA) against hCG, free hCG$\alpha$ and free hCG$\beta$ which have been produced and characterized as described elsewhere [15–17]. MCA (code 19/1) directed against hLH$\beta$ were produced by Dr. Siddle [18].

*2.3. Isolation of total RNA*

Total RNA was extracted either from one gram of testis, term pregnancy human placenta, pituitary or from $2 \times 10^6$ BeWo choriocarcinoma cells and human thyroid epithelial cells by the single step acid guanidium thiocyanate phenol/chloroform method of Chomzynski and Sacchi [19]. Extreme care was taken to avoid specimen contamination during the extraction procedure including using filtered pipet tips and sterile containers. The integrity of RNA was assessed by analysis of 28S and 18S rRNA on ethidium bromide-stained 1% agarose gels (Boehringer-Mannheim, Mannheim, Germany).

*2.4. Reverse transcription*

Between 300–500 ng of total RNA were diluted in 5 $\mu$l DEPC water in a 0.5 ml PCR tube (Perkin-Elmer Cetus, Norwalk, CT), denaturated at 75°C for 3 min in a programmable thermal cycler (TC; Techne, Cambridge, UK), then rapidly cooled to 4°C and the reverse transcription performed in a final volume of 40 $\mu$l under the following reaction conditions: 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine, 10 mM dithiothreitol, 1 mM each of deoxy-ATP (dATP), dCTP, dGTP and dTTP (Promega, Madison, WI, USA), 40 U human placental ribonuclease inhibitor (Promega), 200 pmol random hexamer oligonucleotide (Boehringer-Mannheim, Germany) and 10 U avian myeloblastosis virus reverse transcriptase (Promega). The reaction mix was incubated for 8 min at 20°C and 8 min at 25°C to allow hybridization between the RNA and the hexamer primers. The reverse transcription was then allowed to proceed at 42°C for 30 min. This cycle was repeated four times to increase the amount of cDNA.

*2.5. PCR*

For PCR amplification [20], 5 $\mu$l of each first strand reaction containing the cDNA was placed in a sterile 0.5 ml PCR tube in a final volume of 25 $\mu$l with the following reaction conditions: 10 mM Tris-HCl (pH 9), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM each of deoxy-ATP (dATP), dCTP, dGTP and dTTP, 4 pmol each of the two oligonucleotide primers specific for hCG$\beta$ gene products and 1 U Taq polymerase (Perkin-Elmer Cetus) at pH 9. After an initial denaturation for 3 min at 94°C, 35 cycles of amplification were performed with 1 min denaturation at 95°C, 1 min annealing at 55°C, and 2 min extension at 73°C in a TC. The last cycle had an elongation time of 5 min at 73C.

Three sets of primer pairs either specific for the hCG$\beta$ genes 1 and 2 (5′-CCCCAGTGCTTGCGGAAGATA-3′ located at hCG$\beta$-cDNA −80/−60 and 5′-CCGGCAGGACCCCCTGCAGCA-3′ at position 191/211), or hCG$\beta$ genes 3, 5, 7 and 8 (5′-TCGGGTCACGGCCTCCT-3′ located at −351/−335 and 5′-TCGGGGTGTCCGAGGGC-3′ at 468/484) [8] or the hLH$\beta$ gene (5′-ACCGTCAACACCACCATCT-3′ located at 142/161 and 5′-AGAGCCACAGGGAAGGAGAC-3′ at 299/318), were custom-made by Microsynth (Windisch, Switzerland).

*2.6. Gel electrophoresis and Southern blot analysis*

PCR products were electrophoresed on 2% agarose gels, stained with ethidium bromide and photographed under UV light. Amplified cDNA was then transferred by capillary transfer to a nylon membrane filter (Bio-Rad Laboratories; Richmond, CA, USA), crosslinked by using an UV crosslinker (Stratagene, La Jolla, CA, USA) and, after prehybridization [21], hybridized (37°C, overnight) with a synthetic 18-oligo nucleotide complementary to cDNA nucleotides 126–143 (5′-CTGCCCCGTGTGCATCAC-3′) in exon 2 of the hCG$\beta$ genes. This probe was radioactively labeled to a specific activity of $3 \times 10^6$ cpm/ml using [$\gamma$-$^{32}$P]ATP (NEN Research Products, Boston, MA, USA) and T4 polynucleotide kinase (Promega). Filters were washed twice for 15 min each at room temperature and 58°C with 300 mM NaCl/30 mM sodiumcitrate/0.1% sodiumdodecyl sulfate (SDS) and then exposed to Agfa RP-1 film (Agfa, Vienna, Austria) at −70°C for 3 h.

*2.7. Dot blot analysis*

HLH$\beta$ (177 bp) and hCG$\beta$ 3, 5, 7 and 8 (835 bp) -PCR products were purified using Wizard PCR Preps (Promega), ethanol precipitated, dotted onto nylon membranes, denatured [21] and processed as described for Southern blot analysis (hybridization at 42°C). $^{32}$P-labeled oligonucleotides were complementary to cDNA nucleotides 178–194 specific either for hCG$\beta$ (5′-ACCATGACCCGCGTGCT-3′) or for hLH$\beta$ (5′-ACCATGATGCGCGTGCT-3′). The specific washing steps were performed twice for 3 min at 60°C (hLH$\beta$ probe) or at 61°C (hCG$\beta$ probe).

## 3. Results

### *3.1. hCG, hCGα and hCGβ in serum, hydrocele fluid and testicular extract*

In hydrocele fluids hCG was present only in a single case and free hCG$\alpha$ in 5/11 (mean, 75 pg/ml; range, 0–620 pg/ml). Corresponding serum levels were within the normal range (hCG: < 7pg/ml; hCG$\alpha$ < 196 pg/ml) as defined previously [5]. A clear-cut concentration gradient of hCG$\beta$ could be observed from serum (< 5 pg/ml, all samples) to hydrocele fluids which were positive in 10/11 samples (mean, 226 pg/ml; range, 0–480 pg/ml). In addition, all cytosolic testicular extracts contained free hCG$\beta$ (mean, 12.4 ng/g; range, 2–22.8 ng/g, tissue wet weight) and hLH$\beta$ (mean, 0.9 ng/g; range, 0.07–3.3 ng/g).

### *3.2. Expression of hCGβ and hLHβ genes in human testis*

Proof for the eutopic testicular production of hLH$\beta$ and hCG$\beta$ gene derived molecules has been achived by RT-PCR and consecutive Southern hybridization or dot blot analyses. Both primer pairs specific for products of

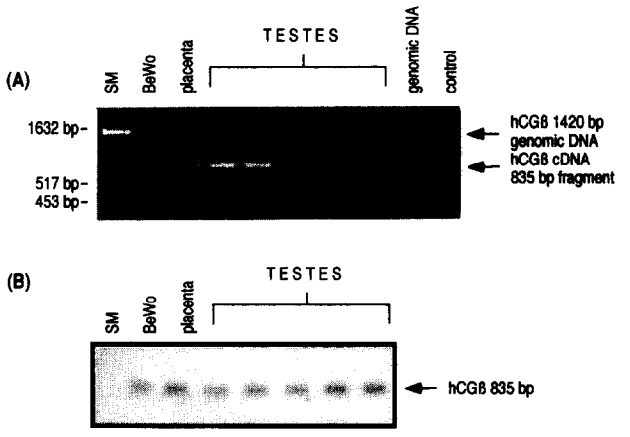


Fig. 1. Detection of mRNA from hCG$\beta$ genes 3, 5, 7 and 8 in human testes by RT-PCR. Five different testes had a positive signal at the predicted size of 835 bp. The cDNA obtained from both human placenta and BeWo choriocarcinoma cells served as a positive control and cDNA derived from human thyroid epithelial cells as a negative control. Amplification of genomic hCG$\beta$ DNA (internal control) resulted in the expected 1420 bp fragment. (A) shows the ethidium-bromide stained agarose gel (2%), and (B) the verification of the hCG$\beta$ RT-PCR product by Southern hybridization with a [$^{32}$P]hCG$\beta$ 18 bp oligonucleotide (position 126–143) which was different from both PCR primers.

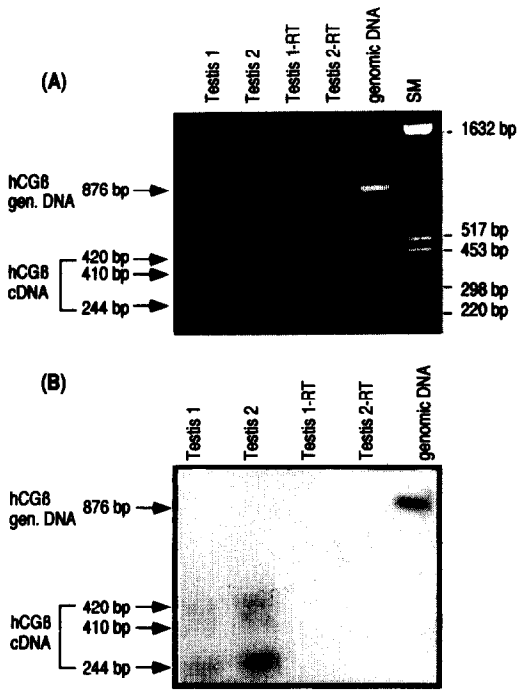


Fig. 2. Detection of hCGβ gene 1&2 mRNA in human testes by RT-PCR. Amplified testicular cDNA generated three transcripts (410, 420 and 244 bp) arising from alternative splicing sites as first described by Bo and Boime [8] for placental tissue. Testicular RNA preparations where reverse transcriptase was omitted from the reaction mixture (testis-RT) contained no cDNA templates. The predicted and observed size of PCR-amplified genomic DNA was 876 bp (internal control). SM, size marker. Panel A shows the ethidium-bromide stained hCGβ gene 1 and 2 RT-PCR products separated with a 2% agarose gel, and panel B the verification of product specificity by Southern hybridization with a [$^{32}$P]hCGβ oligonucleotide (position 126–143) as in Fig. 1B.

hCGβ genes 3, 5, 7 and 8 (835) and hCGβ genes 1 and 2 (244, 410, 420 bp), respectively, yielded fragments of the predicted lengths (Figs. 1A and 2A). It appeared that the testicular hCGβ cDNAs are heterogeneous in nature and include the three alternative splicing products of hCGβ genes 1 and 2 originally described for the placenta [8]. The same results were obtained for the chorion-carcinoma cell line BeWo and term pregnancy placenta which were run in parallel. The hLHβ primer pair generated an expected fragment of 177 bp (Fig. 3). The fragments were not amplified from genomic DNA as otherwise due to intron sequences bands of 1420 bp for hCGβ genes 3, 5, 7 and 8 and 876 bp for hCGβ genes 1 and 2, respectively, and 410 bp for hLHβ would have been generated. HLHβ cDNA was not amplified by the two hCGβ specific primer pairs due to its much shorter 5'-untranslated region. Southern transfer of the hCGβ RT-PCR products and hybridization with a different hCGβ probe (position 126–143) (Figs. 1B and 2B) and dot blot analyses of hLHβ-products with specific hLHβ and hCGβ probes (Fig. 4) validated the specifity of the PCR products. Additional proof of LHβ- and hCGβ-product specificity was obtained by digestions with the restriction enzymes *Hae*III (Boehringer Mannheim) and *Apa*LI (USB, Cleveland, OH) (data not shown).

## 4. Discussion

HCG is a glycoprotein hormone which is eutopically expressed at high levels by trophoblastic cells of the placenta starting at the 6- to 8-cell stage of the blastocyst (for review see [22]). Its classical physiological role is to sustain progesterone secretion by the corpus luteum to maintain pregnancy. Ectopic secretion by a variety of nontrophoblastic tumors made hCG and its free subunits hCGα and hCGβ useful biochemical markers for malignancy [1,2,4]. So far the only biological function of ectopically secreted hCG-like molecules could be ascribed to free hCGα as to be critical for certain transformation phenotypes of human lung tumor cells (ChaGo) [23]. As early as in the 70ies hCG production could be shown in normal tissues (for review see [9,24,25]). However, this remained a highly controversial issue as, except for the pituitary where hCGβ gene expression has been demonstrated by cloning a hCGβ cDNA [26], proof for structural identity between these immunoreactive hCG-like substances and genuine hCG was still lacking. Thus, the pituitary was considered to be the major source of hCG-derived molecules present in minute concentrations in body fluids [1,4–6].

Investigating hCG, hCGα and hCGβ secretion patterns of testicular tumor cells in vivo it appeared that hCG-derivatives were secreted irrespective of histology. In the present study we found a significantly increasing concentration gradient of free hCGβ from serum to *benign* testicular hydrocele fluids. Although free hCGβ and hLHβ could be demonstrated by time resolved fluoroim-

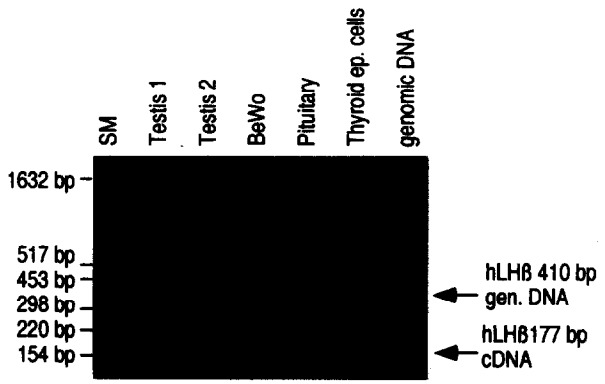


Fig. 3. Amplification of hLHβ cDNA by RT-PCR using the primers described in material and methods. The cDNAs of two different human testes, pituitary derived cDNA and the BeWo choriocarcinoma cDNA yielded a PCR-fragment at the calculated size of 177 bp visualized by ethidium bromide staining after electrophoresis in 2% agarose. Human thyroid epithelial cells served as a negative control. The predicted size of the PCR-generated genomic DNA was 410 bp.

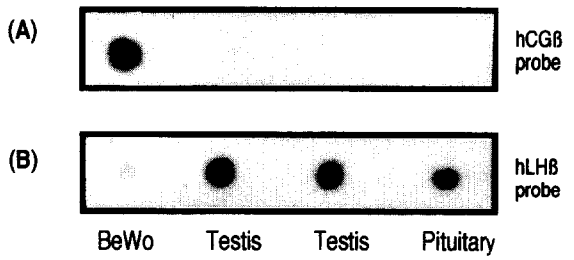


Fig. 4. Dot blot analysis of the 177 bp hLH$\beta$-PCR fragment shown in Fig. 3. Two $\mu$l each of the purified PCR-products were dotted on a nylon membrane and hybridized with either (A) a hCG$\beta$-specific (position 178–194) or (B) a hLH$\beta$-specific (position 178–194) $^{32}$P-labeled oligonucleotide. Clearly, human testes as well as the pituitary but not the choriocarcinoma cell-line BeWo express hLH$\beta$. The positive signal obtained with BeWo cDNA with the hCG$\beta$-probe results from cross-amplification of the highly abundant, homologous hCG$\beta$ nucleotide sequence by the LH$\beta$-primers.

munoassays in cytosolic testicular extracts they might have been produced in the periphery and accumulated locally by testicular LH/CG-receptors. But as proven by the sensitive RT-PCR technique and subsequent Southern blot and Dot Blot analysis hCG$\beta$ and hLH$\beta$ are of genuine testicular origin and not metabolic degradation products of the Leydig cell.

Apart from the primary hypophyseal support of Sertoli and Leydig cell function by follicle stimulating hormone (FSH) and hLH there is increasing evidence that testicular cell–cell interactions are of major importance for testicular function [11]. In addition to testosterone acting as essential paracrine factor for spermatogenesis a number of potential autocrine or paracrine regulatory substances such as $\beta$-endorphin, enkephalins, dynorphins, oxytocin, activin, LH releasing hormone (LHRH), insulin like growth factor I and other growth factors have been identified in the testis of various mamalian species [11–13,27]. Moreover, it is now well recognized that originally pituitary derived hormones are not confined to this organ but are eutopically synthesized, e.g. in the myometrium or in activated T-lymphocytes as it is the case for prolactin [28]. The same is true for the classical placental derived pregnancy hormone hCG and for the pituitary hormone LH: the physiological eutopic production of hCG$\beta$ and hLH$\beta$ in the human testis has now been demonstrated for the first time at both the level of mRNA and the protein level.

Our findings raise two important questions: First, which testicular cells do hCG$\beta$ and hLH$\beta$ originate from and second, what is the biological function of these molecules? As testicular LHRH has been suggested to be a secretory product of the Sertoli cell acting on the Leydig cell [12] one could speculate that the latter is the site of hCG$\beta$ and/or hLH$\beta$ production in the testis. Thus, in analogy to the placenta and the pituitary, hCG- or hLH-production could be stimulated by LHRH in order to sustain basal testosterone secretion. On the other hand, we immunologically have picked up most likely only part of the hCG$\beta$ gene derived proteins as indicated by RT-PCR alternative splicing products which might give rise to entirely different protein sequences [8]. These putative molecules probably will not be able to assemble with hCG$\alpha$ to holo-hCG and therefore might have currently unknown autocrine/paracrine functions. Thus, the paradigms of purely systemical hormonal functions during pregnancy or the female cycle of eutopic placental-derived hCG or pituitary hLH and of accidental ectopic hCG production need to be reassessed.

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