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Expression and Production of Human Chorionic Gonadotropin (hCG) in the Normal Secretory Endometrium: Evidence of *CGB7* and/or *CGB6* Beta hCG Subunit Gene Expression[1]

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ABSTRACT

We have previously confirmed glandular cell *CGB* and *CGA* subunit mRNA gene expression as well as the expression of their dimeric and single-subunit human chorionic gonadotropin (hCG) proteins in normal secretory transformed endometrium. The objective of this study was to investigate the endometrial epithelial gene locus of the human hCG/LH gene cluster from *CGB* genes responsible for gene expression. For this study, endometrial specimens were selected from women characterized using our endometrium score and hCG staining index that had normal secretory transformed endometrium and optimal hCG staining. Using full-length *CGB* mRNA sequence analysis, we found that epithelial *CGB* is (co)expressed as the product of gene locus *CGB7* and *CGB6* (48%), as single *CGB7* (42%), or to a lower percentage as single *CGB6* (10%). In addition to known differences between these genes and *CGB5*, the nucleotide sequence of the mRNA differs between *CGB7* and *CGB6* in the untranslated promoter region and in translated exon 2. Immunohistochemical results show that endometrial joint *CGB7* and *CGB6*, single *CGB7*, and single *CGB6* mRNA expression lead to the release of endometrial hCG. Gene-specific antibodies for CGB7 reveal secretory endometrial hCG production, which is not observed for gene-specific CGB5 antibodies, whereas the placenta is positive for CGB5 and negative for CGB7 antibody as revealed by immunohistochemistry and Western blot hCG isoform analysis. Only endometrial *CGB7* expression seems to be supported specifically by secretory endometrial transcription factors. In conclusion, epithelial hCG is expressed and produced as CGB7 and/or CGB6 but not CGB5, and it is produced together with CGA as a secretory transformation marker in the normal secretory phase endometrium.

endometrium, female reproduction tract, human chorionic gonadotropin (hCG/hCG receptor), menstrual cycle, uterus

INTRODUCTION

Human chorionic gonadotropin (hCG) is composed of two noncovalently bound subunits, alpha-CG (CGA) and beta-CG (CGB) [1]. The *CGA* subunit is encoded by a single gene located on chromosome 6q21.1-q23, but the *CGB* subunit molecule is encoded by any one of the six nonallelic genes, *CGB8*, *CGB7*, *CGB5*, *CGB3 (CGB)*, *CGB2*, and *CGB1*, present on chromosome 19q13.32 [2–4]. Another gene, *CGB6*, has been described previously as an allele of *CGB7* with differences in the 5′ untranslated region sequence within exon 1 [5, 6]. All *CGB* genes are arranged in a gene cluster structure and are linked to a single highly homologous gene encoding the beta subunit of luteinizing hormone (*LHB4*). This *CGB*/*LHB* gene cluster arose from the only ancestral *LHB* gene with segmental duplications, which is evolutionarily conserved and is only expressed in human and nonhuman primates [7]. With regard to evolution, the *CGB7* and *CGB6* subunits are described as the oldest phenotypic *CGB* variations, whereas the *CGB5, CGB3*, and *CGB8* nucleotide structure variations comply with more recent gene locus development for hormone transcription and translation [8, 9].

The specificity of the hCG hormone is mediated by the beta subunit. Trophoblastic tissue almost exclusively expresses *CGB5*, *CGB3*, and *CGB8* (*tCGB* or type II) [1, 4]. However, hCG and/or its subunit CGB are also produced at low quantities by the epithelia of many normal healthy tissues, particularly in the intestinal, urinary, and respiratory tracts and by the pituitary, testis, and fallopian tubes [10–13]. Normal nontrophoblastic tissues such as breast, lung, prostate, bladder, and colon almost exclusively express *CGB7* (*eCGB* or type I) [10]. In healthy men and women who are not pregnant, very low peripheral hCG concentrations and even lower free CGB concentrations have been observed [14]. Human chorionic gonadotropin and LH bind to the hCG/LH receptor that has been identified in the endometrium [15, 16] and in several gonadal and nongonadal tissues [17]. The placental CGA and CGB subunit molecules are characterized by a specific *N*-linked glycosylation pattern, and the CGB molecule is characterized by an additional specific *O*-linked glycosylation pattern in the C-terminal peptide (CTP) [18, 19]. The free CGA and CGB proteins combine to form intact biologically active hCG. The bioactivity and receptor binding depend on glycosylation [20, 21].

In initial immunohistochemical and in situ hybridization experiments, we showed that glandular cells of the normal cyclic healthy endometrium express and produce the CGB subunit primarily during the secretory phase of the uterine epithelium [22, 23]. Recently, we confirmed both *CGA* mRNA and *CGB* mRNA gene expression, detected the CGB and CGA subunits and dimeric hCG protein isoforms by electrophoretic

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separation, measured the hCG and CGB hormone concentrations in endometrial homogenates, and immunohistochemically characterized increases in hCG production in synchrony with the transformation of the secretory endometrium [24]. We found that glandular hCG release is diminished or absent, respectively, in dyssynchronously transforming or nontransforming secretory cycle phase endometria. The demonstrated ability of the cyclic secretory endometrium to produce a high level of epithelial hCG production is intensified in the glandular epithelium of early decidua [25].

The objective of this study was to investigate the origin of epithelial *CGB* mRNA gene expression and hCG production (*CGB7*, *CGB6* or *CGB5*, *CGB8*, and *CGB3*) in gene cluster using gene-specific primers for the total mRNA sequence analysis and our gene-specific CGB7 or CGB5 antibodies for hCG release in normal, healthy transforming secretory endometrium.

MATERIALS AND METHODS

Human Subjects and Tissue Processing

To study gene-specific hCG expression and hormone production, we used endometrial biopsy tissues selected from patients undergoing clinical diagnosis of infertility. Endometrial samples in the patient group were from couples experiencing infertility due solely to tubal damage or a male factor. The specimens were collected after cervical dilatation and endometrial biopsy. Samples from patients who underwent hysterectomy for benign gynecological conditions other than endometrial diseases were added to the study to increase the number of proliferative and in-phase early secretory phase endometrial specimens. Endometrial specimens were disregarded if the women had received exogenous hormones during previous months. All biopsies were obtained after receiving written informed consent from the patient and approval from the Medical Ethics Committee of the University of Leipzig. First, these tissue samples were routinely staged by independent histological scoring by an experienced pathologist using criteria for the secretory endometrium [26]. Progesterone levels were measured on the day of the biopsy.

A total of 581 patient endometrium samples used in our recent study [24], consisting of a variety of normal, dyssynchronous, or missing endometrial secretory transformation specimens, were also analyzed histologically according to our endometrium score parameters proposed in that study (glandular shape, glandular nucleus, stromal differentiation, leukocyte number; each scored 1–6 and summarized). After this analysis only the 145 patient samples identified as normal, synchronously transformed secretory endometria were selected and included in the present study. The samples were subdivided into patient groups of the early secretory (n = 42, score 8–12), midsecretory (n = 35, score 12–16), late secretory (n = 30, score 16–20) and late predecidual secretory (n = 38, score 20–24) endometrial phases [24].

After collection, the endometrial tissue samples were immediately rinsed with saline to remove blood and were divided into several aliquots. For histological and immunohistochemical evaluation, the tissues were immediately fixed in 4% neutral buffered formalin overnight and then embedded in paraffin. For total RNA extraction and subsequent RT-PCR procedure such as *CGB* gene 7 (*CGB7*) and *CGB* gene 6 (*CGB6*) cDNA sequence analyses, samples were immediately submerged in the RNA stabilization reagent RNA-later (Qiagen) and then rapidly frozen and stored at −80°C. Total RNA isolated from the selected endometrial tissues and from the early pregnancy placental tissue as a control was extracted using Trizol reagent (Gibco) according to the manufacturer's instructions. The pellets were dissolved in DE-PC-treated water, and 30-μl RNA samples were quantified and stored as small aliquots at −80°C. For the tissue homogenization and the preparation of supernatants used for Western blot detection, approximately 30 mg of prewashed fresh or −80°C frozen samples was resuspended in 500 μl ice-cold lysis buffer of 50 mM Tris-HCl buffer, pH 7.6, using the Complete Protease Inhibitor Kit (Roche), and were homogenized on ice as previously described [24]. The supernatants were prepared by repeated centrifugation of the homogenates at 19 000 × *g* and 4°C and were frozen immediately at −20°C until examination.

Reverse Transcription and PCR

Total RNA (2 μg) was treated with RNAse-free DNAse (Roche). A 2.5-μl aliquot was heated at 65°C to eliminate DNAse and unfold the RNA, followed by cooling at 4°C. A 2.5-μl aliquot of the cDNA reaction mixture prepared according to the manufacturer's instructions (Roche) was added as previously described [24]. The reverse transcription reaction was carried out using a Perkin Elmer thermocycler under the conditions of 25°C for 10 min, 42°C for 60 min, and finally 5 min at 94°C to destroy the reverse transcriptase activity. The PCR procedure was performed immediately after reverse transcription in the same tube by adding 20 μl of PCR mixture as previously described [24]. Amplification of cDNA was performed using a common *CGB* gene primer pair to capture all of the specific *CGB* subunits expressed from *CGB* gene 3 (*CGB3*), gene 5, gene 6, gene 7, and gene 8 [3, 5–7] that should be expected to appear in amplicons of 548 and 378 bp as well as using a *CGA* primer pair [2] as demonstrated in Table 1 (*CGB*, *CGB3*, NM_000737.2; *CGB5*, NM_033043.1; *CGB6*, M13505.1, X00266; *CGB7*, NM_033142.1, M13503.1; *CGB8*, NM_033183.1; *CGA*, NM_000735). Additional gene-specific *CGB* primer pairs were included to differentiate *CGB5* from joint *CGB7* and *CGB6* gene expression. Additionally, the gene expression of the single *CGB7* or single *CGB6* gene amplicons was determined (Table 1). The resulting PCR amplicons represent the indicated portions of *CGB* RNA because the primers were located in different exons (with the exception of one amplicon localized in a single exon that was detected with and without avian myoblastosis virus reverse transcriptase). To exclude the possibility of amplification of contaminating genomic DNA despite DNAse treatment, PCR was also performed excluding reverse transcriptase from the cDNA step for each primer set. A negative control reaction in which no RNA or cDNA template was added to the reaction mixture was included in each experiment. Placental RNA was applied to each primer set amplification as a positive control. All of the amplification reagents were obtained from Roche. All of the oligonucleotide primer pairs were synthesized by Applied Biosystems. Nine-microliter aliquots of the PCR products were electrophoresed in a 2.0% agarose gel in 50 mM Tris-buffered 150 mM saline (TBS) buffer, pH 7.40, and were imaged for documentation by UV as previously described [24].

Sequence Analysis of the Total Endometrial CGB mRNA Subunit

Endometrium samples from the patient group (n = 145) of normal, synchronously transformed secretory endometria were included in the full-length mRNA sequence analysis of the epithelial *CGB* subunit. Total RNA extraction, cDNA reverse transcription, and RT-PCR were carried out as previously described. Common *CGB* gene primer pairs were chosen (Table 1), resulting in the amplification of the complete *CGB* subunit gene containing amplicons of 548 bp (spanning exons 1–2) and 378 bp (exons 2–3). Both *CGB* amplification reaction products were cut out of the gel and purified using the QIAquick gel extraction kit (Qiagen) to clean up the cDNA fragments. Sequence analysis of the RT-PCR-amplified *CGB* fragments was performed using the same oligonucleotides that were used as PCR primers (548 and 378 bp) to distinguish the expression of different *CGB* genes. The RT-PCR products were sequenced in both the sense and antisense orientations using an ABI 3100 Automated Capillary DNA Sequencer that supports competitive sequencing and cDNA fragment analysis. The samples were processed using the BigDye Terminator Cycle Sequencing Kit from Applied Biosystems.

Production of CGB7- and CGB5-Specific Antibodies

The present commercially available hCG antibodies cannot generally differentiate between the endometrial and placental hCG release. To obtain polyclonal antibodies that specifically recognize the epithelial endometrial and decidual CGB7 subunit or the placental CGB5 subunit, the synthetic oligopeptides P1 and P2 or C1 and C2 were used as antigens with high antigenic activities (Table 2). The resulting CGB7 as well as the CGB5 antibodies are directed against the CGB epitope beta 1 (amino acid [aa] 1–15) on the N-terminus of exon 2 or the CGB epitope beta 9 (aa 110–119) near the CTP of the CGB protein chain in exon 3 [3, 6, 7, 27]. The highly purified peptides P1, P2, C1, and C2 were purchased from Biotrend Corporation in Cologne, which also supported our antibody production. According to the standard immunization protocols for producing polyclonal antibodies, 12-wk-old rabbits were injected i.p. with 500 μl of the following different solutions: two rabbits each received 200 μg of the keyhole limpet hemocyanin carrier-bonded peptides P1 (animals Z51, Z52), C1 (Z53, Z54), P2 (Z55, Z56), or C2 (Z57, Z58) in 0.1% NaCl with 1:1 incomplete adjuvant for the initial immunization injection. After 7 and 14 days, the injections were repeated with the same sample size solution (booster shots). The last shot was administered on day 28. A 20-ml blood sample was removed from each rabbit on day 35. The periodic boosts and bleedings were continued for 4 mo with satisfactory immune responses.

For the antibody titer detection in preimmune and anti-CGB immune serum, the bovine serum albumin-conjugated peptides P1, P2, C1, and C2 diluted to 10 μg/ml in 100 mM sodium carbonate/bicarbonate coating buffer pH

TABLE 1. Oligonucleotide primer pairs for total gene *CGB*, *CGB7*, *CGB6*, *CGB5*, and *CGA*.*

No.	Gene	Primer location bp	Exon	Strand	Nucleotide sequence	Amplicon bp	Paired no.
1	*CGB*	−353/−337	1	Sense	5′-TCGGGTCACGGCCTCCT-3′	548	3
2	*CGB*	108/127	2	Sense	5′-GGCTGTGGAGAAGGAGGGCT-3′	378	4
3	*CGB*	195/178	2,3	Antisense	5′-CAGCACGCGGGTCATGGT-3′		
4	*CGB*	484/468	3	Antisense	5′-TCGGGGTGTCCGAGGGC-3′		
5	*CGA*	83/102		Sense	5′-TGCAGGATTGCCCAGAATGC-3′	231	6
6	*CGA*	313/294		Antisense	5′-CCGTGTGGTTCTCCACTTTG-3′		
7	*CGB7/6*	−346/−325	1	Sense	5′-ACGGCCTCCTCCTGGTTCCCAA-3′	543	3
8	*CGB5*	−346/−325	1	Sense	5′-ACGGCCTCCTCCTGGCTCCCAG-3′	543	3
9	*CGB7/6*	−192/−173	1	Sense	5′-AGACCACTGAGGGGAGAGGA-3′	386	3
10	*CGB5*	−192/−173	1	Sense	5′-GGACCAGTGAGAGGAGAGGG-3′	386	3
11	*CGB7*	53/72	2	Sense	5′-CATGGGCATCCAGGGAGATG-3′	143	3
12	*CGB5*	53/72	2	Sense	5′-CATGGGCATCCAAGGAGCCG-3′	143	3
13	*CGB7/6*	391/410	3	Sense	5′-GATGACCCCCGCTTCCAGGC-3′	94	4
14	*CGB5*	391/410	3	Sense	5′-GATGACCCCCGCTTCCAGGA-3′	94	4
15	*CGB7*	−63/46	1	Sense	5′-CCTTGACGCCCCCACAAA-3′	258	3
16	*CGB6*	−62/46	1	Sense	5′-CTTGCCGCCCCCACAAC-3′	257	3

* The sense and antisense primer pairs used in reverse transcription PCR amplification of endometrial and placental hCG are shown.

9.6 were adsorbed in 50 μl per well onto the Maxisorp ELISA carrier (Nunc) via overnight incubation followed by removal and multiple washes with PBS containing 0.1% Tween 20 (PBS-T) and 0.05% Triton X. The antigen-prepared carrier plates were incubated with the respective immune sera Z51 to Z58 at eight logarithmic dilutions up to 1:250 000, washed with PBS-T, incubated with biotinylated anti-rabbit IgG (Dako) and washed again. To each well, 100 μl of 0.05% hydrogen peroxide, 50 mM natrium acetate, pH 4.5, and 0.12 mg/ml tetramethyl benzidine were added. After a 15-min incubation, the reaction was stopped by adding 500 μl of sulfuric acid. The absorption was determined at 450 nm (with reference to 630 nm).

For the immunoaffinity chromatographic purification of anti-peptide antibodies from antiserum, the respective peptides were immobilized on a gel column in accordance with the manufacturer's procedure (Pierce). The antiserum Z51 was incubated on a CGB7-peptide coupled Sepharose column equilibrated with PBS buffer at room temperature for 60 min. The bound CGB7 antibody was eluted using 100 mM glycine buffer, pH 2.5–3.0. In a second step, the TRIS buffer-neutralized purified CGB7-antibody eluate was applied to a CGB5-peptide coupled Sepharose column under the described conditions to separate it by binding to any remaining CGB5 antibody. Analogously, the monospecific purified *CGB5* antibody was produced using CGB5 and applied to a CGB7 peptide coupled to a Sepharose column for fine purification.

Immunohistochemistry

Paraffin-embedded tissue samples from the normal secretory endometrium and from an early pregnancy placenta as a control were cut into 4-μm-thick serial tissue sections, mounted on superfrost slides, deparaffinized, cleared in xylene, rehydrated in ethanol, and incubated in TBS as previously described [24]. The sections were incubated with 0.3% fresh hydrogen peroxide to block endogenous peroxidase activity. To immunolocalize the general hCG release in the normal secretory endometrium, the epithelial hCG was stained immunohistochemically using the pan-specific polyclonal antibody A0231 (Dako) diluted 1:500 in TBS-T/normal goat serum (NGS) as previously described [24]. The successive incubation and staining steps for the treatment of the tissue sections were carried out according to the manufacturer's instructions (Elite ABC Kit; Vectostain Labs). Negative controls consisted of samples for which the primary hCG antibodies were omitted from the TBS-T/NGS solution, and a positive control section of placental tissue was included for all the primary antibodies used in every staining protocol described for endometrial tissues.

The specific antibodies developed above were used to observe the immunolocalization of either CGB7 or CGB5 subunit-bound hCG. The immunohistochemical staining of normal secretory endometrial hCG was performed using the described CGB7 gene-specific polyclonal antibody Z51. To examine the staining of early gestation placental hCG, the CGB5 gene-specific polyclonal antibody Z53 was used. After blocking the endogenous peroxidase, avidin-biotin, and nonspecific antibody binding activities as described, both primary CGB7 or CGB5 antibodies (diluted 1:5000 in TBS-T/NGS) were incubated with serial tissue sections at 4°C overnight, followed by treatment with biotinylated secondary rabbit anti-mouse IgG antibody and the visualization of peroxidase activity according to the instructions included in the Elite ABC Kit. The placental CGB5 antibody was used as a negative control for endometrial CGB7 staining.

Western Blotting

Fresh or frozen homogenate supernatants of normal secretory endometrium tissues prepared above were examined by Western blot. Endometrial protein concentrations were measured in homogenate supernatants using the BCA Protein Assay (Pierce) with values ranging from 2 to 6 mg/ml. Aliquots of endometrial supernatants, placental supernatants, or dilutions of a pure placental hCG product (Chemicon) were diluted in reducing probe buffer (Roth) containing SDS, glycerol, and mercaptoethanol and boiled at 95°C for 5 min. The hCG controls, endometrial proteins, a low-molecular-weight protein standard mixture, and a rainbow standard (Pharmacia) were size separated by 10% SDS-PAGE and transferred to nitrocellulose by electroblotting. The resulting membranes were incubated at 4°C overnight with polyclonal primary rabbit anti-CGB (A0231; Dako) at a dilution of 1:500, with monoclonal primary mouse anti-CGB (INN22, Serotec) at 1:100, or with polyclonal gene-specific primary rabbit anti-CGB7 (Z51, Z55) and anti-CGB5 (Z53, Z58) antibodies at 1:500, followed by incubation with a biotinylated secondary goat anti-rabbit (1:2000) antibody for 1 h at room temperature. The membranes were then incubated with the ABC complex (Vectostain Labs). The different molecular forms of endometrial and placental hCG were detected by visualization with diaminobenzidine (DAB) staining under the same conditions as used for immunohistochemistry and were correlated with the molecular weight markers. Purified dimeric hCG products were obtained from Chemicon for use as positive and negative controls.

RESULTS

This study showed that the *CGB* gene sequence reported to be expressed in human placental tissue ranging from the transcription start site at −366 bp in the promoter region to the transcription stop site at 495 bp in the CTP of exon 3 [1, 3, 5–7] is also expressed in human secretory endometrial tissue (Figs. 1 and 2A). Different *CGB* primer pairs were selected for the identification of the expressed endometrial and placental *CGB* subunits whose amplicons covered either generally or gene specifically the full length expressed range of exons 1–3 (Table 1). Using pan-specific oligonucleotide primer pairs (for all *CGB5*, *CGB6*, and *CGB7*), we established the *CGB* cDNA amplification products of 548 and 378 bp in the normal secretory transformed endometrium. Whereas the primer pair for the 548-bp product covers the cDNA of exons 1 and 2, the second pair results in the amplification of the 378-bp amplicon of exons 2 and 3. The expression of the *CGA* subunit mRNA was also confirmed in the secretory endometrium (Fig. 2B). The lack of production of *CGA* cDNA when reverse transcriptase was omitted from RT-PCR test reactions demonstrates that hCG may be released in synchronously transformed secretory endometrium. Placental tissues of early pregnancy were used as positive controls for the expression of hCG in the RT-PCR assay (Fig. 2B).

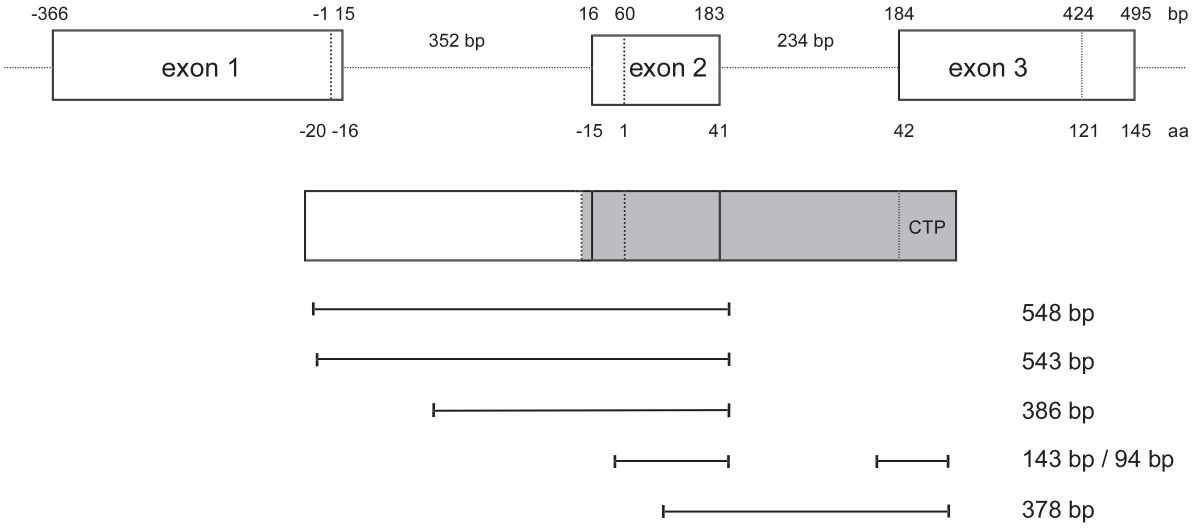


FIG. 1. *CGB* gene expression in the human secretory endometrium. The promoter region (exon 1) and structural genes (exons 2 and 3) are indicated within the full-length beta hCG subunit mRNA from −366 to +495 bp as shown with total gene *CGB* cDNA amplicons (548 and 378 bp), *CGB7*- and *CGB6*- or *CGB5*-specific cDNA amplicons (543, 386, and 94 bp), and *CGB7*- or *CGB5*- and *CGB6*-specific cDNA amplicons (143 bp). This gene leads to the expression of the CGB prehormone subunit (−20 to 145 aa) and the CGB hormone subunit (1 to 145 aa). CTP, C-terminal peptide of the CGB subunit.

CGB7 *and* CGB6 *mRNA Expression with CGA in the Normal Secretory Endometrium*

Only the *CGB* genes 3, 5, 6, 7, and 8 encode a molecule that consists of 145 amino acids. To determine which genes are expressed in the secretory endometrium, we examined the transcription of hCG mRNA and the corresponding release of dimer hCG protein in patients using gene-specific oligonucleotide primers and antibodies. Table 1 lists the gene-specific oligonucleotide primer pairs with their resulting *CGB* amplicon lengths that cover exons 1–3 and indicate gene expression by either *CGB5* or *CGB7* and/or *CGB6* in the control tissue or endometrium samples. The resulting RT-PCR products prove that the *CGB5* subunit (and also *CGB3* and *CGB8*) is expressed in the placenta, without the presence of detectable placental *CGB7* and/or *CGB6* (Fig. 2, D and F). However, in patient samples from normal secretory endometrium amplicons of 543, 386, 143, and 94 bp were detected, but this indicates only that the *CGB7* and/or *CGB6* subunits are expressed (Fig. 2, C and E). The first three endometrial cDNA amplicons always capture at least two of the *CGB* exons 1–3. For the 94-bp amplicon of only exon 3, control amplifications with and without reverse transcriptase indicate the *CGB7* and/or *CGB6* mRNA expression. To separately determine single *CGB7* or single *CGB6* gene expression, the 386-bp amplicon cDNA product (Fig. 2C, line 5) of different endometrial tissue samples is assigned after gel extraction into a cDNA-nested PCR amplification. The oligonucleotide primers 15 or 16 (Table 1) let us recognize solely *CGB7* or *CGB6* gel chromatographically (Fig. 3).

Full-Length Sequence Analysis of Endometrial CGB7 *and* CGB6 *mRNA Gene Expression*

The recently described *CGB*-subunit mRNA in the secretory endometrium [24] was sequenced in this study using the two *CGB* cDNA amplicons of 548 and 378 bp in tissue samples from normal secretory endometrium. The selected pan-specific primers (Table 1) recognize the expression of all of the described genes, which encode endometrial or placental *CGB*. Sequence analyses of 59 patient samples from the normal secretory transformed endometrium group (n = 145) revealed a difference in the distribution of endometrial *CGB* gene expression between *CGB7* alone, *CGB6* alone, and their coexpression. The coexpression of *CGB7* and *CGB6* appears to be most common in secretory endometrial specimens, followed by expression of *CGB7* alone and then *CGB6* alone, but no *CGB5* mRNA was observed (Table 3). The sequence analyses in endometrial specimens of the healthy menstrual cycle confirmed the full-length mRNA expression of the *CGB* subunit from the transcription start of the gene to the CTP in exon 3. Figure 4, B and C, reveals for the first time the endometrial *CGB6* mRNA expression. Figure 4, A–C, compares selected portions of the sequence of the 548-bp cDNA amplicon among the three different single or coexpression patterns of endometrial *CGB6* and *CGB7* gene expression. For the 378-bp amplicon, a nucleotide difference in the endometrial *CGB7* and *CGB6* mRNA sequences at 410 bp in exon 3 (both C versus A in placental *CGB5* mRNA) has been ascertained by sequence analysis (data not shown). The fully analyzed *CGB* mRNA sequences of the secretory endometrium are compiled as *CGB7* and *CGB6* and compared to placental *CGB5* and pituitary *LHB4* in Figure 5. The endometrial *CGB7* mRNA differs from the nucleotide sequence of *CGB5* mRNA mainly in the 5′ untranslated region of exon 1, but also in both the translating exons 2 and 3 of the coding *CGB* gene. The resulting endometrial *CGB7* subunit mRNA that we identified by sequence analysis in exons 2 and 3 differs from placental *CGB5* at +64 bp with AGA, +70 bp with ATG, and +409 bp with GCC instead of AAG, ACC, and GAC respectively (Fig. 5). Additionally, the analyzed *CGB7* and *CGB6* mRNA sequences of the secretory endometrium differ considerably, especially in the gene promoter region and to a lesser extent in the structural genes (Fig. 5).

Immunohistochemistry of Endometrial hCG Using Pan- and Gene-Specific CGB Antibodies

Thus far we have demonstrated endometrial hCG formation immunohistochemically using conventional hCG antibodies. To detect the genetic origin of hCG release in the endometrium, patient samples with a normal secretory endometrium

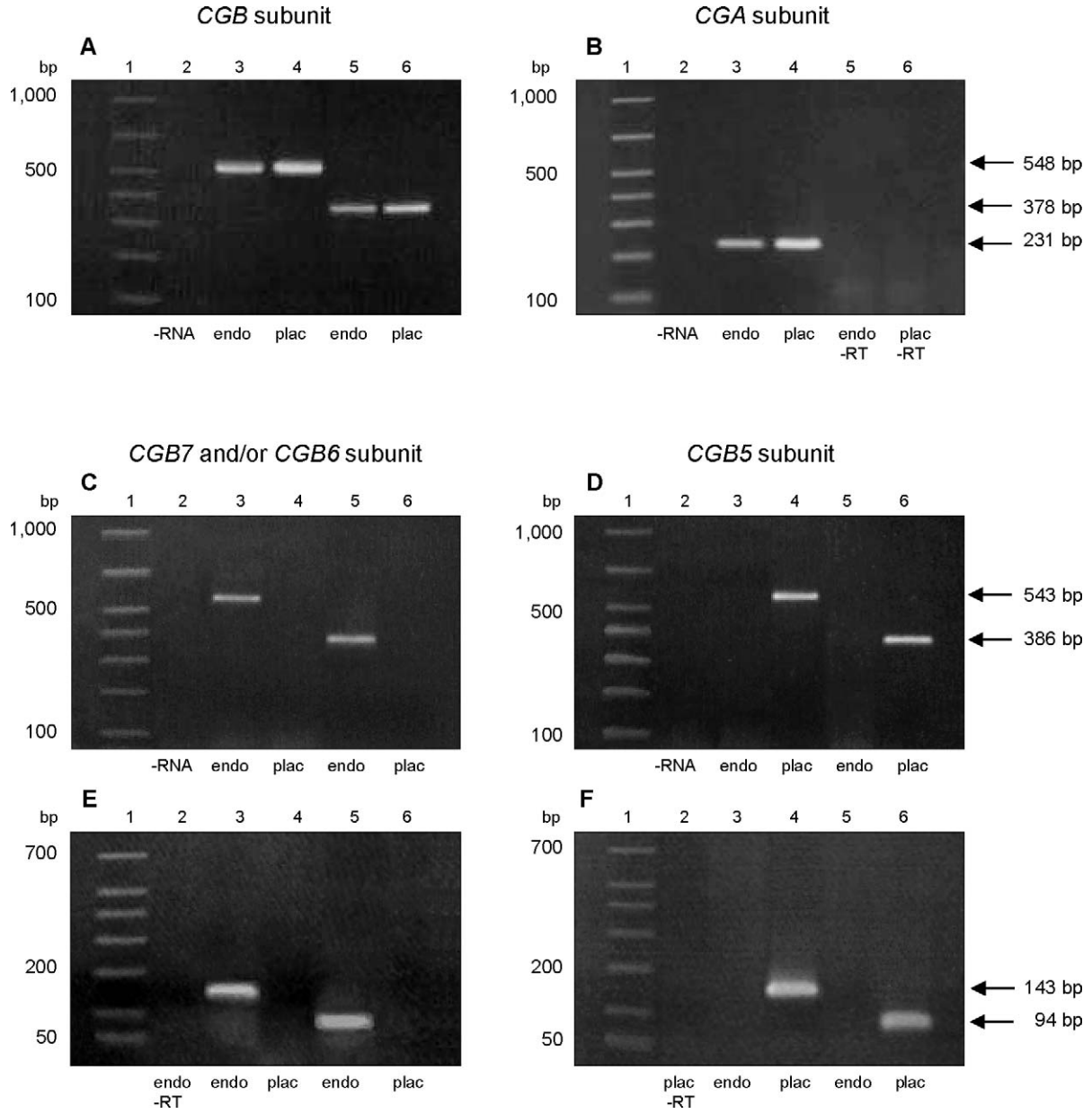


FIG. 2. Expression of full-length *CGB* subunit and *CGA* subunit mRNA (**A**, **B**) and of *CGB7* and/or *CGB6* mRNA (**C**, **E**) in the normal secretory endometrium compared to expression of *CGB5* mRNA (**D**, **F**) in the placenta. RNA isolated from endometrial and placental tissue samples was analyzed by RT-PCR using primer pairs specific to total *CGB* (**A**) or gene-specific to *CGB7*, *CGB6* (**C**, **E**), and *CGB5* (**D**, **F**). The panels show the resulting PCR products from endometrial specimens characterizing the total *CGB* subunit amplicons of 548 and 378 bp (**A**, lanes 3 and 5) and are characterized by the *CGB7* and/or *CGB6* amplicons of 543, 386, and 94 bp (**C**, lanes 3 and 5, and **E**, lane 5) and the *CGB7* amplicon of 143 bp (**E**, lane 3), but no PCR products were obtained using the *CGB5* primer pairs with the endometrial samples (**D** and **F**, lanes 3 and 5). The *CGA* subunit fragments were observed in the endometrial specimens (**B**, lane 3) similar to the placental tissue (**B**, lane 4), in contrast to the failed amplification reaction in the absence of reverse transcriptase activity (−RT, **B**, lanes 5 and 6). The *CGB5* subunit amplicons from early gestation placental tissue are shown as positive control (**D** and **F**, lanes 4 and 6), but no PCR products were obtained using the *CGB7* and/or *CGB6* primer pairs for the placental tissue samples (**C** and **E**, lanes 4 and 6). −RNA, without RNA; endo, endometrium; plac, placenta; −RT, without reverse transcriptase for the 94-bp primer pair assay.

score [24] were selected and stained immunohistochemically for *CGB7* and *CGB6* single or coexpression. The total CGB gene-recognizing hCG antibody A0231 (Dako) was used to confirm the release of secretory endometrial hCG for all of the three hCG expression possibilities determined using sequence analysis (hematoxylin/CD45 staining for endometrium score not shown) (Fig. 6, A–C).

Based on the detected differences in cDNA nucleotide sequence between the endometrial *CGB7* subunit and *CGB5* (Figs. 4 and 5), an endometrial hCG protein with three modified amino acids was translated and secreted in the gland epithelium instead of the placental CGB5. We used the amino acid (aa) differences of aa 2 and aa 4 in CGB exon 1 and aa 117 in exon 3 to produce gene-specific antibodies for both these epitopes. Oligopeptides of the CGB7 and CGB5 amino acid sequence were applied as antigens for immunization, resulting in the production of antibodies against *CGB7* and *CGB5* (Table 2). The antibody titers obtained in the immune serum were determined with CGB7- and CGB5-specific antibody titration assays, and they achieved an antibody titer larger than

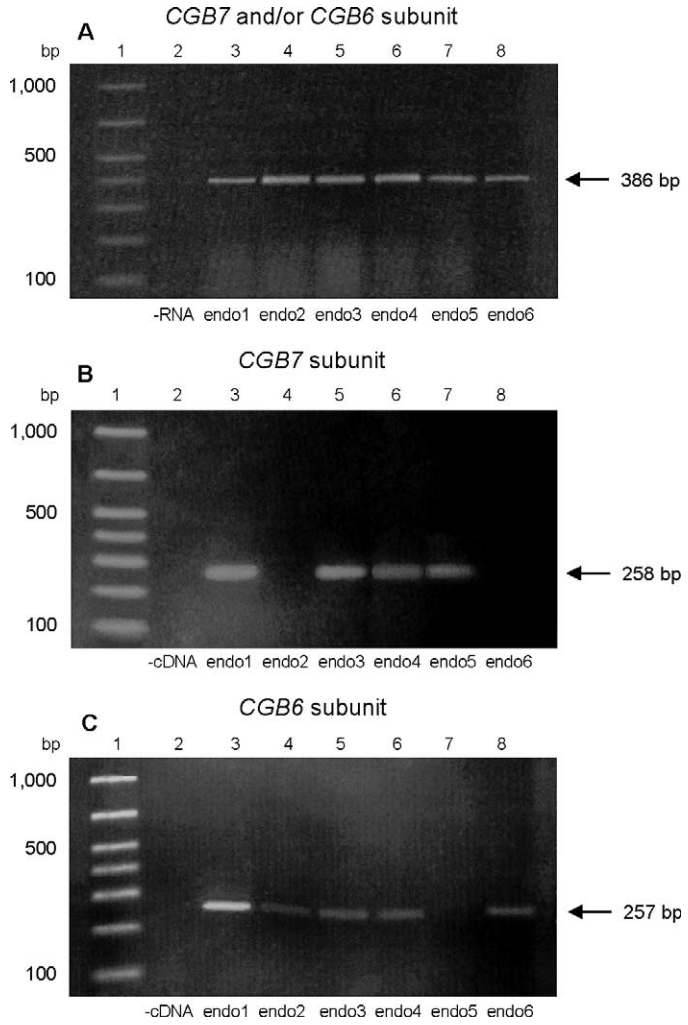


FIG. 3. Differentiation of *CGB7* and *CGB6* mRNA expression in the normal secretory endometrium. RNA isolated from different endometrial tissue samples (endo1 to endo6) was amplified by RT-PCR using the primer pair recognizing both *CGB7* and *CGB6* resulting in a 386-bp PCR product (**A**). The *CGB* amplicons were gel extracted and reamplified using the gene-specific *CGB7* primer pair with a product of 258 bp (**B**) and the gene-specific *CGB6* primer pair with a product of 257 bp (**C**). The endometrial tissue samples in lanes 4, 7, and 8 show single *CGB7* or single *CGB6* gene expression, unlike the coexpression of *CGB7* and *CGB6* seen in lanes 3, 5, and 6. −RNA, without RNA; endo, endometrium; −cDNA, without cDNA.

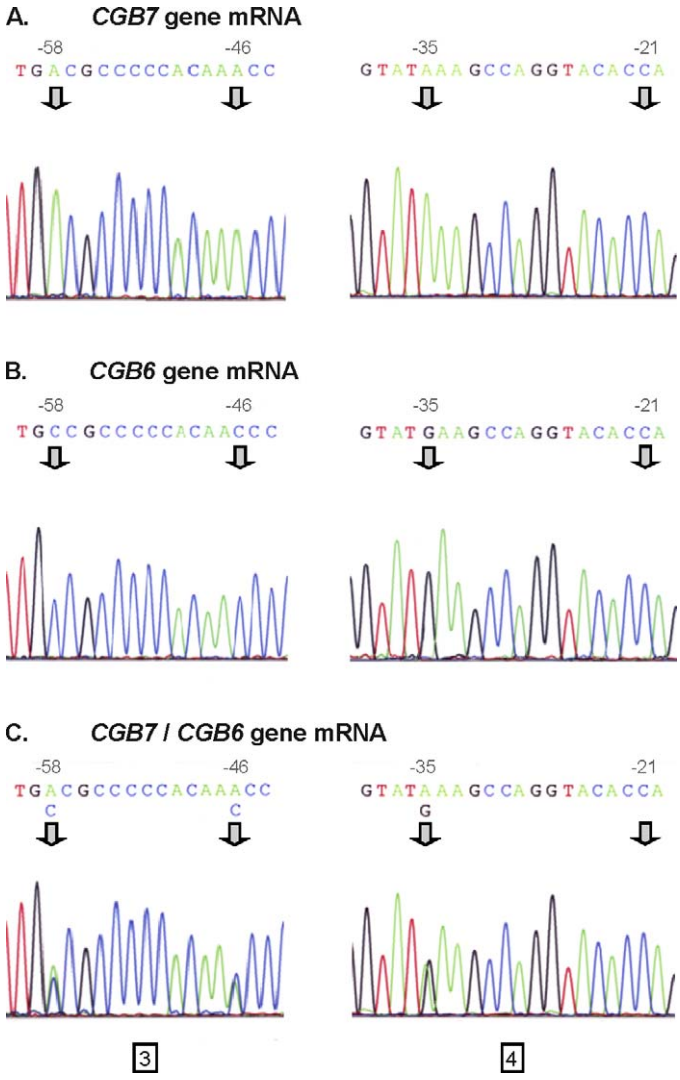


FIG. 4. Sequence analysis of total *CGB* mRNA expression in the normal secretory endometrium. *CGB7* single expression (**A**), *CGB6* single expression (**B**), and *CGB7* and *CGB6* mRNA coexpression (**C**) are observed in selected sequence segments of exon 1 (segments 3 and 4) and further segments (data not shown). The differences between endometrial *CGB7* and *CGB6* and placental *CGB5* gene expression (arrays) and between *CGB7* and *CGB6* gene expression are shown for the chosen 548-bp amplicon sections.

1:150 000 (Supplemental Figure S1, available online at www.biolreprod.org).

The new CGB7 (Z51) and CGB5 antibodies (Z53) were used for the gene-specific immunohistochemical staining of the secreted hCG in samples from normal differentiated endometrium encompassing the early to late secretory cycle phases (Fig. 7). Tissue samples from the normal synchronous secretory endometrium were selected using our endometrial score [24], defined as a sum of four evaluated single scores (hematoxylin/CD45 staining not shown). In Figure 7, staining with our CGB7 hCG-specific antibody confirms the secretion of endometrial hCG containing the CGB7 subunit, whereas no staining of CGB5 was visible in the secretory endometrium

TABLE 2. Oligopeptides for epithelial (CGB7) or placental (CGB5) antibody production.*

No.	*CGB* gene	Peptide	Exon; amino acids	Antibody
P1	*CGB7*	Ser-Arg-Glu-Met-leu-Arg-Pro-Arg-Cys-Arg-Pro-Ile-Asn-Ala-Thr	2; 1–15	Z51, Z52
C1	*CGB5*	Ser-Lys-Glu-Pro-Leu-Arg-Pro-Arg-Cys-Arg-Pro-Ile-Asn-Ala-Thr	2; 1–15	Z53, Z54
P2	*CGB7*	Cys-Asp-Asp-Pro-Arg-Phe-Gln-Ala-Ser-Ser	3; 110–119	Z55, Z56
C2	*CGB5*	Cys-Asp-Asp-Pro-Arg-Phe-Gln-Asp-Ser-Ser	3; 110–119	Z57, Z58

* The oligopeptides P1 and P2 were used as antigens to induce specific CGB7 subunit antibodies, and oligopeptides C1 and C2 were used to induce specific CGB5 subunit antibodies.

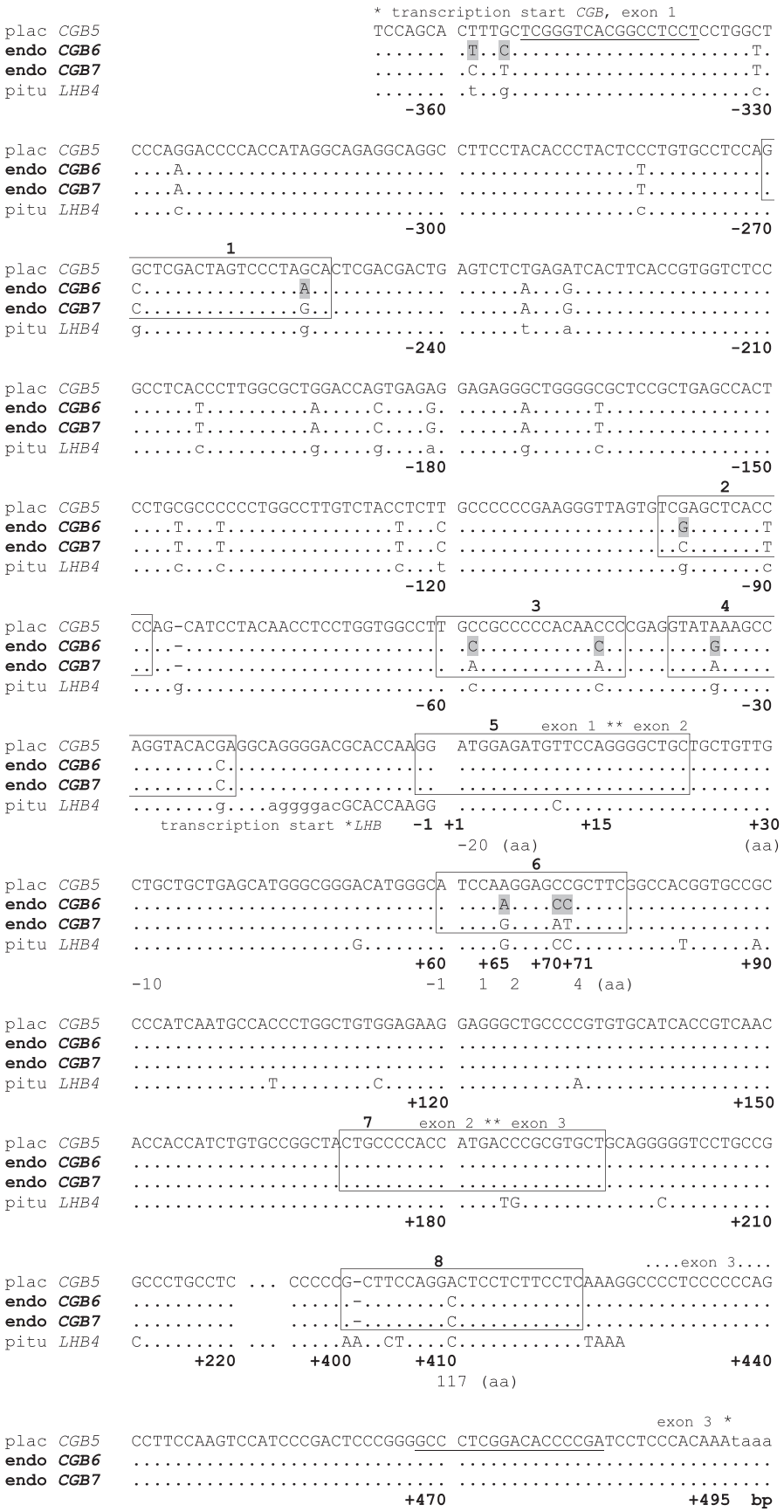


FIG. 5. Detection of *CGB* mRNA sequences in the normal secretory endometrium using the 548- and 378-bp amplicons from RT-PCR. The sequences resulting from the measured endometrial expression of *CGB7* (endo *CGB7*) and *CGB6* (endo *CGB6*) are described between the *CGB* primer 1 to primer 4 sites (underlined sequences) in comparison to the given placental *CGB5* (plac *CGB5*) and pituitary *LHB4* (pitu *LHB4*) sequences ranging from exon 1 to exon 3 (dots indicate that the sequence is identical to that of placental *CGB5*). The endometrial *CGB7* mRNA is distinguished from the placental *CGB5*

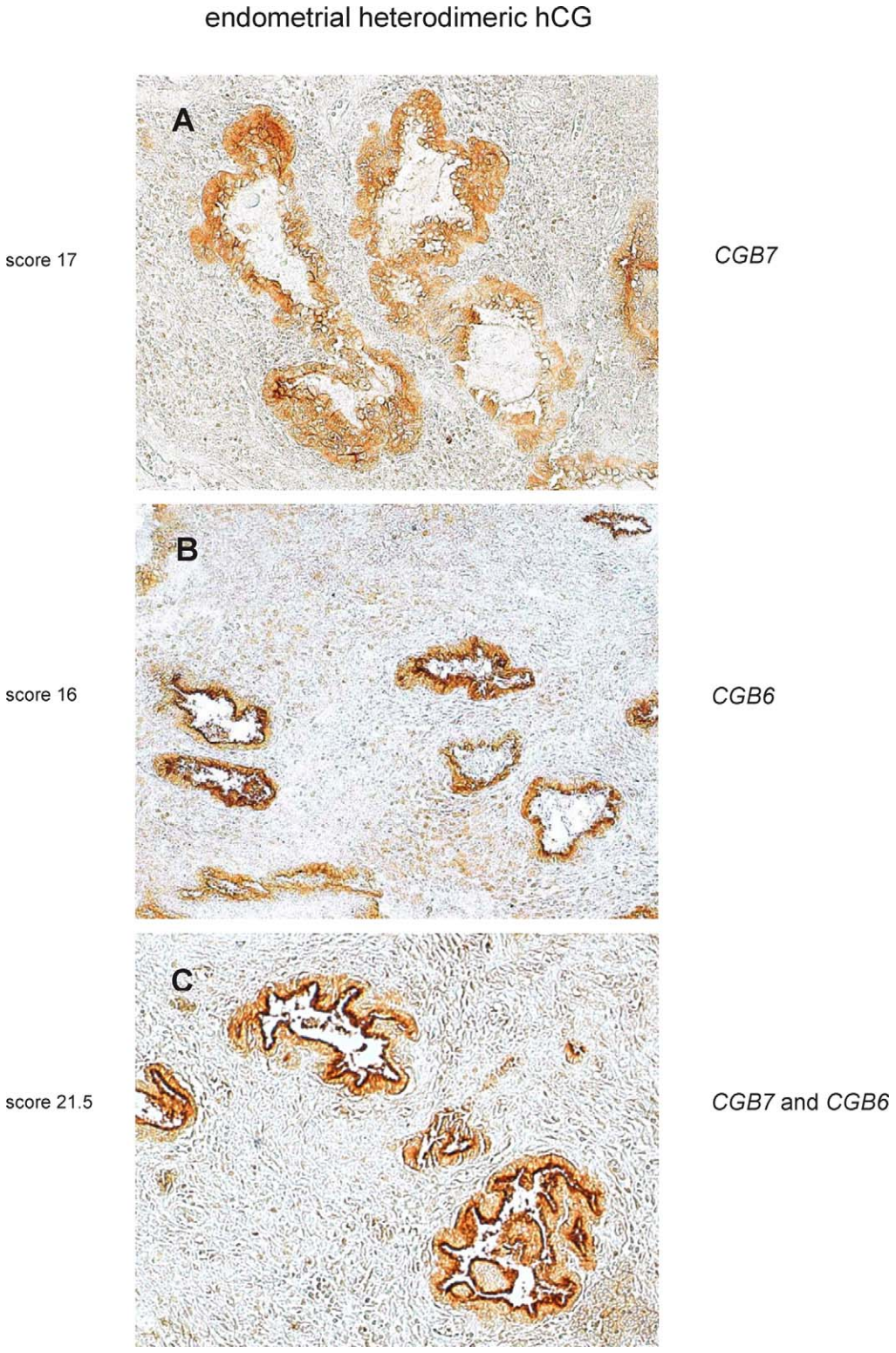
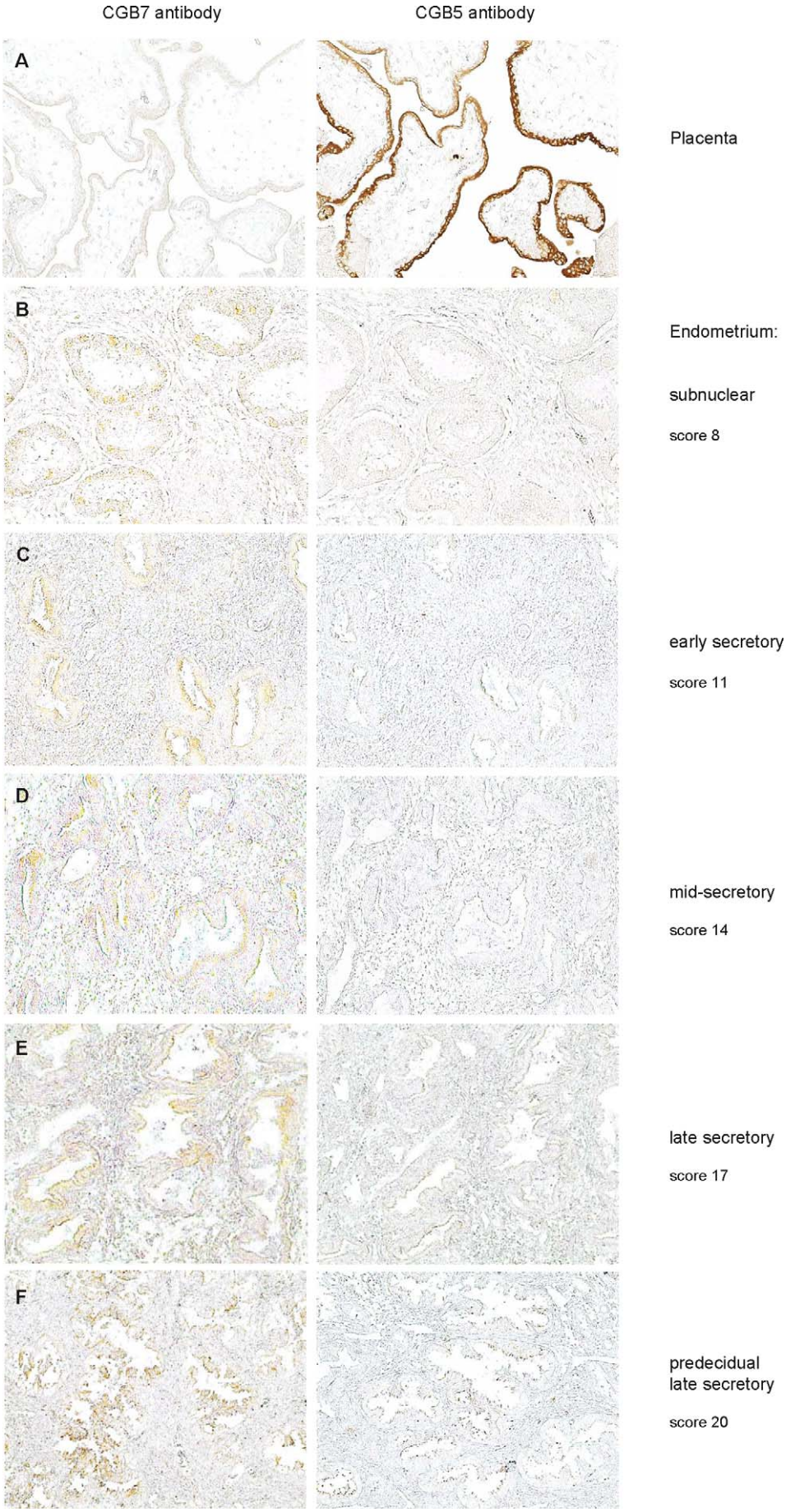


FIG. 6. Immunohistochemical detection of glandular epithelial hCG production in the normal secretory endometrium. Endometrial hCG secretion is demonstrated in patients referring with single gene expression of *CGB7* (**A**), of *CGB6* (**B**), or coexpression of *CGB7* and *CGB6* (**C**) in addition to *CGA* expression. The corresponding *CGB* gene expression patterns were analyzed using sequence analysis. All tissues were stained with the well-established polyclonal total hCG/CGB antibody A0231 (Dako); endometrial scores ranged from 4 to 24, depending on cycle phase [24].

mRNA at 23 nucleotide sites, primarily in the exon 1 region, and at four sites in the structural genes. The endometrial *CGB7* and *CGB6* mRNAs differ at 10 nucleotide sites (shaded). The marked parts of the *CGB* sequence (frames 3 and 4) are demonstrated in Figure 4 or further (frames 1, 2, and 5–8) are omitted from the figure for clarity. The endometrial sequences detected here following nucleotide +220 of exon 3 to near the transcription stop point in exon 3 (+495) are equivalent to the placental mRNA, except for +410, and are partially shown. plac, placenta; endo, endometrium; pitu, pituitary.



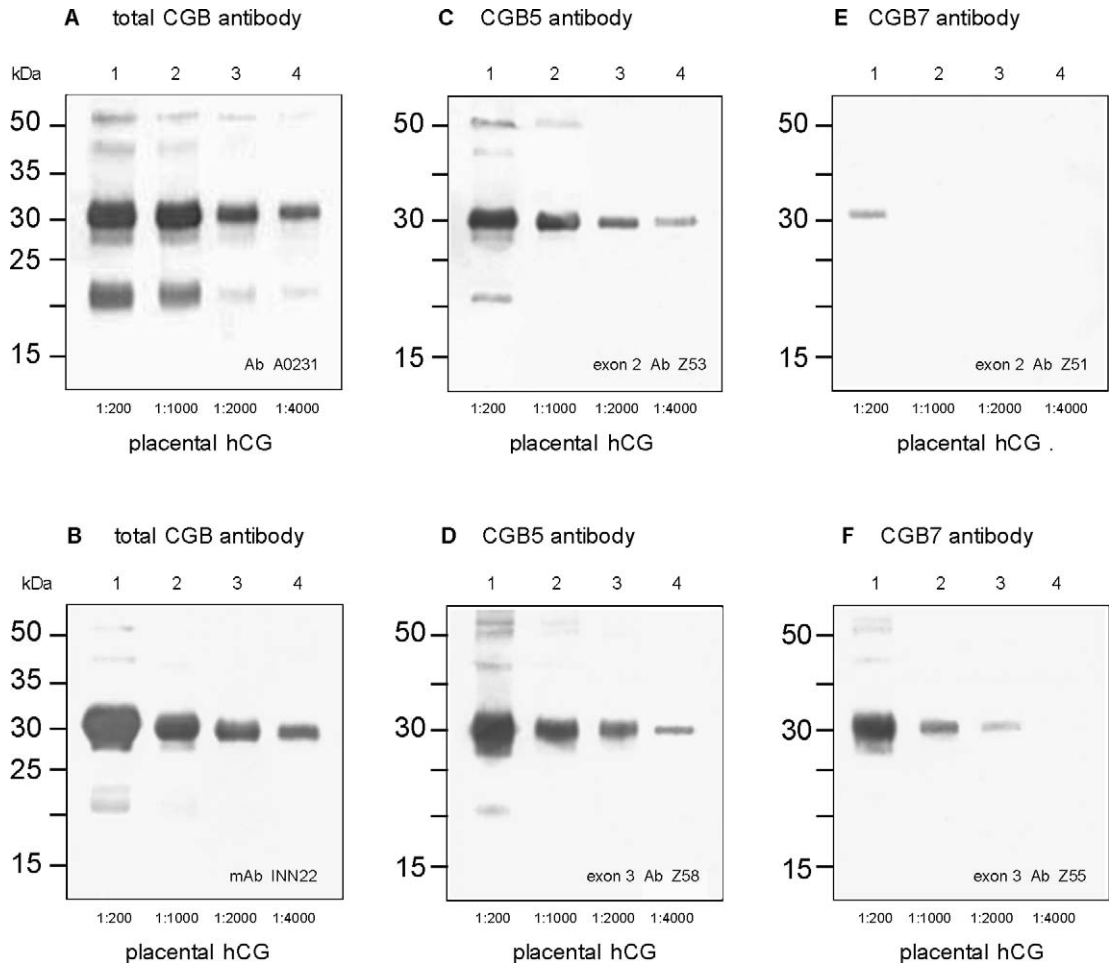


FIG. 8. SDS-polyacrylamide electrophoresis and Western blotting of highly purified placental hCG using the new gene-specific CGB antibodies. The Western immunoblot patterns of a biologically active placental hCG product (Chemicon) at declining dilutions were compared between the different hCG antibodies. The blots were visualized using the polyclonal total hCG/CGB antibody A0231 (Dako; **A**), the monoclonal hCG antibody INN22 (Serotec; **B**) and our polyclonal CGB5-specific (Ab Z53; **C**) and CGB7-specific (Ab Z51; **E**) antibody pair for the exon 2 epitope and the CGB5-specific Ab Z58 (**D**) and CGB7-specific Ab Z55 (**F**) for the exon 3 epitope under reducing conditions, followed by a biotinylated second antibody, ABC complex, and DAB staining. The CGB7 and CGB5 antibody pair raised against aa 1–15 of the current CGB subunit demonstrated sufficient specificity to differentiate gene-specific CGB expression within the physiological concentration range (**C**, **E**), but the CGB5 and CGB7 antibody pair raised against aa 110–119 near the CTP region was only able to differentiate CGB at low concentrations. Ab, antibody; mAb, monoclonal antibody.

with our CGB5 hCG-specific antibody. The CGB5-specific antibody strongly stained the human placenta, which acted as a positive control, but not the endometrium. The CGB7-specific antibody did not react in human placenta as a negative control.

Molecular Isoforms of the CGB Subunits in Western Blot Using Gene-Specific Antibodies

The CGB7- and CGB5-specific antibodies were used in comparison with the conventional total-gene CGB antibodies Ab A0231 and mAb INN22 to detect a highly purified placental hCG product (Fig. 8) and the hCG of placental and secretory endometrium homogenate supernatants (Fig. 9) by Western blot analysis after SDS-PAGE under reducing conditions. Using serial dilutions of pure hCG, the pan-specific CGB antibodies A0231 and INN22 identified the different molecular isoforms of the placental hCG product up to low concentrations (Fig. 8, A and B). In comparison, our CGB5-specific antibody of epitope aa 2 with aa 4 (Z53) identifies the purified placental hCG at comparably low hormone concentrations, whereas the corresponding CGB7-specific antibody for this epitope (Z51) reveals only a very slight cross reaction to placental CGB5 (Fig. 8, C and E, and Table 3). The CGB5-specific antibody for the epitope aa 117 (Z58) also identified the purified placental hCG product under comparable hCG dilution conditions, whereas the respective CGB7-specific antibody of epitope aa 117 (Z55) is able to discriminate

FIG. 7. Immunohistochemical staining using different gene-specific CGB antibodies for visualization of endometrial hCG production. Normal secretory endometrium tissues (**B**–**F**) showing *CGB7* expression and early gestation placenta as a control showing *CGB5* expression (**A**) were treated using our polyclonal CGB7-specific (left) and CGB5-specific (right) hCG antibodies. The endometrial CGB7 staining was first seen in subnuclear glandular vacuoles (**B**) and intensified with increasing glandular hCG secretion in the synchronously transformed early (**C**), middle (**D**), late (**E**), and predecidual late (**F**) secretory endometrium. No CGB staining was seen in the endometrium using the CGB5-specific antibody. The negative placental control shows the expected results using the CGB7-specific antibody. score, endometrial score.

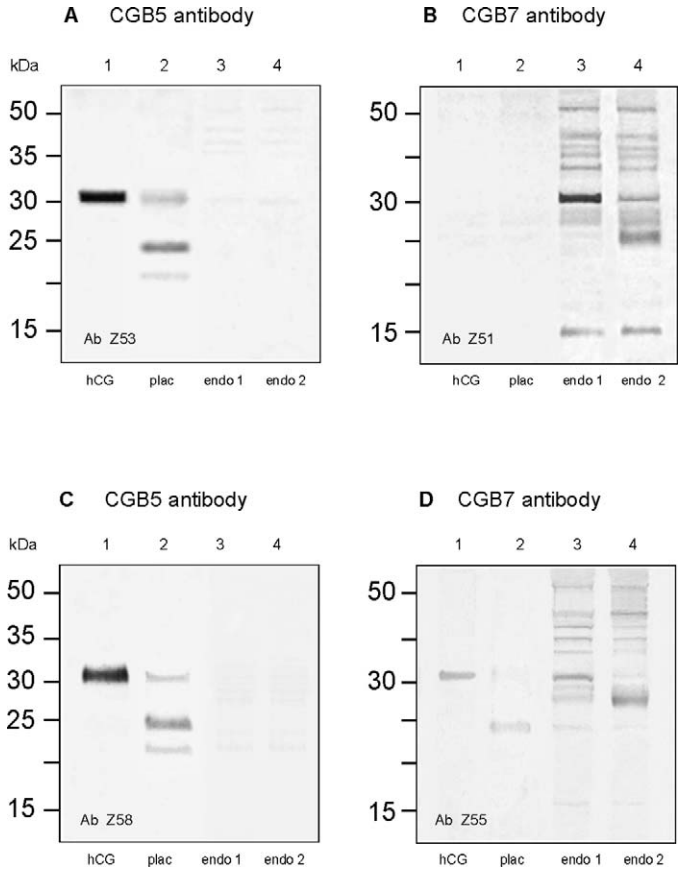


FIG. 9. Western blotting for hCG detection in the normal secretory endometrium using total and gene-specific CGB antibodies. Pure placental hCG product, early gestation placental tissue homogenate, and different normal secretory endometrium homogenate supernatants were identified using CGB5 or CGB7 gene-specific antibodies. The CGB5-specific antibodies against the exon 2 epitope (**A**) and the exon 3 epitope (**C**) recognize the pure hCG and placental homogenate, but not the endometrial homogenate samples, and the CGB7-specific antibodies of exon 2 epitope (**B**) and of exon 3 (**D**, with lower specificity) recognize only the endometrial homogenate samples with their main glycosylated 34-, 32-, and 29-kDa (CGB7-hCG) isoforms. The varying lower CGB hCG molecular isoforms seem to depend on the degree of partial deglycosylation in the CGB5 placental and CGB7 endometrial sample homogenates. Ab, antibody; plac, placenta; endo, endometrium.

endometrial CGB7 from placental CGB5 only when using higher-antigen dilutions (Fig. 8, D and F).

The molecular hCG isoforms can be identified in homogenate supernatants from the secretory endometrium in which *CGB7* mRNA expression alone was detected with the CGB7-specific antibody Z51 by Western blot, whereas these molecular hCG isoforms cannot be identified by the CGB5-specific antibody Z53 (Fig. 9, A and B, and Table 3). Conversely, pure placental hCG and hCG in placental homogenate cannot be identified by the CGB7-specific antibody Z51 but can be identified using the CGB5-specific antibody Z53 (Fig. 9, A and B). Unlike the CGB7- and CGB5-specific antibody pairs against epitopes aa 2 and aa 4, the CGB7 and CGB5 antibody pairs against epitope aa 117 (Z55 and Z58) demonstrate sufficient specificity to differentiate gene-specific *CGB* expression and production, but only with lower hCG antigen concentrations (Fig. 9, C and D). In the endometrium homogenates, the gene-specific CGB7 antibodies confirmed the appearance of the glycosylated CGB7 main molecule isoforms of about 34, 32, and 29 kDa that were also recently found in the endometrium and placenta using the pan-specific hCG antibodies [24, 28]. In addition, numerous glycosylated hCG isoforms of 44, 38, and 35 kDa and partially or totally deglycosylated CGB7 isoforms of 27, 24, 21, and 15 kDa were detected in the endometrium, as has also been described for the trophoblast tissue CGB5 subunit [24, 28–30].

TABLE 3. Gene expression of *CGB* subunit mRNAs in normal secretory endometrial tissue samples.

CGB mRNA expression	n	Percentage
Gene *CGB7* and *CGB6*	28	47.5
Single gene *CGB7*	25	42.4
Single gene *CGB6*	6	10.1
Total endometrium	59	100.0

DISCUSSION

In recent work [24] we demonstrated that the glandular hCG observed there is released optimally in a secretory endometrium of highly synchronous transformation finely tuned between the epithelial, stromal, and mononuclear cell functions. Because secreted hCG is diminished or missing in a dyssynchronous secretory transformation of the endometrium, we found that this glandular hCG is also an implantation marker and have proposed an endometrium score and epithelial hCG staining index for evaluation [24].

Epithelial CGB7 *and* CGB6 *mRNA in the Secretory Endometrium*

This study presented the first characterization of the total mRNA nucleotide sequences of the endometrial *CGB7* and *CGB6* subunits expressed in the glands of the normal secretory endometrium and described the newly investigated endometrial heterodimeric hCG molecule that is unlike trophoblast hCG. No amounts of *CGB3*, *CGB5*, or *CGB8* mRNA were determined from the human hCG/LH gene cluster of six *CGB* genes [3, 5–7]. The numerous nucleotide differences between the *CGB7* and *CGB5* mRNA sequences in the noncoding and encoding gene sections justify that they have a differential impact on the regulation of either the *CGB7* or *CGB5* subunits. On the other hand, the full-length sequence analysis clearly indicates that a further *CGB6* subunit mRNA is expressed in the normal secretory-transformed endometrial tissue, either alone or in combination with the *CGB7* subunit. This additional produced *CGB6* mRNA and hCG protein isoform that we first discovered in the secretory endometrium has been described by Talmadge et al. [5] and Policastro et al. [6] in the trophoblast hCG/LH-gene cluster. Until now, it has been discussed as an allele of the *CGB7* gene, although the *CGB6* gene was classified by Li et al. [8] in their evolutionary phylogenetic analysis of the glycoprotein gene family as the oldest human *CGB* subunit that evolved independently before the creation of *CGB7* or *CGB5*.

All of the gene- and tissue-specific multiple human CGB and CGA subunits of hCG described in this study, including the new endometrial CGB6 subunit molecule, are members of the glycoprotein hormone family that evolved from a single precursor gene through consecutive gene duplications [8, 31]. First, in equine species and marmoset monkeys a separate single *CGB* gene is formed in pituitary and decidual tissues [32, 33]. For the baboon there are three, for the orangutan four, for chimpanzees five, and for humans six *CGB* genes (besides *CGB6*) in addition to *LHB* [3, 7–9]. The *CGB6* described here for the first time in the endometrium evolved as the oldest human *CGB* gene before *CGB7* [5, 6, 8]. The human hCG/LH

gene cluster of *CGB5*, *CGB8*, *CGB3*, and, much less strongly, *CGB7*, encodes placental hCG mRNA expression, but also encodes a nontrophoblastic CGB7-hCG formed in the epithelium of several normal tissues as mentioned before with significantly lower expression levels [10–13, 15, 17, 34]. Our data confirm that an additional epithelial hCG is expressed and produced in the normal secretory endometrium with a CGB7 and/or CGB6 subunit.

Gene-Specific Epithelial hCG Protein in the Secretory Endometrium

We have shown immunohistochemically that these different epithelial forms of hCG (CGB7, CGB6) are released in glands of the synchronously differentiated secretory endometrium as identified by non-gene-specific beta-hCG antibodies. The CGA staining of the epithelial hCG has recently been demonstrated in the normal secretory endometrium [24]. For the first time, we can verify that the CGB7 component of glandularly released hCG was stained immunohistochemically with our gene-specific CGB7 antibody, but not with the trophoblast-specific CGB5 antibody, with the opposite findings for placental tissue staining.

The apparent molecular weights for the endometrial CGB7 subunit determined by Western blot correspond to the molecular weights of the glycosylated placental CGB5 subunit hCG identifying 34- and 29-kDa bands, respectively [15, 28, 29]. Smaller molecular forms of the epithelial CGB7 subunit pattern of hCG represent partially, and to a lesser extent, completely deglycosylated N- or O-glycosidic molecular forms, as has been described for the placental CGB5 subunit of hCG [30]. From this perspective, we can conclude that the epithelial hCG of the normal secretory endometrium characterized by CGA and molecular CGB subunit isoforms is also considered to be a hormone molecule with distinct N- and O-glycosidic glycoprotein binding. Our results showing the endometrial production of glycosylated CGB7-hCG appear to correlate with the findings of a recent paper [35] that glycosylated but not deglycosylated hCG induces massive uterine natural killer (NK) cell proliferation via NK mannose receptor binding as demonstrated in the secretory endometrium and first-trimester decidua [24, 25]. Studies of the endometrial CGB7 molecule showing respective changes in the N- and O-glycosidic glycoprotein structure or in mannose content compared with the placental CGB5 could improve our understanding of the biological function of epithelial hCG [18, 19].

As a result of Western blot analysis using gene-specific CGB7 antibodies in this study and pan-specific antibodies in a previous study [24], we found CGB bands with higher molecular weights of about 35 and 38 kDa in the normal secretory endometrium. Similar bands of 38 kDa have been described for the trophoblastic CGB as potentially hyperglycosylated CGB subunits that are produced immediately after implantation at a maximal level by the villous and extravillous cytotrophoblasts of the early decidua compared with the regular glycosylated syncytiotrophoblast *CGB* subunit of about 34 kDa [15, 28, 29]. But this means that the early production of embryonic hyperglycosylated hCG (CGB5) in decidual extravillous cytotrophoblast cells is accompanied by the additional formation of maternal glycosylated hCG (CGB7) in the glandular epithelium of the early decidua, a new fact that has not been previously considered [24, 36, 37]. We previously presented these documented additional functions of epithelial hCG from the maternal decidua beyond the known role of trophoblast hCG in the continuation of early pregnancy [25]. Thus, both the proven endometrial hCG, even before implantation, and embryonic extravillous hCG can now contribute to stimulating and inhibiting effects on the endometrium and decidua during very early pregnancy [15, 24, 38–42]. This hyperglycosylated extravillous hCG (CGB5) has been shown using specific antibody assays with optimal expression observed for normal and reduced expression observed for disturbed pregnancies [20, 21, 29, 36]. Comparable assay studies in highly secretory transformed endometrium are necessary to explore the respective contribution of maternal endometrial hCG.

Evaluation of Endometrial Receptivity by Candidate Gene Expression

The endometrial hCG glycoprotein studied here is released days before potential embryonic hCG is known to act on the endometrium. This increasing early to midsecretory endometrial hCG (CGB7) formed in the glands correlates to preparing the endometrium for the synchronous secretory transformation and for implantation [24]. Endometrial receptivity is a critical point for the onset of pregnancy. The inability of the endometrium to prepare itself adequately for implantation and to ensure embryonal receptivity is a crucial cause of infertility in women. To date, it was not possible to accurately predict a current receptive cycle [43–45]. The criteria of Noyes et al. [26] for the histological evaluation of timely or days-delayed secretory endometrial development include eight described parameters of endometrial physiology that no longer seem to be suitable indicators for distinguishing between fertile and infertile cycles. Because of growing clinical uncertainty regarding this morphological and histological dating scheme [26], it is more advisable to look for highly increasing mRNA profiles that change dramatically between the early and midsecretory cycle phase and act as mRNA signatures for characterizing the normal secretory endometrium and uterine receptivity. Several molecular transcription profiles (not hCG) have been determined in the endometrium describing the normal early to late secretory cycle phases [45–49] or cycles of normal to suboptimal secretory transformation [44]. Appropriate upregulated candidate genes have been recommended to characterize the date of uterine receptivity. These upregulated genes have been identified that activate epithelial gland secretion, regulate stromal cell decidualization, and affect stromal leukocyte infiltration in the transforming secretory endometrium [45, 46]. In this study, we demonstrate that epithelial endometrial hCG with its CGB7 and CGB6 subunits is strongly expressed and released between the early to midsecretory cycle phase, making it a candidate gene for the evaluation of secretory transformation.

Studies have shown that with the onset of endometrial progesterone action in cooperation with cAMP function, the mRNA and protein expression of the transcription factor FOXO1A rises sharply in the endometrial glands and stromal cells [50–51]. Other favorable candidate genes to exhibit endometrial receptivity and secretory transformation are the transcription factors STAT3 and STAT5 produced in the stromal and glandular cells as the NFκB in the glands of the normal secretory endometrium [45, 52–54]. All of these transcription factors interact with their specific binding sequence on the *CGB7* gene promoter. However, these binding sites are missing from the upstream promoter of the placental *CGB5* subunit of hCG (Gene Cards *CGB*; SABioscience). Thus, some endometrial transcription factors appear to be candidates for involvement in the upregulation of gene-specific *CGB7* formation, the promotion of stromal cell decidualization, and the differentiation of the glandular epithelium in a synchronously healthy endometrium.

Endometrial hCG Subunit CGB7 Is a Marker of Secretory Transformation

Because the induced transcription factors stimulate the expression of endometrial *CGB7*, we can also assume that endometrial *CGB7* is upregulated by the synergistic action of progesterone and the formation of cAMP by G-protein coupled receptor activation of other proteins during the secretory transformation [54, 55]. Recently, the transcription factor p53 was shown to play a normal physiological role in the preparation of the normal secretory endometrium and the receptivity to the embryo [55–57]. Further maker genes supporting implantation can be upregulated in various normal reproductive tissues by the cAMP-induced nuclear transcription factor p53 [58, 59]. The p53 protein binds to the LIF promoter in glandular epithelial endometria and considerably stimulates gene expression and release of its protein at implantation [56]. Reduced fertilization rates have been observed along with reduced LIF secretion in p53-deficient mice and in human patients with allele mutations of p53, who have a higher risk of experiencing recurrent implantation failure [60, 61]. The DNA-binding domain of p53 recognizes specific transcription factor binding sites in the epithelial *CGB7* gene promoter, but not in the *CGB5* gene promoter (Gene Cards *CGB*). Normally, a regulatory molecule binds to the p53 protein and should block its binding to target promoters, reducing its transcriptional activity [62]. This p53-regulatory complex is now believed to be able to induce transcription, even in unstressed cells, by binding to other promoter target sequences besides the usual p53 consensus sequence with reduced binding capacity. Eight p53 binding sites are present in the epithelial *CGB7* promoter gene, which activates *CGB7* gene expression [62]. Consequently, the *CGB7* subunit of endometrial hCG seems to be upregulated not only by the transcription factors discussed so far, but also by p53 in the normal secretory endometrium, where p53 is expressed and released in stromal and epithelial cells at low levels depending on cAMP [55–57]. It would be of interest to perform further research to determine the role of p53 in the regulation of gene expression of the epithelial CGB7-hCG in healthy and impaired secretory endometrium and in the epithelium of other normal tissues. The CGB7-hCG formed in normal epithelium may be a marker of epithelial health and may support regenerative processes generally [34].

In summary, this study provides the first evidence that the glycosylated form of hCG, which is expressed and released during the normal secretory cycle phase in the endometrium, contains CGB7 and/or CGB6 subunits. This epithelial hCG contributes to ensure optimal secretory conditions already from the early to midsecretory phase and is involved in preparing the endometrium for implantation. With the first determination of the full-length mRNA sequences of the endometrial *CGB7* and *CGB6* subunits and the production of gene-specific CGB7 antibody, endometrial hCG can now be used as a diagnostic marker and a candidate gene for the molecular and biochemical evaluation of receptivity in the early to midsecretory phase, perhaps as a part of the endometrial hCG staining index that we have proposed. With our specific CGB5 antibody we can also differentiate first between trophoblastic and endometrial hCG. Future investigations should focus on the endometrial transcription factors that activate only the gene-specific *CGB7* promoter region for the induction of the endogenous epithelial hCG release leading to secretory glandular transformation, stromal differentiation, and leukocyte infiltration for decidualization of the secretory endometrium as a precondition for implantation.

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