MOLECULAR AND CELLULAR BIOLOGY, Dec. 1988, p. 5100–5107
0270-7306/88/125100-08$02.00/0

Vol. 8, No. 12

Isolation and Characterization of the Human Chorionic Gonadotropin β Subunit (CGβ) Gene Cluster: Regulation of a Transcriptionally Active CGβ Gene by Cyclic AMP

J. LARRY JAMESON* AND CHRISTINE M. LINDELL

Thyroid Unit, Massachusetts General Hospital, and Harvard Medical School, Boston, Massachusetts 02114

Received 4 April 1988/Accepted 30 August 1988

The α and β subunit genes encoding chorionic gonadotropin (CG) are regulated transcriptionally in placental cells by cyclic AMP (cAMP). The regulatory response sequences of the α gene have been studied extensively. Similar studies of the CG β subunit (CGβ) gene have not been possible because transcriptionally active sequences have not been identified in the clones isolated to date. The CGβ subunit genes form a complex cluster of seven structurally similar genes that include six CGβ-like genes and a single luteinizing hormone β subunit (LHβ) gene. We isolated overlapping clones containing the entire CGβ/LHβ gene cluster (68 kilobases) from a human genomic cosmid library. The organization of the gene cluster was similar to that found in previous analyses, as determined by Southern blots of genomic DNA, but differed from some of the gene assignments, as determined by fragments cloned in λ phage. The 5′-flanking sequence of the most active CGβ gene (CGβ5) was linked to the chloramphenicol acetyltransferase (CAT) coding sequence for analyses of transient expression in different cell types. CGβCAT was expressed preferentially in JEG-3 choriocarcinoma cells, and expression was markedly stimulated by treatment with 8-bromo-cAMP. Deletion mutagenesis of the CGβ 5′-flanking sequence revealed that multiple regions were required for maximal expression. The kinetics for cAMP stimulation of αCAT and CGβCAT expression were different, suggesting that different pathways may be involved in cAMP-stimulated expression of the α and CGβ genes.

Chorionic gonadotropin (CG) is a placental hormone that stimulates steroidogenesis and maintains the corpus luteum of pregnancy (17). CG comprises noncovalently bound α and β subunits that are encoded by separate genes (12). The α gene is a single-copy gene, whereas there are multiple copies of structurally similar CG β subunit (CGβ) genes or pseudogenes in humans (3, 12, 30, 31). Southern blot analyses indicate that there are six CGβ genes and a single copy of the luteinizing hormone (LHβ) gene clustered within a 60-kilobase (kb) region of chromosome 19 (31).

The majority of the DNA encoding the CGβ/LHβ gene cluster has been isolated from λ phage DNA libraries (3, 12, 30). However, there is evidence that some of the cloned DNA fragments contain rearrangements of homologous regions of different CGβ genes (31). In addition, approximately 7 kb of DNA in the central region of the gene cluster is not present in the phage isolates but has been isolated recently from a cosmid library derived from human choriocarcinoma cells (15). This central region of the gene cluster includes the 5′-flanking region of CGβ gene 5 (CGβ5), the member of the CGβ gene family that is expressed in the greatest amounts in placenta (23, 35).

The CGα and CGβ subunit genes are regulated transcriptionally in placental cells by cyclic AMP (cAMP) (21, 29), leading to 15- to 30-fold stimulation of steady-state mRNA levels after 24 to 48 h (6, 21, 29). The kinetics for transcriptional stimulation of the CGα and CGβ genes are different, with rapid induction of the CGα gene followed by delayed induction of CGβ gene transcription (29). The DNA sequences mediating cell-specific expression and cAMP responsiveness in the CGα gene have been delineated by examining the activity of various mutants in transient-expression assays (9–11, 20, 33). Similar analyses have not been feasible for the CGβ gene owing to lack of transcriptional activity in constructions containing all of the currently available CGβ 5′-flanking region (282 base pairs [bp]) (21). These results suggest either that additional 5′-flanking sequences are required for expression or that sequences downstream from the site of transcriptional initiation may act as enhancers for CGβ gene expression.

It would be useful to obtain transcriptionally active CGβ and LHβ DNA regulatory regions to allow direct comparisons of α and β gene regulatory elements when linked to an identical heterologous reporter, such as chloramphenicol acetyltransferase (CAT). To this end, we isolated the entire CGβ/LHβ gene cluster, including the central region that includes the 5′-flanking region of CGβ gene 5. We found that a CGβ-*cat* fusion gene containing this additional sequence was expressed in JEG-3 choriocarcinoma cells, albeit at a lower level than the α gene. Expression of both the α and CGβ fusion genes was markedly stimulated by cAMP, and the time course for induction occurred with different kinetics.

MATERIALS AND METHODS

Screening of human genomic DNA for the CGβ/LHβ gene cluster. A cosmid library prepared from human female placenta DNA was generously provided by J. Collins (25). The library was propagated in *Escherichia coli* JM109 (*rec*) and screened by the high-density colony hybridization technique (16, 27). Replicate filters were screened initially with three different oligonucleotides (Fig. 1) that are directed toward distinct regions of the CGβ5 gene. After secondary and tertiary screening of the positive colonies, restriction enzyme digestions and Southern blot analyses (34) of the isolated cosmid DNAs (2) indicated that 19 of the 23 isolates contained portions of the CGβ/LHβ gene cluster and that at least four distinct genomic fragments were present. Three of

* Corresponding author.

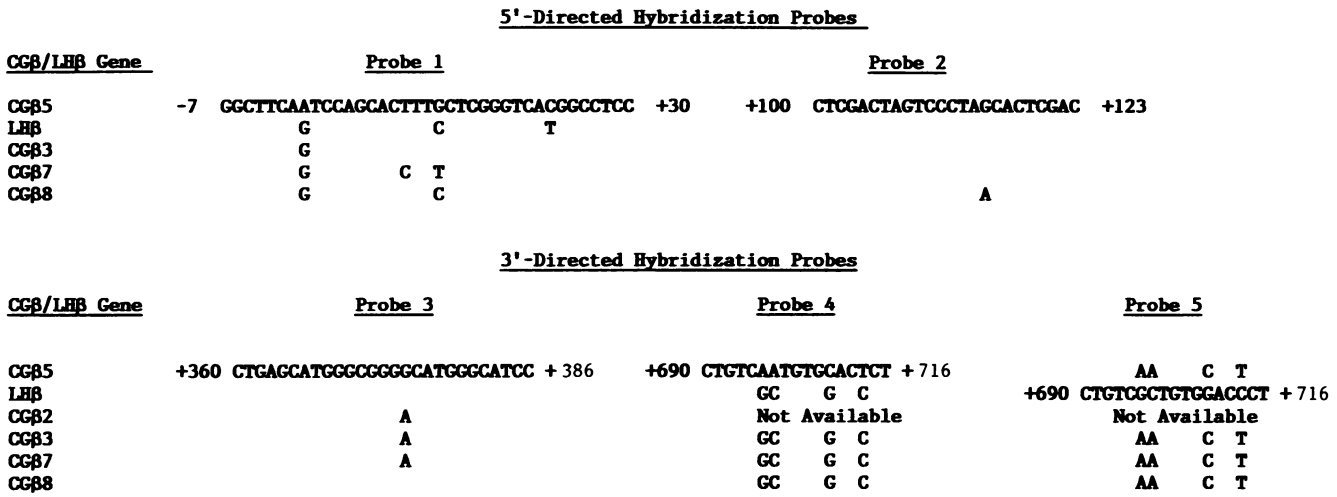

FIG. 1. CGβ/LHβ hybridization probes. Sequences are oriented in the 5'→3' direction and correspond to the sense strand. The actual probes used were complementary to the sequences illustrated to allow independent hybridization analyses of expressed mRNA. The 5'-directed probes are numbered relative to the start of transcription (22). The 3'-directed probes are numbered relative to the start of transcription in the processed mRNA. The entire sequences are shown for CGβ5; only bases differing from that sequence are shown for the other genes. Only genes for which sequences have been published are listed (12, 30, 31). The CGβ8 sequences are tentatively assumed to correspond to the sequences published for CGβ6 (31).

these DNA fragments overlapped and appeared to encompass the entire gene cluster (see Results). (The nomenclature used for the CGβ/LHβ gene cluster is that originally defined by Boorstein et al. [3]. It should be noted that CGβ6 appears to be a result of recombination during cloning [31] and that CGβ4 corresponds to the LHβ gene. In addition, the CGβ genes isolated by Policastro et al. [30] correspond to this nomenclature as follows: CGβa = CGβ7; CGβe left = CGβ2; CGβe center = CGβ3; CGβe right = LHβ.)

Plasmid construction. Construction of an expression vector (αCAT) containing 1.5 kb of 5'-flanking sequence and 44 bp of the 5' untranslated region from the human glycoprotein hormone α subunit gene linked to the chloramphenicol acetyltransferase (CAT) coding sequence has been described before (21). This vector was modified by subcloning a fragment of the αCAT fusion gene (*Bgl*II-*Bam*HI fragment) into pGEM-3. The resulting fusion gene, referred to as −846 αCAT in this report, contains 846 bp of α gene 5'-flanking sequence linked to *cat*. Construction of −282 CGβCAT has been described before (21). This vector contains 282 bp of 5'-flanking sequence and 273 bp of the 5'-untranslated region derived from the CGβ5 gene (12) that was isolated from a λ phage library. The CGβ-*cat* fusion gene (CGβCAT) was subcloned into the polylinker site of pGEM-3 to facilitate subsequent modifications. A vector with additional 5'-flanking sequence from the CGβ5 gene (−3.7 CGβCAT) was prepared by isolating from Cos 2 the 3.8-kb *Kpn*I-*Spe*I fragment of the CGβ5 gene 5'-flanking sequence and ligating this fragment into the −282 CGβCAT vector that had been digested with *Kpn*I (polylinker site) and *Spe*I (+104 of CGβ 5' untranslated region). Additional CGβ 5'-untranslated sequences that are homologous to the LHβ promoter were inserted into this plasmid by using restriction enzyme fragments and synthetic DNA sequences. The resulting plasmid, referred to as −3.7 CGβCAT, contained approximately 3.7 kb of 5'-flanking sequence and 362 bp of the 5' untranslated region from the CGβ5 gene fused to the *cat* coding sequence. To verify the validity of the construction and to ensure that the CGβ gene sequence was actually that of gene 5, the nucleotide sequence (32) of 800 bp of the CGβ5 gene was determined through the junction with the *cat* coding sequence. The CGβ nucleotide sequence was identical to that reported previously (12), with the exception of a G for A substitution at −45 bp. Additional deletion mutants were derived from −3.7 CGβCAT by using exonuclease III or restriction enzyme sites. The plasmid α-CGβCAT was constructed by inserting a region of the α gene that contains basal and cAMP-responsive enhancer sequences (−846 to +44) (10, 11, 33) 71 bp upstream from the −282 CGβCAT fusion gene at an *Aat*II site located in pBR322. The α gene fragment was inserted in both orientations by blunt-end ligation.

The plasmid pA10CAT (kindly provided by B. Howard) is a derivative of pSV2CAT in which the simian virus 40 (SV40) enhancer has been deleted, but the basal promoter elements and 21-bp repeated sequences have been retained (24). The following sequences were ligated into the *Bgl*II site of pA10CAT immediately upstream from the 21-bp repeats: (i) the α gene *Bgl*II-*Bam*HI fragment consisting of 5'-flanking sequence between −846 and +44 in both orientations; (ii) CGβ5 gene 5'-flanking sequence between −282 and +273 inserted in both orientations by blunt-end ligation; (iii) a 3.3-kb fragment of the CGβ5 gene that includes 282 bp of 5'-flanking sequence, all three exons, and 1 kb of 3'-flanking sequence inserted in both orientations by blunt-end ligation; and (iv) two copies of the SV40 72-bp enhancer sequence containing *Bam*HI linkers.

Cell culture, DNA transfection, and CAT assays. JEG-3 (HTB 36) and BHK (CCL 10) cells were obtained from the American Type Culture Collection. Cells were plated onto 60-mm dishes 24 h before transfection and grown in Dulbecco modified Eagle medium containing 10% fetal calf serum (Gibco Laboratories), penicillin (100 U/ml), and streptomycin (100 μg/ml). Plasmid DNA preparations were purified twice on CsCl gradients and transfected into JEG-3 and BHK cells by the calcium phosphate precipitation technique (14, 39). GH4 and INR1-G9 cells were transfected by using DEAE-dextran (26). In experiments in which 8-bromo-cAMP was added, cells were incubated for 28 h after transfection and then exposed to 1 mM 8-bromo-cAMP for

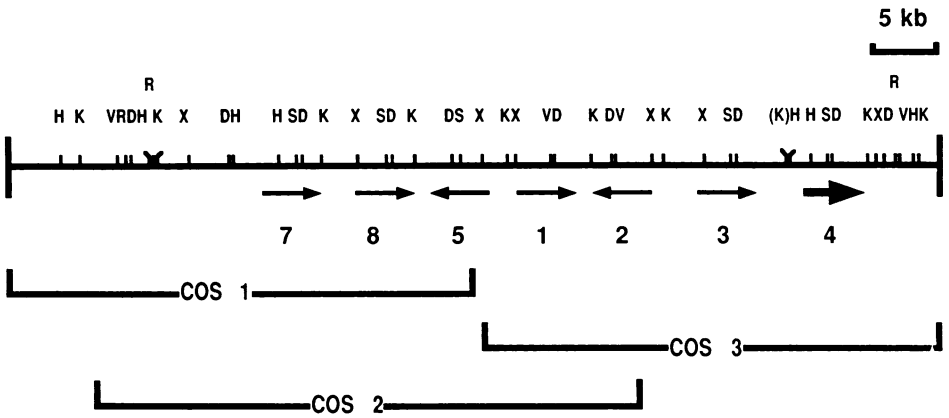

FIG. 2. Map of the human CGβ/LHβ gene cluster. The orientation and arrangement of the CGβ genes are indicated by arrows. The single LHβ gene is denoted by the large arrow. Restriction enzyme sites are abbreviated as follows: H, *Bam*HI; K, *Kpn*I; V, *Eco*RV; R, *Eco*RI; D, *Hin*dIII; X, *Xba*I; S, *Spe*I. The *Kpn*I site in parentheses represents a polymorphic site not present in our cosmid isolate. The regions of the gene cluster present in the overlapping cosmid fragments are indicated at the bottom of the figure.

20 h before preparation of extracts for CAT assays. GH4 cells were provided by Henry Kronenberg (Massachusetts General Hospital, Boston), and INR1-G9 cells were obtained from R. Takaki (Medical College of Oita, Japan). In the absence of cAMP treatment, cell extracts were prepared for CAT assays 48 h after transfection.

CAT enzyme assays were performed as described previously (13). Samples were diluted to allow measurements within the linear range of the assay (<40% conversion). Protein content of cellular extracts was determined by the method of Bradford (Bio-Rad Laboratories kit) (5), and CAT activity is expressed as the percentage of chloramphenicol converted to acetylated derivatives per microgram of protein per hour times 100.

RESULTS

CGβ5 gene sequences cloned previously were not sufficient for expression in JEG-3 cells. Substantial evidence indicates that CGβ gene 5 is an actively expressed member of the CGβ gene family (23, 35). We have shown previously that CGβCAT fusion genes containing all of the available 5′-flanking sequence (282 bp of 5′-flanking sequence and 273 bp of 5′ untranslated region) of CGβ gene 5 are not expressed in JEG-3 cells (21). Before we cloned additional CGβ upstream sequences, the downstream regions of CGβ5 were screened for enhancer elements that would be absent in the fusion genes by testing for activation of a heterologous SV40 promoter (24). Although the α gene 5′-flanking sequence activated the SV40 promoter and conferred responsiveness to cAMP, neither the CGβ5 5′-flanking sequence (−282 to +273 in either orientation) nor the CGβ5 gene and 3′-flanking sequence (−282 to +3000 in either orientation) activated the SV40 promoter in JEG-3 cells (data not shown). These results suggest that CGβ regulatory elements that are necessary for expression reside upstream from the available 5′-flanking sequence for CGβ5 and prompted us to isolate the uncloned regions of the CGβ gene cluster.

Isolation and characterization of the human CGβ/LHβ gene cluster. Because the repeated copies of structurally related CGβ genes may predispose to recombination during propagation in *E. coli*, a human genomic cosmid library with minimal amplification was propagated in a *rec* mutant of *E. coli* (JM109). Nineteen clones containing portions of the CGβ/LHβ gene cluster were isolated by hybridization to three different oligonucleotide probes as described in Materials and Methods.

Preliminary analyses of cosmid DNA isolates with oligonucleotide probes specific for the 3′ end of the CGβ (probe 4) or LHβ (probe 5) gene (Fig. 1) indicated that Cos 1 and 2 contained one end of the CGβ gene cluster (CGβ 7, 8, 5, and 1) and that Cos 3 contained the other end of the gene cluster (CGβ 1, 2, and 3 and LHβ) (Fig. 2). Similarities in structure of independent isolates suggested that major rearrangements were not present.

Because previous analyses differed in determining the number of copies and arrangement of the CGβ genes (3, 12, 15, 30, 31), our cosmid isolates were characterized in detail and compared with gene assignments that were based on Southern blot analyses of total genomic DNA (31). Each of the cosmid isolates was digested with seven different restriction enzymes in single and multiple combinations. Digested DNA was then hybridized to oligonucleotides specific for either the 5′-flanking region or the 3′ coding sequence of the CGβ or LHβ gene. The high degree of homology allowed each of the CGβ genes to be detected with the hybridization probes (Fig. 1). The cosmid isolates spanned 68 kb and included all seven members of the CGβ/LHβ gene cluster.

An example of selected restriction enzyme digestions of Cos 2 and Cos 3 is shown in Fig. 3a and provides evidence for several salient features of the gene cluster. The combined restriction enzyme digestions with *Kpn*I-*Spe*I and *Kpn*I-*Eco*RV divided the structurally similar members of the CGβ gene cluster into fragments with characteristic lengths that could be readily resolved (Fig. 3b). Cos 2 contained the leftward end of the gene cluster, including CGβ genes 7, 8, 5, and 1 and the 3′ end of CGβ2. Cos 3 overlapped Cos 2 and contained the rightward end of the cluster, including CGβ genes 1, 2, and 3 and the LHβ gene. The region of overlap of Cos 2 and Cos 3 was most apparent in the *Eco*RV digest, in which a 4.9-kb fragment containing the 3′ ends of CGβ genes 1 and 2 was found in both the Cos 2 and 3 isolates. It was of particular importance to clone a 7-kb region of the CGβ gene cluster between CGβ genes 5 and 1, as these sequences were not isolated from several different human genomic DNA libraries cloned in λ phage (12, 30). This segment of the gene cluster was represented in its entirety by the region of overlap between Cos 2 and 3. The single copy of the LHβ gene was identified by using a pair of oligonucleotides (Fig.

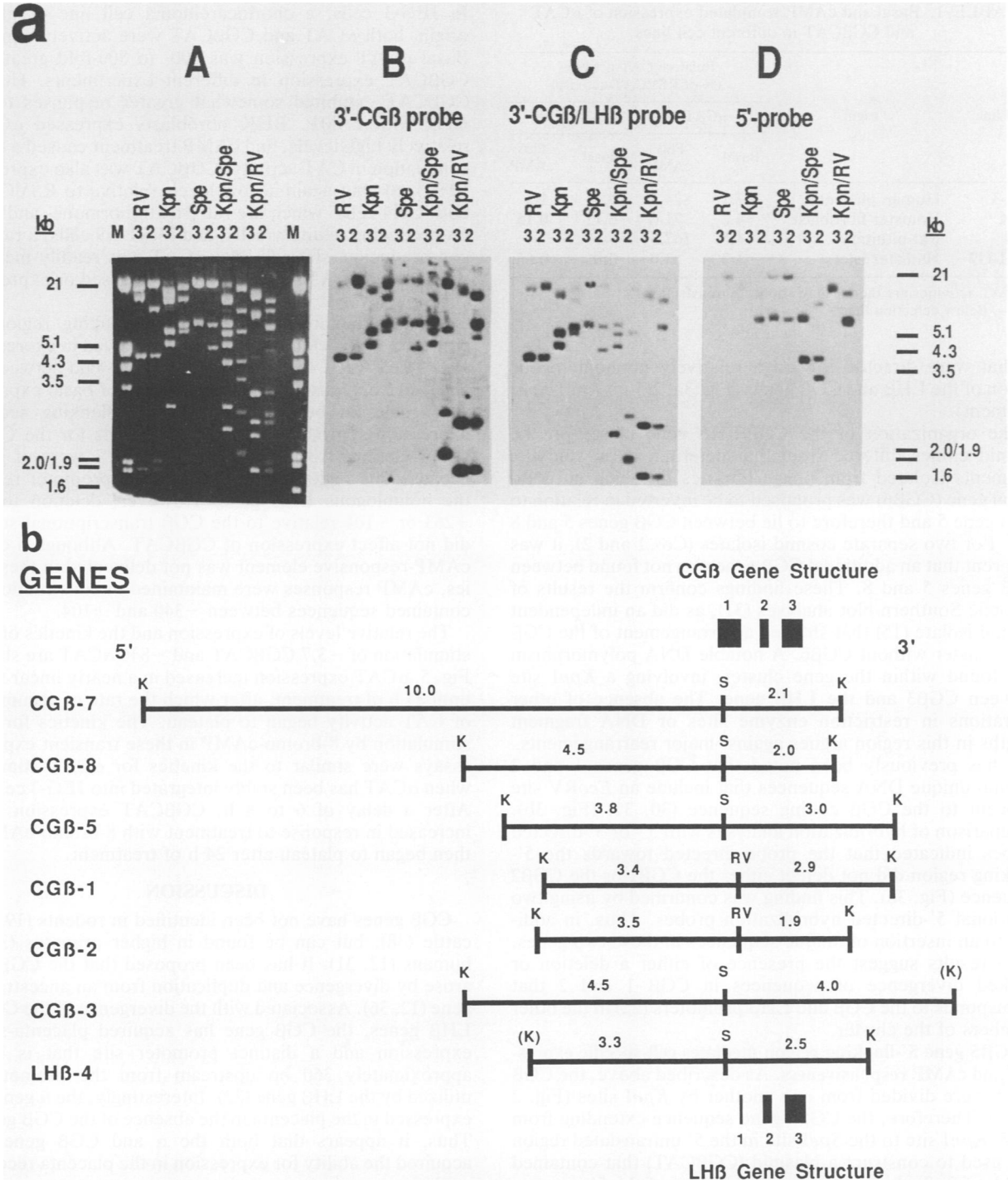

FIG. 3. (a) Southern blot analyses of the CGβ/LHβ gene cluster. A, Cosmids 2 and 3 (see Fig. 2) were digested with *Eco*RV (RV), *Kpn*I (Kpn), *Spe*I (Spe), *Kpn*I and *Eco*RV (Kpn/RV), or *Kpn*I and *Spe*I (Kpn/Spe) and subjected to electrophoresis through a 0.8% agarose gel containing ethidium bromide. The digested DNA was transferred to a Nytran membrane, which was sequentially hybridized with specific ^{32}P-labeled oligonucleotide probes. λ DNA digested with *Eco*RI and *Hin*dIII was used as size markers (lane M). B, 3'-Directed probe (probe 4 in Fig. 1) that detects CGβ but not LHβ sequences. C, 3'-Directed probe (probe 3 in Fig. 1) that detects both CGβ and LHβ sequences; D, 5'-directed probe (probe 1 in Fig. 1) that detects, under low stringency, all of the members of the CGβ/LHβ gene cluster except CGβ genes 1 and 2. (b) Restriction enzyme fragments that characterize the individual CGβ and LHβ genes. The genes are listed according to the left-to-right orientation in the gene cluster (Fig. 2). The *Kpn*I (K) sites that separate each of the genes and the *Spe*I (S) or *Eco*RV (RV) sites that divide the 5'-flanking region from the coding sequence of each of the genes are indicated. The *Kpn*I site shown in parentheses at the 3' end of CGβ3 and at the 5' end of the LHβ gene is a polymorphic variant. The length (in kilobases) of each fragment is indicated and provides a means of identifying and isolating individual gene sequences from the various members of the cluster. The locations of the three exons in the CGβ gene and the LHβ gene are shown above and below the gene sequences.

TABLE 1. Basal and cAMP-stimulated expression of αCAT and CGβCAT in different cell lines

Cell line	Origin	Relative CAT activity[a] (% of RSVCAT activity)			
		αCAT		CGβCAT	
		Basal	Plus cAMP	Basal	Plus cAMP
JEG-3	Human placenta	107.1	824.5	0.13	1.5
BHK	Hamster fibroblast	14.2	71.0	0.11	0.15
GH4	Rat pituitary	4.5	167.3	—[b]	—
INR1-G9	Hamster islet	1.2	3.0	0.06	0.06

[a] CAT activities are the mean of triplicate transfections.
[b] —, Below detection limit.

1) that were directed toward a relatively nonhomologous region of the LHβ and CGβ genes (Fig. 3a; 2.5-kb *Kpn*I-*Spe*I fragment).

The organization of the CGβ/LHβ gene cluster in the cosmid clones differed from that determined by studying fragments isolated from phage libraries in which an additional gene (CGβ6) was proposed to be inverted in relation to CGβ gene 5 and therefore to lie between CGβ genes 5 and 8 (12). For two separate cosmid isolates (Cos 1 and 2), it was apparent that an additional CGβ gene was not found between CGβ genes 5 and 8. These findings confirm the results of genomic Southern blot analyses (31), as did an independent cosmid isolate (15) that showed an arrangement of the CGβ gene cluster without CGβ6. A notable DNA polymorphism was found within the gene cluster, involving a *Kpn*I site between CGβ3 and the LHβ gene. The absence of other alterations in restriction enzyme sites or DNA fragment lengths in this region argues against major rearrangements.

It has previously been noted that CGβ genes 1 and 2 contain unique DNA sequences that include an *Eco*RV site adjacent to the CGβ coding sequence (30, 31) (Fig. 3b). Comparison of hybridization analyses with 5′- or 3′-directed probes indicated that the probe directed towards the 5′-flanking region did not detect either the CGβ1 or the CGβ2 sequence (Fig. 3a). This finding was confirmed by using two additional 5′-directed hybridization probes. Thus, in addition to an insertion of unique sequences in these two genes, these results suggest the presence of either a deletion or marked divergence of sequences in CGβ 1 and 2 that corresponds to the CGβ and LHβ promoters (22) in the other members of the cluster.

CGβ5 gene 5′-flanking region mediates cell-specific expression and cAMP responsiveness. As described above, the CGβ genes were divided from one another by *Kpn*I sites (Fig. 2 and 3). Therefore, the CGβ5 gene sequence extending from the 5′ *Kpn*I site to the *Spe*I site in the 5′ untranslated region was used to construct a plasmid (CGβCAT) that contained 3.7 kb of 5′-flanking sequence and 362 bp of the 5′ untranslated region linked to the *cat* coding sequence. Initial experiments indicated that −3.7 CGβCAT was expressed in JEG-3 cells and that expression was stimulated as much as 50-fold by treatment with 8-bromo-cAMP.

CGβCAT was transfected in cell lines with different phenotypic characteristics to examine whether it was broadly expressed or relatively restricted to placental cell lines (Table 1). For comparison, αCAT was also transfected into each cell line, and responses to treatment with 8-bromo-cAMP were determined. The activities of αCAT and CGβCAT are expressed relative to a plasmid that contains the Rous sarcoma virus promoter linked to CAT (RSVCAT). In JEG-3 cells, a choriocarcinoma cell line of placental origin, both αCAT and CGβCAT were actively expressed. Basal αCAT expression was 200- to 800-fold greater than CGβCAT expression in different experiments. However, CGβCAT exhibited somewhat greater responses to treatment with cAMP. BHK fibroblasts expressed αCAT at relatively high levels, and cAMP treatment caused a fivefold stimulation in CAT activity. CGβCAT was also expressed in BHK cells but again at low levels relative to RSVCAT. In both GH4 cells, which are rat growth hormone- and prolactin-producing pituitary cells, and INR1-G9 cells, a rat glucagon-producing islet cell line, αCAT was readily measured, whereas CGβCAT was either not expressed or expressed at very low levels.

Deletion mutants of the CGβ 5′-flanking region were constructed to delineate sequences involved in expression in JEG-3 cells (Fig. 4). Deletion from the 5′ end between −3.7 and −612 bp caused a progressive loss of basal expression. After deletion beyond 340 bp of 5′-flanking sequence, expression approached background levels for the CAT assay. Deletions from the 3′ end of exon 1 were of interest because this region corresponds to the promoter region of the homologous LHβ gene. However, deletion to either +263 or +104 relative to the CGβ transcriptional start site did not affect expression of CGβCAT. Although a discrete cAMP-responsive element was not delineated in these studies, cAMP responses were maintained in constructions that contained sequences between −340 and +104.

The relative levels of expression and the kinetics of cAMP stimulation of −3.7 CGβCAT and −846 αCAT are shown in Fig. 5. αCAT expression increased in a nearly linear manner until 24 h of treatment, after which the rate of accumulation of CAT activity began to plateau. The kinetics for αCAT stimulation by 8-bromo-cAMP in these transient expression assays were similar to the kinetics for αCAT stimulation when αCAT has been stably integrated into JEG-3 cells (21). After a delay of 6 to 8 h, CGβCAT expression rapidly increased in response to treatment with 8-bromo-cAMP and then began to plateau after 24 h of treatment.

DISCUSSION

CGβ genes have not been identified in rodents (19, 37) or cattle (38), but can be found in higher primates (37) and humans (12, 31). It has been proposed that the CGβ genes arose by divergence and duplication from an ancestral LHβ gene (12, 36). Associated with the divergence of the CGβ and LHβ genes, the CGβ gene has acquired placenta-specific expression and a distinct promoter site that is located approximately 360 bp upstream from the promoter site utilized by the LHβ gene (22). Interestingly, the α gene is not expressed in the placenta in the absence of the CGβ gene (7). Thus, it appears that both the α and CGβ genes have acquired the ability for expression in the placenta recently in evolution.

We found that αCAT was expressed transiently in nonplacental cell lines at unexpectedly high levels relative to a promoter with broad specificity, such as RSVCAT. CGβCAT expression was much lower than that of αCAT and appeared to be relatively restricted to cell lines of placental origin. Both the α and CGβ genes are expressed ectopically in certain neoplasms. However, expression of the α gene is sometimes observed in the absence of the CGβ gene, consistent with the idea that CGβ expression may be more restricted than that of the α gene in nonplacental cells.

The reason for lower basal levels of CGβCAT expression relative to αCAT expression has not been elucidated, and

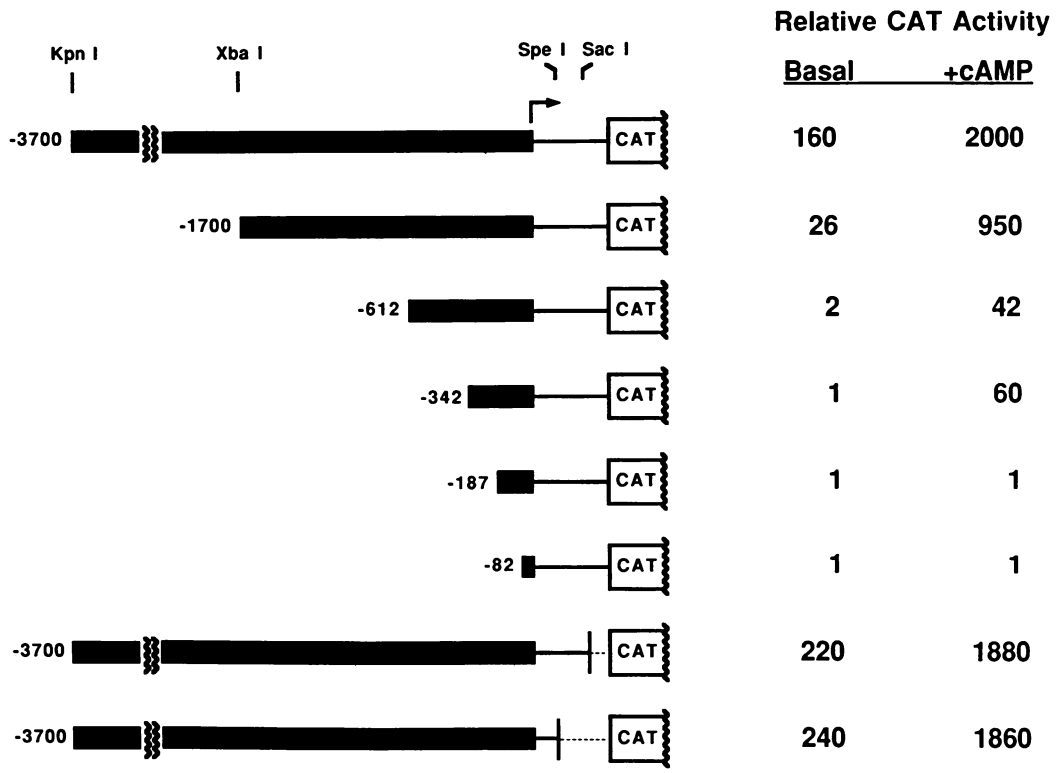

FIG. 4. Expression of CGβCAT deletion mutants in JEG-3 cells. The structures of the CGβCAT fusion genes are illustrated at the left. DNA was transfected into JEG-3 cells in the absence or presence of 8-bromo-cAMP as described in the text. CAT activity values are the mean of triplicate transfections and expressed relative to −82 CGβCAT activity.

this difference may account in part for the inability to detect CGβCAT expression in nonplacental cells. Direct measurements of RNA synthesis from the endogenous genes show that the basal transcription rate of the α gene is no more than fourfold greater than that of the CGβ gene (21, 29). Similarly, the steady-state levels of α mRNA are only two- to threefold greater than CGβ mRNA levels (6, 21, 29). We have recently observed that our JEG-3 cells are producing a greater ratio of CGα/CGβ mRNA (L. Jameson, unpublished data), raising

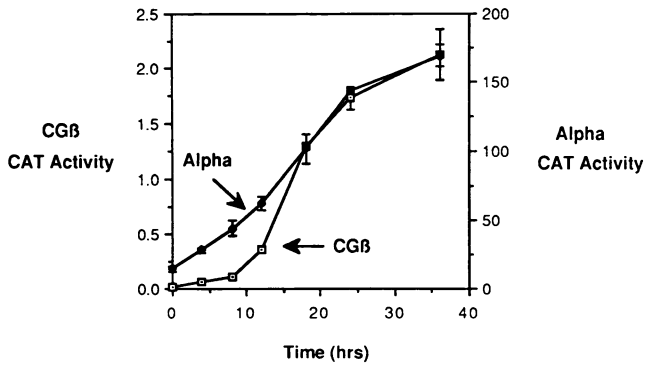

FIG. 5. Time course for transcriptional activation of CGβCAT and αCAT expression by 8-bromo-cAMP. CGβCAT (10 μg) and αCAT (10 μg) were transfected into JEG cells. After 24 h, the medium was changed and cells were treated for various lengths of time (0 to 36 h) with 1 mM 8-bromo-cAMP. Cell extracts from triplicate transfections were assayed for CAT activity. A similar time course for cAMP stimulation of αCAT and CGβCAT expression was found in an independent experiment.

the possibility that the cellular factors involved in CGβ gene expression have been altered during repeated passage of the cells. Alternatively, it is possible that additional CGβ gene regulatory elements that have not yet been identified contribute to the basal transcriptional activity of the CGβ gene. For example, such sequences could occur more than 3.7 kb upstream from the transcriptional start site of the CGβ5 gene. In this instance, the regulatory elements would be more within the domain of the adjacent CGβ gene 1 (Fig. 2), which is likely a pseudogene.

Deletion mutagenesis of the CGβ5 5′-flanking sequence indicates that several different regions are required for maximal expression, including elements more than 2 kb upstream from the transcriptional start site. The requirement for multiple regulatory DNA elements is typical of many other genes (28). In some instances, including the α gene (10, 11, 33), repeated elements are identified, whereas in other cases distinct elements appear to act in a complementary manner (28). Further delineation of the CGβ regulatory elements by fine mapping will be of interest to better understand how the individual elements function to increase expression.

Because the CGβ gene has acquired a transcriptional start site that is upstream from the promoter recognition site in the homologous LHβ gene (22), it seemed possible that basal regulatory elements from the vestigial LHβ-like promoter might now exist in the CGβ 5′ untranslated sequence. However, deletion of the CGβ 5′ untranslated region that is homologous to the LHβ promoter did not affect expression of −3.7 CGβCAT. Thus, the sequences involved in CGβ expression appear to reside far upstream from the vestigial

LHβ promoter and function independently of this region of the gene.

A number of studies implicate cAMP as a second messenger that stimulates CG biosynthesis and secretion (6, 8, 18, 21, 29). It is clear that both the CGα and CGβ gene 5′-flanking regions contain DNA sequences that mediate transcriptional responses to cAMP (Fig. 5) (21, 29). The cAMP-responsive element in the α gene has been well characterized and involves a palindromic enhancer element, TGACGTCA. In our initial studies of the CGβ gene, the cAMP-responsive element(s) has not been clearly delineated. Responses to treatment with cAMP were still observed after deletion to −340, at which the level of basal expression was very low. Similarly, cAMP responses were preserved despite deletion of the 5′ untranslated region beyond +104. At this point, the low levels of expression in transient assays precluded further delineation of any cAMP-responsive regions between −340 and +104. Insertion of the SV40 enhancer upstream from deleted CGβ promoter elements did not prove to increase basal expression levels (J. L. Jameson, unpublished data).

Studies of the kinetics for induction of α and CGβ mRNAs showed a slightly different time course for accumulation of the steady-state mRNAs, with the increase in α mRNA preceding that of CGβ mRNA by 1 to 2 h (6, 21, 29). Similarly, studies of RNA synthesis by nuclear runoff assays indicated that transcription of the α gene is activated by cAMP prior to that of the CGβ gene (29). These studies suggest that cAMP may activate α and CGβ gene transcription via fundamentally different mechanisms. In light of these findings, it was of interest to examine the kinetics for cAMP stimulation of α and CGβ fusion genes in transient expression assays (Fig. 5). Under these experimental conditions, any effects of cAMP on mRNA stability are mitigated by using a common reporter sequence (CAT) to assess transcriptional activity. We found that the kinetics for cAMP stimulation of α-*cat* and CGβ-*cat* fusion genes were different. There was an apparent lag of 8 h preceding CGβCAT stimulation that was not observed for αCAT.

These findings raise the possibility that the α and CGβ genes respond to the cAMP signaling system via distinct pathways. In this regard, it is of interest that there were no sequences within the initial 600 bp of the CGβ 5′-flanking sequence that were homologous to the α gene cAMP-responsive element. Additional regions of the CGβ gene also have no cleavage sites for *Aat*II, an enzyme that characteristically cleaves within the consensus 8-bp palindrome (TGACGTCA) that includes the α gene cAMP-responsive element (L. Jameson, unpublished observations). Physiological evidence for distinct regulation of the α and CGβ genes is exemplified by changes in the relative amounts of their mRNAs during pregnancy. For example, the ratio of α to CGβ mRNA changes from approximately 2 during the first trimester to at least 12 at the termination of pregnancy (4). This change in the relative amounts of the mRNAs is associated with the onset of placental steroidogenesis and a decline in CG biosynthesis after the first trimester (1). The cellular mechanisms involved in unbalanced expression of the α and CGβ genes are not known but may involve differential production of distinct *trans*-acting factors. The goal of future studies will be to identify the cellular factors that interact with the regulatory sequences of both the α and CGβ genes.

ACKNOWLEDGMENTS

We thank John Collins for generously providing the human cosmid library. We are indebted to Joel Habener for helpful discussions and for allowing us to perform the initial library screening in his laboratory. We are grateful to Vivian Fuh for assistance with the CAT assays and to Rose Mooradian and Ruth DiBlasi for secretarial assistance.

This research was supported by Public Health Service grant HD23519 from the National Institutes of Health and by the Chugai and Upjohn Scholar Awards to J. Larry Jameson.

LITERATURE CITED

1. **Aspilliga, M. O., P. G. Whittaker, A. Taylor, and T. Lind.** 1983. Some new aspects of the endocrinological response to pregnancy. Br. J. Obstet. Gynecol. **90**:596–603.
2. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
3. **Boorstein, W. R., N. C. Vamvakopoulos, and J. C. Fiddes.** 1982. Human chorionic gonadotropin β-subunit is encoded by at least eight genes arranged in tandem and inverted pairs. Nature (London) **300**:419–422.
4. **Boothby, M., J. Kukowska, and I. Boime.** 1983. Imbalanced synthesis of human choriogonadotropin α and β subunits reflects the steady state levels of the corresponding mRNAs. J. Biol. Chem. **258**:9250–9253.
5. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **42**:248–254.
6. **Burnside, J., S. B. Nagelberg, S. S. Lippman, and B. D. Weintraub.** 1985. Differential regulation of hCG α and β subunit mRNAs in JEG-3 choriocarcinoma cells by 8-bromo-cAMP. J. Biol. Chem. **260**:12705–12709.
7. **Carr, F. E., and W. W. Chin.** 1985. Absence of detectable chorionic gonadotropin subunit messenger ribonucleic acid in the rat placenta throughout gestation. Endocrinology **116**:1151–1157.
8. **Chou, J. Y., S. S. Wang, and J. C. Robinson.** 1978. Regulation of the synthesis of human chorionic gonadotropin by 5-bromo-2′-deoxyuridine and dibutyrl cyclic AMP in trophoblastic and nontrophoblastic tumor cells. J. Clin. Endocrinol. Metab. **47**:46–51.
9. **Darnell, R. B., and I. Boime.** 1985. Differential expression of the human gonadotropin α gene in ectopic and eutopic cells. Mol. Cell. Biol. **5**:3157–3167.
10. **Delegeane, A. M., L. H. Ferland, and P. L. Mellon.** 1987. Tissue-specific enhancer of the human glycoprotein hormone α-subunit gene: dependence on cyclic AMP-inducible elements. Mol. Cell. Biol. **7**:3994–4002.
11. **Deutsch, P. J., J. L. Jameson, and J. F. Habener.** 1987. Cyclic AMP responsiveness of human gonadotropin-α gene transcription is directed by a repeated 18-base pair enhancer. α-Promoter receptivity to the enhancer confers cell-preferential expression. J. Biol. Chem. **262**:12169–12174.
12. **Fiddes, J. C., and K. Talmadge.** 1984. Structure, expression, and evolution of the genes for the human glycoprotein hormones. Recent Prog. Horm. Res. **40**:43–78.
13. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
14. **Graham, F., and A. Van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–457.
15. **Graham, M. Y., T. Otani, I. Boime, M. V. Olson, G. F. Carle, and D. D. Chaplin.** 1987. Cosmid mapping of the human chorionic gonadotropin β subunit genes by field-inversion electrophoresis. Nucleic Acids Res. **15**:4437–4448.
16. **Hanahan, D., and M. Meselson.** 1983. Plasmid screening at high colony density. Methods Enzymol. **100**:333–342.
17. **Hanson, F. W., J. E. Powell, and V. C. Stevens.** 1971. Effects of hCG and human pituitary LH on steroid secretion and functional life of the human corpus luteum. J. Clin. Endocrinol. Metab. **32**:211–215.

18. **Hussa, R. O.** 1980. Biosynthesis of human chorionic gonadotropin. Endocrinol. Rev. **1**:268–294.
19. **Jameson, L., W. W. Chin, A. N. Hollenberg, A. S. Chang, and J. F. Habener.** 1984. The gene encoding the β-subunit of rat luteinizing hormone. J. Biol. Chem. **259**:15474–15480.
20. **Jameson, J. L., R. C. Jaffe, P. J. Deutsch, C. Albanese, and J. F. Habener.** 1988. The gonadotropin α-gene contains multiple protein binding domains that interact to modulate basal and cAMP-responsive transcription. J. Biol. Chem. **263**:9879–9886.
21. **Jameson, J. L., R. C. Jaffe, S. L. Gleason, and J. F. Habener.** 1986. Transcriptional regulation of chorionic gonadotropin α and β-subunit gene expression by 8-bromo-adenosine 3′,5′-monophosphate. Endocrinology **119**:2560–2567.
22. **Jameson, J. L., C. M. Lindell, and J. F. Habener.** 1986. Evolution of different transcriptional start sites in the human luteinizing hormone and chorionic gonadotropin beta subunit genes. DNA **5**:227–234.
23. **Jameson, J. L., C. M. Lindell, and J. F. Habener.** 1987. Gonadotropin and thyrotropin α and β subunit gene expression in normal and neoplastic tissues characterized using specific mRNA hybridization probes. J. Clin. Endocrinol. Metab. **64**: 319–327.
24. **Laimins, L. A., G. Khoury, C. Gorman, B. Howard, and P. Gruss.** 1982. Host-specific activation of transcription by tandem repeats from simian virus 40 and Moloney murine sarcoma virus. Proc. Natl. Acad. Sci. USA **79**:6453–6457.
25. **Lindenmaier, W., H. Hauser, I. G. de Wilke, and G. Schutz.** 1982. Gene shuttling: moving of cloned DNA into and out of eukaryotic cells. Nucleic Acids Res. **10**:1243–1256.
26. **Lopata, M. A., D. W. Cleveland, and B. Sollner-Webb.** 1984. High level transient expression of a chloramphenicol acetyl transferase gene by DEAE-dextran mediated DNA transfection coupled with dimethyl sulfoxide or glycerol shock treatment. Nucleic Acids Res. **12**:5707–5717.
27. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Maniatis, T., S. Goodbourn, and J. A. Fischer.** 1987. Regulation of inducible and tissue-specific gene expression. Science **236**: 1237–1245.
29. **Milstead, A., R. P. Cox, and J. H. Nilson.** 1987. Cyclic AMP regulates transcription of the genes encoding human chorionic gonadotropin with different kinetics. DNA **6**:213–219.
30. **Policastro, P., C. E. Ovitt, M. Hoshina, H. Fukuoka, M. R. Boothby, and I. Boime.** 1983. The β subunit of human chorionic gonadotropin is encoded by multiple genes. J. Biol. Chem. **258**: 11492–11499.
31. **Policastro, P. F., S. Daniels-McQueen, G. Carle, and I. Boime.** 1986. A map of the hCGβ-LHβ gene cluster. J. Biol. Chem. **251**: 5907–5916.
32. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
33. **Silver, B. J., J. A. Bokar, J. B. Virgin, E. A. Vallen, A. Milsted, and J. H. Nilson.** 1987. Cyclic AMP regulation of the human glycoprotein hormone α-subunit gene is mediated by an 18-base-pair element. Proc. Natl. Acad. Sci. USA **84**:2198–2202.
34. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
35. **Talmadge, K., W. R. Boorstein, N. C. Vamvakopoulos, M. J. Gething, and J. C. Fields.** 1984. Only three of seven human chorionic gonadotropin beta subunit genes can be expressed in the placenta. Nucleic Acids Res. **12**:8415–8436.
36. **Talmadge, K., N. C. Vamvakopoulos, and J. C. Fiddes.** 1984. Evolution of the genes for the β subunits of human chorionic gonadotropin and luteinizing hormone. Nature (London) **307**: 37–40.
37. **Tepper, M. A., and J. L. Roberts.** 1984. Evidence for only β-luteinizing hormone and no β-chorionic gonadotropin gene in the rat. Endocrinology **115**:385–391.
38. **Virgin, J. B., B. J. Silver, A. R. Thomason, and J. H. Nilson.** 1985. The gene for the β subunit of bovine luteinizing hormone encodes a gondoatropin mRNA with an unusually short 5′ untranslated region. J. Biol. Chem. **260**:7072–7077.
39. **Wigler, M., A. Pellicer, S. Silverstein, R. Axel, G. Urlaub, and L. Chasin.** 1979. DNA-mediated transfer of the adenosine phosphoribosyltransferase locus into mammalian cells. Proc. Natl. Acad. Sci. USA **76**:1373–1376.