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Expression of the Glycoprotein Hormone α-Subunit Gene in the Placenta Requires a Functional Cyclic AMP Response Element, whereas a Different *cis*-Acting Element Mediates Pituitary-Specific Expression

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The single-copy gene encoding the α subunit of glycoprotein hormones is expressed in the pituitaries of all mammals and in the placentas of only primates and horses. We have systematically analyzed the promoter-regulatory elements of the human and bovine α-subunit genes to elucidate the molecular mechanisms underlying their divergent patterns of tissue-specific expression. This analysis entailed the use of transient expression assays in a chorionic gonadotropin-secreting human choriocarcinoma cell line, protein-DNA binding assays, and expression of chimeric forms of human or bovine α subunit genes in transgenic mice. From the results, we conclude that placental expression of the human α-subunit gene requires a functional cyclic AMP response element (CRE) that is present as a tandem repeat in the promoter-regulatory region. In contrast, the promoter-regulatory region of the bovine α-subunit gene, as well as of the rat and mouse genes, was found to contain a single CRE homolog that differed from its human counterpart by a single nucleotide. This difference substantially reduced the binding affinity of the bovine CRE homolog for the nuclear protein that bound to the human α CRE and thereby rendered the bovine α-subunit promoter inactive in human choriocarcinoma cells. However, conversion of the bovine α CRE homolog to an authentic α CRE restored activity to the bovine α-subunit promoter in choriocarcinoma cells. Similarly, a human but not a bovine α transgene was expressed in placenta in transgenic mice. Thus, placenta-specific expression of the human α-subunit gene may be the consequence of the recent evolution of a functional CRE. Expression of the human α transgene in mouse placenta further suggests that evolution of placenta-specific *trans*-acting factors preceded the appearance of this element. Finally, in contrast to their divergent patterns of placental expression, both the human and bovine α-subunit transgenes were expressed in mouse pituitary, indicating differences in the composition of the enhancers required for pituitary- and placenta-specific expression.

The glycoprotein hormone family consists of luteinizing hormone, follicle-stimulating hormone, thyroid-stimulating hormone, and chorionic gonadotropin. These hormones control diverse biological functions, including sexual function, pregnancy, and metabolism. They are structurally similar, each consisting of an α subunit, common to all four hormones, and a unique, noncovalently associated β subunit (27). Expression of the glycoprotein hormone genes is controlled by a number of hormones, including sex steroids (21, 23, 26), thyroid hormone (30), hypothalamic hormones (15, 16, 25), and intracellular signals that act through cyclic AMP (cAMP)-dependent protein kinase A and protein kinase C (1, 6–8, 31). The genes are also under strict tissue-specific control. All mammals synthesize luteinizing, follicle-stimulating, and thyroid-stimulating hormones in the pituitary, whereas synthesis of chorionic gonadotropin occurs in the placentas of only primates and horses (5, 10). Thus, in primates and horses, synthesis of the full spectrum of glycoprotein hormones requires expression of the single-copy gene in two locations, pituitary and placenta.

Recent studies with human choriocarcinoma cells indicate that placenta-specific expression of the α-subunit gene requires at least two sequence elements (6, 20, 31). These elements are located in the 5′-flanking region between nucleotides −169 and −100 relative to the start site of transcription. One of these elements, located between −169 and −154 and referred to as the upstream regulatory element (URE), binds a protein present in choriocarcinoma cells and presumably human placenta (6, 20). The enhancer activity of the URE depends on an adjacent sequence element located between positions −145 and −111. This sequence is an 18-base-pair direct repeat; a single copy mediates stimulation of transcription by cAMP and is referred to as the cAMP response element (CRE) (31). The CRE binds a protein, CREB, which is distinct from the protein(s) that binds to the URE and appears to be highly conserved across tissues and species (2, 17, 28, 33). The CRE also differs from the URE because it functions independently to confer cAMP responsiveness to a heterologous promoter. However, the CRE mediates placenta-specific expression only when linked to the URE, suggesting that this property requires an interaction between the proteins that bind to the URE and CRE. Consequently, we use the term placenta-specific enhancer to refer to the bipartite, *cis*-acting element composed of both the URE and CRE.

Why is the α-subunit gene expressed in the placentas of primates and horses, whereas its expression is confined to the pituitary in other mammals? Acquisition of placental expression may be the result of a recent evolutionary event whereby multiple *cis*-acting elements combined to form a

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new enhancer. The bipartite character of the placenta-specific enhancer is consistent with such a mechanism. Moreover, if placenta-specific expression of the α-subunit gene is a recent evolutionary event, then other *cis*-acting elements, distinct from those required for placenta-specific expression, may govern pituitary-specific expression. To test this hypothesis, we systematically analyzed the promoter-regulatory elements of the human and bovine α-subunit genes to further explore the sequence requirements for pituitary- and placenta-specific expression. This comparison entailed the use of protein-DNA binding assays, transient expression of chimeric reporter genes in choriocarcinoma cells, and transgenic animals. Results reported herein support the view that the formation of the α-subunit placenta-specific enhancer is a recent evolutionary event stemming from the formation of a functional CRE from an ancestral homolog. We also propose that a distinct *cis*-acting element is required for pituitary-specific expression of the α-subunit gene.

MATERIALS AND METHODS

Materials. [^{14}C]chloramphenicol (60 mCi/mmol), [α-^{32}P]-dCTP (800 Ci/mmol), and [γ-^{32}P]ATP (3,000 Ci/mmol) were purchased from Dupont, NEN Research Products. Nonradioactive deoxyribonucleotides, acetyl coenzyme A, and 8-Br-cAMP were obtained from Sigma Chemical Co. Proteinase K, Klenow fragment, T4 polynucleotide kinase, and restriction enzymes were purchased from Boehringer Mannheim Biochemicals. Sequenase was obtained from U.S. Biochemical Corp. Silica gel thin-layer chromatography plates were purchased from J. T. Baker Chemical Co. Nitrocellulose (BA-85) was obtained from Schleicher & Schuell, Inc. All other materials were of the highest grade available.

Synthesis of oligodeoxyribonucleotides. Oligodeoxyribonucleotides were synthesized with an Applied Biosystems 380A DNA synthesizer. The following oligodeoxyribonucleotides were designed to have *Xba*I-compatible ends to facilitate cloning. Their specific sequences are as follows: α18, 5′-ctagAAATTGACGTCATGGTAA-3′ and 5′-ctagTTACCATGACGTCAATTT-3′; α18μ8, 5′-ctagAAATTGATGTCATGGTAA-3′ and 5′-ctagTTACCATGATGTCTTAAA-3′; and α18μ6-11, 5′-ctagAAATTCTTCGGATGGTAA-3′ and 5′-ctagTTACCATCCGAAGAATTT-3′.

Construction of plasmids. Plasmids pHαCAT, pXSV1 CAT, and pRSVCAT are described by Silver et al. (31). pHα(−290)CAT was made by isolating sequences −290 to +21 of the human α-subunit gene by digesting pHαCAT with *Alu*I. This restriction fragment was ligated into the *Hin*dIII site of pSV0CAT (13), using *Hin*dIII linkers. A *Bgl*II-*Xho*II restriction fragment containing bovine α-subunit gene sequences from −313 to +48 was isolated from the bovine α-subunit genomic clone pBα$_1$ (12). This restriction fragment was ligated into the *Hin*dIII site of pSV0CAT by using *Hin*dIII linkers to generate pBαCAT. These constructs were used to generate the remaining constructs shown in Fig. 3 and 4 (with the exception of pBα*CAT) by standard molecular biology techniques. To make pBα*CAT, a double-stranded oligodeoxyribonucleotide containing the sequence of the bovine gene from −166 to −115 with a T-to-C substitution at position −136 and containing a *Hin*dIII-compatible 5′ end was chemically synthesized. This fragment was ligated to a *Rsa*I-*Hin*dIII (−114 to +48) fragment from pBαCAT, and the products were redigested with *Hin*dIII. A 222-bp fragment recovered after electrophoresis represents bovine sequences −166 to +48, with a T-to-C substitution at −136. This fragment was inserted into the *Hin*dIII site of pSV0CAT. The plasmids illustrated in Fig. 5 were made by ligating the double-stranded oligodeoxyribonucleotides α18, α18μ8, and α18μ6-11 into the *Xba*I site of pXSV1CAT. The sequences of the regions of plasmids that were generated by using oligodeoxyribonucleotides were verified by dideoxy sequencing of supercoiled plasmid DNA.

Cell culture, transfection, and CAT assays. Cell culture, transfections, and chloramphenicol acetyltransferase (CAT) assays of cell lysates were all performed as previously described (31). Control and cAMP-treated samples were always derived from single transfected plates, which were subsequently passaged 1:2 to control for any variability in transfection efficiency.

Preparation of nuclear extracts. BeWo nuclear extracts were prepared by the method of Dignam et al. (9), with the modification that the extract was brought to 50% saturation with $(NH_4)_2SO_4$. The pellet was resuspended in buffer D (9) and dialyzed against 100 volumes of buffer D before storage at −80°C. Protein concentrations were determined by the method of Bradford (3), using Bio-Rad reagents. Nuclear extract was heated at 65°C for 1 min before use in the band shift assay to inactivate a non-sequence-specific binding protein (2).

Gel retardation assays. Probes for the gel retardation assays were prepared by annealing synthetic oligodeoxyribonucleotides for 60 min in 150 mM NaCl–10 mM Tris (pH 7.6), 1 mM EDTA and labeling with T4 polynucleotide kinase and [γ-^{32}P]ATP. Binding assays were performed in a volume of 20 μl consisting of 0.2 ng of probe (14 fmol; ~100,000 cpm), 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.6), 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 50 mM NaCl, and 4 μg of nuclear protein. Incubations were carried out at room temperature for 20 min. Bromophenol blue was added to each sample, and the samples were applied to a 6% polyacrylamide gel (44:0.8, acrylamide/bisacrylamide) in 25 mM Tris–0.19 M glycine (pH 7.9). Electrophoresis was performed at room temperature at 35 mA for 2 h, with buffer recirculation. The gel was transferred to Whatman 3MM paper, dried, and analyzed by autoradiography.

Production and identification of transgenic mice. Plasmids pHαCAT and pBαCAT were digested with *Bam*HI (just 3′ to the CAT gene). Linearized DNA was purified by agarose gel electrophoresis and was suspended in 10 mM Tris–1 mM EDTA at a concentration of 2 μg/ml. Linearized HαCAT solution (2 pl) was microinjected into the male pronuclei of fertilized mouse eggs obtained from C57BL6/SJL mice as described by Wagner et al. (32). Injected eggs were reimplanted in oviducts of pseudopregnant females and allowed to develop to term. After birth and subsequent weaning, mice were analyzed for possession of the transgene by a slot blot of DNA isolated from tail sections (24), using a CAT-specific DNA probe (23).

CAT assays of tissues from mice. Mice were sacrificed by asphyxiation in a chamber of dry ice. Tissue samples (approximately 3 mm^3 in size) were removed and immediately placed in ice-cold 0.25 M Tris hydrochloride (pH 7.8). Placentas were collected from pregnant females between days 12 and 20 of pregnancy. Placentas were always harvested with a small amount of uterine tissue attached to ensure that the syncytiotrophoblast was obtained. Tissues were homogenized with a disposable Kontes pellet pestle, and lysates were prepared by three freeze-thaw cycles. Cellular debris was pelleted by centrifugation in a microfuge

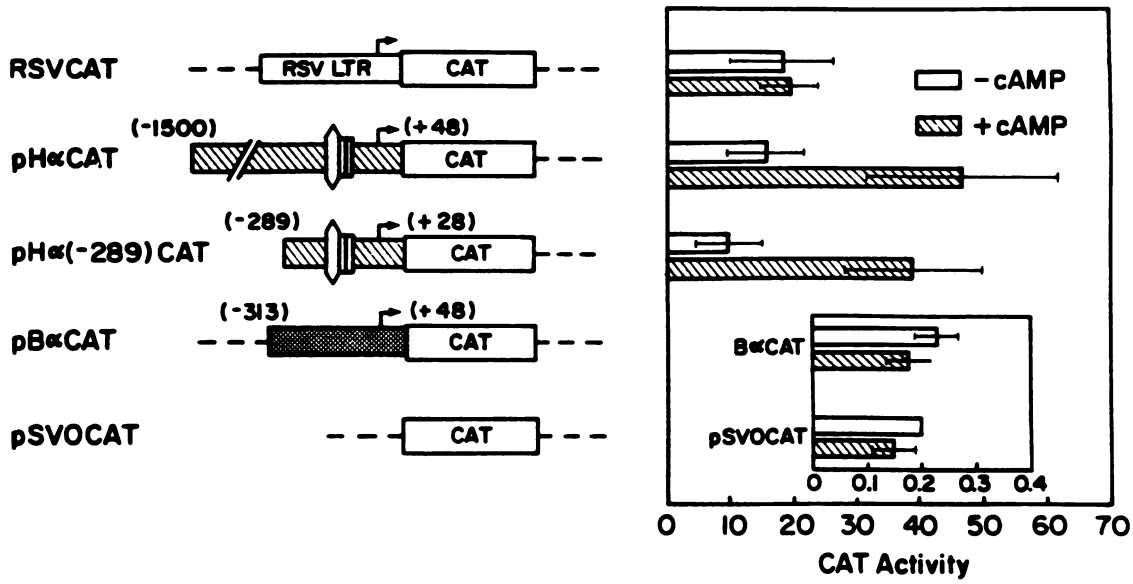

FIG. 1. Differential activities of the human and bovine α-subunit promoters in choriocarcinoma cells. The indicated promoter-regulatory regions from either Rous sarcoma virus, the human α-subunit gene, or the bovine α-subunit gene were fused to the CAT-coding sequence by ligation into the *Hin*dIII site in pSV0CAT (14). The resulting constructs were transfected into BeWo choriocarcinoma cells. Each transfected plate was subcultured; half of the cells were treated with medium containing 1 mM 8-Br-cAMP, and the other half were given control medium. Cell lysates were prepared and subsequently tested for CAT activity. The values reported for CAT activity are means and standard errors of the mean from an average of six transfections (minimum of three). CAT activity is expressed as percent conversion per hour per 100 μg of protein.

for 5 min at 4°C. The lysate was then heated at 65°C to remove endogenous CAT-like activity. Denatured proteins were pelleted by centrifugation in a microfuge for 10 min at 4°C, and CAT assays were performed on the supernatant. Samples containing 25 to 100 μg of protein (as determined by a protein assay [3] with Bio-Rad reagents) were diluted to 150 μl with 0.25 M Tris hydrochloride (pH 7.8). The CAT reaction was initiated by addition of 30 μl of 1 M Tris hydrochloride (pH 7.8), 20 μl of 40 mM acetyl coenzyme A, and 1 μl of [^{14}C]chloramphenicol. The reaction was allowed to proceed overnight (approximately 20 h) at 37°C. The samples were extracted with 1 ml of ethyl acetate, concentrated, and analyzed by thin-layer chromatography (14).

RESULTS

Differential expression of human α- and bovine α-CAT fusion genes in choriocarcinoma cells. The α-subunit gene may be silent in bovine placenta for a number of reasons, including lack of critical *cis*-acting elements in the 5′ promoter-regulatory region, the presence of negative regulatory elements, the absence of necessary *trans*-acting factors, or combinations of all three possibilities. To distinguish between these alternatives, we carried out a series of transfection experiments in human choriocarcinoma cells. These cells were selected because they express their endogenous α and chorionic gonadotropin β genes; thus, they contain all of the *trans*-acting factors required for placenta-specific expression. The presence of placenta-specific *trans*-acting factors means that the bovine α promoter-regulatory region can be tested directly for positive or negative *cis*-acting regulatory elements.

To test whether the bovine α promoter-regulatory region contains the *cis*-acting elements required for placenta-specific expression, a chimeric gene containing 313 bp of bovine α 5′-flanking sequence linked to the reporter gene encoding bacterial CAT was transfected into BeWo cells, a human choriocarcinoma cell line (Fig. 1). Activity of this construct was no greater than the activity of pSV0CAT, a promoterless parent vector that served as a negative control. Neither vector responded to cAMP. Two vectors containing either 1,500 (pHαCAT) or 290 [pHα(−290)CAT] bp of 5′-flanking sequence from the human α-subunit gene were expressed at levels approaching that of RSVCAT, a positive control that utilizes the Rous sarcoma virus promoter-enhancer to direct efficient expression of the CAT gene. Both human α-CAT vectors were responsive to cAMP, indicating the presence of a functional placenta-specific enhancer. These results are also consistent with the pattern of expression of the human and bovine α-subunit genes in vivo; the human α-subunit gene is actively transcribed in placenta, whereas bovine placenta lacks detectable α-subunit mRNA (unpublished observation).

Inactivity of the bovine α-subunit promoter in human choriocarcinoma cells suggests that it either lacks a placenta-specific enhancer or contains negative *cis*-acting elements that suppress its activity in placenta. The latter possibility is unlikely on the basis of transfection experiments in which the bovine α 5′-flanking region from pBαCAT was inserted 3′ to the CAT gene in pHαCAT; such relocation failed to attenuate activity of the human α-subunit promoter (data not shown).

The sequences of the human and bovine α promoter-regulatory regions are homologous but contain critical differences in the regions corresponding to the URE and CRE. As indicated earlier, the placenta-specific enhancer of the human α-subunit gene contains two different *cis*-acting elements, the URE and CRE. Both are required for placental activity. Sequence comparison of the human and bovine α 5′-flanking regions indicates a sequence identity of 85% out to position −313, with the exception of two gapped regions

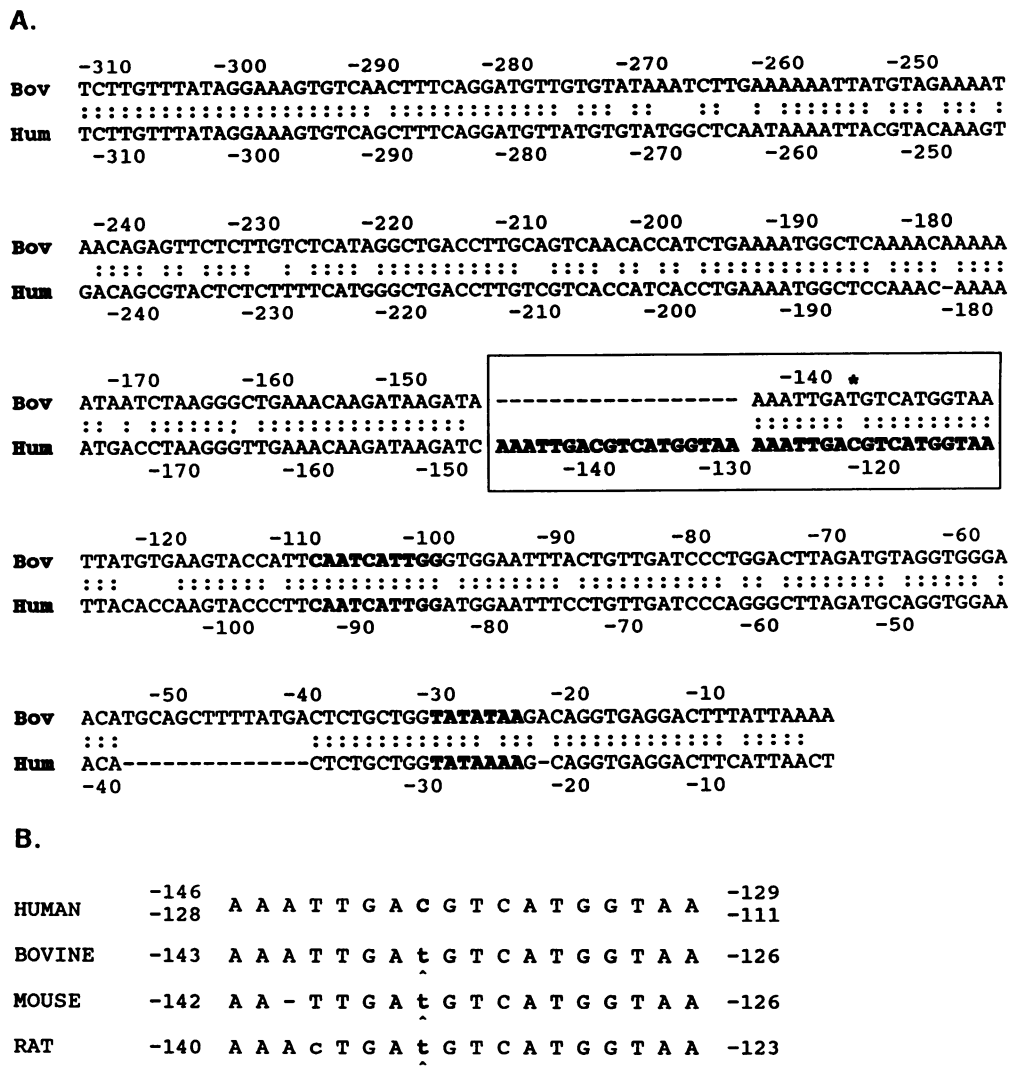

FIG. 2. Comparison of the 5'-flanking sequences of the human and bovine α-subunit genes. (A) Sequence of the bovine α-subunit 5'-flanking region (12). The sequence of the human α-subunit 5'-flanking region was determined by the dideoxy-chain termination method (29). The sequences were aligned for maximum homology; gapped regions (−) and nucleotides that are identical for the two genes (:) are indicated. The boldface region near −30 corresponds to the TATA consensus element. The boldface region near −110 of the bovine sequence represents the CCAAT homology. The two boxes extending from −146 to −111 in the human sequence contain the human α CRE and the homologous bovine region. *, The only nucleotide in the CRE region that is different between the human and bovine genes. (B) Sequence of the human α-subunit CRE and sequences from homologous regions in the bovine, murine (13), and rat (4) genes. Lowercase letters and the dash represent differences and a deletion, respectively, relative to the human sequence. The carat indicates the conserved nucleotide difference relative to the human sequence.

(Fig. 2A). The bovine sequence shows striking differences within the region of the human placenta-specific enhancer (−169 to −100). For example, whereas the human α-subunit gene contains an 18-bp direct repeat (two tandem CRE) between positions −146 and −111, the bovine gene contains only one CRE homolog. Moreover, the bovine homolog to the human α CRE contains a single-base mismatch centrally located in the palindromic core sequence (thymine rather than cytosine). Such a transition could disrupt activity of the CRE. Interestingly, both the rat and mouse α-subunit genes are also silent in placenta, and both contain a single CRE homolog with the same C→T transition (Fig. 2B). In addition to differences noted for the CRE, the 30-bp region immediately upstream of the bovine CRE homolog contains four single-base mismatches relative to the human sequence. The URE is found within this region of the human α-subunit gene. Accordingly, the bovine α gene may be silent in placenta because of a nonfunctional URE or CRE, either of which would disrupt activity of the placenta-specific enhancer.

The C→T transition renders the bovine CRE homolog nonfunctional. The CRE in the human α gene interacts sequence specifically and with high affinity ($K_d = \sim 10^{-10}$ M) with a nuclear protein(s) found in a number of cell types (2). To test whether the C→T transition in the bovine α CRE homolog affects its ability to bind the same nuclear protein(s) as the human α CRE, we performed competition gel mobility shift assays. A double-stranded oligodeoxyribonucleotide corresponding to the 18-bp human α CRE (α18) was radiolabeled and incubated with nuclear protein prepared from BeWo cells. Additional nonradiolabeled human α CRE (α18) or the homologous 18-bp region of the bovine α-subunit gene

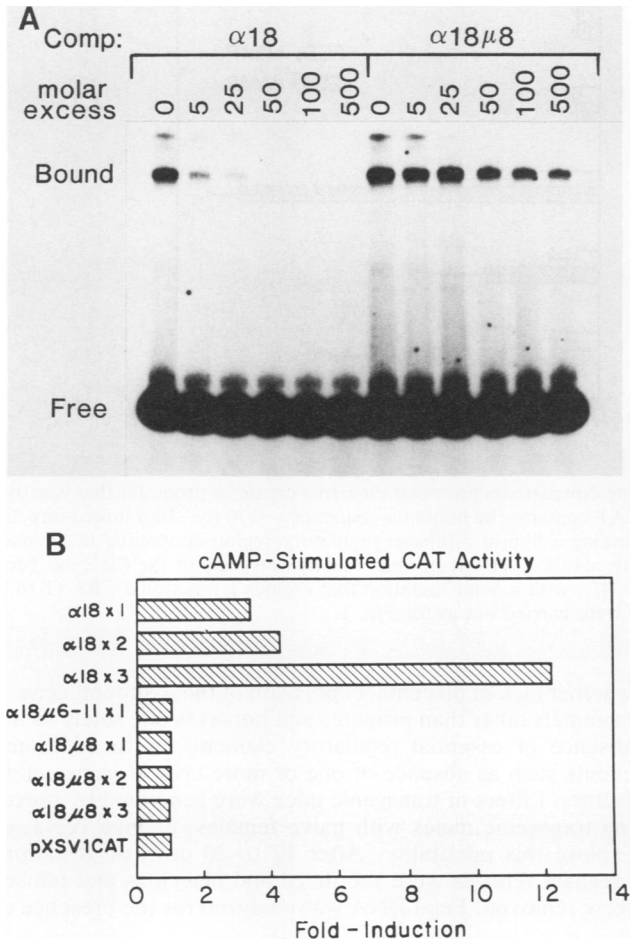

FIG. 3. Comparison of the ability of the human α CRE and the bovine CRE homolog to bind a nuclear protein from BeWo cells and to mediate cAMP-inducible transcription. (A) Autoradiograph of a competition gel mobility shift assay. Each lane contained 0.2 ng of radiolabeled α18 incubated for 20 min with 4 μg of heat treated nuclear extract, followed by electrophoresis on a nondenaturing Tris-glycine polyacrylamide gel. The amount of unlabeled α18 or α18μ8 included in the incubation mixture is indicated above each lane as the molar excess of unlabeled competitor relative to probe. Sequences of the oligodeoxyribonucleotides used are given in Materials and Methods. (B) CAT activity. One to three copies of either α18 or α18μ8 or a single copy of α18μ6-11 was inserted into the *Xba*I site of pXSV1CAT, and the constructs were transfected into BeWo cells. Relative CAT activity is reported as for Fig. 1. Values are means of an average of six transfections.

(α18μ8) was present in parallel reactions to test for the ability of these sequences to compete with radiolabeled α18 for specific binding (Fig. 3A). A fivefold molar excess of α18 completely competed for binding of the sequence-specific nuclear protein to radiolabeled α18. In contrast, a 1,000-fold molar excess of the homologous bovine sequence (α18μ8), differing by only a single base pair, competed weakly for binding. Therefore, the affinity of the bovine α CRE homolog for a sequence-specific DNA-binding protein in BeWo nuclei is at least 200 times lower than that of the human α CRE. Moreover, the affinity of the bovine CRE homolog (α18μ8) was identical to that of a negative control oligodeoxyribonucleotide that had a 6-bp substitution within the core consensus region of the CRE (α18μ6-11; data not shown).

The functional significance of the C→T transition in the bovine α CRE homolog was tested by transfection. One to three copies of either α18 (the human α CRE) or α18μ8 (the bovine α CRE homolog) or a single copy of α18μ6-11 (negative control) was inserted upstream of the enhancerless simian virus 40 (SV40) early promoter in the vector pXSV1CAT. A single copy of α18 conferred cAMP responsiveness to the modified SV40 promoter, whereas multiple copies conferred an even greater induction (Fig. 3B). Both the negative control sequence (α18μ6-11) and one to three copies of the bovine CRE homolog (α18μ8) failed to confer cAMP responsiveness to the SV40 promoter. We conclude that the inability of the bovine α CRE homolog to bind CREB is one reason why the bovine α promoter is inactive in choriocarcinoma cells and presumably in placenta in vivo.

A functional CRE restores activity of the bovine α-subunit promoter in human choriocarcinoma cells. To determine more precisely the sequence requirements for activation of the bovine α-subunit promoter, the human URE-CRE, contained within the region −170 to −100, was linked to the bovine proximal promoter region, −114 to +48 (pHu BαCAT; see Fig. 4). The 5′-flanking sequence of this vector is similar to the bovine wild-type sequence except for the following: (i) it contains two functional CRE rather than one nonfunctional CRE, (ii) there are two point mutations in the region just upstream from the CRE, and (iii) there are four additional mismatches downstream of the CRE (however, this region is not thought to be a part of the tissue-specific enhancer). These changes increased activity of the bovine promoter approximately fourfold relative to the activity of either the promoterless pSV0CAT parent vector or the intact pBαCAT construct (Fig. 4). An additional 10-fold increase in activity resulted after addition of cAMP. Thus, the bovine promoter can function when linked in *cis* to a bona fide placenta-specific enhancer. In other experiments (data not shown), we demonstrated that the bovine α-subunit promoter initiates transcription from the correct start site when linked to the heterologous human α enhancer. Consequently, it is the bovine α-subunit promoter rather than a cryptic promoter that directs transcription.

Two additional vectors, p(Δ18)HuBαCAT and pBα*CAT, were used to determine whether the C→T transition in the bovine α CRE homolog was solely responsible for inactivity of the bovine α-subunit promoter in human choriocarcinoma cells. The former vector is similar to pHuBαCAT but contains only a single copy of the CRE. In untreated cells, p(Δ18)HuBαCAT was inactive, as indicated by comparison with the two negative controls, pSV0CAT and pBαCAT. However, addition of cAMP stimulated activity of p(Δ18)Hu BαCAT by approximately threefold, whereas activity of the two negative controls remained unchanged. The construct p(Δ18)HuBαCAT is a critical control because it indicates the level of activity expected when a functional URE (from the human α promoter) is linked to one rather than two functional CRE. Thus, the activity of pBα*CAT, which contains the bovine α 5′-flanking sequence (to −170) with a single point mutation that converts the bovine α CRE homolog to a functional CRE, can be compared directly with the activity of p(Δ18)HuBαCAT. Such a comparison reveals that the C→T transition within the bovine α CRE homolog restored full transcriptional activity to the bovine α-subunit promoter. The nearly identical profiles of these two vectors underscores the importance of the CRE regarding placenta-specific expression and also suggests that the bovine α-subunit promoter-regulatory region contains a functional

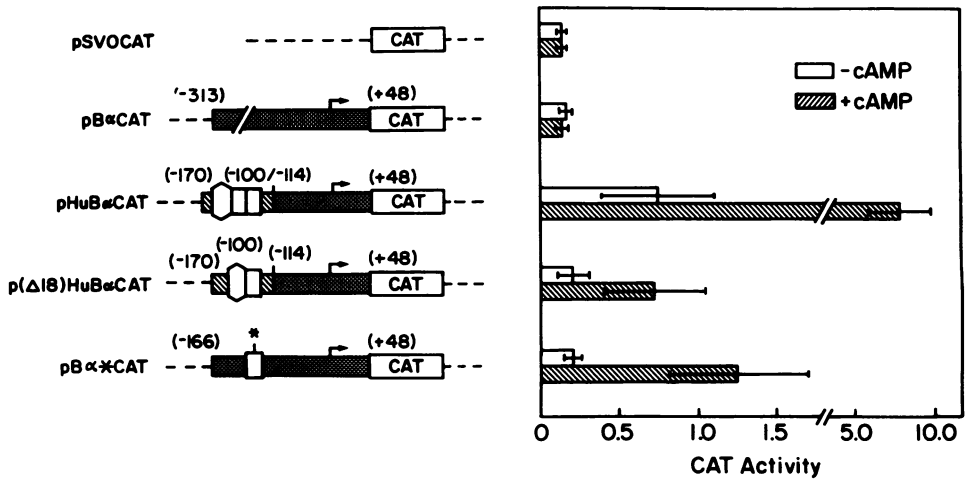

FIG. 4. Demonstration that fusion of the human URE-CRE to the bovine downstream promoter elements creates a promoter that is active in BeWo cells. pSV0CAT and pBαCAT are described in Fig. 1. pHuBαCAT contains the human α sequence (−170 to −100) linked directly to the downstream sequence (−114 to +48) of the bovine promoter, forming a hybrid promoter-regulatory region containing the human URE-CRE linked to the bovine promoter. p(Δ18)HuBαCAT is identical to pHuBαCAT except that exactly one copy of the CRE has been deleted. pBα*CAT contains the bovine α 5′-flanking sequence (−166 to +45), with a point mutation that creates a functional CRE (T to C at position −136), linked to CAT. Transfections and CAT determination were carried out as for Fig. 1.

URE despite two nucleotide differences relative to the human URE.

Pituitary-specific expression of the bovine α promoter in transgenic mice occurs despite the absence of a functional CRE. The α-subunit gene is expressed in the pituitary of all mammals. To determine whether pituitary-specific promoter-regulatory elements are distinct from placenta-specific elements, we constructed transgenic mice harboring either pHαCAT, with 1,500 bp of human α 5′-flanking sequence (containing the tandemly repeated CRE), or pBαCAT, with 313 bp of bovine α 5′-flanking sequence (lacking a functional CRE). Founder mice containing either of the transgenes were bred with naive (CF1) mice, and the progeny were screened by slot blot hybridization with a CAT-specific probe. Resulting progeny were analyzed for expression of the CAT reporter gene in a variety of tissues (Fig. 5). The only tissues that had detectable levels of CAT activity were pituitary and brain. The latter result was not entirely surprising given reports of immunoreactive thyroid-stimulating hormone (18) and luteinizing hormone (19) in brain.

All other tissues tested lacked detectable CAT activity. Similar results were obtained in 14 of 24 mice tested in two distinct lines (different sites of integration) of mice containing the pHαCAT transgene and in 10 of 12 mice from three distinct lines (or sites of integration) containing the pBαCAT transgene (Table 1). Progeny from a fourth line of transgenic mice failed to express the bovine transgene in any tissue (see Discussion). Tissues were also removed from naive mice and analyzed for CAT activity; no tissues were positive, including pituitary and brain (data not shown). We use these collective results to suggest that the proximal 1,500 bp of 5′-flanking region of the human α-subunit gene and the proximal 313 bp of the bovine α-subunit gene each contains sufficient information to permit pituitary-specific expression. Pituitary-specific expression of pBαCAT occurred despite the absence of a functional CRE, consistent with the possibility that the compositions of the pituitary- and placenta-specific enhancers are different.

Only the human α-subunit 5′-flanking sequences direct placenta-specific expression in transgenic mice. It is unclear whether lack of placental expression of the α-subunit gene in mammals other than primates and horses is due solely to the absence of essential regulatory elements or to additional events such as absence of one or more critical *trans*-acting factors. Litters of transgenic mice were generated by breeding transgenic males with naive females, or vice versa, to explore this possibility. After 12 to 20 days of gestation, pregnant females were sacrificed and placentas and fetuses were removed. Fetal DNA was analyzed for the presence of

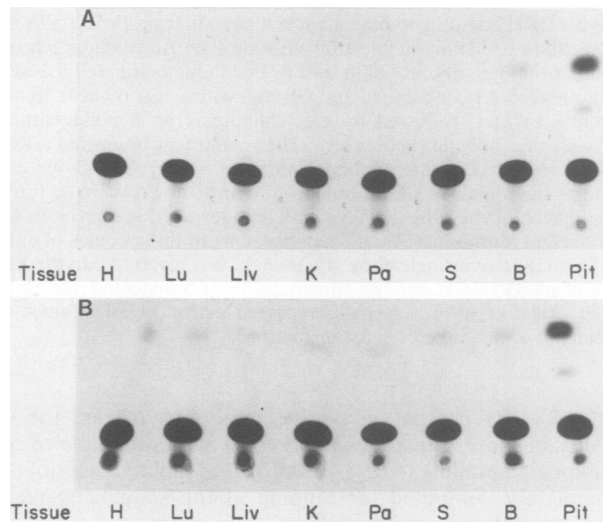

FIG. 5. Tissue-specific expression of the human and bovine α-subunit gene promoters in transgenic mice. Transgenic mice harboring either HαCAT or BαCAT were produced. Upon reaching sexual maturity, the mice were sacrificed and the following tissues were collected: heart (H), lung (Lu), liver (Liv), kidney (K), pancreas (Pa), spleen (S), brain (B), and pituitary (Pit). Cell lysates were prepared from each tissue, and equivalent amounts of protein were assayed for CAT activity. (A) CAT activity in tissues prepared from mouse 241 from the 3CHα2 line; (B) CAT activity in tissues prepared from mouse 200 from the 5ABα1 line.

TABLE 1. Expression of human and bovine α transgenes in transgenic mice

Mouse line[a]	No. with CAT+ pituitary/no. sampled[b]	No. of litters	No. of transgenic pups	No. with CAT+ placenta[c]
pHα(−1500)CAT				
3AHα1	6/7	4	45	17
3CHα2	8/17	9	38	34
pBα(−314)CAT				
5ABα1A	6/6	12	61	0
5ABα1B	2/3	1	4	0
5ABα3	2/3			
6BBα1	0/3	2	21	0
CF1	0/8			

[a] Lines of transgenic mice were derived from founder animals carrying the pHαCAT (3AHα1 and 3CHα2) or pBαCAT (5ABα1A, 5ABα1B, 5ABα3, and 6BBα1) transgenes.

[b] Pituitaries, nonpituitary tissues, and placentas were analyzed for CAT activity. Tissues were determined to express the transgene in the following manner. For each mouse, samples of heart, lung, liver, kidney, pancreas, spleen, brain, and pituitary were collected and tested for CAT activity. A particular tissue sample was considered to be positive for expression (CAT+) if its CAT activity was greater than 3 standard deviations above the mean of the six tissues (heart, lung, liver, kidney, pancreas, and spleen) from the same mouse. In no instance did the activity of one of these six tissues ever fit the criterion for positive expression.

[c] A placenta from a transgenic pup was considered to be positive for expression if its CAT activity was greater than 3 standard deviations above the mean for the nontransgenic placentas from the same litter. No nontransgenic placentas ever fit the criterion for positive expression.

the transgene by slot blot hybridization (Fig. 6). This analysis permitted a correlation between inheritance of the transgene with CAT activity in placenta. For example, in the initial breeding experiment (Fig. 6A), we observed a perfect correlation between these two parameters. Extended analysis of transgenic pups propagated by breeding several different animals from this line of mice indicated that 34 of 38 placentas from fetuses that harbored the human α-CAT transgene had levels of CAT activity that were at least 3 standard deviations greater than that present in the placentas obtained from nontransgenic pups (Table 1). Analysis of an independently derived line of human α-CAT transgenic mice indicated more variable levels of placental expression, with 17 of 45 pups meeting the criterion described above. This is a conservative estimate because 3 standard deviations above the control is a rigorous statistical criterion, approaching the 99.9% confidence interval for 45 animals. Perhaps more important, CAT activity has never been detected in placentas from pups that do not contain the transgene. Therefore, placenta-specific expression of CAT is likely governed by a DNA-regulatory element and not the result of integration into a favorable chromosomal site.

In contrast to human α-CAT transgenic mice, CAT activity was not detected in the placentas of mice containing the bovine α-CAT transgene (Fig. 6B, Table 1). Fifteen litters containing a total of 86 transgenic pups were analyzed. These litters were derived from two different founder animals. The bovine α transgene integrated into multiple sites in one of the founders (5ABα1). Two lines of mice (5ABα1A and 5ABα1B; Table 1) representing different integration sites were derived by subsequent breeding of this mouse. The other founder, 6BBα1, harbors a single integration site. None of the subsequent progeny derived from either founder expressed the bovine α transgene in placenta. A large proportion of the progeny derived from 5ABα1 expressed

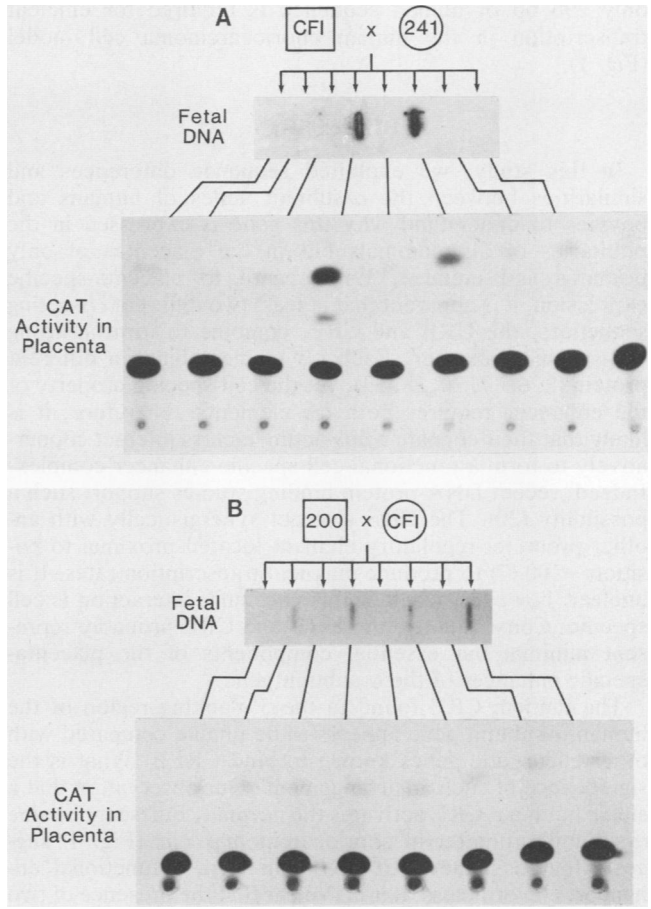

FIG. 6. Expression of the human but not the bovine α-subunit promoter region in placentas of transgenic mice. (A) A transgenic female mouse (241) harboring the HαCAT construct was bred with a naive male (CF1). At approximately 2 weeks of gestation, the pregnant female was sacrificed, and the pups and placentas were removed. DNA isolated from each pup was analyzed for CAT by slot blot hybridization with a CAT-specific probe. Cell lysates were prepared from the placentas, and equivalent amounts of protein from each placenta were assayed for CAT activity. The resulting autoradiographs of the DNA hybridization and the corresponding thin-layer chromatographs are shown. Nontransgenic placentas from mouse 241 had a mean activity of (0.095% conversion per μg of protein) × 100, with a standard deviation of 0.114. Therefore, to fit the criterion of positive expression, a placenta must have an activity of at least (0.440% conversion per μg of protein) × 100. The values for the placentas used in this experiment were (left to right) 0.120, 0.051, 0.008, 10.560, 0.080, 2.220, 0.00, and 0.310. (B) A transgenic male (200) harboring the BαCAT construct was bred with a naive female mouse (CF1). The pups and placentas were analyzed as for panel A. Nontransgenic placentas from mouse 200 had a mean activity of (0.127% conversion per μg of protein) × 100, with a standard deviation of 0.054. Therefore, to fit the criterion of positive expression, a placenta must have an activity of at least (0.300% conversion per μg of protein) × 100. The values for the placentas used in this experiment were (left to right) 0.111, 0.130, 0.073, 0.00, 0.123, 0.173, 0.202, and 0.082.

CAT specifically in the pituitary. Therefore, it is unlikely that lack of placenta-specific expression is due to an aberrant integration event. If elements upstream of −313 are necessary for placenta-specific expression, then such elements would be absent in the bovine construct but present in the human construct. This seems unlikely, however, because

only 290 bp of human sequence is required for efficient transcription in the human choriocarcinoma cell model (Fig. 1).

DISCUSSION

In this study, we exploited sequence differences and similarities between the α-subunit genes of humans and bovines to understand why this gene is expressed in the pituitaries of all mammals but in the placentas of only primates and equidae. With regard to placenta-specific expression, it is apparent that at least two different *cis*-acting sequences, the URE and CRE, combine to form a strong cell-specific enhancer. Each *cis* element binds a different protein (2, 6, 17, 20, 28, 33), yet the cell-specific property of the enhancer requires both *cis* elements. Therefore, it is likely that their cognate *trans*-acting factors interact cooperatively to form a functional cell-specific enhancer complex. Indeed, recent DNA-protein binding studies support such a possibility (20). The CRE also act synergistically with another promoter-regulatory element located proximal to position −100 (7) to produce maximal transcription rates. It is unclear, however, whether this particular interaction is cell specific. Consequently, the URE and CRE probably represent minimal but essential components of the placenta-specific enhancer of the α-subunit gene.

The tandem CRE found in the 5′-flanking region of the human α-subunit gene appears to be unique compared with other eucaryotic genes known to bind CREB. What is the significance of such an arrangement? Our observation that a single human α CRE activates the normally quiescent bovine α-subunit promoter in choriocarcinoma cells (Fig. 4) suggests that a single CRE-URE can form a functional enhancer. Nevertheless, it is also clear that the presence of two CRE imparts a synergistic effect on promoter activity that is tissue specific (6).

Our observation that the proximal 5′-flanking regions of the bovine, rat, and mouse α-subunit genes all have single CRE homologs rather than tandem CRE is consistent with the possibility that the human α CRE is a recent product of primate evolution. Although the bovine α CRE homolog contains a 17-of-18-bp match to its human counterpart, the C→T transition in the bovine α CRE lowers its binding affinity for CREB by at least 200-fold. The rat and mouse α CRE homologs have the same C→T transition and thus are also likely to bind CREB with much lower affinity. Although it is possible that the bovine α CRE homolog is a functional CRE in another tissue or species, this seems unlikely because sequence requirements for binding the nuclear protein are highly conserved among tissues and species (2, 28). For example, the C→T transition of the bovine α CRE is not found in the CRE of at least eight other cAMP-responsive genes expressed in a variety of tissues (28). Thus, the cytosine in the fourth position of the highly conserved core palindrome (TGACGTCA) of mammalian CRE may be an essential requirement for CREB binding.

We were surprised to find that the CRE is an essential component of the placenta-specific enhancer of the human α-subunit gene, especially given the ubiquitous distribution of CREB and its probable role as a mediator the A-kinase signal transduction pathway. Nevertheless, it is also important to recognize that additional sequence elements may also contribute to the transcriptional activity of the α-subunit promoter in placenta. For example, although the human URE-CRE can activate the bovine promoter in BeWo cells (pHuBαCAT; Fig. 4), the resulting chimeric construct remains about 10% as active as the pHαCAT construct in untreated cells and 20% as active in cAMP-stimulated cells. As stated above, previous studies indicate that the human α-subunit promoter region contains an element downstream of the CRE which is capable of interacting with the URE-CRE. The bovine α-subunit promoter region may lack this element or, alternatively, the interaction may be disrupted in the bovine promoter constructs because of an unfavorable spatial orientation between upstream and downstream promoter-regulatory elements. In this regard, it is noteworthy that the human α 5′-flanking region contains a 15-bp deletion relative to the bovine sequence (between the CCAAT and TATA boxes; Fig. 2).

Placenta-specific expression of pHαCAT, but not pBα CAT, in transgenic mice is consistent with results of transfection experiments using choriocarcinoma cells and correlates with the pattern of expression of these genes in the species from which the constructs were derived. This finding suggests that mouse placenta contains a sufficient complement of *trans*-acting factors necessary for activation of the human α-subunit gene. Therefore, a nonfunctional CRE is likely to be the primary cause underlying lack of expression of the endogenous α-subunit gene in mouse placenta. These findings are also consistent with our hypothesis that placenta-specific expression resulted from the formation of a placenta-specific enhancer rather than from acquisition of new *trans*-acting factors.

In contrast to our results, a recent study (11) concluded that the human α-subunit gene is expressed in pituitaries but not in placentas of transgenic mice. The reason for this discrepancy is not clear; however, it may be the result of a difference in sensitivity of detection of α-subunit mRNA relative to detection of CAT enzymatic activity. Another reason for this discrepancy may be the way in which placentas were collected. A small amount of uterus must remain attached to the placenta to ensure that the entire syncytiotrophoblast is harvested. In our experiments, CAT was detected only in placentas obtained from transgenic fetuses and did not correlate with the genotype of the mother; thus, it is unlikely that CAT was expressed in the uterine tissue.

Until recently, lack of a pituitary cell line that expresses the α-subunit gene prevented analysis of the *cis*-acting elements involved in pituitary-specific expression of this gene. Our results from transgenic mice indicate that 1,500 nucleotides of human α 5′-flanking sequence or 313 nucleotides of bovine α 5′-flanking sequence is sufficient to direct pituitary-specific expression of a reporter gene. Thus, essential components of the pituitary-specific enhancer are located within the proximal-promoter regulatory region. This result extends the recent report of Fox and Solter (11) indicating that the entire human α-subunit gene (17 kilobase pairs), including 5.7 kilobase pairs of 5′-flanking sequence and 1.9 kilobase pairs of 3′-flanking sequence, is expressed solely in the pituitaries of transgenic mice.

Interestingly, the CRE, which is essential for placenta-specific expression, is not required for pituitary-specific expression of the α-subunit gene, since the bovine promoter-regulatory region present in pBαCAT lacks such an element. This implies that the *cis*-acting elements required for pituitary-specific expression of the α-subunit gene are different from those required for placenta-specific expression. An alternative explanation is that the bovine 17-of-18-bp match to the human CRE is a functional CRE in pituitary due to a difference in sequence specificity of a pituitary *trans*-acting factor. This is unlikely, however, because of the strict

requirement for cytosine at the fourth nucleotide of the palindromic core region of the CRE.

It is unclear why a significant proportion of transgenic mice fail to express the CAT gene in pituitary and placenta. Lack of expression is not due to differences in sites of integration, because both expressing and nonexpressing mice were found within lines of transgenic mice derived from single integration sites as determined by Mendelian inheritance patterns and intensities of slot blots. In this regard, other laboratories have reported similar inconsistencies in tissue-specific expression of transgenes (22, 24). One explanation for inconsistent tissue-specific expression in pituitary and placenta could be a variation in genetic background that could arise from strain differences in oocytes used for microinjection or could occur during the outbreeding of founder animals with nontransgenic CF1 mice. With respect to placenta, another complicating factor could be the wide range in ages of the fetuses used for the placental study (days 12 to 20). Finally, it is also possible that the human and bovine constructs contain only the minimal number of *cis*-acting elements required for tissue-specific expression. Other auxiliary regulatory elements may be necessary for full transcriptional activity and hence consistent patterns of expression. Nevertheless, it is important to emphasize that pituitary- and placenta-specific expression of the α-CAT transgene has been observed in at least two independently derived lines of transgenic mice, further supporting the view that tissue-specific expression is a consequence of specific sequence information contained within the α 5′-flanking region rather than integration into an advantageous site.

In summary, we conclude that the human α-subunit gene contains at least one and possibly several functional *cis*-acting sequences that are not present in the bovine α-subunit promoter-regulatory region. The human placenta-specific enhancer is likely to have evolved after mammalian radiation by a point mutation that created a functional CRE and by subsequent duplication of this element. Although other sequence differences exist between these genes and contribute to the level of expression of the human α-subunit gene in placenta, evolution of the directly repeated CRE appears to have been a critical step for maximal expression of α subunit in human placenta. If this is correct, it may be possible to find examples of a single functional CRE in the α-subunit genes from other primate species.

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LITERATURE CITED

1. **Andersen, B., A. Milsted, G. Kennedy, and J. H. Nilson.** 1988. Cyclic AMP and phorbol esters interact synergistically to regulate expression of the chorionic gonadotropin genes. J. Biol. Chem. **263**:15578–15583.
2. **Bokar, J. A., W. J. Roesler, G. R. Vandenbark, D. M. Kaetzel, R. W. Hanson, and J. H. Nilson.** 1988. Characterization of the cAMP responsive elements from the genes from the α subunit of glycoprotein hormones and phosphoenolpyruvate carboxykinase (GTP): conserved features of nuclear protein binding between tissues and species. J. Biol. Chem. **263**:19740–19747.
3. **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. **72**:248–256.
4. **Burnside, J., P. R. Buckland, and W. W. Chin.** 1988. Isolation and characterization of the gene encoding the α-subunit of the rat pituitary glycoprotein hormones. Gene **70**:67–74.
5. **Chin, W. W.** 1985. Organization and expression of the glycoprotein hormone genes, p. 103–125. *In* H. Imura (ed.), The pituitary gland. Raven Press, New York.
6. **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.
7. **Deutsch, P. J., J. P. Hoeffler, J. L. Jameson, and J. F. Habener.** 1988. Cyclic AMP and phorbol ester-stimulated transcription mediated by similar DNA elements that bind distinct proteins. Proc. Natl. Acad. Sci. USA **85**:7922–7926.
8. **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-bp enhancer. J. Biol. Chem. **262**:12169–12174.
9. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11**:1475–1489.
10. **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.
11. **Fox, N., and D. Solter.** 1988. Expression and regulation of the pituitary- and placenta-specific human glycoprotein hormone alpha-subunit gene is restricted to the pituitary in transgenic mice. Mol. Cell. Biol. **8**:5470–5476.
12. **Goodwin, R. G., C. L. Moncman, F. R. Rottman, and J. H. Nilson.** 1983. Characterization and nucleotide sequence of the gene for the common α subunit of the bovine pituitary glycoprotein hormones. Nucleic Acids Res. **11**:6873–6882.
13. **Gordon, D. F., W. M. Wood, and E. C. Ridgeway.** 1988. Organization and nucleotide sequence of the mouse α-subunit gene of the pituitary glycoprotein hormones. DNA **7**:679–690.
14. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1055.
15. **Haisenleder, D. J., S. Khoury, S. M. Zmeili, S. Papavasiliou, G. A. Ortolano, C. Dee, J. A. Duncan, and J. C. Marshall.** 1987. The frequency of gonadotropin-releasing hormone secretion regulates expression of α and luteinizing hormone β-subunit messenger ribonucleic acids in male rats. Mol. Endocrinol. **1**:834–838.
16. **Hamernik, D. L., M. E. Crowder, J. H. Nilson, and T. M. Nett.** 1986. Measurement of messenger ribonucleic acid for luteinizing hormone β-subunit, α-subunit, growth hormone, and prolactin after hypothalamic pituitary disconnection in ovariectomized ewes. Endocrinology **119**:2704–2710.
17. **Hoeffler, J. P., T. E. Meyer, Y. Yun, J. L. Jameson, and J. F. Habener.** 1988. Cyclic AMP-responsive DNA-binding protein: structure based on a cloned placental cDNA. Science **242**: 1430–1433.
18. **Hojvat, S., G. Baker, L. Kirsteins, and A. M. Lawrence.** 1982. TSH in the rat and monkey brain. Neuroendocrinology **34**: 327–332.
19. **Hostetler, G., A. Eaton, M. Carnes, J. Gildner, and M. S. Brownfield.** 1987. Immunocytochemical distribution of luteiniz-

ing hormone in rat central nervous system. Neuroendocrinology **46**:185–193.

20. **Jameson, J. L., R. J. 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. **Landefeld, T. D., J. Kepa, and F. J. Karsch.** 1983. Regulation of α-subunit synthesis by gonadal steroid feedback in the sheep anterior pituitary. J. Biol. Chem. **258**:2390–2393.
22. **McGrane, M. M., J. deVente, J. Yun, J. Bloom, E. Park, A. Wynshaw-Boris, T. Wagner, F. M. Rottman, and R. W. Hanson.** 1988. Tissue-specific expression and dietary regulation of a chimeric phosphoenolpyruvate carboxykinase/bovine growth hormone gene in transgenic mice. J. Biol. Chem. **263**:11443–11451.
23. **Nilson, J. H., M. T. Nejedlik, J. B. Virgin, M. E. Crowder, and T. M. Nett.** 1983. Expression of α subunit and luteinizing hormone β genes in the ovine anterior pituitary: estradiol suppresses accumulation of mRNAs from both α subunit and luteinizing hormone β. J. Biol. Chem. **258**:12087–12090.
24. **Palmiter, R. D., J. Y. Chen, and R. L. Brinster.** 1982. Differential regulation of metallothionein-thymidine kinase fusion genes in transgenic mice and their offspring. Cell **29**:701–710.
25. **Papavasiliou, S. S., S. Zmeili, S. Khoury, T. D. Landefeld, W. W. Chin, and J. C. Marshall.** 1986. Gonadotropin-releasing hormone differentially regulates expression of the genes for luteinizing hormone α and β subunits in male rabbits. Proc. Natl. Acad. Sci. **83**:4026–4029.
26. **Phillips, C. L., L. W. Lin, J. C. Wu, K. Guzman, A. Milsted, and W. L. Miller.** 1988. 17β-Estradiol and progesterone inhibit transcription of the genes encoding the subunits of ovine follicle stimulating hormone. Mol. Endocrinol. **2**:641–649.
27. **Pierce, J. G., and T. F. Parsons.** 1981. Glycoprotein hormones: structure and function. Annu. Rev. Biochem. **50**:465–495.
28. **Roesler, W. J., G. R. Vandenbark, and R. W. Hanson.** 1988. Cyclic AMP and the induction of eukaryotic gene transcription. J. Biol. Chem. **263**:9063–9066.
29. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
30. **Shupnik, M. A., W. W. Chin, J. F. Habener, and E. C. Ridgway.** 1985. Transcriptional regulation of the thyrotropin subunit genes by thyroid hormone. J. Biol. Chem. **258**:15120–15124.
31. **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 is mediated by an 18-bp element. Proc. Natl. Acad. Sci. **84**:2198–2202.
32. **Wagner, T. E., P. C. M. Hoppe, J. D. Jollick, D. R. Scholl, R. Hodinka, and J. G. Gault.** 1981. Microinjection of a rabbit β-globin gene into zygotes and its subsequent expression in adult mice and their offspring. Proc. Natl. Acad. Sci. USA **78**:6376–6380.
33. **Yamamoto, K. K., G. A. Gonzalez, W. H. Biggs, and M. R. Montminy.** 1988. Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. Nature **334**:494–498.