MOLECULAR AND CELLULAR BIOLOGY, May 1992, p. 2143–2153
0270-7306/92/052143-11$02.00/0

Vol. 12, No. 5

Tissue-Specific Gene Expression in the Pituitary: The Glycoprotein Hormone α-Subunit Gene Is Regulated by a Gonadotrope-Specific Protein

FRIEDEMANN HORN,† JOLENE J. WINDLE,‡ KERRY M. BARNHART, AND PAMELA L. MELLON*

The Salk Institute, La Jolla, California 92037, and Departments of Reproductive Medicine and Neurosciences and the Center for Molecular Genetics, University of California, San Diego, La Jolla, California 92093-0674

Received 31 October 1991/Accepted 17 February 1992

The molecular mechanisms for the development of multiple distinct endocrine cell types in the anterior pituitary have been an area of intensive investigation. Though the homeodomain protein Pit-1/GHF-1 is known to be involved in differentiation of the somatotrope and lactotrope lineages, which produce growth hormone and prolactin, respectively, little is known of the transcriptional regulators important for the gonadotrope cell lineage, which produces the glycoprotein hormones luteinizing hormone and follicle-stimulating hormone. Using transgenic mice and transfection into a novel gonadotrope lineage cell line, we have identified a regulatory element that confers gonadotrope-specific expression to the glycoprotein hormone α-subunit gene. A tissue-specific factor that binds to this element is purified and characterized as a 54-kDa protein which is present uniquely in cells of the gonadotrope lineage and is not Pit-1/GHF-1. The human and equine α-subunit genes are also expressed in placental cells. However, the previously characterized placental transcription factors designated TSEB and α-ACT are not found in the pituitary gonadotrope cells, indicating that independent mechanisms confer expression of these genes in the two different tissues.

The temporal and tissue-specific control of gene expression in mammals remains a central question in developmental biology. The anterior pituitary has served as an excellent model in which to study the specification of gene expression. Five distinct endocrine cell types arise, each specialized to produce different hormones. These are, in developmental order of appearance, as follows: corticotropes, which produce pro-opiomelanocortin; thyrotropes, which produce thyroid-stimulating hormone (TSH); gonadotropes, which produce both luteinizing hormone (LH) and follicle-stimulating hormone (FSH); somatotropes, which produce growth hormone (GH); and lactotropes, which produce prolactin.

Substantial information is available concerning the molecular events important for regulation of GH and prolactin gene expression as a result of the availability of the somatotrope/lactotrope GH cultured cell lines (50). A specific POU-homeodomain protein (22), termed Pit-1 or GHF-1, activates expression of these genes in somatotropes and lactotropes (2, 12, 31). Pit-1/GHF-1 is also required for development of the somatotrope and lactotrope cell lineages. Dwarf mice in which the Pit-1/GHF-1 gene is mutated or deleted fail to develop these cell types (30). Curiously, Pit-1/GHF-1 expression is not restricted to these two lineages. Though Pit-1/GHF-1 is first detected after the expression of TSH, it is found in thyrotropes, and this cell type is also eliminated from dwarf mice (30). Moreover, the mRNA for Pit-1/GHF-1 is also expressed in the remaining two cell types, gonadotropes and corticotropes, but the protein is not detectable (47), indicating a potential role for translational control (12).

Much less is known about the mechanisms determining expression of the other pituitary hormone genes. Three of these hormones, TSH, LH, and FSH, form a family of glycoprotein hormones each of which is a heterodimer composed of a common α subunit and a distinct β subunit. The ontogenic appearance of the individual subunits is not coordinated. Surprisingly, the α-subunit gene is first expressed on embryonic day 11.5 (day e11.5) of rat development in the placode of somatic ectoderm, which beginning on day 12 will form Rathke's pouch, the anlagen of the anterior pituitary (47). This expression is maintained in thyrotropes and gonadotropes but may be restricted from the cells destined for other lineages (47). TSH β mRNA is detected on day e15.5, while LH β and FSH β mRNAs appear even later, on days e16.5 and e17.5, respectively.

The related glycoprotein hormone, human chorionic gonadotropin (hCG), is formed in the human placenta by combination of the common α subunit with a fourth β subunit specific to hCG. In equine species, the α-subunit gene is also activated in the placenta to form a component of pregnant mare serum gonadotropin (13, 49). The tissue-specific expression of the human and equine α-subunit genes of the glycoprotein hormones is thus tightly limited to two very distinct tissues, the placenta and the anterior pituitary. Within the placenta, expression of this gene is specifically directed to the trophoblasts. However, many other mammals (including rodents) do not produce a placental gonadotropin hormone (51). In these species, the α-subunit gene is expressed only in pituitary but in the same two endocrine cell types, the thyrotropes and gonadotropes. Thus, a single human or equine α-subunit gene must confer the specificity for three distinct cell types within two diverse tissues; in contrast, the rodent α-subunit gene confers expression only to the pituitary (gonadotropes and thyrotropes).

The molecular basis for placenta-specific expression of the human α-subunit gene has been studied by transfection of human placental trophoblast cell lines that express hCG and are derived from choriocarcinoma tumors (9). The placental

* Corresponding author.
† Present address: Institut für Biochemie an der RWTH, Aachen, D-5100 Aachen, Germany.
‡ Present address: Division of Cellular Biology, Cancer Therapy and Research Center, San Antonio, TX 78229.

specificity of the human gene is determined by a composite enhancer composed of a trophoblast-specific element (TSE; also termed URE) (26) that binds a protein present specifically in placenta, TSEB (10); a GATA element that binds a protein (α-ACT) (49) related to the family of GATA-binding factors (41, 56); and two cyclic AMP-responsive elements (CREs) (10, 46) that bind the ubiquitous protein CREB (15, 19). In addition, a specific CCAAT-binding protein (28) and a factor binding a site just downstream of the CREs termed the junctional regulatory element (1a) may also participate in regulation of the human gene in placenta. In contrast, the equine gene carries no TSE or CRE, though it does contain two GATA elements that bind the protein α-ACT from placental cells (49), while the mouse gene has no TSE, CREs, or GATA elements, consistent with its lack of expression in placental cells. In thyrotrope tumor cells, expression of the mouse α-subunit gene appears to be conferred by two large proximal regions which are bound by tissue-specific proteins (39, 42).

The molecular basis of the tissue specificity of the α-subunit gene in gonadotropes could not be investigated heretofore because of the lack of an appropriate cell line. The human gene shows expression after transfection into primary pituitary cells (6), but these studies are quite difficult and employ a heterogeneous population of cells. In transgenic mice, an 18-kb DNA fragment carrying the entire human α-subunit gene has been demonstrated to direct tissue-specific expression to the pituitary (14), as did a 1.5-kb fragment of the 5′-flanking region of the α-subunit gene (3). We have also demonstrated that 1.8 kb of the 5′-flanking region from this gene can direct pituitary-specific expression of the simian virus 40 T-antigen oncogene (Tag) in transgenic mice (55). Most importantly for the studies presented here, the tumors derived from these mice were cultured to derive clonal cell lines (e.g., αT3-1 cells) which continue to produce the endogenous mouse α subunit and express the human α-Tag transgene. These cells also display functional gonadotropin-releasing hormone receptors (21) (though they do not respond to thyrotropin-releasing hormone), indicating their origin as the gonadotrope lineage (55). We have proposed that these cells represent a precursor to the gonadotrope lineage which is immortalized by the early expression of Tag directed by the α-subunit gene upstream region during development perhaps as early as day e11.5.

The mechanism by which expression of one gene can be directed specifically to two developmentally very different tissues might be based on the presence in pituitary cells of the same tissue-specific regulator(s) that has been shown to regulate the α-subunit gene in placenta (e.g., TSEB) or, alternatively, on a distinct protein(s) unique to pituitary or perhaps even unique to gonadotropes versus thyrotropes. In this report, we present analysis of the pituitary tissue specificity of the glycoprotein hormone α-subunit gene, using introduction of hybrid genes into transgenic mice and into the cell line of pituitary gonadotrope lineage derived from tumors generated in transgenic mice (55). We find that, as is the case in placental cells (10, 25, 46), deletion of the CREs destroys expression of the human α-subunit gene in pituitary cells. However, specification of expression of the human α-subunit gene to the anterior pituitary gonadotrope does not involve TSEB (10), α-ACT (49), or Pit-1/GHF-1 (2, 31), which are all absent from this gonadotrope precursor cell. Instead, it is at least partially due to a DNA-binding protein (GSEB1) found uniquely in these cells which binds to a site just upstream of the TSE, the gonadotrope-specific element (GSE).

MATERIALS AND METHODS

Plasmid constructions. The α-cat plasmids were described previously by Delegeane et al. (10). Additional upstream truncations were prepared as described previously (10). Cloning of the equine and mouse α-subunit promoter regions was described previously by Steger et al. (49). The truncations of the mouse gene were prepared by cleavage at restriction sites and religation. The mutation in the GSE sequence was created by replacing the *Xba*I-to-*Mae*III fragment (−224 to −210) with a synthetic oligonucleotide carrying the two C-to-T mutations at positions −215 and −216. GSE-TK-CAT and $(GSE)_2$-TK-CAT were constructed by cloning the GSE oligonucleotide into the *Bam*HI site of pTK-CAT (44).

Microinjection and DNA analysis. The α-Tag plasmid is described in reference 55. The α168-Tag and α152-Tag transgenes were constructed by replacing the 1.8-kb 5′-flanking sequence with the truncated fragments derived from α168-cat and α152-cat constructions (10). The α-Tag transgene was excised from the plasmid by digestion with *Eco*RI and *Sal*I, while the α168-Tag and α152-Tag transgenes were excised with *Xba*I and *Sal*I and purified by agarose gel electrophoresis and binding to glass beads (Geneclean; Bio 101 Inc., La Jolla, Calif.). Approximately 1 to 2 pl of a solution of DNA at a concentration of 2 μg/ml was microinjected into the pronuclei of fertilized one-cell mouse embryos (20). The F_2 embryos were derived from matings of CB6F1/J (C57BL/6J × BALB/cJ) males and females, obtained from the Jackson Laboratory. The injected embryos were reimplanted into CD-1 pseudopregnant mice (20). The presence of the transgene in the resulting mice was determined by preparing genomic DNA from a small piece of tail and assaying by Southern blot analysis (34).

Cell culture, transfections, and CAT assays. Cell lines were maintained in Dulbecco modified Eagle medium with 5% fetal bovine serum, 5% equine serum, glucose (4.5 mg/ml), penicillin (100 U/ml), and streptomycin (0.1 mg/ml). Transfections were performed by using calcium phosphate precipitates (35) containing 10 μg of plasmid DNA and 2.5 μg of the internal control plasmid, pucLac (8), and were incubated for 5 h; the cells were then rinsed, and the medium was changed. Cells were harvested after 48 h. Protein extracts were prepared by freeze-thawing as described previously (16), and protein concentrations were determined by using Bio-Rad protein assay reagent (4). Chloramphenicol acetyltransferase (CAT) assays were performed as previously described (16, 45), and β-galactosidase assays were performed as described by Delegeane et al. (10). Units of CAT activity were normalized to the internal control β-galactosidase activity.

Oligonucleotides. Oligonucleotides were purified on acrylamide gels prior to use. The GSE oligonucleotide had the sequence 5′-CTAGTGGGCTGACCTTGTCGTCACCATCA CCTGCTAG-3′. This oligonucleotide corresponds to positions −224 to −196 of the glycoprotein hormone α-subunit gene, plus *Xba*I linkers, and was used in the construction of transfection vectors (Fig. 9), in the Southwestern (DNA-protein) analysis (Fig. 7), and as a competitor (Fig. 5).

The 19-bp GSE oligonucleotide had the sequence 5′-AAT TCTGACCTTGGTCAATT-3′. This oligonucleotide corresponds to positions −220 to −210 of the α-subunit gene, plus *Eco*RI linkers, and was used as both a probe and competitor in Fig. 8.

The TSE oligonucleotide had the sequence 5′-CTAGAA AAATGACCTAAGGGTTGAAACAAGATAAGCTAG-3′. This oligonucleotide corresponds to positions −180 to −150

of the α-subunit gene, plus *Xba*I linkers, and was used as a competitor in Fig. 5.

The Pit-1 oligonucleotide had the sequence 5′-GATCCCA TGCATAAATGTACACAGGATC-3′. This oligonucleotide corresponds to positions −89 to −70 of the human GH gene, plus *Bam*HI linkers, and was used as a competitor in Fig. 5 and 8.

Nuclear extract preparation and DNase I protection analysis. Nuclear extracts were prepared according to the method of Dignam et al. (11) as described by Delegeane et al. (10), with the modification that the ammonium sulfate precipitation was performed with 55% saturated ammonium sulfate. DNA probes for the human α-subunit gene were prepared from plasmids pICα−290/+3 and pICα−441/−151, which were generated by subcloning the *Alu*I-to-*Pst*I fragment (−290 to +3) of α391-CAT (10) into pIC20R (33) cut with *Sma*I and *Pst*I and the *Xba*I-to-*Sau*3AI fragment (−442 to −151) of α441-CAT (10) into pIC20R (33) cut with *Xba*I and *Bam*HI. The plasmids were labeled at the *Eco*RI sites by using the Klenow fragment of DNA polymerase I and [α-^{32}P]dATP (3,000 Ci/mmol) and recut at *Hin*dIII, or vice versa for the opposite strand. End-labeled probes for the equine and murine α-subunit genes were prepared from pBSK(Eα) (49) and pBSK(Mα) (49) by labeling at the *Eco*RI site directly flanking the upstream end of these fragments and subsequently digesting at the *Hin*dIII site in the vector. These labeled fragments were purified from a 6% native polyacrylamide gel. DNase I protection was performed by using 1 to 2 fmol (i.e., 5,000 to 10,000 cpm) of purified DNA fragment per reaction and as described previously (40), with the following modifications: salmon sperm DNA was substituted for poly(dI-dC) as carrier DNA, and 10 μg of bovine serum albumin (BSA) was included in the binding incubations.

Gel retardation assays. The gel retardation assay was slightly modified from the method of Sawadogo et al. (43). Binding reactions were performed in 20 μl in 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-KOH (pH 7.8)–50 mM KCl–5 mM spermidine–1 mM EDTA–5 mM dithiothreitol–0.5 mM phenylmethylsulfonyl fluoride–10% (vol/vol) glycerol. BSA (2 μg/μl)–poly(dI-dC) · poly(dI-dC) (5 ng/μl) or BSA (1 μg/μl)–poly(dI-dC) · poly(dI-dC) (0.1 μg/μl) was also included in the binding reactions using purified proteins and crude nuclear extracts, respectively. Where indicated, oligonucleotide probes were used. DNA fragments or oligonucleotides were labeled with the Klenow fragment of DNA polymerase I and [α-^{32}P]dATP (3,000 Ci/mmol) and purified from a 6% native acrylamide gel. Unlabeled competitor oligonucleotides were also treated with Klenow fragment and unlabeled deoxynucleoside triphosphates to fill the overhanging ends. The indicated amount of nuclear extract or partially or affinity-purified protein was preincubated with the reaction mixture for 5 to 10 min at 20°C. The probes (5,000 cpm, 1 to 2 fmol) and competitors were added, and binding was allowed to proceed for a further 10 min at 20°C. To resolve the complexes, the reaction mixtures were applied to 4% native acrylamide gels (20:1 acrylamide/bisacrylamide) in 0.25× Tris-acetate-EDTA (pH 8.0)–10% (vol/vol) glycerol for 2 h at 10 V/cm at room temperature and dried for autoradiography.

Methylation interference and Southwestern blotting. The methylation interference experiment was carried out according to Tsai et al. (53). Southwestern blotting was performed by using a procedure modified from that of Miskimins et al. (36). Crude or partially purified nuclear extracts were applied to denaturing electrophoresis on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel (29), and the proteins were subsequently transferred to nitrocellulose filters by using a semidry electroblotting apparatus. The filters were immersed in binding buffer (20 mM HEPES [pH 7.9], 50 mM KCl, 1 mM dithiothreitol, 10% glycerol, 0.1% Nonidet P-40) for 10 min at room temperature to allow the proteins to renature and were blocked for 10 min by gentle shaking in 5% nonfat dry milk in binding buffer. The milk was rinsed off by three washes in binding buffer. Binding was carried out overnight at 4°C in a sealed plastic bag with gentle shaking, using 10^6 cpm of oligonucleotide probe per ml of binding buffer which contained 0.5% nonfat dry milk and 1 μg of salmon sperm DNA per ml. The filters were washed three times for 5 min each with binding buffer, dried, and autoradiographed. For probes for the experiment in Fig. 7, the long GSE and GSE mutant probes were digested with *Mae*III, and the upstream fragments of the oligonucleotides were labeled with the Klenow fragment of DNA polymerase I and [α-^{32}P]dATP (3,000 Ci/mmol) and isolated on a native 20% polyacrylamide gel.

Purification of GSEB1. A 150-mg sample of αT3-1 nuclear extract was loaded onto a heparin-Sepharose (Pharmacia LKB, Piscataway, N.J.) column equilibrated with 50 mM KCl in column buffer (20 mM HEPES [pH 7.8], 12.5 mM $MgCl_2$, 1 mM EDTA, 20% glycerol, 0.1% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol). The column was washed with 10 volumes of 50 mM KCl followed by 5 volumes of 250 mM KCl, both in column buffer, and eluted with 450 mM KCl in column buffer. The collected fractions were analyzed in a gel retardation assay using a labeled GSE oligonucleotide as a probe. Fractions containing GSEB1 activity were pooled, adjusted to 100 mM KCl by dilution with column buffer, and applied to a nonspecific DNA-cellulose column which was prepared by coupling calf thymus DNA to CNBr-activated cellulose and equilibrated in column buffer containing 50 mM KCl. The column was washed with 10 volumes of 100 mM KCl and eluted with 350 mM KCl, both in column buffer. For affinity chromatography, 250 μg of a GSE oligonucleotide was phosphorylated and ligated. The 5′ overhangs of the highly multimerized GSE oligonucleotides were then filled with the Klenow fragment of DNA polymerase I and biotinylated dUTP (Sigma, St. Louis, Mo.) and coupled to 2 ml of strepavidin-agarose (Sigma) as described previously (48). After adjustment to 100 mM KCl with column buffer and addition of 2 μg of poly(dI-dC) per ml, the combined active fractions from the DNA-cellulose chromatography step were loaded onto the GSE oligonucleotide affinity column equilibrated with 100 mM KCl in column buffer. The column was washed with 10 volumes of 250 mM KCl and eluted with 550 mM KCl, both in column buffer. The fractions containing GSEB1 were combined, diluted to 100 mM KCl, and further purified by a second passage over the affinity column under the conditions described above except that the poly(dI-dC) concentration was 0.5 μg/ml. The overall procedure yielded 7.5 μg of apparently homogeneous GSEB1.

RESULTS

Sequences upstream of the placenta-specific elements are required for pituitary expression of the human α-subunit promoter in transgenic mice. Transgenes in which the human α-subunit gene 5′-flanking region directs expression show tight pituitary specificity (3, 55). To investigate the role of the upstream regions in pituitary specificity, transgenic mice containing transgenes composed of the α-subunit promoter

TABLE 1. Pituitary tumors in transgenic mice[a]

Mouse	Pituitary tumors at indicated days of age	Ectopic tumors	Reproductive status[a]
αT-1	93		Infertile
αT-2	92		Infertile
αT-3	49		Sexually immature
αT-4	93		Infertile
αT-5 line	None		Fertile
αT-6	143		1 litter of 2 pups
αT-6-2	101		Infertile
αT-7	272	Thymus	Fertile
αT-7 line	~110–250	Pancreas (rare)	M fertile, F infertile
αT-8 line	None		Fertile
αT-9	105		Fertile; no transmission
αT-10	178		Infertile
αT-11	98		Infertile
αT-12	134		Fertile
αT-12 line	~140		M fertile, F infertile
αT-13	43		ND
αT-14	420		Fertile
αT-14 line	~210–300	Pancreas (rare)	Fertile
αT-15 line	None		Fertile
αT-16 line	None		Fertile
αT-17	252		Infertile
α168T-1	170		Fertile; small testes
α168T-1 line	~147–211	Prostate, pancreas	Fertile
α168T-2 line	None		Fertile
α168T-3	None	Brain	Fertile
α168T-3 line	None	Pancreas	Fertile
α168T-4	None	Brain	Fertile
α168T-4 line	None	Pancreas	Fertile
α168T-5	98	Brain	Infertile
α152T-1	None		Fertile; no transmission
α152T-2	330		Fertile
α152T-2 line	None		Fertile
α152T-3 line	None		Fertile
α152T-6	None		Fertile; no transmission
α152T-7 line	None		Fertile
α152T-8	None	Brain	Fertile
α152T-8 line	Single animal at 435	Pancreas	Fertile
α152T-9 line	None		Fertile

[a] The incidence of pituitary and other tumors is shown individually for founder transgenic mice and for the lines of mice. The age in days (for the founders) and the range of ages (for the lines) at sacrifice due to pituitary tumor formation are shown. No significant difference in age of tumor formation was seen between male (M) and female (F) mice. Four founder mice were fertile but failed to transmit the transgene, indicating that they were mosaic (no transmission). ND, Not determined.

truncated to either −168 or −152 linked to the Tag gene were generated for comparison with the original α-Tag transgene, which used 1.8 kb of α-subunit 5′-flanking DNA (Table 1). A 168-bp segment of 5′-flanking DNA is sufficient for placental expression in cell culture (10); however, most but not all pituitary specificity was lost with the −168 transgene, since three of five α168-Tag founder mice instead developed brain tumors at a relatively young age. Only rarely did these mice develop pituitary tumors; instead, they frequently developed other tumors of the prostate, pancreas, or brain. All types of tumors examined expressed Tag mRNA, while only the pituitary tumors also expressed the α-subunit mRNA. Essentially all pituitary specificity was lost with the −152 transgene, since only two isolated animals developed pituitary tumors, while several developed brain and pancreatic tumors and several lines failed to produce tumors. In contrast, brain tumors were never found in the α-Tag mice (Table 1), and tumors in other tissues were rarely observed.

The redirection of Tag expression to brain and pancreas with the −168 and −152 truncations may reflect a natural tissue preference for expression of Tag, which becomes dominant when the attached promoter has been sufficiently weakened. Brinster et al. have observed that Tag driven by its own promoter and enhancer predominantly causes tumors of the choroid plexus in the brain (5). However, removal of the simian virus 40 enhancer and promoter results in loss of this specificity, and instead tumors of the pancreas and liver predominate. Since our constructs lack simian virus 40 promoter and enhancer elements, the incidence of pancreatic tumors may be explained by the specificity of the Tag gene. It is also possible that the truncated α-subunit promoter accounts for weak specificity for brain tissue.

The human α-subunit 5′-flanking DNA directs specific expression in the gonadotrope lineage pituitary cell line. By using transfection, high-level expression of the human α-subunit gene has been demonstrated to be specific to placental cell lines (10). The mouse α-subunit gene has also been demonstrated to be specifically expressed after transfection into mouse thyrotrope tumor cells dispersed in culture after passage in mice (39). In contrast, expression of the human gene in cell lines derived from a variety of other tissues (such as CV-1 fibroblasts, GC pituitary somatotrope/lactotrope cells, PC12 adrenocortical cells, and HeLa cervical carcinoma cells) is very low or undetectable (10). The third cell type in which the human α-subunit gene is specifically expressed is the gonadotrope of the anterior pituitary. To determine the elements important for specification of α-subunit gene expression to these cells, we transfected a series of truncated constructions of the human α-subunit 5′-flanking region (linked to the bacterial CAT gene) into mouse pituitary tumor cells of the gonadotrope lineage, αT3-1 cells (55) (Fig. 1A). The decrease in expression with progressively larger deletions is more gradual than is found in placental cells, indicating stronger contribution from sequences upstream of −168. The upstream regions of the human gene which contribute most strongly to expression in the gonadotrope cells lie between −442 and −391 and between −224 and −168 (Fig. 1A).

The elements necessary for placental expression of the human α-subunit gene (10, 24) include the pair of CREs (between −111 and −146), the GATA element between −141 and −161, and the TSE between −159 and −182. In pituitary cells, deletion of the region from −224 to −168, which carries the TSE, decreases expression by approximately twofold, while deletion of the region of the GATA element has less impact on expression. Internal deletion of the CREs decreases expression of the human gene in αT3-1 cells dramatically (Fig. 1A), indicating an important role for this element in expression in both cell types. This role is likely to be unique to the human α-subunit gene, however, since this CRE sequence (TGACGTCA) is mutated in the α-subunit genes of nonprimate species to TGATGTCA and thus fails to bind CREB (3, 13, 49). Finally, gonadotrope-specific expression is also dependent on sequences from −442 to −391 upstream of those required for placental expression.

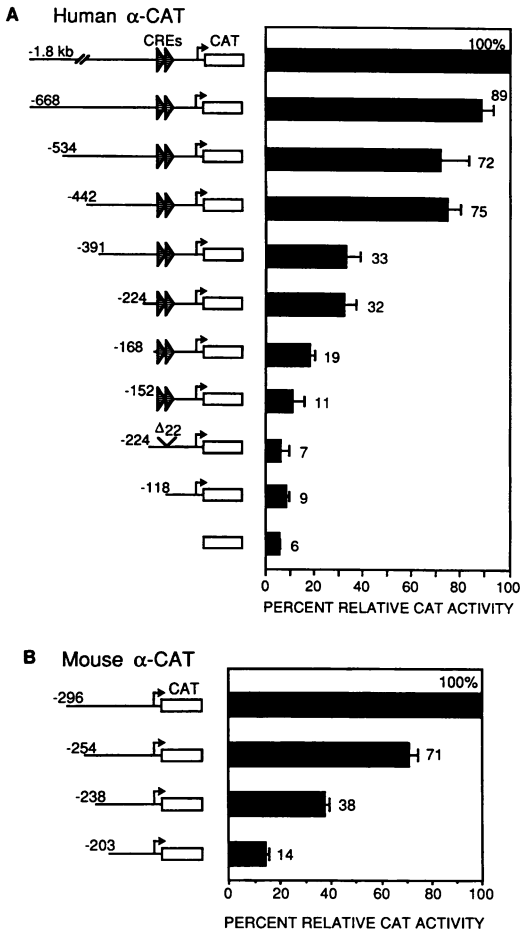

FIG. 1. Analysis of the regions important for α-subunit gene expression in pituitary cells. (A) Transfection of the human α-subunit gene into αT3-1 cells. A series of 5′ truncations of the human α-subunit regulatory region in the CAT expression system were transfected into the pituitary gonadotrope lineage cell line αT3-1, using the calcium phosphate method. CAT enzyme activity values were corrected for the levels of β-galactosidase activity from an internal control β-galactosidase expression vector and expressed as percentages of the activity from the 1.8-kb regulatory region. All values are means of three independent transfection experiments, with error bars representing ± standard errors of the means. (B) Transfection of the mouse α-subunit gene into αT3-1 cells. Four CAT expression vector constructions with 5′-truncated regulatory regions from the murine α-subunit gene were analyzed as in panel A.

As noted above, the mouse α-subunit 5′-flanking sequence differs from the human sequence in the absence of the binding sites for CREB and the placental proteins, TSEB and α-ACT, as well as several other sequence changes. Noting that the gonadotrope cell line used for these studies is of mouse origin, we investigated the regions important for gonadotrope expression of the mouse α-subunit by using a more limited set of truncations spanning the proximal 5′-flanking region (Fig. 1B). Expression was substantially decreased by truncation from −257 to −240 and from −240 to −206. Thus, the regions important for expression of the human and mouse genes in αT3-1 cells show some overlap with each other and with those required for expression of the mouse gene in thyrotrope tumor cells, which were mapped from −480 to −417 and −254 to −177 by using a different set of truncation end points (39, 42).

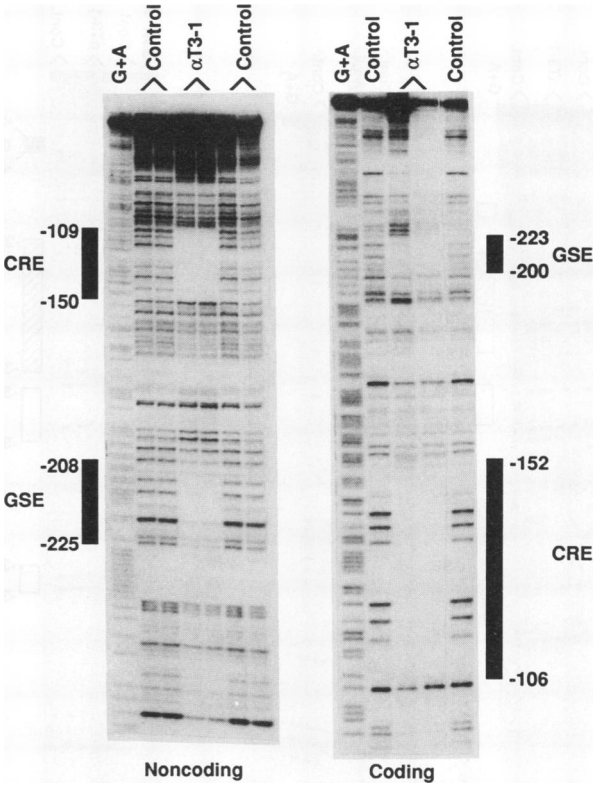

FIG. 2. DNase I footprint analysis of αT3-1 nuclear proteins binding to the human α-subunit promoter. ^{32}P-end-labeled fragment −290 to +3 of the α-subunit promoter was incubated with (αT3-1) or without (control) 80 μg of αT3-1 nuclear extract, partially digested by DNase I, and separated on a 6% sequencing gel as described in Materials and Methods. G+A represents the Maxam-Gilbert sequence ladder. Coding strand (mRNA identical strand) and noncoding strand are labeled. Filled boxes indicate strongly protected regions.

DNase I footprints reveal DNA-binding proteins present in pituitary cells. To determine whether the patterns of expression in gonadotropes, thyrotropes, and placental trophoblasts were due to common or different DNA-binding proteins, we analyzed the nuclear proteins from αT3-1 cells binding to the human α-subunit gene promoter in vitro. As shown in Fig. 2, two prominent footprints appeared when an end-labeled probe containing −290 to +4 of the human α-subunit gene was incubated with nuclear extract from αT3-1 cells, followed by partial digestion by DNase I. The footprint located at −150 to −106 covers the direct repeat of two CREs, indicating the presence of CRE-binding proteins in αT3-1 cells (which may be CREB or members of the CREB/ATF family) (17, 18). A second strong footprint extends from −225 to −208 on the coding (−223 to −200 on the noncoding) strand. In contrast, the regions protected by placenta cell extracts (JEG-3) corresponding to the binding of the TSEB (−182 to −159) and α-ACT (−161 to −142) proteins are not protected using the pituitary cell extracts (αT3-1).

Further upstream (using a probe −442 to −151), multiple footprints within the region from −382 to −244 became evident (Fig. 3). However, deletion of this region (−391 to −224) had little or no effect on expression (Fig. 1A). In contrast to the proximal footprints described above, these

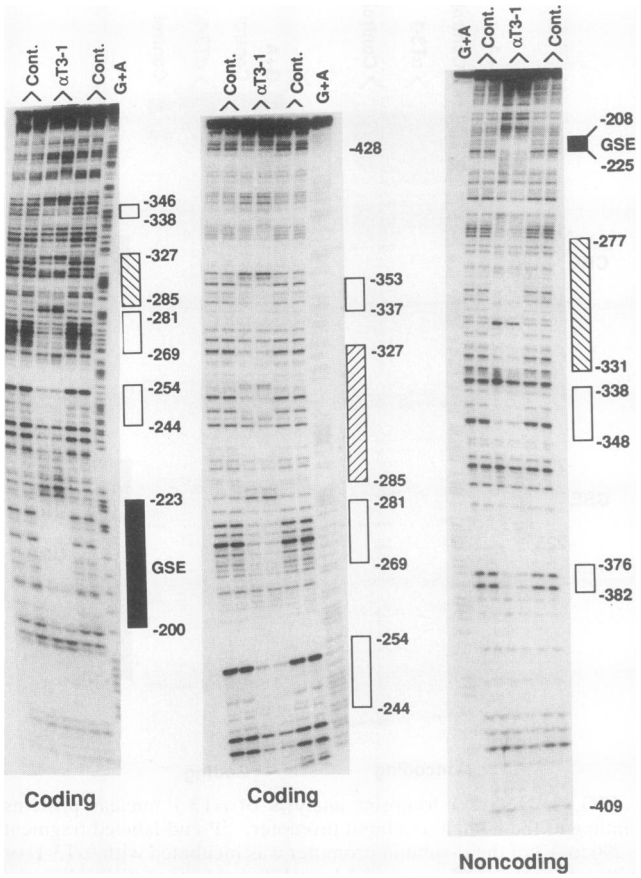

FIG. 3. DNase I footprint analysis of αT3-1 nuclear proteins binding to the human α-subunit upstream region. ^{32}P-end-labeled fragment −442 to −151 of the α-subunit 5′-flanking region was incubated with or without 80 μg of αT3-1 nuclear extract, partially digested by DNase I, and separated on a 6% sequencing gel as described in Materials and Methods. G+A represents the Maxam-Gilbert sequence ladder. Filled boxes indicate the strongly protected GSE, open boxes indicate weakly protected sequences, and hatched boxes indicate regions of alternating weak protection and hypersensitive sites. Cont., control.

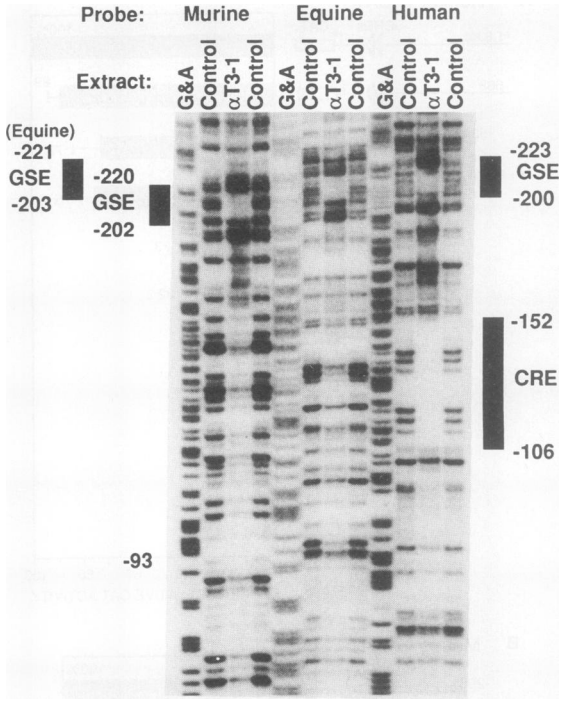

FIG. 4. Comparison of footprints on the murine, equine, and human α-subunit genes. ^{32}P-end-labeled fragments of the murine, equine, and human α-subunit promoters (−296 to +56, −297 to +63, and −300 to +63, respectively) (49) were incubated with or without 80 μg of αT3-1 nuclear extract, partially digested by DNase I, and separated on a 6% sequencing gel as described in Materials and Methods. Noncoding strand is shown.

more distal footprints are characterized by only partial protection, indicating either lower abundance of the binding proteins or lower affinity of binding. Two footprints were observed at −254 to −244 and −281 to −269 on the coding strand. A cluster of alternating protected regions and hypersensitive sites extends from −327 to −285 (−331 to −277 on the noncoding strand), and further footprints are located at −353 to −337 on the coding and at −348 to −338 and −382 to −376 on the noncoding strand. In contrast, the region from −442 to −391, deletion of which caused over a twofold loss of activity (Fig. 1A), failed to be protected by proteins in the αT3-1 nuclear extracts (Fig. 3).

A conserved element in the α-subunit genes from several species binds a gonadotrope-specific DNA-binding protein (GSEB1). The well-protected region from −225 to −208 is highly conserved in the α-subunit genes from all of the species thus far sequenced. To further demonstrate the conservation of this sequence element, we tested the affinity of the αT3-1 nuclear protein by footprinting the human, mouse, and equine genes in parallel. Figure 4 demonstrates that this region shows identical footprinting with the αT3-1 nuclear extract over the homologous sequences in the human, murine, and equine genes (−220 to −202, −221 to −203, and −223 to −200, respectively). An additional footprint is observed in all three species over the region corresponding to a CCAAT sequence at approximately −93 in the human gene. This region of the human gene is thought to be bound by a placenta-specific protein in JEG-3 cells which therefore must have a counterpart in pituitary cells (28). The CRE footprint present in the human gene is absent from the murine and equine genes, indicating that αT3-1 cells do not contain proteins that bind to their altered CRE-like sequence (13, 49). In addition, the α-ACT footprint is absent from the human (−161 to −142) and equine (−157 to −124) genes, and as shown above, the placental TSEB footprint is absent from the human gene (TSEB is known not to bind the mouse or equine genes) (49).

The footprints observed by using the pituitary cell extracts (αT3-1) are also quite different from those found in thyrotrope tumor cells on the mouse α-subunit gene (39), for which two broad footprints were detected over the regions from −213 to −170 and −158 to −101. The upstream footprint in thyrotropes (−213 to −170) overlaps slightly but differs substantially from the αT3-1 footprint, which covers the much more narrow area from −220 to −202, indicating that these represent different activities and that the protein which binds over −220 to −202 in αT3-1 extracts is absent from thyrotrope tumor cells. This region of the mouse gene is also unprotected by JEG-3 cell extracts (49). The proximal thyrotrope footprint on the mouse gene (−158 to −101) (39) is absent or very weak in αT3-1 nuclear extracts. A subset of

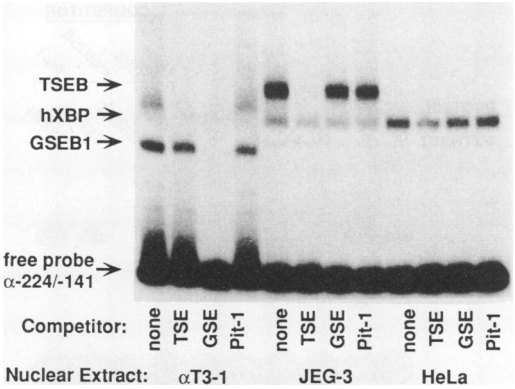

FIG. 5. Gel retardation analysis of protein complexes with the human α-subunit regulatory region in different cell types. The gel retardation assay used the ^{32}P-end-labeled fragment −224 to −148 from the human gene as a probe. Nuclear extract (1 μg) from αT3-1, JEG-3, or HeLa cells was incubated with a ^{32}P-end-labeled DNA fragment as described in Materials and Methods. A 100-fold excess of each of the indicated unlabeled oligonucleotide competitors was added to the incubation mixture.

this region (−146 to −122) is protected by a protein in JEG-3 cells which is unrelated to CREB (49).

The differences in regulation of α-subunit gene expression in gonadotropes versus placental cells appears to be reflected by the binding of different nuclear proteins to the regulatory region of the gene. To investigate the tissue specificity of these binding activities, gel retardation assays were performed with a probe (−224 to −148) from the human gene which includes the TSE and the upstream sequence protected by αT3-1 cell nuclear extracts (−223 to −200). Figure 5 demonstrates that nuclear extracts from αT3-1 cells, human JEG-3 placenta cells, and human HeLa cervical carcinoma cells form distinctly different retarded complexes with this DNA fragment. As expected, a major complex formed with JEG-3 cells is the TSEB. A second JEG-3 complex is in common with HeLa cell extracts (but not the αT3-1 extracts) and is not competed for by the specific oligonucleotides. This protein binds over a CRE-like sequence, TGTCGTCA, found from −219 to −212, just downstream from the major αT3-1 footprint and may be the human homolog of the protein mXBP (1, 23, 27). The major complex formed with the αT3-1 extracts is not formed with extracts from the other cell types and is specifically competed for by an oligonucleotide representing the footprinted region from −225 to −208. Thus, we term this element the gonadotrope-specific element (GSE), and the tissue-specific DNA-binding protein which binds the GSE is termed GSEB1.

Pituitary specific expression of the GH and prolactin genes is conferred by the protein Pit-1/GHF-1. This specific POU-homeodomain protein (22) activates expression of these genes in somatotropes and lactotropes (2, 12, 31). Though the GSE site bears no nucleotide sequence homology to the binding site for this protein, we tested an oligonucleotide containing the binding sequence for competition with the GSE (Fig. 5). This oligonucleotide did not compete for binding, indicating that GSEB1 is not Pit-1/GHF-1. Indeed, expression of the gene encoding Pit-1/GHF-1 is very low or absent in αT3-1 cells, since the mRNA is undetectable by Northern (RNA) blotting (at least 100-fold lower than is found in GC or GH_4 somatotrope/lactotrope cell lines [29a]).

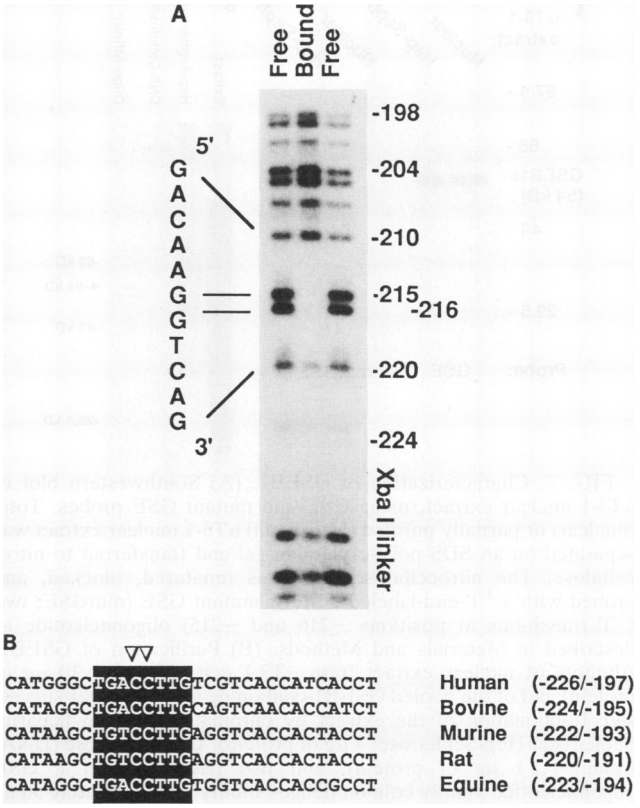

FIG. 6. Methylation interference of the nucleotides contacted by GSEB1 in binding to the GSE region. (A) Analysis of the GSEB binding site by methylation interference. A ^{32}P-end-labeled fragment (−224 to −100) was partially methylated with dimethylsulfate. After binding to αT3-1 nuclear extract partially purified by heparin-Sepharose chromatography, the protein-DNA complexes were separated by electrophoresis through a 4% native polyacrylamide gel as described for gel retardation assays. The shifted GSEB band (bound) and the free probe were excised, and the DNA was isolated, chemically cleaved by formic acid-piperidine, and separated on a 10% sequencing gel. (B) Sequence comparison of the conserved human, bovine, murine, rat, and equine, α-subunit promoter GSE regions. Triangles indicate protected nucleotides, and the black box indicates bases in the contacted region.

GSEB1 is a 54-kDa tissue-specific protein which recognizes the GSE sequence. We further characterized the binding site for GSEB1 by using methylation interference with gel retardation. As shown in Fig. 6A, methylation of the G residues at −216 and −215 on the noncoding strand interferes with GSEB1 binding. In contrast, methylation of the G residues at the borders (−210 or −220) or outside of this region does not strongly interfere with binding. This result indicates that the contacted regions are located in the sequences from −219 to −211 (TGACCTTGT). This element is highly conserved in other mammalian α-subunit genes, with both of the contacted G residues remaining unchanged (Fig. 6B; the third nucleotide can be either an A as in most species or a T as in rodents). The first half of this element, TG(A/T)CC, is homologous to half of the binding site for certain members of the nuclear steroid hormone receptor superfamily (38, 52, 54).

To determine the size and binding specificity of GSEB1, we performed Southwestern blotting. Nuclear extracts from αT3-1 cells or heparin-Sepharose fractions enriched for

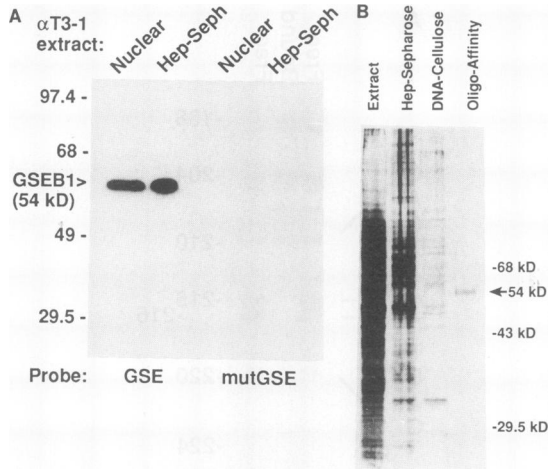

FIG. 7. Characterization of GSEB1. (A) Southwestern blot of αT3-1 nuclear extract, using GSE and mutant GSE probes. Total (nuclear) or partially purified (Hep-Seph) αT3-1 nuclear extract was separated on an SDS-polyacrylamide gel and transferred to nitrocellulose. The nitrocellulose filter was renatured, blocked, and probed with a ^{32}P-end-labeled GSE or mutant GSE (mutGSE; two C-T transitions at positions −216 and −215) oligonucleotide as described in Materials and Methods. (B) Purification of GSEB1. Aliquots of nuclear extract from αT3-1 cells (Extract; 10 μg of protein) and of the pooled GSEB1-containing fractions after successive fractionation of the extract by chromatography on heparin-Sepharose (Hep-Sepharose; 4 μg of protein), DNA-cellulose (DNA-Cellulose; 1 μg of protein), and two passages over a GSE oligonucleotide affinity column (Oligo-Affinity; approximately 50 ng of protein) were analyzed by electrophoresis on a denaturing 10% polyacrylamide gel (29). Protein bands were visualized by silver staining. Molecular sizes are indicated as determined by molecular weight protein markers; the arrow indicates 54-kDa GSEB1.

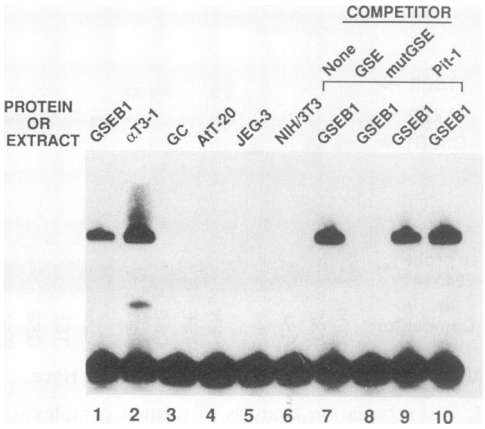

FIG. 8. Distribution of GSEB1 in pituitary cell lines and specificity of binding by purified GSEB1. Nuclear protein extracts (5 μg) from αT3-1, GC, AtT-20, JEG-3, and NIH 3T3 cells or affinity-purified GSEB1 were analyzed in a gel retardation assay using the 19-bp GSE oligonucleotide as a probe. Reaction mixtures contained either no competitor (lanes 1 to 7) or a 100-fold excess of the GSE, mutant GSE, or Pit-1/GHF-1 oligonucleotide as a competitor (lanes 8 to 10).

GSEB1 were electrophoresed in a denaturing protein gel and blotted onto nitrocellulose. Oligonucleotides representing the GSE site or a mutant GSE containing two C-to-T mutations at positions −215 and −216 (in accordance with the contact sites determined by methylation interference; Fig. 6B) were used as probes. A single 54-kDa band was detected with the native probe in both the nuclear extract and partially purified protein preparations (Fig. 7A). The mutant probe failed to detect bands in either protein preparation. We further purified the heparin-Sepharose fractions containing GSEB1 by multiple passages over a specific oligonucleotide affinity column. Figure 7B shows that a single band of 54 kDa is present in the highly purified GSEB1 preparation, consistent with the result obtained with Southwestern blotting.

Though the GSE region is not footprinted by human JEG-3 placental cells or human HeLa cervical carcinoma cells, the GSEB1 protein might be more broadly distributed in the pituitary or might be specific to mouse cells. To address these issues, tissue distribution was further investigated by testing nuclear extracts from a variety of pituitary and nonpituitary cell types, using an oligonucleotide representing only the protected sequence as probe in a gel retardation assay. Incubation of αT3-1 nuclear extract produces one major retarded band which comigrates with the complex produced with affinity-purified GSEB1 (Fig. 8). Nuclear proteins from the rat somatotrope/lactotrope cell line GC, the mouse corticotrope cell line AtT-20, the human placental cell line JEG-3, and the mouse fibroblast cell line NIH 3T3 failed to form retarded complexes with the GSE oligonucleotide. In accordance with the results shown in Fig. 5 and 7, binding of affinity-purified GSEB1 is completely abolished by the inclusion of the GSE element as competitor but unaffected by oligonucleotide carrying the CC-to-TT mutations or by the Pit-1/GHF-1 oligonucleotide. These results demonstrate that GSEB1-binding activity is specific to the cells of the gonadotrope lineage, is not Pit-1/GHF-1, and specifically binds to the GSE.

The GSE enhances expression of the human α-subunit gene in gonadotrope lineage pituitary cells in a cell-specific manner. The role of GSEB1 in regulating α-subunit gene expression in pituitary cells was further investigated by the introduction of the two C-to-T transitions into the native 5′-flanking sequence of the construction α224-CAT. These mutations eliminate the binding of GSEB1 in Southwestern blotting and gel retardation experiments (Fig. 5 and 7). Transfection of this mutant gene in parallel with the intact (α224-CAT) and 5′-truncated (α168-CAT) constructions demonstrates that the decrease in expression caused by the elimination of the GSEB binding site is of the same magnitude as that due to truncation of this region (Fig. 9A).

To determine whether the sequence context of the GSE is important for tissue-specific expression, the GSE site was cloned in one or two copies upstream of the heterologous promoter from the herpes simplex virus thymidine kinase (TK) gene in a CAT expression vector. Transfection of these constructions into mouse NIH 3T3 fibroblasts showed no effect of the GSE oligonucleotides. In contrast, the GSE element enhanced expression in αT3-1 cells, demonstrating its potential as an independent tissue-specific regulatory element (Fig. 9B).

DISCUSSION

Study of the α-subunit gene of the glycoprotein hormones provides an opportunity to address the mechanisms involved in directing expression of an individual gene to multiple distinct cell types. Tissue-specific regulation of gene expression has been found to be conferred by the binding of tissue

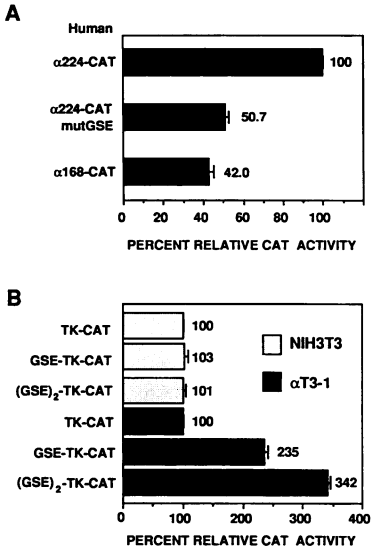

FIG. 9. Enhancement of transcriptional activity by the GSE. (A) Transfection of the human α-subunit gene with mutated GSE into αT3-1 cells. The GSE element in the −224 truncated human α-subunit regulatory region was mutated by the substitution of two T nucleotides for the two C nucleotides (−224 mutGSE) (see Fig. 6B). The activity of this mutated regulatory region was compared with the activity of the wild-type −224 and −168 truncated regulatory regions by transfection into the pituitary gonadotrope lineage cell line αT3-1, using the calcium phosphate method. CAT activity values were corrected for the levels of β-galactosidase activity from an internal control β-galactosidase expression vector and expressed as percentages of the activity from the 1.8-kb regulatory region. All values are means of three independent transfection experiments, with error bars representing ± standard errors of the means. (B) Analysis of the activity of the GSE on the heterologous promoter, TK. The GSE oligonucleotide was inserted in a polylinker 5′ to the herpes simplex virus TK promoter (at −109) on a CAT expression vector in one (GSE-TK-CAT) or two [$(GSE)_2$-TK-CAT] copies and transfected into either NIH 3T3 or αT3-1 cells by the calcium phosphate method as described above.

specifically expressed transcriptional regulatory proteins to enhancer sequences (32, 37). Thus, specification to multiple cell types could be based on the presence of a single enhancer-binding protein in all of the expressing cell types or different proteins binding to the same or alternative enhancer sequences in each cell type.

The α-subunit gene is expressed in three cell types in humans: in the trophoblasts of the placenta (an extraembryonic tissue) and in the gonadotropes and thyrotropes of the anterior pituitary. In this study, we have addressed the molecular mechanism for directing expression of the α-subunit gene to the gonadotropes of the anterior pituitary. We find that this specificity is determined by different tissue-specific DNA-binding proteins in the three different cell types. Expression of the human gene in both gonadotropes and placental trophoblasts is dependent on the CRE but is specified at least partly by a highly conserved GSE in gonadotropes. This site is bound by a specific 54-kDa nuclear protein (GSEB1) which is not found in placental cells or fibroblasts or in other pituitary cell types such as corticotropes, somatotropes/lactotropes, or thyrotropes and is not identical to the pituitary specific transcription factor Pit-1/GHF-1.

Tissue-specific expression of the α-subunit gene is conferred by different DNA-binding proteins in three cell types. In the gonadotrope lineage cells αT3-1, 442 bp of the human α-subunit 5′ regulatory region is sufficient for expression, and 257 bp of the mouse gene also shows expression. In placental cells, the human gene is fully active when truncated to −224 (10), while the mouse gene is completely inactive (49). In thyrotropes, the mouse α-subunit gene requires 480 bp of 5′-flanking DNA (39) (the human gene has not been studied). In agreement with these observations, the specific elements important for expression in each cell type are quite different. Placenta-specific expression of the human α-subunit gene is primarily determined by the trophoblast-specific protein TSEB cooperating with the more widely expressed proteins CREB and α-ACT. In thyrotropes, two binding activities are detected in the proximal region of the mouse α-subunit gene, −158 to −101 (also partially protected by mouse L-cell fibroblast extracts but unlikely to be related to CREB) and −213 to −170 (39). In contrast, we find that in gonadotrope lineage cells, tissue-specific expression is conferred at least in part by a specific protein, GSEB1, which is not detected in either of the aforementioned cell types or in unrelated cells. Furthermore, the DNA-binding activities important for expression in placental cells (TSEB) and thyrotropes (−158 to −101, −213 to −170, and −447 to −419) are absent from αT3-1 nuclear extracts.

The specific GSE DNA-binding protein detected only in cells of the gonadotrope lineage is a single species of 54 kDa. It contacts the sequence TGACCTTGT, which is highly conserved in the mammalian α-subunit genes. Its binding is highly specific in that mutation of two bases in the GSE site eliminates the interaction. In addition, it does not bind to the site for the somatotrope/lactotrope-specific factor Pit-1/GHF-1. The GSE contains the sequence TG(A/T)CC, known to comprise a half-site for binding of certain members of the nuclear steroid hormone receptor superfamily. Members of this family bind to hormone-responsive elements that are divergent inverted or direct repeats of this sequence (38, 52, 54). However, the GSE and surrounding sequences contain no candidate second half-site.

In placental cells, the TSE acts in conjunction with the CREs, and potentially with the GATA-binding site (10, 49), to confer placenta-specific expression to the human α-subunit gene. As in placenta, deletion of the CREs from the human α-subunit gene decreases gonadotrope expression to background levels. However, in contrast to the TSE, which has little or no independent activity when tested on the TK promoter (10), the GSE does enhance the TK promoter, indicating that it has independent activity in gonadotrope cells and does not depend on interaction with other elements to act as a tissue-specific enhancer.

Developmental role of cell-specific factors in pituitary cell lineage. The POU-homeodomain protein Pit-1/GHF-1, which binds to multiple sites in the upstream regions of the GH and prolactin genes, is known to confer somatotrope/lactotrope specificity. However, the mechanisms by which GH is later targeted only to the somatotrope and prolactin only to the lactotrope are not known. Not only does Pit-1/GHF-1 protein regulate expression of the genes which mark the endpoints of differentiation for these cell types, it is also required for their differentiation cell division in vivo (30) and in vitro (7). Naturally occurring mutations which affect the gene for Pit-1/GHF-1 in mice eliminate the two cell types from the pituitary. Surprisingly, the thyrotrope cell is eliminated as well. Though Pit-1/GHF-1 is not known to regulate either the common α-subunit or the TSH β-subunit gene

directly, it must play a crucial role in the differentiation or maintenance of these cells. Corticotropes (which secrete adrenocorticotropin) and gonadotropes are not affected.

Much less is known of the lineage relationships and developmental mechanisms important for the gonadotrope lineage. The early gonadotrope precursor cell line αT3-1 does not express Pit-1/GHF-1, indicating that the early lineage of the gonadotrope cell does not involve this homeobox protein. It will be of great interest to determine whether the protein that we have found to be important for the tissue-specific regulation of the α-subunit gene in gonadotropes, GSEB1, plays a similarly crucial role in the developmental lineage of the gonadotrope. It is intriguing that expression of the α-subunit gene occurs so early in the development of the pituitary (47). Perhaps GSEB1 will serve as an even earlier marker for the pituitary anlagen, providing a close link between GSEB1 and very early pituitary development.

ACKNOWLEDGMENTS

We are grateful to Jesse Marron and Kathleen Huber for technical assistance and to Joachim Altschmied and Claudia Stauber for critical reading of the manuscript.

This work was supported by NIH grants HD20377 and HD23818, by the March of Dimes (1-1182), and by NCI core grant CA14195. F.H. was a recipient of a fellowship from the Deutsche Forschungsgemeinschaft, J.J.W. was a fellow of the American Cancer Society, California Division (S-60-89), and K.M.B. was supported by NCI postdoctoral training grant CA09370.

REFERENCES

1. **Altschmied, J., F. Horn, and P. L. Mellon.** Unpublished data.
1a. **Andersen, B., G. Kennedy, and J. Nilson.** 1990. A *cis*-acting element located between the cAMP response elements and CCAAT box augments cell-specific expression of the glycoprotein hormone α subunit gene. J. Biol. Chem. **265:**21874–21880.
2. **Bodner, M., J. Castrillo, L. Theill, T. Deerinck, M. Ellisman, and M. Karin.** 1988. The pituitary-specific transcription factor GHF-1 is a homeobox-containing protein. Cell **55:**505–518.
3. **Bokar, J. A., R. A. Keri, T. A. Farmerie, R. A. Fenstermaker, B. Andersen, D. L. Hamernik, J. Yun, T. Wagner, and J. H. Nilson.** 1989. Expression of the glycoprotein hormone alpha-subunit gene in the placenta requires a functional cyclic AMP response element, whereas a different *cis*-acting element mediates pituitary-specific expression. Mol. Cell. Biol. **9:**5113–5122.
4. **Bradford, M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. Anal. Biochem. **72:**248–254.
5. **Brinster, R. L., H. Y. Chen, A. Messing, T. van Dyke, A. J. Levine, and R. D. Palmiter.** 1984. Transgenic mice harboring SV40 T-antigen genes develop characteristic brain tumors. Cell **37:**367–379.
6. **Burrin, J., and J. Jameson.** 1989. Regulation of transfected glycoprotein hormone α-gene expression in primary pituitary cell cultures. Mol. Endocrinol. **3:**1643–1651.
7. **Castrillo, J.-L., L. Theill, and M. Karin.** 1991. Function of the homeodomain protein GHF1 in pituitary cell proliferation. Science **253:**197–199.
8. **Collins, S., J. Altschmied, O. Herbsman, M. G. Caron, P. L. Mellon, and R. J. Lefkowitz.** 1990. A cAMP response element in the β_2-adrenergic receptor gene confers transcriptional autoregulation by cAMP. J. Biol. Chem. **265:**19330–19335.
9. **Darnell, R. B., and I. Boime.** 1985. Differential expression of the human gonadotropin alpha 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 alpha-subunit gene: dependence on cyclic AMP-inducible elements. Mol. Cell. Biol. **7:**3994–4002.
11. **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.
12. **Dolle, P., J.-L. Castrillo, L. E. Theill, T. Deerinck, M. Ellisman, and M. Karin.** 1990. Expression of GHF-1 protein in mouse pituitaries correlates both temporally and spatially with the onset of growth hormone gene activity. Cell **60:**809–820.
13. **Fenstermaker, R., T. Farmerie, C. Clay, D. Hamernik, and J. Nilson.** 1990. Different combinations of regulatory elements may account for expression of the glycoprotein hormone α-subunit gene in primate and horse placenta. Mol. Endocrinol. **4:**1480–1487.
14. **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.
15. **Gonzalez, G. A., K. K. Yamamoto, W. H. Fischer, D. Darr, P. Menzel, W. Briggs, W. Vale, and M. Montminy.** 1989. A cluster of phosphorylation sites on the cyclic AMP-regulated nuclear factor CREB predicted by its sequence. Nature (London) **337:**749–752.
16. **Gorman, C.** 1985. High efficiency gene transfer into mammalian cells, p. 143–190. *In* D. M. Glover (ed.), DNA cloning: a practical approach. IRL Press, Oxford.
17. **Habener, J.** 1990. Cyclic AMP response element binding proteins: a cornucopia of transcription factors. Mol. Endocrinol. **4:**1087–1094.
18. **Hai, T., F. Liu, W. J. Coukos, and M. R. Green.** 1989. Transcription factor ATF cDNA clones: an extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers. Genes Dev. **3:**2083–2090.
19. **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.
20. **Hogan, B., F. Costantini, and E. Lacy.** 1986. Manipulating the mouse embryo: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
21. **Horn, F., L. Bilezikjian, M. Perrin, M. Bosma, J. Windle, K. Huber, A. Bount, B. Hille, W. Vale, and P. Mellon.** 1991. Intracellular responses to GnRH in a clonal cell line of the gonadotrope lineage. Mol. Endocrinol. **5:**347–355.
22. **Ingraham, H. A., V. R. Albert, R. P. Chen, E. Crenshaw, H. P. Elsholtz, X. He, M. S. Kapiloff, H. J. Mangalam, L. W. Swanson, M. N. Treacy, and M. G. Rosenfeld.** 1990. A family of POU-domain and Pit-1 tissue-specific transcription factors in pituitary and neuroendocrine development. Annu. Rev. Physiol. **52:**773–791.
23. **Ivashkiv, L. B., H.-C. Liou, C. J. Kara, W. W. Lamph, I. M. Verma, and L. H. Glimcher.** 1990. mXBP/CRE-BP2 and c-Jun form a complex which binds to the cyclic AMP, but not to the 12-*O*-tetradecanoylphorbol-13-acetate, response element. Mol. Cell. Biol. **10:**1609–1621.
24. **Jameson, J. L., C. Albanese, and J. F. Habener.** 1989. Distinct adjacent protein-binding domains in the glycoprotein hormone alpha gene interact independently with a cAMP-responsive enhancer. J. Biol. Chem. **264:**16190–16196.
25. **Jameson, J. L., P. J. Deutsch, G. D. Gallagher, R. C. Jaffe, and J. F. Habener.** 1987. Trans-acting factors interact with a cyclic AMP response element to modulate expression of the human gonadotropin alpha gene. Mol. Cell. Biol. **7:**3032–3040.
26. **Jameson, J. L., R. C. Jaffe, P. J. Deutsch, C. Albanese, and J. F. Habener.** 1988. The gonadotropin alpha-gene contains multiple protein binding domains that interact to modulate basal and cAMP-responsive transcription. J. Biol. Chem. **263:**9879–9886.
27. **Kara, C. J., H.-C. Liou, L. B. Ivashkiv, and L. H. Glimcher.** 1990. A cDNA for a human cyclic AMP response element-binding protein which is distinct from CREB and expressed preferentially in brain. Mol. Cell. Biol. **10:**1347–1357.
28. **Kennedy, G. C., B. Andersen, and J. H. Nilson.** 1990. The human alpha subunit glycoprotein hormone gene utilizes a unique CCAAT binding factor. J. Biol. Chem. **265:**6279–6285.
29. **Laemmli, U.** 1970. Cleavage of structural proteins during the

assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.

29a. **Lew, D.** Personal communication.

30. **Li, S., E. Crenshaw, E. Rawson, D. Simmons, L. Swanson, and M. Rosenfeld.** 1990. Dwarf locus mutants lacking three pituitary cell types result from mutations in the POU-domain gene *pit*-1. Nature (London) **347**:528–533.
31. **Mangalam, H. J., V. R. Albert, H. A. Ingraham, M. Kapiloff, L. Wilson, C. Nelson, H. Elsholtz, and M. G. Rosenfeld.** 1989. A pituitary POU domain protein, Pit-1, activates both growth hormone and prolactin promoters transcriptionally. Genes Dev. **3**:946–958.
32. **Maniatis, T., S. Goodbourn, and J. Fischer.** 1987. Regulation of inducible and tissue-specific gene expression. Science **236**:1237–1245.
33. **Marsh, J., M. Ertle, and E. Weykes.** 1984. The pIC plasmid and phage vectors with versatile cloning sites for recombinant selection by insertional inactivation. Gene **32**:481–485.
34. **Meinkoth, J., and G. Wahl.** 1984. Hybridization of nucleic acids immobilized on solid supports. Anal. Biochem. **138**:267–284.
35. **Mellon, P. L., V. Parker, Y. Gluzman, and T. Maniatis.** 1981. Identification of DNA sequences required for transcription of the human alpha$_1$ globin gene using a new SV40 host-vector system. Cell **27**:279–288.
36. **Miskimins, W., M. Roberts, A. McClelland, and F. Ruddle.** 1985. Use of a protein blotting procedure and a specific DNA probe to identify nuclear proteins that recognize the promoter region of the transferrin receptor gene. Proc. Natl. Acad. Sci. USA **82**:6741–6744.
37. **Mitchell, P. J., and R. Tjian.** 1989. Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. Science **245**:371–378.
38. **Näär, A., J.-M. Boutin, S. Lipkin, V. Yu, J. Holloway, C. Glass, and M. Rosenfeld.** 1991. The orientation and spacing of core DNA-binding motifs dictate selective transcriptional responses to three nuclear receptors. Cell **65**:1267–1279.
39. **Ocran, K., V. Sarapura, W. Wood, D. Gordon, A. Gutierrez-Hartmann, and E. Ridgway.** 1990. Identification of cis-acting promoter elements important for expression of the mouse glycoprotein hormone α-subunit gene in thyrotropes. Mol. Endocrinol. **4**:766–772.
40. **Ohlsson, H., and T. Edlund.** 1986. Sequence-specific interactions of nuclear factors with the insulin gene enhancer. Cell **45**:35–44.
41. **Orkin, S.** 1990. Globin gene regulation and switching: circa 1990. Cell **63**:665–672.
42. **Sarapura, V., W. Wood, D. Gordon, K. Ocran, M. Kao, and E. Ridgway.** 1990. Thyrotrope expression and thyroid hormone inhibition map to different regions of the mouse glycoprotein hormone α-subunit gene promoter. Endocrinology **127**:1352–1361.
43. **Sawadogo, M., M. W. Van Dyke, P. D. Gregor, and R. G. Roeder.** 1988. Multiple forms of the human gene-specific transcription factor USF. J. Biol. Chem. **263**:11985–11993.
44. **Schüle, R., M. Muller, H. Otsuka-Murakami, and R. Renkawitz.** 1988. Cooperativity of the glucocorticoid receptor and the CACCC-box binding factor. Nature (London) **332**:87–90.
45. **Seed, B., and J. Sheen.** 1988. A simple phase-extraction assay for chloramphenicol acyltransferase activity. Gene **67**:271–277.
46. **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 alpha-subunit gene is mediated by an 18-base-pair element. Proc. Natl. Acad. Sci. USA **84**:2198–2202.
47. **Simmons, D. M., J. W. Voss, H. A. Ingraham, J. M. Holloway, R. S. Broide, M. G. Rosenfeld, and L. W. Swanson.** 1990. Pituitary cell phenotypes involve cell-specific Pit-1 mRNA translation and synergistic interactions with other classes of transcription factors. Genes Dev. **4**:695–711.
48. **Singh, R., and V. Natarajan.** 1987. A rapid affinity method for isolation and characterization of sequence specific DNA binding factor. Biochim. Biophys. Res. Commun. **147**:65–71.
49. **Steger, D., J. Altschmied, M. Büscher, and P. Mellon.** 1991. Evolution of placenta-specific gene expression: comparison of the equine and human gonadotropin α-subunit genes. Mol. Endocrinol. **5**:243–255.
50. **Tashjian, A. H. J., Y. Yasumura, L. Levine, G. H. Sato, and M. L. Parker.** 1968. Establishment of clonal strains of rat pituitary tumor cells that secrete growth hormone. Endocrinology **82**:342–352.
51. **Tepper, M. A., and J. L. Roberts.** 1984. Evidence for only one β-luteinizing hormone and no β-chorionic gonadotropin gene in the rat. Endocrinology **115**:385–391.
52. **Truss, M., G. Chalepakis, E. Slater, S. Mader, and M. Beato.** 1991. Functional interaction of hybrid response elements with wild-type and mutant steroid hormone receptors. Mol. Cell. Biol. **11**:3247–3258.
53. **Tsai, S., I. Sagami, H. Wang, M.-J. Tsai, and B. O'Malley.** 1987. Interactions between a DNA-binding transcription factor (COUP) and a non-DNA binding factor (S300-II). Cell **50**:701–709.
54. **Umesono, K., K. Murakami, C. Thompson, and R. Evans.** 1991. Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D_3 receptors. Cell **65**:1255–1266.
55. **Windle, J., R. Weiner, and P. Mellon.** 1990. Cell lines of the pituitary gonadotrope lineage derived by targeted oncogenesis in transgenic mice. Mol. Endocrinol. **4**:597–603.
56. **Yamamoto, M., L. Ko, M. Leonard, H. Beug, S. Orkin, and J. Engel.** 1990. Activity and tissue-specific expression of the transcription factor NF-E1 multigene family. Genes Dev. **4**:1650–1662.