

## Activation of the Glycoprotein Hormone $\alpha$ -Subunit Promoter by a LIM-Homeodomain Transcription Factor

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Recently, a pituitary-specific enhancer was identified within the 5' flanking region of the mouse glycoprotein hormone  $\alpha$ -subunit gene. This enhancer is active in pituitary cells of the gonadotrope and thyrotrope lineages and has been designated the pituitary glycoprotein hormone basal element (PGBE). In the present studies, we sought to isolate and characterize proteins which interact with the PGBE. Mutagenesis experiments identified a 14-bp imperfect palindrome that is required for binding of a factor which is present in cells of gonadotrope and thyrotrope lineages but not in other cells. Screening of a mouse cDNA library with a DNA probe containing the imperfect palindrome resulted in the isolation of a LIM-homeodomain transcription factor. The cDNA predicts a mouse protein which is 94% identical to the recently described rat LIM-homeodomain protein LH-2. LH-2 contains two zinc fingers (LIM domain) and a consensus homeodomain. Hybridization analysis revealed relatively high expression of LH-2 mRNA in the central nervous system and in pituitary cells of the gonadotrope and thyrotrope lineages. Lower or nondetectable levels of LH-2 mRNA were found in other pituitary cells and tissues, including placental cells. Recombinant LH-2 homeodomain was found to selectively bind to the previously identified imperfect palindrome in the PGBE. Point mutations in the PGBE resulted in parallel losses in the binding of a nuclear factor from a cell line of the gonadotrope lineage and recombinant LH-2-binding activity. Use of an antibody to LH-2 provided evidence that endogenous PGBE-binding activity from cells of the gonadotrope lineage involves a protein which is immunologically related to LH-2. Expression of LH-2 in two heterologous cell types resulted in activation of a reporter gene containing the mouse  $\alpha$  promoter. These data suggest that the LIM-homeodomain factor LH-2 plays a role in stimulating tissue-specific expression of the mouse glycoprotein hormone  $\alpha$  subunit. The finding that a LIM-homeodomain protein can stimulate expression of one of the earliest markers of pituitary differentiation raises the possibility that this factor plays a role in cell lineage determination in the pituitary.

The glycoprotein hormones are a family of heterodimeric proteins which consist of a common  $\alpha$  subunit noncovalently associated with a hormone-specific  $\beta$  subunit (37). The glycoprotein hormones include the pituitary hormones, luteinizing hormone, follicle-stimulating hormone, and thyroid-stimulating hormone. In addition, some species also synthesize a chorionic gonadotropin within the placenta. Within a species, the glycoprotein hormones all share a common  $\alpha$  subunit, while the unique  $\beta$  subunits specify the biological activity of the heterodimer. In the pituitary gland, luteinizing hormone and follicle-stimulating hormone are synthesized within cells which are designated gonadotropes, while thyroid-stimulating hormone is synthesized in thyrotropes. Thus, the glycoprotein hormone  $\alpha$ -subunit gene is expressed within two different cell types in the pituitary and in some species within the placenta. The mechanisms mediating the tissue-specific expression of the  $\alpha$ -subunit gene have been the focus of a large number of studies. While a number of DNA elements which are important for  $\alpha$ -subunit expression in the placenta have been identified (3, 4, 11, 12, 25, 28, 34, 46), much less is known concerning requirements for expression in the pituitary. It has been demonstrated that reporter genes containing various

amounts of the 5' flanking sequence from the  $\alpha$ -subunit gene can be targeted to the pituitary (4, 15). Transgenic animal studies have also provided evidence that different DNA elements are required for expression of the  $\alpha$ -subunit in thyrotropes and gonadotropes of the pituitary (20, 27). The development of clonal cell lines representing the gonadotrope (55) and thyrotrope (2) lineages has greatly facilitated more detailed studies of DNA elements required for tissue-specific expression of the  $\alpha$ -subunit gene. Studies using these cell lines have provided information concerning DNA regions important for expression in the pituitary (36, 42). This has led to the identification of a gonadotrope-specific DNA element and a factor which interacts with this element (22).

Recently, we identified DNA sequences which are important for pituitary-specific expression of the mouse  $\alpha$  promoter (43, 44). These studies found that sequences between -344 to -300 were sufficient and necessary to direct maximal expression of the mouse  $\alpha$ -subunit promoter to cells of the gonadotrope and thyrotrope lineages. This DNA element is distinct from the gonadotrope-specific element identified by Horn et al. (22). As the element that we identified is required for basal expression in the pituitary but not in the placenta, we designated this region of the mouse  $\alpha$ -subunit gene as the pituitary glycoprotein hormone basal element (PGBE). In this study, we have examined factors which interact with the PGBE. These studies have resulted in the identification of a cDNA which encodes a LIM-homeodomain factor, LH-2. LH-2 binds specifically to the PGBE and is able to stimulate expression of the glycoprotein hormone  $\alpha$ -subunit promoter.

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## MATERIALS AND METHODS

**Cell culture.**  $\alpha$ T3-1, JEG-3, and Cos 1 cells were grown in monolayer culture in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum.  $\alpha$ TSH cells were grown in spinner culture in Joklik's medium containing 10% neonatal calf serum. GH<sub>3</sub> cells were grown in monolayer culture in DMEM containing 15% horse serum and 2.5% fetal bovine serum. AtT20 cells were grown in monolayer culture in DMEM containing 10% neonatal calf serum.

**Preparation of nuclei and DNA affinity chromatography.** Nuclei from all cell types were prepared when cells were approximately 80% confluent essentially as described previously (19). Briefly, cells were washed twice in ice-cold *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-buffered saline and allowed to swell in 60 mM KCl–15 mM NaCl–0.15 mM spermine–0.5 mM spermidine–15 mM HEPES (pH 7.8)–14 mM  $\beta$ -mercaptoethanol (buffer A) containing 0.3 M sucrose and the following protease inhibitors (subsequently referred to as protease inhibitors; all from Sigma): leupeptin and pepstatin (2.5  $\mu$ g/ml), phenylmethylsulfonyl fluoride (0.2 mM), and benzamide (5 mM). The swollen cells were then Dounce homogenized vigorously (tight pestle) in the absence of detergent until 90% of the cells appeared disrupted as determined by phase-contrast light microscopy. The cell homogenate was layered on a cushion of buffer A containing 0.9 M sucrose and protease inhibitors and centrifuged for 10 min at 4,500 rpm. The nuclear pellet was resuspended in buffer A containing 0.3 M sucrose and protease inhibitors, Dounce homogenized an additional three times, and again centrifuged through a 0.9 M sucrose cushion in the same buffer. The partially purified nuclei were resuspended in a buffer containing 75 mM NaCl, 0.5 mM EDTA, 20 mM Tris (pH 7.9), 1 mM dithiothreitol, 50% glycerol, and protease inhibitors. Nuclei were stored at  $-70^{\circ}\text{C}$  until further use.

For DNA affinity purification of PGBE-binding proteins, biotinylated oligonucleotides representing sequences  $-317$  to  $-353$  of the mouse  $\alpha$  promoter were prepared and annealed as described previously (19). Binding reaction mixtures contained nuclei suspended in buffer containing 10 mM Tris (pH 7.5), 5% glycerol, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 80  $\mu$ g of poly(dI-dC) per ml, and protease inhibitors. Biotinylated annealed oligonucleotides were added, and the mixture was incubated with gentle agitation for 2 h at  $4^{\circ}\text{C}$ . Nuclei were sedimented by centrifugation (4,500 rpm, 15 min), and the supernatant was passed over a streptavidin agarose column equilibrated with binding buffer described above. The column was washed with binding buffer containing 200 and 400 mM NaCl, and binding activity was eluted in buffer containing 600 mM NaCl. Fractions were stored at  $-80^{\circ}\text{C}$ , and specific binding activity was evaluated by electrophoretic mobility shift assay.

**Electrophoretic mobility shift and DNase I protection assays.** Wild-type and mutant DNA probes containing sequences for  $-424$  to  $-288$  of the mouse  $\alpha$  promoter were prepared by PCR amplification. For electrophoretic mobility shift assays, reaction mixtures contained 6,000 to 8,000 cpm of  $^{32}\text{P}$ -labeled DNA probes, various amounts of affinity-purified nuclear proteins, 30  $\mu$ g of bovine serum albumin (BSA), 400 ng of poly(dI-dC), 10 mM Tris (pH 7.5), 5% glycerol, 50 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol in a total volume of 25  $\mu$ l. Reaction mixtures were incubated for 5 min in the absence of radiolabeled probe and then for 20 min in the presence of probe at room temperature. The reaction mixture was then electrophoresed through a nondenaturing polyacrylamide gel in  $0.25 \times$  TBE buffer (22 mM Tris-borate [pH 8.3], 0.5 mM

EDTA) at  $4^{\circ}\text{C}$ , dried, and analyzed by autoradiography. In some experiments, antiserum to LH-2 was incubated with affinity-purified nuclear proteins and the DNA probe before electrophoresis. Antiserum to chicken LH-2 was prepared by immunizing rabbits with a recombinant fusion protein containing glutathione *S*-transferase linked to a carboxy-terminal fragment of LH-2. The antigen was prepared by subcloning a 430-bp *HincII-SmaI* fragment from the chicken LH-2 cDNA into pGEX-3X, with expression and purification of the recombinant fusion protein as described previously (48). The rabbit antiserum to chicken LH-2 was absorbed against an acetone powder prepared from bacteria expressing glutathione *S*-transferase.

DNase I protection assays were prepared as described above, with the following modifications. Following the 20-min incubation with  $^{32}\text{P}$ -labeled DNA probes, 0.075 U of RQ1 DNase (Promega) in 100 mM Tris (pH 7.5)–35 mM  $\text{MgCl}_2$  was added, and the incubation continued for an additional 8 min. The reaction was terminated by the addition of 3  $\mu$ l of 0.5 M EDTA, extracted with phenol, and precipitated with ethanol. The precipitated products were collected by centrifugation and electrophoresed through a polyacrylamide gel containing 8 M urea, dried, and analyzed by autoradiography.

**Site-directed mutagenesis and transient-transfection assays.** Mutations in the mouse  $\alpha$  promoter were made by oligonucleotide-directed mutagenesis as described previously (23, 43). Mutations were confirmed by nucleotide sequence analysis (41) and subcloned into the luciferase reporter constructs (8, 10). For transfection experiments, 10  $\mu$ g of mouse  $\alpha$ -luciferase or thymidine kinase (TK)-luciferase reporter fusion gene and 5  $\mu$ g of Bluescript carrier DNA were transfected into cells, using a single electrical pulse at 220 V and 960  $\mu$ F as described previously (9, 43). Cell lysates were prepared 20 to 24 h after transfection, and luciferase activity was determined (10).

**Screening of an  $\alpha$ T3-1  $\lambda$ gt11 cDNA library for PGBE-binding proteins.**  $\lambda$ gt11 cDNA expression libraries were prepared from  $\alpha$ T3-1 cell polyadenylated RNA essentially as described previously (45). Two different primary libraries were prepared; each contained approximately  $0.9 \times 10^6$  independent cDNA clones. All screens were conducted with unamplified cDNA libraries. Expression screening for clones which contained PGBE-binding activity was conducted as described by Vinson et al. (53), using a radiolabeled PGBE DNA probe which was prepared as described previously (21). Following identification of a partial cDNA clone by using the expression screen, the initial clone was used as a hybridization probe to identify additional clones (40). The nucleotide sequences of both strands of cloned cDNAs were determined by the dideoxy chain termination technique (41).

**Expression and purification of recombinant LH-2.** A fragment of the mouse LH-2 cDNA encoding lysine 147 through the carboxy terminus of LH-2 was subcloned in pET16B (Novagen), an inducible bacterial expression vector which encodes an amino-terminal extension containing 10 histidine residues. Growth, induction, and collection of bacterial cells were performed as described previously (49). Cell pellets were resuspended in 10 mM Tris (pH 8.0)–50 mM NaCl, and the cells were lysed in a French press. Cellular debris was removed by centrifugation, and supernatants were adjusted to contain 30 mM Tris (pH 8.0) and 0.6 M ammonium sulfate. The recombinant LH-2 was then purified by nickel chelate chromatography (1). Nickel chelate agarose was added to absorb the protein (0.25 ml/liter of bacterial culture), and the mixture was incubated on a rotary mixer for 1.5 h at  $4^{\circ}\text{C}$ . After washing with 10 mM Tris (pH 8.0)–0.6 M ammonium sulfate and 10

mM Tris (pH 8.0)–75 mM imidazole, the resin was eluted with 10 mM Tris (pH 6.8) containing 0.5 M imidazole. Fractions containing recombinant LH-2 were identified by gel electrophoresis. The eluate was dialyzed against 10 mM Tris (pH 7.5)–50 mM NaCl–5% glycerol–1 mM EDTA–1 mM dithiothreitol, aliquoted, and stored at  $-80^{\circ}\text{C}$ .

**Hybridization analysis of LH-2 mRNA.** Total cellular RNA was isolated by solubilization in guanidine HCl and sedimentation through CsCl as described previously (18). Equal amounts of RNA were electrophoresed on agarose gels containing formaldehyde (51) and transferred to nitrocellulose filters by capillary transfer. The nitrocellulose filters were baked and then prehybridized for 8 h in 50% deionized formamide–0.75 M NaCl–0.075 M sodium citrate–0.04% BSA–0.04% Ficoll–0.04% polyvinylpyrrolidone–20 mM HEPES (pH 7.4)–1 mg of salmon sperm DNA per ml–0.1% sodium PP<sub>i</sub> at  $42^{\circ}\text{C}$ . DNA probes for LH-2 and  $\beta$ -actin were radiolabeled by random primer method (14) to a specific activity of  $10^9$  cpm/ $\mu\text{g}$  of DNA or greater. Following prehybridization, radiolabeled probe was added, and hybridization continued for 12 h. The blots were then washed extensively, air dried, and analyzed by autoradiography.

## RESULTS

**$\alpha\text{T3-1}$  and  $\alpha\text{TSH}$  cells contain a factor which interacts specifically with the PGBE.** Our previous analysis of the mouse  $\alpha$  promoter revealed that the PGBE of the mouse  $\alpha$ -subunit gene was sufficient to function as a tissue-specific enhancer (43). The present studies have characterized factors which interact with this DNA element. Biotinylated oligonucleotides representing the PGBE were incubated with nuclei from the gonadotrope-derived  $\alpha\text{T3-1}$  cell line (55) or the thyrotrope-derived  $\alpha\text{TSH}$  cell line (2). The biotinylated DNA was then bound to streptavidin agarose beads, and proteins were eluted with increasing ionic strength. The presence of DNA-binding proteins in the eluate of the DNA affinity column was examined by electrophoretic mobility shift assay (Fig. 1). These studies revealed two discrete DNA-protein complexes formed when wild-type PGBE sequences were incubated with affinity-purified extracts from  $\alpha\text{T3-1}$  or  $\alpha\text{TSH}$  cells. To test the sequence specificity of this binding activity, competition studies and mutant radiolabeled probes were used in mobility shift assays. The addition of a 50-fold molar excess of unlabeled wild-type competitor DNA resulted in a specific loss of both shifted complexes. Affinity-purified proteins from  $\alpha\text{T3-1}$  or  $\alpha\text{TSH}$  cells failed to bind a probe containing an 8-bp mutation at positions  $-337$  to  $-330$  (probe 1), a mutation which we previously found substantially reduced basal  $\alpha$ -subunit expression (43). An 8-bp mutation at positions  $-350$  to  $-343$  (probe 2) resulted in only a minimal loss of binding activity, consistent with the finding that this mutation has little effect on  $\alpha$ -subunit expression (43). These results identify a specific complex which is dependent on DNA sequences in the  $-337$  to  $-330$  region, the same region which we have previously shown is crucial for transcriptional activation in pituitary cells of the gonadotrope and thyrotrope lineages (43).

**The factor which binds to the PGBE has a restricted cell distribution.** To examine cell specificity for PGBE-binding activity, nuclear extracts from several different cells were fractionated by oligonucleotide affinity purification. Equal amounts of nuclei from several different cell types were subjected to the DNA affinity purification procedure. The cells were representative of several different pituitary cell lineages, including cells of the gonadotrope ( $\alpha\text{T3-1}$ ) and thyrotrope ( $\alpha\text{TSH}$ ) lineages, which express the endogenous  $\alpha$ -subunit

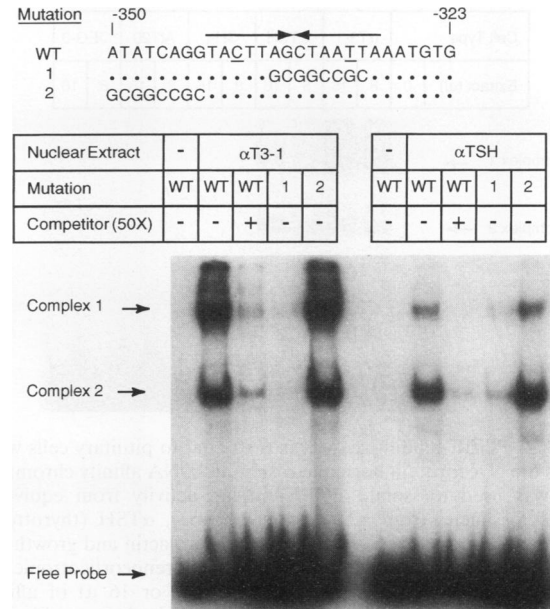


FIG. 1. Analysis of the binding of nuclear factors from  $\alpha\text{T3-1}$  and  $\alpha\text{TSH}$  cells to the PGBE. Radiolabeled wild-type probe (WT) and mutant DNA probes (probes 1 and 2) containing the  $-424$  to  $-288$  region of the mouse  $\alpha$ -subunit gene were used in an electrophoretic mobility shift assay. Mutations were placed in the  $-350$  to  $-323$  region as indicated. Oposposing arrows above the wild-type sequence indicate the presence of an imperfect palindrome. Radiolabeled probes were incubated in either the absence or the presence of affinity-purified nuclear extracts from  $\alpha\text{T3-1}$  and  $\alpha\text{TSH}$  cells; some reaction mixtures contained a 50-fold molar excess of unlabeled wild-type probe. Complexes were resolved by nondenaturing polyacrylamide gel electrophoresis and autoradiography. Two different complexes and the free probe are indicated by arrows at the left.

gene, and pituitary cells, which express other pituitary hormones such as growth hormone and prolactin (GH<sub>3</sub> cells) and adrenocorticotrophic hormone (AtT20 cells). Placental cells which express the  $\alpha$  subunit (JEG-3 cells) were also tested. The affinity-purified nuclear extracts were then assayed for DNA-binding activity by mobility shift assay (Fig. 2). Distinct DNA-protein complexes were observed in extracts from  $\alpha\text{T3-1}$  or  $\alpha\text{TSH}$  cells but not in extracts derived from the other pituitary and placental cell types. Similar studies using crude extracts of  $\alpha\text{T3-1}$ ,  $\alpha\text{TSH}$ , GH<sub>3</sub>, and JEG-3 cells produced similar, tissue-specific bands, although several minor bands were observed (data not shown). Furthermore, the crude extracts of all of the cell types demonstrated specific binding to a consensus cyclic AMP (cAMP) response element, demonstrating that all of the extracts contained active DNA-binding proteins (data not shown).

**Mutations which disrupt binding of a factor to the PGBE also affect transcriptional activation.** Inspection of DNA sequence in the region which was important for factor binding and transcriptional activation revealed the presence of a 14-bp imperfect palindromic sequence, TACTTAGCTAATTA (positions  $-343$  to  $-330$ ). It seemed likely that this imperfect palindrome represented the factor-binding site. As an initial approach to explore this issue, we prepared a variety of point mutations in this region and tested both factor binding and transcriptional activation (Fig. 3). Ten mutations within this region were tested. Most of the mutations within the palindromic sequence decreased DNA-binding activity, as evi-

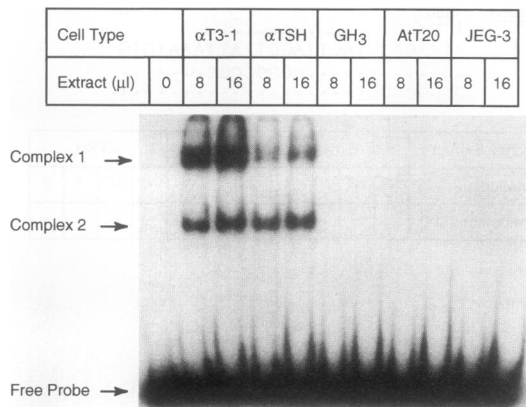


FIG. 2. PGBE-binding activity is restricted to pituitary cells which express the glycoprotein hormone  $\alpha$  subunit. DNA affinity chromatography was used to isolate PGBE-binding activity from equivalent numbers of nuclei from  $\alpha$ T3-1 (gonadotrope),  $\alpha$ TSH (thyrotrope), GH<sub>3</sub> (mammosomatotropes which produce prolactin and growth hormone), AtT20 (corticotropes which produce adrenocorticotrophic hormone), and JEG-3 (placental) cells. Either 8 or 16  $\mu$ l of affinity-purified extracts was then used for the electrophoretic mobility shift assay. The radiolabeled DNA probe contained the PGBE (as part of a DNA fragment representing the mouse  $\alpha$  -424 to -288 region). DNA-protein complexes were analyzed by nondenaturing polyacrylamide gel electrophoresis and autoradiography. Two different complexes and the free probe are indicated by the arrows at the left.

denced by a decrease in both complex 1 and complex 2. Interestingly, mutations at the +5 and -5 positions (relative to the center of palindrome), in which the wild-type sequence contains a disruption of a perfect palindrome, had a rather variable effect. For instance, a mutation at the +5 position creates a perfect palindrome and decreased factor binding (probe 7). In contrast, a mutation at position -5 which also created a perfect but different palindrome (probe 9) appeared to modestly increase binding. This finding suggests that a perfect, 14-bp palindrome with the sequence TAATTAGCTA ATTA may be the preferred binding site for the factor(s). Importantly, there was an excellent correlation between effects of the mutations on both complexes (Fig. 3B) and expression of the  $\alpha$ -subunit promoter (Fig. 3C). These findings suggest that complex formation at the palindromic sequence is important for transcriptional activation of the mouse  $\alpha$ -subunit gene.

**Cloning of a factor which binds to the PGBE.** In an attempt to clone factors which bind to the PGBE, an  $\alpha$ T3-1 cDNA library was screened with a radiolabeled PGBE probe. An initial screen of  $6 \times 10^5$  plaques yielded 36 positive signals. Rescreening with both a PGBE probe and an unrelated probe yielded a single signal which specifically bound to the PGBE. Following plaque purification, the cDNA which yielded this positive signal was used as a hybridization probe to screen a random primed  $\alpha$ T3-1 cDNA library. The largest cDNA identified by this screen predicted a 427-amino-acid open reading frame. Consistent with the predicted open reading frame, in vitro transcription and translation of the cDNA resulted in the synthesis of an approximately 47-kDa protein (data not shown). Comparison with other known amino acid sequences (GenBank) revealed this protein to be a member of the LIM-homeodomain family of transcription factors (38). LIM-homeodomain factors contain both a putative zinc finger (the LIM domain) and a homeodomain (Fig. 4A). The homeodomain of these factors is sufficient for specific DNA binding (57). The LIM domain contains putative zinc finger-like struc-

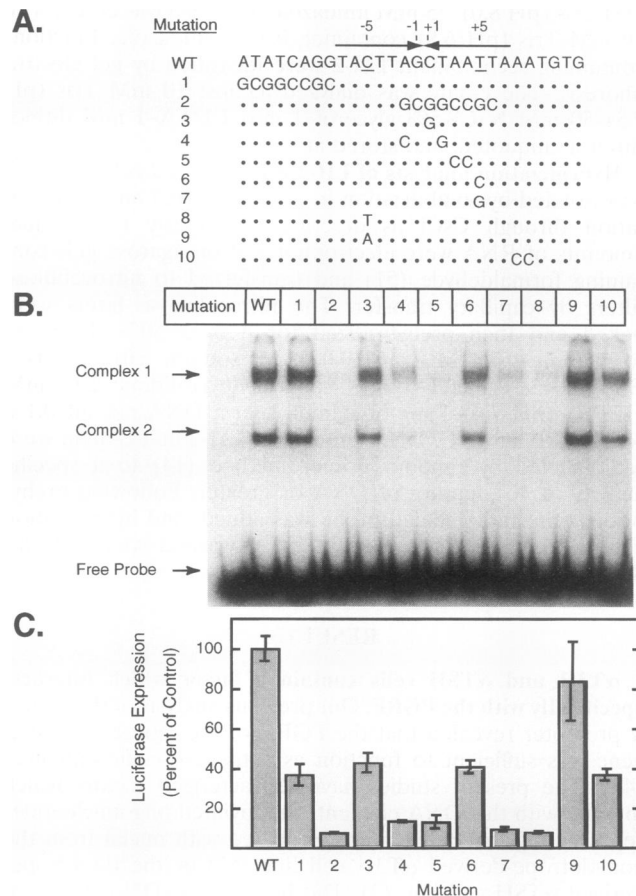


FIG. 3. Effects of mutations in the PGBE on in vitro factor binding and in vivo expression of the mouse  $\alpha$ -subunit promoter in  $\alpha$ T3-1 cells. Radiolabeled wild-type probe (WT) and mutant DNA probes (probes 1 to 10) containing the -424 to -288 region of the mouse  $\alpha$ -subunit gene were used in an electrophoretic mobility shift assay. Mutations were placed in the -350 to -323 region as indicated (A). Opposing arrows above the wild-type sequence indicate the presence of an imperfect palindrome. The imperfect palindrome is numbered relative to the center of the palindrome. Affinity chromatography-purified  $\alpha$ T3-1 extracts were incubated with the individual wild-type or mutant probes, and the resulting complexes were analyzed by nondenaturing polyacrylamide gel electrophoresis (B). Two different complexes and the free probe are indicated by the arrows on the left. To test the effects of mutations in the PGBE on expression of the  $\alpha$ -subunit promoter, the mutations were incorporated within the context of a DNA fragment representing positions -507 to +46 of the mouse  $\alpha$ -subunit promoter, and this fragment was linked to luciferase. The wild-type and mutant  $\alpha$ -subunit/luciferase constructs (10  $\mu$ g) were transfected into  $\alpha$ T3-1 cells, and luciferase expression was determined (C). The data are expressed as a percentage of the wild-type values and are the mean values  $\pm$  standard errors from nine transfections for each construct from three separate experiments. The histograms are aligned with the mobility shift data for each mutation.

tural motifs, and it has been shown that the LIM domain is capable of binding zinc (30, 35). However, it seems likely that the LIM domain is not a DNA-binding domain, as it may actually inhibit DNA binding (57). Rather, the available evidence suggests that LIM domains may function as protein-protein interaction domains (39). When we isolated this clone, no other sequence in GenBank was identical to that of the newly isolated cDNA. However, as we proceeded with the characterization of this clone, Xu et al. described the cloning of

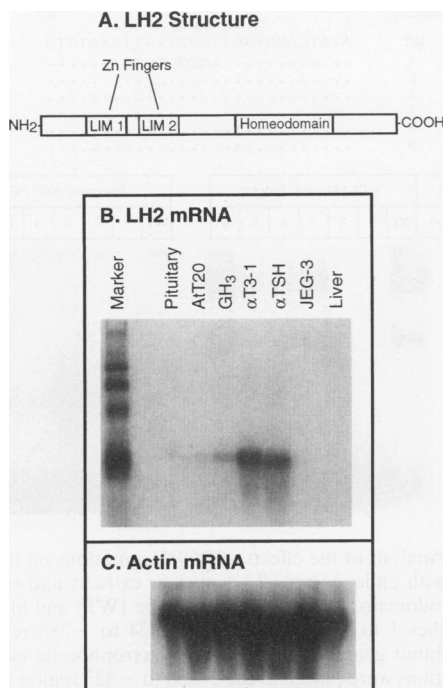


FIG. 4. Analysis of the distribution of LH-2 mRNA. LH-2 is a 47-kDa protein characterized by two LIM domains (putative zinc finger domains) which are located in a region close to the amino terminus of the protein and a homeodomain toward the carboxy terminus (A). The distribution of mRNA encoding LH-2 in mouse pituitary and liver as well as clonal cell lines of pituitary cell lineage was analyzed by denaturing gel electrophoresis and hybridization to a radiolabeled LH-2 probe (B). Twenty micrograms of total cellular RNA from each tissue or cell type was subjected to agarose gel electrophoresis in gels containing formaldehyde, and then the RNA was transferred to nitrocellulose membranes (51). Radiolabeled LH-2 cDNA was hybridized to the filter, and after washing, specific LH-2 mRNA species were identified by autoradiography. A DNA size marker (marker lane) was prepared from *Hind*III-digested  $\lambda$  DNA. Parallel samples were analyzed for actin mRNA (C).

a cDNA which they designated the rat LIM-homeodomain protein, LH-2 (56). The predicted amino acid sequence of LH-2 is 94% identical to the sequence of the protein predicted by the mouse cDNA which we isolated. Thus, the cDNA clone which we isolated by screening for binding to the PGBE appears to be the mouse homolog of LH-2.

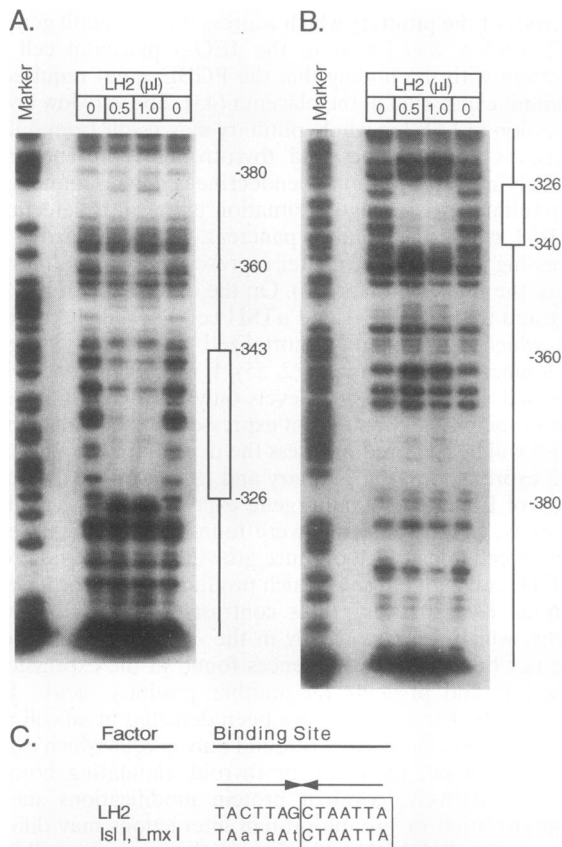
**LH-2 mRNA is found in relatively high concentrations in pituitary cell lines which express the  $\alpha$ -subunit gene and in the central nervous system.** Previously it was found that LH-2 has a rather restricted tissue distribution, with expression in some cells of the lymphoid system and within specific areas of the brain (56). To examine LH-2 expression in cells which express the  $\alpha$ -subunit gene, total cellular RNA was prepared from whole rat pituitaries, AtT20, GH<sub>3</sub>,  $\alpha$ T3-1,  $\alpha$ TSH, and JEG-3 cells, as well as mouse liver. Hybridization to a radiolabeled LH-2 probe after fractionation by denaturing gel electrophoresis and transfer to nitrocellulose, demonstrated the presence of an approximately 2.3-kb transcript in some of the samples. The LH-2 mRNA was most abundant in  $\alpha$ T3-1 and  $\alpha$ TSH cells, less abundant in whole rat pituitaries, AtT20 cells, and GH<sub>3</sub> cells, and nondetectable in JEG-3 cells and mouse liver (Fig. 4B). In addition, the mouse LH-2 transcript was abundantly present in mouse brain (data not shown). These findings show that relatively high levels of LH-2 mRNA were found in the clonal

cell types of the pituitary which express the  $\alpha$ -subunit gene. No LH-2 mRNA was found in the JEG-3 placental cell line, consistent with the finding that the PGBE is not required for  $\alpha$ -subunit expression in the placenta (43). The very low level of expression of LH-2 in whole pituitary may result from dilution effects, as gonadotropes and thyrotropes represent only a relatively small proportion of endocrine cell types found within the pituitary. An analogous situation exists with detection of the Isl-1 transcript in adult pancreas, in which case  $\beta$  cells expressing the insulin promoter represent only a small proportion of the total cell mass (26). On the other hand, it has been suggested that the  $\alpha$ T3-1 and  $\alpha$ TSH cell lines likely represent cells which have been immortalized at an early stage in development of the pituitary (2, 55). It is possible that LH-2 is expressed at relatively high levels only during early development of the pituitary and is not expressed in the adult. Further studies will be required to assess the developmental pattern of LH-2 expression in the pituitary and to determine the contribution of LH-2 to  $\alpha$ -subunit gene expression in the adult. In this study, LH-2 transcripts were found at very low levels in pituitary cell lines which produce growth hormone and prolactin (GH<sub>3</sub> cells) and in cells which produce adrenocorticotrophic hormone (AtT20 cells). This contrasts with PGBE-binding activity, which was found only in the  $\alpha$ T3-1 and  $\alpha$ TSH cells. This may be similar to differences found in the expression of transcripts and proteins for another pituitary factor, Pit-1. Although Pit-1 transcripts have been detected in all cell types of the pituitary, the protein is found only in cells which express growth hormone, prolactin, or thyroid-stimulating hormone (47). Alternatively, covalent protein modifications such as phosphorylation or protein-protein interactions may differentially regulate LH-2 DNA-binding activity in various cell types.

**The LH-2 homeodomain is sufficient for selective binding to the PGBE.** To examine the specificity of mouse LH-2 binding to the  $\alpha$ -subunit promoter, a carboxy-terminal fragment of LH-2 encoding residues 147 to 427 (LH-2<sub>147-427</sub>) was prepared by using a bacterial expression system. This portion of LH-2 contains the homeodomain but not the LIM domain. Recombinant LH-2<sub>147-427</sub> was used to determine sequence specificity of binding to the mouse  $\alpha$ -subunit promoter by DNase I protection assay (Fig. 5). Recombinant LH-2<sub>147-427</sub> protected DNA sequences at positions -343 to -326 of the mouse  $\alpha$  promoter on the upper strand (Fig. 5A) and a similar region on the other strand (Fig. 5B). Particularly on the upper strand, stronger protection was found at the downstream portion of the footprint. The protected region overlaps the imperfect palindrome which is located at positions -342 to -329. These results provide evidence that the LH-2 homeodomain is sufficient for specific DNA binding in the absence of the LIM domain. Analysis of other mammalian LIM-homeodomain protein-binding sites (Fig. 5C) reveals that the sequence CTAATTA which is present in the downstream half of the mouse  $\alpha$ -subunit palindrome is also present in the binding sites for Lmx I and Isl-1 in the insulin promoter (17, 26). The similarity in these binding sites offers further evidence for a role for LH-2 in specific binding to the PGBE of the mouse  $\alpha$ -subunit gene.

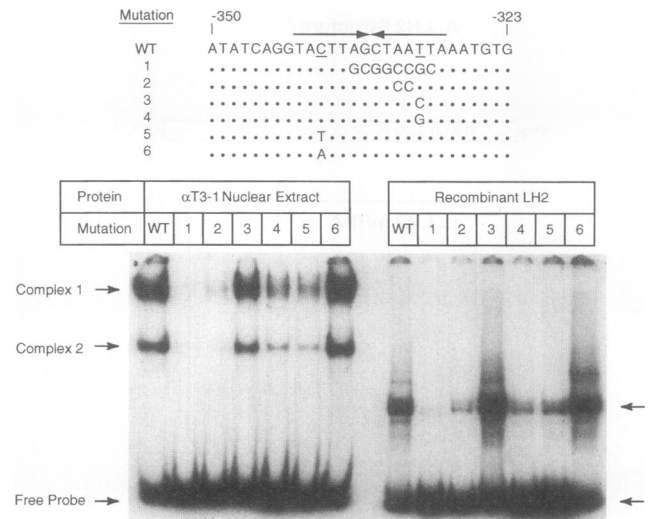
Electrophoretic mobility shift assays were used to examine whether recombinant LH-2<sub>147-427</sub> showed a binding selection similar to that of the factor which is present in  $\alpha$ T3-1 cells (Fig. 6). Wild-type and six mutant PGBE probes were used for these studies. In general, the patterns of binding to the wild-type and mutant probes were similar for the endogenous binding activity from  $\alpha$ T3-1 cells and recombinant LH-2<sub>147-427</sub>. Mutations within the imperfect palindrome of the PGBE tended to have a greater effect on LH-2<sub>147-427</sub> binding than on the endoge-





**FIG. 5.** Recombinant LH-2 homeodomain binds specifically to the mouse  $\alpha$ -subunit PGBE. DNase I protection experiments were used to examine the sequence specificity of LH-2 interaction with the mouse  $\alpha$ -subunit promoter. Recombinant LH-2<sub>147-427</sub> containing the homeodomain was expressed in *Escherichia coli* as a fusion protein containing a polyhistidine amino-terminal extension. The recombinant protein was partially purified by chromatography over nickel chelate agarose. A DNA probe representing the mouse  $\alpha$  -424 to -288 region was radiolabeled at either terminus and incubated with increasing amounts of the partially purified recombinant LH-2<sub>147-427</sub> preparation. The binding reaction was partially digested with DNase I and then analyzed by denaturing polyacrylamide gel electrophoresis and autoradiography. Regions protected by the recombinant LH-2 preparation are indicated by open boxes. DNA fragments were also subjected to the chemical modification and cleavage reactions of Maxam and Gilbert (33) for use as markers. The region of the mouse  $\alpha$ -subunit which is protected by LH-2 was aligned with the binding sites for Isl-1 and Lmx I from the insulin promoter (C).

nous binding activity. This finding suggests that the remainder of the LH-2 molecule, which is not contained in recombinant LH-2<sub>147-427</sub>, may have some effect on binding activity. Alternatively, the interaction of LH-2 with other proteins in  $\alpha$ T3-1 cells may alter binding activity. It is evident that a single major complex is obtained with the recombinant LH-2 homeodomain preparation, while two major complexes were always obtained with endogenous  $\alpha$ T3-1 cell protein. Presumably, the complex obtained with the recombinant preparation has a different mobility than either of the two endogenous complexes because the recombinant protein represents only a portion of LH-2. The presence of only a single major complex with the recombinant LH-2 was detected at several different concentrations of protein. As the binding site contains an imperfect palindrome, it is certainly possible that binding involves homodimers or



**FIG. 6.** Analysis of the effects of PGBE mutations on formation of complexes with endogenous  $\alpha$ T3-1 nuclear extracts and recombinant LH-2 homeodomain. Radiolabeled wild-type (WT) and mutant DNA probes (probes 1 to 6) containing the -424 to -288 region of the mouse  $\alpha$ -subunit gene were used in an electrophoretic mobility shift assay. Mutations were placed in the -350 to -323 region as indicated (A). Opposing arrows above the wild-type sequence indicate the presence of an imperfect palindrome. PGBE-binding activity from  $\alpha$ T3-1 nuclear extracts was prepared by DNA affinity chromatography. Recombinant LH-2<sub>147-427</sub> was expressed in *E. coli* and partially purified by chromatography over nickel chelate agarose. DNA-protein complexes were resolved by nondenaturing polyacrylamide gel electrophoresis and analyzed by autoradiography. Two different protein-DNA complexes obtained with  $\alpha$ T3-1 nuclear extracts and free probe are indicated by the arrows on the left. DNA-protein complexes from recombinant LH-2 and free probe are indicated by the arrows on the right.

heterodimers. All of the mutations which have effects on binding to the PGBE appear to have similar effects on both complexes formed from  $\alpha$ T3-1 proteins (Fig. 3 and 6), suggesting that the two complexes may involve cooperative interactions.

**$\alpha$ T3-1 nuclear extracts contain a PGBE-binding protein which is immunologically related to LH-2.** To determine whether LH-2 is a component of the endogenous,  $\alpha$ T3-1 nuclear proteins which interact with the PGBE, an electrophoretic mobility shift experiment was performed in the presence of antiserum to LH-2 (Fig. 7). Antiserum to LH-2 resulted in the loss of the faster-migrating complex (complex 2) and the appearance of two new complexes (indicated by open triangles). Presumably, the new complexes represent PGBE-LH-2 complexes which are specifically bound to antibodies. The increased intensity of the faster-migrating complex in the presence of antiserum to LH-2 is similar to results observed with MEC-3 (57) and may indicate that antibodies directed toward the carboxy-terminal domain of these LIM factors can affect DNA binding. Antisera to the cAMP response element-binding protein (CREB) or Pit-1 did not appreciably alter the mobility of the complexes. These findings suggest that a protein which is recognized by antiserum to LH-2 contributes to the PGBE-binding activity from  $\alpha$ T3-1 cells.

**LH-2 expression can activate the mouse  $\alpha$  promoter in heterologous cell types.** A cotransfection assay was used to test the ability of LH-2 to activate the  $\alpha$ -subunit promoter. A mammalian expression vector encoding full-length LH-2 was

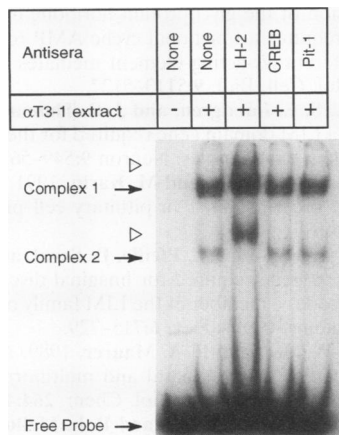


FIG. 7. The PGBE-binding activity from  $\alpha$ T3-1 cells is immunologically related to LH-2. A DNA probe representing positions -424 to -288 of the mouse  $\alpha$ -subunit gene (containing the PGBE) was radiolabeled and used in an electrophoretic mobility shift assay. The radiolabeled DNA probe was incubated with affinity-purified nuclear extracts from  $\alpha$ T3-1 cells in the absence or presence of 5  $\mu$ l of antiserum to LH-2 (1:2,000 dilution), CREB (1:200 dilution), or Pit-1 (1:200 dilution). Complexes were resolved by nondenaturing polyacrylamide gel electrophoresis and analyzed by autoradiography. The migration of major complexes and the free DNA probe are indicated by closed arrows. Open arrows indicate the complexes with altered mobility which were observed in the presence of antiserum to LH-2.

transfected with reporter genes into Cos 1 (kidney origin) cells. Two reporter genes were used as a target for the LH-2 molecule. One reporter contained the mouse  $\alpha$ -subunit promoter (-507 to +46) linked to luciferase, while the other reporter contained three copies of the PGBE upstream of the TK promoter linked to luciferase (3  $\times$  PGBE-luc). In Cos cells, LH-2 activated the  $\alpha$ -subunit promoter approximately twofold and the 3  $\times$  PGBE reporter five- to sixfold compared with transfection with a control vector encoding globin (Fig. 8). The effects were promoter specific, as the LH-2 expression vector did not affect the activity of a TK-luciferase construct. Essentially identical results were obtained in JEG-3 cells (data not shown).

## DISCUSSION

These studies suggest that the LIM-homeodomain protein LH-2, which was previously found to be present in the central nervous system and some lymphoid cells (56), is a strong candidate as an activator of the glycoprotein hormone  $\alpha$ -subunit gene. We identified an LH-2 cDNA clone through screening a bacterial expression library with a tissue-specific enhancer sequence from the mouse  $\alpha$ -subunit gene. LH-2 mRNA is present at relatively high levels in cells which express the glycoprotein hormone  $\alpha$ -subunit gene. Recombinant LH-2 selectively binds to a 14-bp imperfect palindrome in the  $\alpha$ -subunit gene which appears to be the core of a pituitary-specific enhancer. Point mutations in the imperfect palindrome which disrupt binding of LH-2 also decrease expression of the mouse  $\alpha$ -subunit promoter. Mobility shift experiments in the presence of antiserum to LH-2 suggest that LH-2 or a closely related protein is likely a component of the endogenous PGBE-binding activity of  $\alpha$ T3-1 cells. Cotransfection assays in heterologous cells demonstrate that LH-2 is able to activate expression of the mouse  $\alpha$ -subunit promoter.

Like other transcription factors, LIM-homeodomain pro-

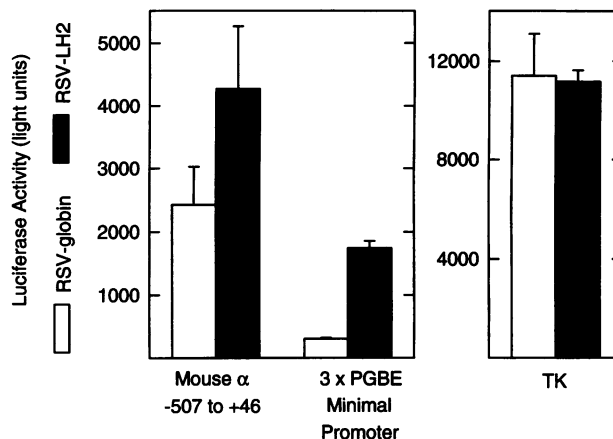


FIG. 8. LH-2 activates the mouse  $\alpha$  promoter or a promoter containing multimers of the PGBE. Cos 1 cells were transfected with an LH-2 expression vector (5  $\mu$ g) and a luciferase reporter gene (10  $\mu$ g) containing either the mouse glycoprotein hormone  $\alpha$ -subunit -507 to +46 region or three copies of the PGBE upstream of the TK promoter (3  $\times$  PGBE). The TK promoter coupled to luciferase was used as a control construct. The unshaded bars indicate values from reporter constructs cotransfected with a control Rous sarcoma virus (RSV)-globin expression plasmid, and shaded bars indicate values from reporter constructs cotransfected with the RSV-LH-2 expression plasmid. Data are reported as mean luciferase activity  $\pm$  standard error of the mean for nine transfections for each construct from three separate experiments.

teins have been found to synergistically interact with other factors. These interactions may involve either the LIM domain or the homeodomain. For instance, there is evidence for cooperative DNA binding by the LIM-homeodomain factor, MEC-3, and a POU factor, UNC-86. The synergistic DNA binding is dependent on the homeodomain of MEC-3 (57). It has also been shown that the putative zinc fingers of LIM domains may mediate protein-protein interaction. A synergy between the LIM-homeodomain protein Lmx I and the basic helix-loop-helix protein shPan-I is required for the transactivation of an insulin promoter in fibroblast cells (17). Other studies indicate that for cytoskeletal proteins which contain LIM domains, the LIM domain may serve as a protein-protein interaction surface which facilitates heterodimer formation (39). Of course, LIM-homeodomain proteins may also make important protein-protein contacts which do not involve the LIM domain or the homeodomain.

In view of the functional interactions of LIM-homeodomain factors with other transcription factors, it is interesting that the PGBE and a different, unrelated DNA element of the mouse  $\alpha$ -subunit gene are required to permit a response to the peptide hormone, gonadotropin-releasing hormone (GnRH) (44). This finding raises the possibility that interactions involving LH-2 and other transcription factors are required for responses to GnRH. A parallel situation has been observed for Isl-1 and the regulation of the somatostatin gene. Functional interaction between the LIM-homeodomain factor, Isl-1, and CREB has recently been observed for the somatostatin gene (29). A requirement for the functional interaction of ubiquitous factors which respond to signal transduction pathways with tissue-restricted factors such as LH-2 or Isl-1 may play a role in limiting hormonal regulation of specific genes to the appropriate cell types. It will be particularly interesting to test for physical interactions between LH-2 and other factors which are important for regulating expression of the  $\alpha$ -subunit gene

such as the factor required for GnRH responsiveness (44) and the factor which binds to the gonadotrope-specific element (22). A lack of specific protein-protein interactions may also explain the finding that LH-2 provides only a modest (twofold) transcriptional activation of the  $\alpha$ -subunit promoter in heterologous cells. This may indicate that heterologous cells lack appropriate protein targets which contribute to the ability of LH-2 to activate the  $\alpha$ -subunit promoter in gonadotropes and thyrotropes.

The identification of a LIM-homeodomain protein as an activator of the  $\alpha$ -subunit gene has intriguing implications for pituitary development. A number of LIM-homeodomain factors, including MEC-3 and LIN-11 from *Caenorhabditis elegans* (16, 54), *apterous* from *Drosophila melanogaster* (5, 7), and the *Xenopus* factor Xlim-1 (50), appear to have a role in the regulation of embryogenesis and the determination of cell fate. Expression of the glycoprotein hormone  $\alpha$ -subunit gene is the earliest known marker of pituitary differentiation. The commitment of somatic ectoderm into primordial pituitary cells is marked by the expression of the  $\alpha$ -subunit transcript prior to the formation of a definitive Rathke's pouch, the tissue which ultimately forms the pituitary (47). In view of the role of LH-2 in stimulating  $\alpha$ -subunit transcription and the early appearance of  $\alpha$ -subunit gene expression in pituitary development, a possible role of LH-2 in differentiation of the pituitary seems reasonable. Previous studies with other pituitary cell types have clearly established a role for homeodomain proteins in regulating both differentiation and specific target gene expression in the pituitary. The POU-homeodomain protein Pit-1 has been shown to activate hormone genes which are markers for the differentiation of particular pituitary cell lineages (24, 32). Pit-1 has been shown to be essential for the growth and differentiation of this cell lineage (6, 31). Whatever role LH-2 may play in regulating development of the pituitary, it certainly must be involved in other activities, as it is expressed at relatively high levels in the central nervous system (56). In this regard, the tissue distribution of LH-2 is rather similar to that of another LIM-homeodomain protein, Isl-1. Isl-1 was originally cloned as a factor which binds to the insulin enhancer (26). Subsequently it has been shown that Isl-1 is expressed in subsets of neurons and endocrine cells, leading to the suggestion that Isl-1 may be involved in the specification or maintenance of particular endocrine and neural cell lineages (13, 52). The present findings are consistent with the possibility of a similar role for LH-2.

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#### REFERENCES

- Abate, C., D. Luk, R. Gentz, F. J. Rauscher III, and T. Curran. 1990. Expression and purification of the leucine zipper and DNA-binding domains of fos and jun: both fos and jun contact DNA directly. *Proc. Natl. Acad. Sci. USA* **87**:1031-1036.
- Akerblom, I. E., E. C. Ridgway, and P. L. Mellon. 1990. An  $\alpha$ -subunit-secreting cell line derived from a mouse thyrotrope tumor. *Mol. Endocrinol.* **4**:589-596.
- Andersen, B., G. C. Kennedy, and J. H. Nilson. 1990. A cis-acting element located between the cAMP response elements and CCAAT box augments cell-specific expression of the glycoprotein hormone  $\alpha$  subunit gene. *J. Biol. Chem.* **265**:21874-21880.
- Bokar, J. A., R. A. Keri, T. A. Farmerie, R. A. Fenstermaker, B. Anderson, B. 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.
- Bourgouin, C., S. E. Lundgren, and J. B. Thomas. 1992. *apterous* is a Drosophila LIM domain gene required for the development of a subset of embryonic muscles. *Neuron* **9**:549-561.
- Castrillo, J.-L., L. E. Theill, and M. Karin. 1991. Function of the homeodomain protein GHF1 in pituitary cell proliferation. *Science* **253**:197-199.
- Cohen, B., M. E. McGuffin, C. Pfeifle, D. Segal, and S. M. Cohen. 1992. *apterous*, a gene required for imaginal disc development in *Drosophila* encodes a member of the LIM family of developmental regulatory proteins. *Genes Dev.* **6**:715-729.
- Day, R. N., J. Walder, and R. A. Maurer. 1989. A protein kinase inhibitor gene reduces both basal and multihormone-stimulated prolactin gene transcription. *J. Biol. Chem.* **264**:431-436.
- 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.
- d'Emden, M. C., Y. Okimura, and R. A. Maurer. 1992. Analysis of functional cooperativity between individual transcription-stimulating elements in the proximal region of the rat prolactin gene. *Mol. Endocrinol.* **6**:581-588.
- Deutsch, P. J., J. L. Jameson, and J. F. Habener. 1987. Cyclic AMP responsiveness of human gonadotropin- $\alpha$  gene transcription is directed by a repeated 18-base pair enhancer.  $\alpha$ -promoter receptivity to the enhancer confers cell-preferential expression. *J. Biol. Chem.* **262**:12169-12174.
- de Wet, J. R., K. V. Wood, M. DeLuca, D. R. Helinski, and S. Subramani. 1987. Firefly luciferase gene: structure and expression in mammalian cells. *Mol. Cell. Biol.* **7**:725-737.
- Dong, J., S. L. Asa, and D. J. Drucker. 1991. Islet cell and extrapancreatic expression of the LIM domain homeobox gene *isl-1*. *Mol. Endocrinol.* **5**:1633-1641.
- Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**:6-13.
- 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.
- Freyd, G., S. K. Kim, and H. R. Horvitz. 1990. Novel cysteine-rich motif and homeodomain in the product of the *Caenorhabditis elegans* cell lineage gene *lin-11*. *Nature (London)* **344**:876-879.
- German, M. S., J. Wang, R. B. Chadwick, and W. J. Rutter. 1992. Synergistic activation of the insulin gene by a LIM-homeo domain protein and a basic helix-loop-helix protein: building a functional insulin minienhancer complex. *Genes Dev.* **6**:2165-2176.
- Glisin, V., R. Crkvenjakov, and C. Byus. 1974. Ribonucleic acid isolated by cesium chloride centrifugation. *Biochemistry* **13**:2633-2637.
- Hagenbuechle, O., and P. K. Wellauer. 1992. A rapid method for the isolation of DNA-binding proteins from purified nuclei of tissues and cells in culture. *Nucleic Acids Res.* **20**:3555-3559.
- Hamernik, D. L., R. A. Keri, C. M. Clay, J. N. Clay, G. B. Sherman, H. R. Sawyer, Jr., T. M. Nett, and J. H. Nilson. 1992. Gonadotrope- and thyrotrope-specific expression of the human and bovine glycoprotein hormone  $\alpha$ -subunit genes is regulated by distinct cis-acting elements. *Mol. Endocrinol.* **6**:1745-1755.
- Harline, M. C., J. C. Kandala, R. D. Sage, R. V. Guntaka, and A. DeAngelo. 1992. Improved method for screening cDNA expression libraries for DNA-binding proteins. *BioTechniques* **13**:388-390.
- Horn, F., J. J. Windle, K. M. Barnhart, and P. L. Mellon. 1992. Tissue-specific gene expression in the pituitary: the glycoprotein hormone  $\alpha$ -subunit gene is regulated by a gonadotrope-specific protein. *Mol. Cell. Biol.* **12**:2143-2153.
- Hutchison, C. A., III, S. Phillips, M. H. Edgell, S. Gillam, P. Jahnke, and M. Smith. 1978. Mutagenesis at a specific position in a DNA sequence. *J. Biol. Chem.* **253**:6551-6560.
- Ingraham, H. A., R. Chen, H. J. Mangalam, H. P. Elsholtz, S. E.



- Flynn, C. R. Lin, D. M. Simmons, L. Swanson, and M. G. Rosenfeld. 1988. A tissue-specific transcription factor containing a homeodomain specifies a pituitary phenotype. *Cell* 55:519–529.
25. Jameson, J. L., A. C. Powers, G. D. Gallagher, and J. F. Habener. 1989. Enhancer and promoter element interactions dictate cyclic adenosine monophosphate mediated and cell-specific expression of the glycoprotein hormone  $\alpha$ -gene. *Mol. Endocrinol.* 3:763–772.
  26. Karlsson, O., S. Thor, T. Norberg, H. Ohlsson, and T. Edlund. 1990. Insulin gene enhancer binding protein Isl-1 is a member of a novel class of proteins containing both a homeo- and a cys-his domain. *Nature (London)* 344:879–882.
  27. Kendall, S. K., T. L. Saunders, L. Jin, R. V. Lloyd, L. M. Glode, T. M. Nett, R. A. Keri, J. H. Nilson, and S. A. Camper. 1991. Targeted ablation of pituitary gonadotropes in transgenic mice. *Mol. Endocrinol.* 5:2025–2036.
  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. Leonard, J., P. Serup, G. Gonzalez, T. Edlund, and M. Montminy. 1992. The LIM family transcription factor Isl-1 requires cAMP response element binding protein to promote somatostatin expression in pancreatic islet cells. *Proc. Natl. Acad. Sci. USA* 89:6247–6251.
  30. Li, P. M., J. Reichert, G. Freyd, H. R. Horvitz, and C. Walsh. 1991. The LIM region of a presumptive *Caenorhabditis elegans* transcription factor is an iron-sulfur- and zinc-containing metal domain. *Proc. Natl. Acad. Sci. USA* 88:9210–9213.
  31. Li, S., E. B. Crenshaw III, E. J. Rawson, D. M. Simmons, L. W. Swanson, and M. G. 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.
  32. 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.
  33. Maxam, A. M., and W. Gilbert. 1977. A new method for sequencing DNA. *Proc. Natl. Acad. Sci. USA* 74:560–564.
  34. Mellon, P. L., C. H. Clegg, L. A. Correll, and G. S. McKnight. 1989. Regulation of transcription by cyclic AMP-dependent protein kinase. *Proc. Natl. Acad. Sci. USA* 86:4887–4891.
  35. Michelsen, J. W., K. L. Schmeichel, M. C. Beckerle, and D. R. Winge. 1993. The LIM motif defines a specific zinc-binding protein domain. *Proc. Natl. Acad. Sci. USA* 90:4404–4408.
  36. Ocran, K. W., V. D. Sarapura, W. M. Wood, D. F. Gordon, A. Gutierrez-Hartmann, and E. C. Ridgway. 1990. Identification of cis-acting promoter elements important for expression of the mouse glycoprotein hormone  $\alpha$ -subunit gene in thyrotropes. *Mol. Endocrinol.* 4:766–772.
  37. Pierce, J. G., and T. F. Parsons. 1981. Glycoprotein hormones: structure and function. *Annu. Rev. Biochem.* 50:465–495.
  38. Rabbitts, T. H., and T. Boehm. 1990. LIM domains. *Nature (London)* 346:418.
  39. Sadler, I., A. W. Crawford, J. W. Michelsen, and M. C. Beckerle. 1992. Zyxin and cCRP: two interactive LIM domain proteins associated with the cytoskeleton. *J. Cell Biol.* 119:1573–1587.
  40. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
  41. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74:5463–5467.
  42. Sarapura, V. D., W. M. Wood, D. F. Gordon, K. W. Ocran, M. Y. Kao, and E. C. Ridgway. 1990. Thyrotrope expression and thyroid hormone inhibition map to different regions of the mouse glycoprotein hormone  $\alpha$ -subunit gene promoter. *Endocrinology* 127:1352–1361.
  43. Schoderbek, W. E., K. E. Kim, E. C. Ridgway, P. L. Mellon, and R. A. Maurer. 1992. Analysis of DNA sequences required for pituitary-specific expression of the glycoprotein hormone  $\alpha$ -subunit gene. *Mol. Endocrinol.* 6:893–903.
  44. Schoderbek, W. E., M. S. Roberson, and R. A. Maurer. 1993. Two different DNA elements mediate gonadotropin releasing hormone effects on expression of the glycoprotein hormone  $\alpha$ -subunit gene. *J. Biol. Chem.* 268:3903–3910.
  45. Showers, M. O., and R. A. Maurer. 1986. A cloned bovine cDNA encodes an alternate form of the catalytic subunit of the cAMP-dependent protein kinase. *J. Biol. Chem.* 261:16288–16291.
  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. Smith, D. B., and K. S. Johnson. 1988. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene* 67:31–40.
  49. Studier, F. W., and B. A. Moffatt. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* 189:113–130.
  50. Taira, M., M. Jamrich, P. J. Good, and I. B. Dawid. 1992. The LIM domain-containing homeo box gene *Xlim-1* is expressed specifically in the organizer region of *Xenopus* gastrula embryos. *Genes Dev.* 6:356–366.
  51. Thomas, P. S. 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. USA* 77:5201–5205.
  52. Thor, S., J. Ericson, T. Brännström, and T. Edlund. 1991. The homeodomain LIM protein Isl-1 is expressed in subsets of neurons and endocrine cells in the adult rat. *Neuron* 7:881–889.
  53. Vinson, C. R., K. L. LaMarco, P. F. Johnson, W. H. Landschulz, and S. L. McKnight. 1988. In situ detection of sequence-specific DNA binding activity specified by a recombinant bacteriophage. *Genes Dev.* 2:801–806.
  54. Way, J. C., and M. Chalfie. 1988. *mec-3*, a homeobox-containing gene that specifies differentiation of the touch receptor neurons in *C. elegans*. *Cell* 54:5–16.
  55. Windle, J. J., R. I. Weiner, and P. L. Mellon. 1990. Cell lines of the pituitary gonadotrope lineage derived by targeted oncogenesis in transgenic mice. *Mol. Endocrinol.* 4:597–603.
  56. Xu, Y., M. Baldassare, P. Fisher, G. Rathbun, E. M. Oltz, G. D. Yancopoulos, T. M. Jessell, and F. W. Alt. 1993. LH-2: a LIM/homodomain gene expressed in developing lymphocytes and neural cells. *Proc. Natl. Acad. Sci. USA* 90:227–231.
  57. Xue, D., Y. Tu, and M. Chalfie. 1993. Cooperative interactions between the *Caenorhabditis elegans* homeoproteins UNC-86 and MEC-3. *Science* 261:1324–1328.