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Molecular biology of gonadotrophins

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Regulation of gonadotrophin synthesis involves a complex interaction between hypothalamic and gonadal hormones. Chronic administration of oestrogens and androgens to gonadectomized animals blocked the postcastration rise in amounts of mRNA encoding gonadotrophin subunits. Removal of endogenous GnRH decreased amounts of mRNA encoding gonadotrophin subunits. Pulsatile administration of GnRH to GnRH-deficient animals increased amounts of mRNA encoding gonadotrophin subunits. Studies using transgenic mice and transient transfection assays identified at least eight *cis*-acting DNA sequences in the proximal 350 bp of 5′ flanking sequence of the human α subunit gene that directed expression to gonadotrophs or conferred responsiveness to oestrogens, androgens or GnRH. Unique DNA-binding proteins were also identified which directed expression of the human α subunit gene specifically to the pituitary. Pituitary cell lines that express bovine gonadotrophin subunit genes are not currently available; thus, relatively little is known about the molecular mechanisms that regulate expression of bovine gonadotrophin subunit genes. Recent studies with transgenic mice harbouring bovine α, LHβ, or FSHβ subunit transgenes revealed that DNA sequences important for gonadotroph-specific expression and hormonal regulation resided within the proximal 5′ flanking sequences.

Introduction

Reproduction in mammals depends on synthesis and secretion of gonadotrophins (LH and FSH) from the anterior pituitary gland. LH and FSH stimulate steroidogenesis and gametogenesis in males and females, and are members of the glycoprotein hormone family that also includes the pituitary hormone TSH. Syncytiotrophoblasts of primates and horses produce a fourth glycoprotein hormone, chorionic gonadotrophin (CG). Glycoprotein hormones consist of a common α subunit and a hormone-specific β subunit that confers biological specificity (Pierce and Parsons, 1981). Whereas pituitary gonadotrophin subunits are encoded by single-copy genes located on different chromosomes, there are six CGβ subunit genes arranged in a cluster that spans approximately 50 kb in humans (Jameson and Hollenberg, 1993). The α subunit gene is unique because it is expressed in the pituitary gland of all mammals and in the placenta of primates and horses. Recently, cDNA and genomic clones for gonadotrophin subunits have been characterized from a number of species including cattle (reviewed by Gharib *et al.*, 1990).

Synthesis of gonadotrophins in the pituitary is thought to occur in a manner similar to that described for other glycoproteins (Chappel *et al.*, 1983). In general, gonadotrophin genes are transcribed in the nucleus to yield specific mRNA molecules that are processed (i.e. addition of the 5′ CAP structure, splicing of intervening nucleotide sequences and addition of the 3′ polyA tail) and transported to the cytoplasm where translation of mRNA to protein occurs on ribosomes. Further processing occurs on ribosomes and in the endoplasmic reticulum and involves removal of the signal peptide and

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glycosylation. Additional modifications of carbohydrate moieties occur in the Golgi apparatus. Newly synthesized proteins are stored in secretory granules (Chappel *et al.*, 1983). Although each of these steps is a potential site of quantitative or qualitative regulation, this review will focus on pretranslational regulation of gonadotrophin synthesis in ruminants. Additional details of regulated expression of gonadotrophin genes in rodents and humans will be presented when such information in ruminants is lacking.

Regulation of Amounts of mRNA Encoding Gonadotrophin Subunits

Although much was known about regulation of gonadotrophin secretion, only in the last ten years have significant strides been made in understanding regulation of gonadotrophin synthesis. In most physiological situations, amounts of mRNA encoding gonadotrophin subunits are coordinately regulated. In general, expression of gonadotrophin subunit genes is enhanced by GnRH and inhibited by chronic administration of gonadal steroids or inhibin (reviewed by Gharib *et al.*, 1990; Mercer, 1990; Nett, 1990).

Amounts of mRNA encoding gonadotrophins change during the ovine oestrous cycle and during pregnancy and the early postpartum period in sheep (Nett, 1990). However, not all periods of anoestrus are characterized by insufficient amounts of mRNA encoding gonadotrophin subunits. Steady-state amounts of mRNA encoding α and LHβ subunits are not limiting during sexual maturation in beef heifers (Roberson *et al.*, 1992). Changes in amounts of mRNA encoding gonadotrophin during the ovine oestrous cycle and pregnancy may result from changes in serum concentrations of gonadal steroids or changes in pulsatile secretion of GnRH.

Regulation of mRNA encoding gonadotrophin subunits by ovarian steroids and GnRH

After ovariectomy there is a gradual increase in amounts of mRNA encoding gonadotrophin subunits in the pituitary (Gharib *et al.*, 1990). Chronic administration of oestradiol to ovariectomized ewes decreased amounts of mRNA encoding gonadotrophin subunits, while chronic administration of midluteal phase concentrations of progesterone did not affect amounts of mRNA encoding gonadotrophin subunits (Gharib *et al.*, 1990; Mercer, 1990; Nett, 1990). Studies with at least three different experimental models revealed that oestradiol and progesterone acted directly at the anterior pituitary gland to regulate amounts of mRNA encoding gonadotrophin subunits. In primary cultures of sheep pituitaries, oestradiol or progesterone inhibited steady-state amounts of mRNA encoding α and FSHβ subunits (Phillips *et al.*, 1988). Treatment of ovariectomized and hypothalamic–pituitary disconnected ewes with pulsatile administration of GnRH and chronic administration of oestradiol demonstrated that oestradiol acted directly at the anterior pituitary to regulate amounts of mRNA encoding FSHβ subunit negatively and positively regulate amounts of mRNA encoding α subunit without affecting amounts of mRNA encoding LHβ subunit (Mercer *et al.*, 1988, 1989). In contrast, Nett *et al.* (1990) administered oestradiol and progesterone to ovariectomized ewes for 69 days and then gave pulsatile GnRH for an additional 42 days. Pulses of exogenous GnRH did not increase amounts of mRNA encoding α or LHβ subunits. Thus, oestradiol and progesterone inhibited amounts of mRNA encoding α and LHβ subunits by acting directly at the anterior pituitary gland.

Because oestradiol and progesterone acted at the hypothalamus to decrease secretion of GnRH in sheep (Karsch *et al.*, 1987), it is possible that ovarian steroids regulated amounts of mRNA encoding gonadotrophin subunits indirectly by inhibiting secretion of GnRH which was needed to stimulate gonadotrophin synthesis. Relative amounts of mRNA encoding gonadotrophin subunits decreased after removal of endogenous GnRH by hypothalamic–pituitary disconnection in ovariectomized ewes (Hamernik *et al.*, 1986; Mercer *et al.*, 1988). Administration of a GnRH antagonist, which occupied GnRH receptors and prevented endogenous GnRH from stimulating gonadotrophs, decreased amounts of mRNA encoding gonadotrophin subunits in ovariectomized ewes (Sanchez *et al.*, 1994). Immunoneutralization of endogenous GnRH by active immunization of ovariectomized beef cows to GnRH also resulted in decreased amounts of mRNA encoding α and LHβ subunits (Stumpf *et al.*, 1992). Hence,

ovarian steroids regulated amounts of mRNA encoding gonadotrophin subunits both by acting directly at the anterior pituitary gland and by acting indirectly at the hypothalamus to decrease secretion of GnRH, which is required to stimulate amounts of mRNA encoding gonadotrophin subunits.

Regulation of mRNA encoding gonadotrophin subunits by androgens

In contrast to inhibitory effects of oestradiol and progesterone on mRNA encoding gonadotrophin subunits, androgens negatively regulated only mRNA encoding α and LHβ subunits and had little or no effect on mRNA encoding FSHβ subunit in male rodents (Gharib *et al.*, 1990). In another study, androgen stimulated mRNA encoding FSHβ subunit but decreased mRNA encoding α and LHβ subunits in castrate male rats treated with a GnRH antagonist (Wierman and Wang, 1990). Thus, androgen could potentially act directly at the anterior pituitary gland to positively regulate amounts of mRNA encoding FSHβ subunit. Although regulation of gonadotrophin gene expression in male ruminants has not been extensively characterized, it seems likely that androgens may act directly at the anterior pituitary gland, as well as indirectly at the hypothalamus to control synthesis of gonadotrophins.

Regulation of mRNA encoding gonadotrophin subunits by gonadal polypeptides

Production of polypeptides (inhibin, activin, follistatin) by developing ovarian follicles during late dioestrus and the preovulatory period provides a mechanism for differential control of LH and FSH synthesis and secretion. Administration of charcoal-stripped follicular fluid (containing inhibin) to ovariectomized heifers (Beard *et al.*, 1989) and ewes (Mercer *et al.*, 1987; Nett, 1990) specifically decreased mRNA encoding FSHβ subunit, but not mRNA encoding α or LHβ subunits. Likewise, inhibin decreased amounts of mRNA encoding FSHβ subunit, but not mRNA encoding α or LHβ subunits in ovariectomized ewes that received pulsatile GnRH after hypothalamic–pituitary disconnection (Mercer *et al.*, 1987). Purified porcine inhibin also decreased amounts of mRNA encoding FSHβ subunit in primary cultures of rat pituitaries (Gharib *et al.*, 1990). Thus, inhibin acted directly at the anterior pituitary gland to suppress amounts of mRNA encoding FSHβ subunit specifically.

In primary cultures of rat pituitaries, activin stimulated amounts of mRNA encoding FSHβ subunit but did not affect amounts of mRNA encoding α or LHβ subunits (Gharib *et al.*, 1990). Follistatin was identified in ovarian follicular fluid and may regulate synthesis of FSH by binding activin to prevent activin from stimulating production of FSH (Findlay, 1993). Administration of purified porcine follistatin to primary cultures of rat pituitaries decreased amounts of mRNA encoding FSHβ subunit but did not affect amounts of mRNA encoding α or LHβ subunits (Gharib *et al.*, 1990). In addition to ovarian follicles, rodent and primate gonadotrophs were also a source of activin and follistatin (Roberts *et al.*, 1989; Attardi *et al.*, 1992; Bilezikjian *et al.*, 1993; DePaolo *et al.*, 1993). Thus, activin and follistatin may regulate amounts of mRNA encoding FSHβ subunit by paracrine actions.

Transcriptional and post-transcriptional regulation of gonadotrophin synthesis

The aforementioned studies described measurements of steady-state amounts of mRNA encoding gonadotrophins in various physiological states. The limitation to these studies was that transcriptional and post-transcriptional regulation of gonadotrophin synthesis was not investigated directly. Transcriptional regulation of gonadotrophin subunit genes was studied further by isolating nuclei from pituitaries and conducting nuclear run-off assays to measure rates of transcription directly. Estimates of gonadotrophin mRNA stability were determined by measuring RNA turnover in the presence of drugs that block synthesis of RNA.

Transcriptional and post-transcriptional regulation of gonadotrophins by gonadal steroids. Chronic administration of oestradiol to ovariectomized rats inhibited transcription of gonadotrophin genes *in vivo* (Gharib *et al.*, 1990). Administration of oestradiol or progesterone to ovine pituitary cells *in vitro* inhibited transcription of α and FSHβ subunit genes (Phillips *et al.*, 1988). Transcription of α and

LHβ subunit genes was reduced by administration of testosterone to castrate male rats (Paul *et al.*, 1990). However, stimulatory effects of testosterone on amounts of mRNA encoding FSHβ subunit in castrate male rats did not involve increased transcription of the gene encoding FSHβ subunit and probably resulted from increased stability of mRNA encoding FSHβ subunit (Paul *et al.*, 1990). Thus, gonadal steroids acted directly at the anterior pituitary gland to regulate transcription of gonadotrophin subunit genes and stability of mRNA encoding gonadotrophin.

Transcriptional and post-transcriptional regulation of gonadotrophins by GnRH. Pulsatile administration of GnRH stimulated transcription of genes encoding α, LHβ and FSHβ subunits in rat pituitaries *in vitro* (Gharib *et al.*, 1990). Administration of a GnRH antagonist to castrate male rats inhibited rates of transcription of α, LHβ and FSHβ subunit genes (Paul *et al.*, 1990). A mouse gonadotroph cell line (αT3-1 cells) that expresses the endogenous α subunit gene and GnRH receptors was developed by Windle *et al.* (1990). The αT3-1 cells do not express LHβ, FSHβ or TSHβ subunit genes or TRH receptors (Windle *et al.*, 1990). Rates of transcription and stability of mRNA encoding α subunit was stimulated by GnRH in αT3-1 cells (Chedrese *et al.*, 1994). Effects of GnRH on stability of mRNA encoding gonadotrophin subunits have not yet been investigated *in vivo*. Thus, GnRH stimulated gonadotrophin synthesis *in vivo* by increased rates of transcription of gonadotrophin subunit genes and increased stability of mRNA encoding gonadotrophin subunits.

Transcriptional and post-transcriptional regulation of gonadotrophins by gonadal polypeptides. Inhibin decreased the rate of transcription of the FSHβ subunit gene, in sheep; however, post-transcriptional mechanisms may also be required to account fully for the suppressive effects of inhibin on amounts of mRNA encoding FSHβ subunit (Clarke *et al.*, 1993). Inhibin or follistatin reduced steady-state amounts of mRNA encoding FSHβ subunit in primary cultures of rat pituitaries by increased degradation of mRNA encoding FSHβ subunit (Attardi and Winters, 1993). Thus, effects of gonadal peptides on mRNA encoding FSHβ subunits appear to be regulated primarily by post-transcriptional processes. In summary, GnRH and gonadal hormones regulate amounts of mRNA encoding gonadotrophin subunits by altering rates of transcription of gonadotrophin subunit genes and by influencing stability of mRNA encoding gonadotrophin subunits.

Characterization of *Cis*-acting DNA Sequences and *Trans*-acting DNA Binding Proteins that Regulate Transcription of Genes Encoding Gonadotrophins

Identification of *cis*-acting regulatory DNA sequences, *trans*-acting nuclear transcription factors and the mechanisms regulating expression of genes in specific cells or in response to extracellular signals has been the focus of work in several laboratories in recent years. The general scheme for these studies involves (i) isolation of putative regulatory regions, (ii) modification of native DNA sequences (i.e. mutations or deletions), (iii) construction of chimaeric genes by linking the putative regulatory regions to reporter genes, (iv) transfer of chimaeric genes into cells in culture (i.e. transient transfection assays) or into the mouse genome (i.e. transgenic mice), and (v) assay of reporter gene activity as a measure of promoter function.

Transient transfection of mouse or human α subunit promoters in αT3-1 cells or production of transgenic mice harbouring rat, bovine or human gonadotrophin α and β subunit genes were used to identify promoter-regulatory sequences (necessary for the correct start site of transcription) and enhancer sequences (important for quantitative changes in transcription). Our current understanding of the *cis*-acting DNA sequences important for gonadotroph-specific expression and hormonal regulation of genes encoding gonadotrophin subunits are shown in Fig. 1. Much of what is known about the DNA regulatory sequences and DNA binding proteins important for transcription of the α subunit gene was obtained by transient transfection of the human α subunit 5′ flanking region linked to the bacterial gene encoding chloramphenicol acetyltransferase (CAT) into αT3-1 cells or human choriocarcinoma cells (cell lines of human placental origin that express endogenous α and CGβ subunit genes).

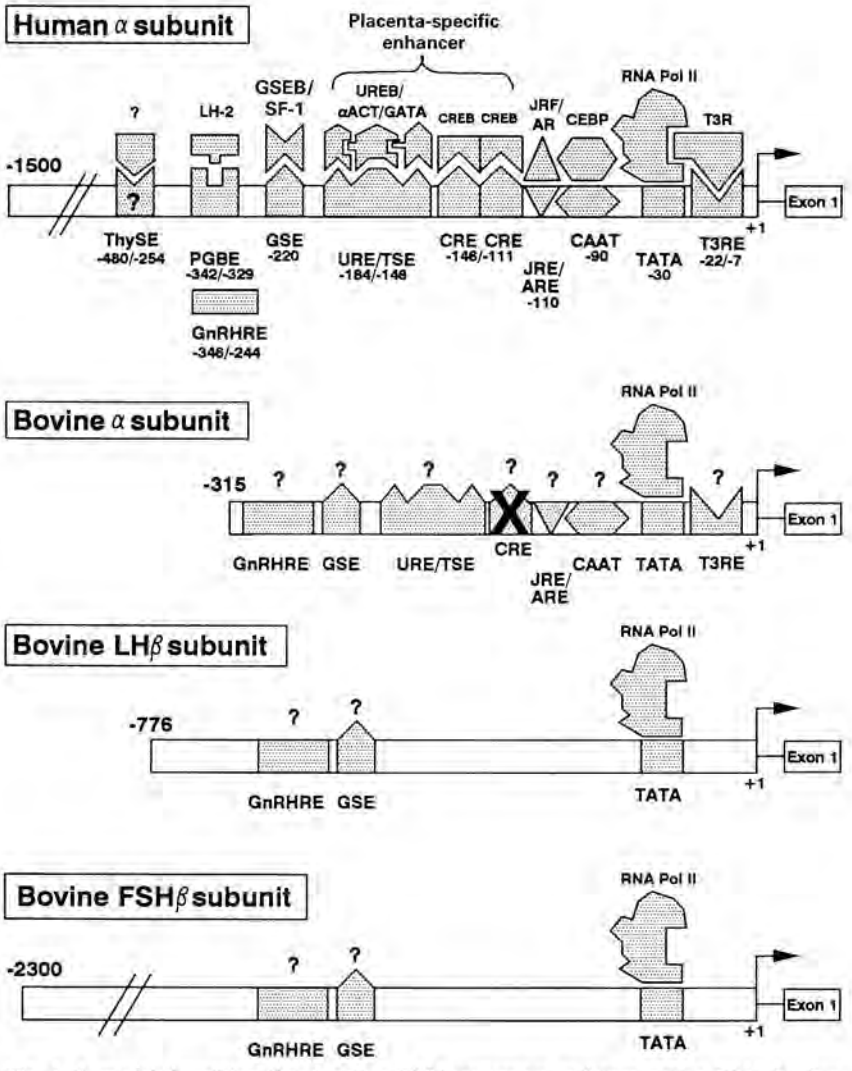

Fig. 1. A model describing the *cis*-acting DNA sequences and *trans*-acting DNA-binding proteins that regulate expression of gonadotrophin subunit genes. Expression of the human α subunit gene requires the following: (i) TATA sequence and the RNA polymerase II complex of transcription factors (RNA Pol II), (ii) CAAT sequence and CAAT enhancer binding protein (CEBP), (iii) the junctional regulatory element (JRE) and JRE binding factor (JRF) in placenta or androgen response element (ARE) and androgen receptor (AR) in pituitary, (iv) tandem cAMP response elements (CRE) and CRE binding protein (CREB), (v) the upstream regulatory element (URE) or trophoblast-specific element (TSE) and URE binding proteins (UREB, αACT or GATA binding proteins), (vi) a gonadotroph-specific element (GSE) and GSE-binding protein (GSEB or steroidogenic factor 1, SF-1), (vii) a pituitary glycoprotein hormone basal element (PGBE) and LIM-homeodomain transcription factor 2 (LH-2), and (viii) a GnRH responsive element (GnRHRE). The thyroid hormone response element (T3RE) binds thyroid hormone–thyroid hormone receptor complex (T3R) and represses transcription by preventing binding of RNA polymerase II to the human α subunit gene *in vitro*. A thyrotroph-specific element (ThySE) was defined *in vitro* but was not functional in transgenic mice. The bovine α subunit gene lacks a functional CRE and shares nucleotide sequence similarity with several regulatory sequences in the human α subunit gene; however, it is not yet known whether these sequences are functional in the bovine α subunit gene. Functional *cis*-acting regulatory sequences have not yet been reported in the bovine gonadotrophin β subunit genes. The arrow indicates the transcription initiation site (+ 1).

The minimal DNA sequences needed for expression of the human α subunit gene in placenta are located within the proximal 169 bp of 5′ flanking sequence and consist of (i) the TATA sequence that binds the RNA polymerase II complex of transcription factors to initiate transcription correctly (ii) a CAAT sequence that binds a placenta-specific member of the ubiquitous CCAAT enhancer binding protein (CEBP) family (Kennedy *et al.*, 1990), (iii) a junctional regulatory element (JRE) which binds a specific protein in placenta (junctional regulatory factor, JRF; Andersen *et al.*, 1990) or the androgen receptor in the pituitary (Clay *et al.*, 1993), (iv) two copies of a consensus cAMP response element (CRE) which bind the ubiquitous CRE binding protein (CREB) and confers responsiveness to activators of protein kinase A and C pathways (reviewed by Jameson and Hollenberg, 1993) and (v) an upstream regulatory element (URE) or trophoblast-specific element (TSE) which binds placenta-specific proteins (Jameson and Hollenberg, 1993; Steger *et al.*, 1993; Pittman *et al.*, 1994; Steger *et al.*, 1994). Sequences important for gonadotroph-specific expression (GSE) are discussed in detail below. A thyroid hormone responsive element (T3RE) is located near the transcription initiation site and, when bound by thyroid hormone–thyroid hormone receptor complex, represses transcription by preventing binding of RNA polymerase II (Chatterjee *et al.*, 1989). A DNA sequence important for expression of the human or mouse α subunit promoters in thyrotrophs (ThySE) was reported from studies *in vitro* (Ocran *et al.*, 1990; Sarapura *et al.*, 1990) but was not functional in transgenic mice (Hamernik *et al.*, 1992). The discrepancy between transfection analyses and studies with transgenic mice are intriguing and may result from more rigid requirements for correct cell-specific expression of the transgene during development in transgenic mice.

Pituitary cell lines that express bovine or ovine gonadotrophin subunit genes are not yet available; thus, much less is known about *cis*-acting DNA sequences and *trans*-acting DNA binding proteins that regulate expression of bovine or ovine gonadotrophin subunit genes. Human and bovine α subunit genes share approximately 85% identity in nucleotide sequence within the proximal 315 bp of 5′ flanking sequence (Bokar *et al.*, 1989). Thus, it seems likely that the TATA, T3RE, CAAT, JRE, URE and GSE sequences are functional in the bovine α subunit promoter. In summary, the human α subunit gene contains DNA sequences that are (i) common to many other eukaryotic genes (i.e. TATA and CAAT), (ii) unique to genes expressed in gonadotrophs (i.e. GSE), and (iii) important for hormonal regulation (i.e. CRE, T3RE and JRE).

Gonadotroph-specific expression

Gonadotrophin β subunit genes are expressed solely in gonadotrophs, while the common α subunit gene is expressed in gonadotrophs and thyrotrophs in the anterior pituitary gland of all mammals. Studies with transient transfection of the human or mouse α subunit promoters into αT3-1 cells have recently identified enhancer sequences and DNA-binding proteins that are important for expression of α subunit in gonadotrophs. DNA sequences approximately 340 bp upstream of the transcriptional start site in the mouse α subunit promoter are important for expression of the mouse α subunit promoter in gonadotrophs and thyrotrophs (Schoderbek *et al.*, 1992). This DNA sequence is important for basal expression of the mouse α subunit gene in the pituitary and was designated the pituitary glycoprotein hormone basal element (PGBE; Roberson *et al.*, 1994). A transcription factor of the LIM*-homeodomain family, LH-2, bound specifically to the PGBE and stimulated expression of the mouse α subunit gene in gonadotrophs and thyrotrophs (Roberson *et al.*, 1994). A gonadotroph-specific element (GSE) approximately 220 bp upstream of the transcription initiation site that bound a protein unique to gonadotrophs (gonadotroph-specific element binding protein, GSEB) was also reported by Horn *et al.* (1992). GSEB was shown to be the orphan nuclear receptor steroidogenic factor-1 (SF-1), which is a primary regulator of genes encoding steroidogenic enzymes in the adrenal gland and gonads (Barnhart and Mellon, 1994). DNA-binding proteins of the GATA family bound to specific DNA sequences in the URE and may be important for expression of α subunit in the pituitary (Steger *et al.*, 1994). Thus, expression of the human α subunit gene in the pituitary requires several specific DNA sequences (i.e. PGBE, GSE and URE) and unique DNA-binding proteins (i.e. LH-2, GSEB/SF-1 and GATA binding proteins).

*LIM is an acronym for *lin-11*, *isl-1*, and *mec-3*. These genes encode proteins that share structural similarities in a region called the LIM domain. The LIM domain comprises putative zinc finger-like structures and regulates transcription by participating in protein–protein interactions.

A transgenic mouse model was developed to investigate further the regulated expression of the human or bovine α subunit promoters in a physiological setting. The transgenic mouse model was important because the bovine α subunit promoter was not expressed after transient transfection in αT3-1 cells. In transgenic mice, pituitary-specific expression was directed by about 1500 bp of human or 315 bp of bovine α subunit 5′ flanking sequence (Bokar *et al.*, 1989). These findings were significant because they narrowed the location of important regulatory sequences in an α subunit genomic clone of approximately 17 kb to only 1500 bp of human or 315 bp of bovine α subunit 5′ flanking sequence. Hamernik *et al.* (1992) used gonadectomy and hormone-replacement paradigms and found that 1500 bp of human and 315 bp of bovine α subunit 5′ flanking sequence directed expression specifically to gonadotrophs in transgenic mice. We subsequently constructed a second line of transgenic mice that harboured 1500 bp of the human α subunit gene linked to the bacterial *lacZ* gene (encoding β-galactosidase) to directly test for cell-specific expression of the human α subunit promoter. Colocalization studies were conducted by staining cross-sections of pituitaries from transgenic mice for β-galactosidase and performing immunocytochemistry with antibodies to either LH or TSH to identify gonadotrophs or thyrotrophs, respectively, that express the human α subunit transgene. Consistent with our hormone-replacement paradigms, cell-specific staining in pituitaries of transgenic mice revealed that the human α subunit promoter was expressed in gonadotrophs, but not in thyrotrophs (Hamernik *et al.*, 1992). Another line of transgenic mice containing the bovine α subunit promoter linked to the diphtheria toxin A-chain structural gene demonstrated severe hypogonadal phenotype owing to almost complete ablation of gonadotrophs; other cell types in the anterior pituitary, including thyrotrophs, were normal (Kendall *et al.*, 1991). Collectively, our studies with transgenic mice demonstrated that the proximal 1500 bp of human or 315 bp of bovine α subunit 5′ flanking sequence contained enough information to direct expression specifically to gonadotrophs in transgenic mice.

Compared with α subunit, relatively little is known about the molecular mechanisms underlying gonadotroph-specific expression of gonadotrophin β subunit genes. Lack of gonadotroph cell lines that express gonadotrophin β subunits has limited progress in this area. To begin to investigate regulated expression of the LHβ subunit gene, Kim *et al.* (1990) transiently transfected 1.7 kb of 5′ flanking sequence of the rat LHβ subunit gene and found promoter activity in primary cultures of rat pituitary cells but not in human choriocarcinoma cells or GH_3 cells (growth hormone and prolactin producing cells). Transgenic mice have recently been constructed that contain about 2 kb of rat, 1.9 kb of ovine, or 776 bp of bovine LHβ subunit 5′ flanking sequence (Fallest and Shupnik, 1994; Brown *et al.*, 1993; Keri *et al.*, 1994, respectively). The LHβ subunit promoter directed expression to gonadotrophs in all lines of transgenic mice. Assuming that mechanisms underlying gonadotroph-specific expression are conserved across species, then sequences necessary for gonadotroph-specific expression must reside within the proximal 776 bp of 5′ flanking sequence of the LHβ subunit gene. Using 2.3 kb of bovine FSHβ subunit 5′ flanking sequence, Markkula *et al.* (1993) reported expression of the transgene in the pituitary and testis of transgenic mice. It seems likely that gonadotrophin β subunit genes may contain DNA sequences similar to those found in α subunit that direct expression specifically to gonadotrophs (i.e. GSE). Additional studies using transgenic mice or transient transfection assays in cell lines that express gonadotrophin β subunit genes with mutations in the gonadotrophin β subunit promoters will be required to define more precisely *cis*-acting DNA sequences and DNA-binding proteins directing expression of gonadotrophin β subunit genes to gonadotrophs.

Oestrogen-responsive DNA sequences

Chronic administration of oestradiol to ovariectomized animals decreased the amounts of mRNA encoding gonadotrophin subunits (Gharib *et al.*, 1990; Mercer, 1990; Nett, 1990). However, the molecular mechanisms through which oestradiol inhibited transcription of gonadotrophin subunit genes have not been well understood. In general, steroid hormones regulate transcription by binding to nuclear receptors to form steroid hormone receptor complexes that interact with specific DNA sequences. The highly conserved oestrogen responsive element (ERE) consists of the sequence: AGGTCA*nnn*TGACCT (where *n* is any nucleotide) and binds the purified oestrogen receptor complex with high affinity (Beato, 1989). The human α subunit gene lacks a high-affinity binding site for the oestrogen receptor (Keri *et al.*,

1991). In addition, oestradiol failed to regulate 1500 bp of the human α subunit 5′ flanking sequence following transient transfection in cells that contain endogenous oestrogen receptor (MCF-7 cells, a human breast tumour cell line) or lack endogenous oestrogen receptor (BeWo, human choriocarcinoma cells; Keri *et al.*, 1991). Adult female transgenic mice were ovariectomized and treated chronically with oestradiol for 2 weeks to determine whether the 5′ flanking regions of the human or bovine α subunit genes were responsive to oestradiol. Activity of human or bovine α subunit promoters was inhibited approximately 75% in pituitaries of oestradiol-treated transgenic mice (Keri *et al.*, 1991). From these studies, it was concluded that oestradiol regulated expression of the α subunit gene by a mechanism that did not involve direct binding of the oestrogen receptor complex to the α subunit gene. The indirect mechanism whereby oestradiol regulated expression of the α subunit gene probably involved altered secretion of GnRH (see discussion below).

In contrast to the α subunit gene, a high-affinity binding site for the oestrogen receptor complex was found in the rat LHβ subunit gene. A DNA sequence that was similar to the consensus ERE described above was localized between 1388 and 1105 bp upstream of the transcription initiation site in the rat LHβ subunit gene (Shupnik *et al.*, 1989). Surprisingly, oestradiol stimulated (rather than inhibited) transcription of the rat LHβ subunit promoter following transient transfection into GH_3 cells (Shupnik *et al.*, 1989). In transgenic mice expressing the rat or bovine LHβ subunit transgenes in the pituitary, chronic administration of oestradiol to gonadectomized animals reduced activity of the LHβ subunit promoter to values comparable to those of intact animals (Fallest and Shupnik, 1994; Keri *et al.*, 1994). Thus, oestradiol could potentially regulate transcription of the LHβ subunit gene by a direct stimulatory action or an indirect inhibitory action that involves decreased secretion of GnRH from the hypothalamus.

Androgen-responsive DNA sequences

Like oestrogens, chronic administration of testicular androgens to castrated animals suppressed amounts of α and LHβ subunit mRNA (Gharib *et al.*, 1990). However, the molecular mechanisms through which androgens inhibit expression of gonadotrophin subunit genes appear to be distinct from those of oestrogen. Whereas the oestrogen receptor complex failed to bind directly to the human α subunit gene (Keri *et al.*, 1991), androgen receptor complex bound with high affinity to the human α subunit promoter and inhibited transcription of the human α subunit promoter after transient transfection in αT3-1 cells (Clay *et al.*, 1993).

The first indication that human and bovine α subunit 5′ flanking sequences might be regulated by androgen directly was the presence of a region similar to the consensus androgen response element (ARE; GGTACA*nnn*TGTTCT; Beato, 1989) about 101 bp upstream of the transcription initiation site. This region was previously defined as the JRE (Andersen *et al.*, 1990). Gel-mobility shift assays were subsequently conducted to demonstrate that the JRE/ARE in the human α subunit promoter bound purified androgen receptor with high affinity *in vitro* (Clay *et al.*, 1993). The functional importance of the JRE/ARE in conferring transcriptional repression to the human α subunit gene was demonstrated by cotransfecting the human α subunit promoter with human androgen receptor expression vector in αT3-1 cells (Clay *et al.*, 1993). In addition, chronic administration of androgen to castrated animals prevented the post-castration rise in activity of the human or bovine α subunit promoters in pituitaries of transgenic male mice (Fig. 2, Clay *et al.*, 1993). The suppressive effects of dihydrotestosterone (a nonaromatizable androgen) on the expression of the transgene were not as marked as the effects of testosterone (Fig. 2). Suppression of α subunit transgene expression in testosterone-treated transgenic male mice may have resulted from direct effects of androgen (DHT) and indirect effects occurring after aromatization of testosterone to oestrogens (Clay *et al.*, 1993). The ability of the androgen receptor to bind directly to the human α subunit promoter implies that androgen may act directly at the pituitary gland, in addition to acting indirectly at the hypothalamus to inhibit secretion of GnRH, and inhibit expression of the α subunit gene.

In transgenic mice harbouring rat or bovine LHβ subunit 5′ flanking sequences, promoter activity was increased after castration and suppressed by chronic administration of androgen to castrate males (Fallest and Shupnik, 1994; Keri *et al.*, 1994). Preliminary evidence (Keri *et al.*, 1994) indicated that the

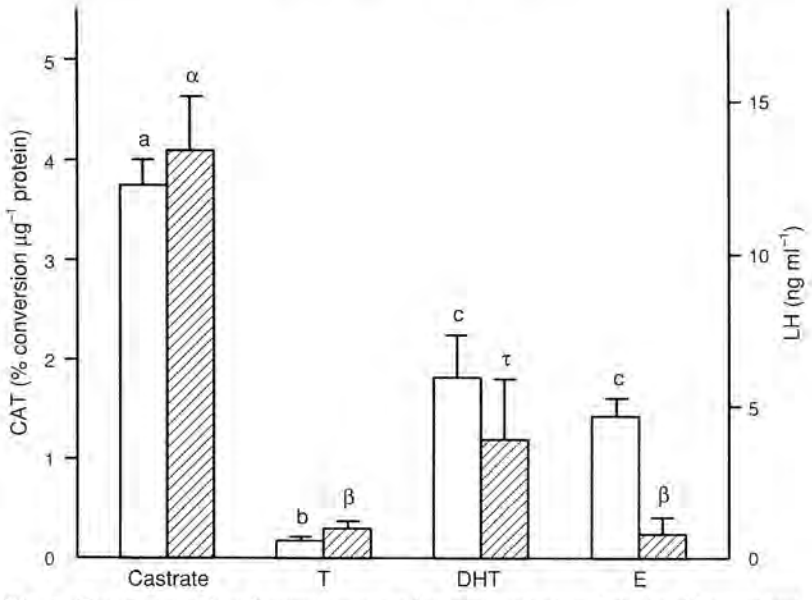

Fig. 2. Oestrogen and androgens suppress the post-castrational rise in chloramphenicol acetyltransferase (CAT) activity in the pituitary (□) and serum concentrations of LH (▨) in transgenic mice containing the proximal 315 bp of 5′ flanking sequence of the bovine α subunit gene linked to the bacterial gene encoding CAT. Adult male, transgenic mice were castrated and implanted with constant release pellets containing inert carrier (castrate), testosterone (T), 5α-dihydrotestosterone (DHT) or oestradiol (E) for two weeks. Lettered superscripts above vertical bars represent statistical differences in CAT activity. Greek symbols above vertical bars represent statistical differences in serum concentrations of LH. Reprinted from Clay *et al.* (1993) with permission.

bovine LHβ subunit 5′ flanking sequence lacked a high-affinity binding site for the androgen receptor; thus, regulation of LHβ subunit gene expression by androgen probably involves an indirect action of androgens to decrease secretion of GnRH from the hypothalamus.

Chronic administration of androgen to transgenic mice expressing the human FSHβ subunit gene resulted in profound suppression of human, but lesser suppressive effects on mouse, mRNA encoding FSHβ subunit in pituitary (Kumar and Low, 1993). Suppressive effects of androgen on FSHβ subunit gene expression may have resulted from species differences in FSHβ subunit gene regulation. It is possible that the rodent FSHβ subunit gene lacked direct androgen regulatory sequences or that the human FSHβ subunit gene was more dependent on GnRH stimulation for basal expression than was the mouse FSHβ subunit gene (Kumar and Low, 1993). Further studies will be needed to understand differential regulation of human, rodent and ruminant FSHβ subunit genes by androgens more fully.

GnRH-responsive DNA sequences

Pulsatile GnRH stimulated amounts of gonadotrophin subunit mRNA in rodents and ruminants (Gharib *et al.*, 1990; Mercer, 1990; Nett, 1990). A GnRH responsive region spanning nucleotides -346 to -244 of the human α subunit promoter was identified by transient transfection in αT3-1 cells (Kay and Jameson, 1992). Nucleotides -416 to -385 of the mouse α subunit promoter were also responsive to GnRH after transient transfection in αT3-1 cells (Schoderbek *et al.*, 1993). To determine whether human or bovine α subunit 5′ flanking sequences contained GnRH-responsive regions, we conducted ovariectomy and hormone replacement studies with chronic oestradiol and pulsatile GnRH in transgenic mice (Fig. 3). Pulsatile administration of GnRH in the continued presence of oestradiol completely

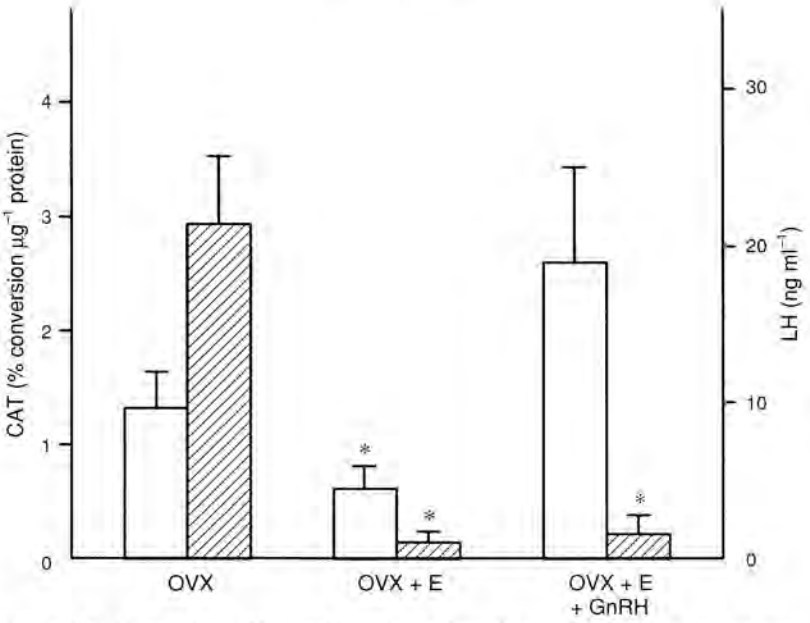

Fig. 3. GnRH stimulates chloramphenicol acetyltransferase (CAT) activity in pituitary of transgenic mice (□) containing the proximal 315 bp of 5′ flanking sequence of the bovine α subunit gene linked to the bacterial gene encoding CAT. Adult female, transgenic mice were ovariectomized (OVX), treated with daily injections of oestradiol (E) for 14 days (OVX + E), and saline or GnRH every other hour for the last 7 days of oestradiol treatment (OVX + E + GnRH). Serum and pituitaries were collected 30 min after the last injection of GnRH. (▨) Serum concentrations of LH. *Significantly different from OVX. Reprinted from Hamernik *et al.* (1992) with permission.

restored activity of the human or bovine α subunit promoters to values similar to those of ovariectomized mice (Hamernik *et al.*, 1992). From these studies, we concluded that the proximal 1500 bp of human or 315 bp of bovine α subunit 5′ flanking sequence contained sufficient information to confer responsiveness to GnRH in transgenic mice.

Studies with the human and mouse promoters in αT3-1 cells that identified a GnRH-responsive region upstream of 329 bp are difficult to reconcile with our results demonstrating GnRH responsiveness in transgenic mice containing only 315 bp of the bovine α subunit promoter. It is possible that there are subtle differences between species in nucleotide sequences or that studies with transgenic mice represent a more physiological model to study cell-specific expression and hormonal regulation of gonadotrophin subunit genes because stringent requirements are imposed on DNA sequences to direct correct temporal and spatial expression of the transgene during development. Specific DNA sequences in the α subunit promoter that confer responsiveness to GnRH in transgenic mice remain to be identified.

What second messenger system mediates GnRH-stimulated transcription of gonadotrophin subunit genes? There is considerable evidence that GnRH-induced secretion of LH involved activation of the calcium or protein kinase C pathways (Conn *et al.*, 1987); however, relatively little is known about the intracellular mediators of GnRH-induced transcription of α subunit. Tandem CRE were found in the human α subunit 5′ flanking sequence and mediated transcriptional induction after activation of the protein kinase A or C pathways in human choriocarcinoma cells (Jameson and Hollenberg, 1993); thus, it is possible that GnRH activates transcription of the human α subunit promoter through the CRE. The bovine α subunit 5′ flanking region contains a CRE-like sequence that failed to bind CREB and failed to confer cAMP responsiveness after transient transfection in human choriocarcinoma cells (Bokar *et al.*, 1989), yet the bovine α subunit promoter was transcriptionally stimulated by GnRH in transgenic mice (Hamernik *et al.*, 1992). It seems likely that DNA sequences other than the CRE, but within the proximal

315 bp of the bovine α subunit promoter, are needed for GnRH-induced transcription of the bovine α subunit gene in transgenic mice. Amounts of mRNA for LHβ subunit in rat pituitaries *in vitro* were stimulated by GnRH and activation of the protein kinase C pathway (Andrews *et al.*, 1988). In addition, administration of a GnRH antagonist to transgenic mice harbouring the bovine LHβ subunit gene resulted in decreased activity of the promoter compared with ovariectomized transgenic mice (Keri *et al.*, 1994). The proximal 800 bp of 5′ flanking sequence of the bovine LHβ subunit gene lacked a consensus CRE but contained enough information to confer responsiveness to GnRH in transgenic mice. Specific DNA sequences and DNA-binding proteins mediating GnRH-stimulated transcription of gonadotrophin α and β subunit genes remain to be characterized.

Conclusions

Transgenic mice and transient transfection assays with mouse gonadotroph cell lines expressing the endogenous mouse α subunit gene have proved valuable in identifying DNA sequences in mouse and human gonadotrophin α subunit genes that direct expression specifically to gonadotrophs and mediate hormone responsiveness. Development of gonadotroph cell lines that express bovine or ovine gonadotrophin α or β subunit genes or *in vivo* gene transfer studies into cattle or sheep will be needed to define further molecular mechanisms that regulate cell-specific expression and hormone responsiveness of bovine or ovine gonadotrophin genes. A better understanding of the molecular mechanisms that regulate synthesis of gonadotrophins may lead to methods to for enhancing reproductive efficiency in domestic animals.

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