REVIEW

Regulation of gonadotropin subunit gene transcription

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Abstract

Reproductive function in mammals is regulated by the pituitary gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH and FSH are secreted by the gonadotrope cell and act on the gonad in a sequential and synergistic manner to initiate sexual maturation and maintain cyclic reproductive function. The synthesis and secretion of LH and FSH are regulated mainly by the pulsatile release of the hypothalamic decapeptide hormone gonadotropin-releasing hormone (GnRH). The control of differential LH and FSH synthesis and secretion is complex and involves the interplay between the gonads, hypothalamus and pituitary. In this review, the transcriptional regulation of the gonadotropin subunit genes is discussed in a physiologic setting, and we aimed to examine the mechanisms that drive those changes.

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Introduction

Dynamic regulation of the pituitary gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) is essential for mammalian reproduction. LH and FSH are comprised of two glycoprotein subunits, α (common to both), and LH β and FSH β , which are coded by three genes located on separate chromosomes (Chin 1987, Gharib *et al.* 1990b). LH and FSH are secreted by the pituitary gonadotropes and act on the gonad in a sequential and synergistic manner to initiate sexual maturation and maintain cyclic reproductive function (Bäckström *et al.* 1982, Marshall & Kelch 1986, Wu *et al.* 1990). The synthesis and secretion of the gonadotropins are regulated primarily by the hypothalamic decapeptide gonadotropin-releasing hormone (GnRH), which is secreted into the hypophyseal-portal circulation in a pulsatile manner (Clarke & Cummins 1982, Levine & Ramirez 1982). The control of LH and FSH synthesis and secretion is complex and involves interplay between the gonads, pituitary and hypothalamus. LH and FSH act on the ovaries and

the testes to regulate folliculogenesis, ovulation, spermatogenesis and steroidogenesis. Gonadal steroids and peptides, in turn, act at the hypothalamus and/or pituitary to regulate either positively or negatively LH and FSH synthesis and secretion. The aims of this review are to present the transcriptional regulation of the gonadotropin subunit genes in a physiologic setting and examine the mechanisms that drive those changes.

Physiologic changes in subunit gene transcription

Estrous cycle

Changes in gonadotropin subunit mRNA expression have been determined over the course of the estrous cycle in rats and mice. In rats, we have shown that LH β and FSH β mRNAs increase during the time of preovulatory gonadotropin surge. The increase in LH β mRNA precedes that of FSH β ; LH β mRNA began increasing around 1400 h, before the beginning of the LH surge; was maximal at 1700 h; and had returned to basal by

2200 h (Zmeili *et al.* 1986, Kerrigan *et al.* 1995). FSH β mRNA did not increase until about 2200 h, 2 h after the increase in serum FSH, and was maximal at 0200 h during estrus and had returned to basal by 0800 h (Ortolano *et al.* 1988). In contrast, α mRNA did not change during the preovulatory surge (Zmeili *et al.* 1986). The periovulatory changes in the β -subunit mRNAs are coincident with increased GnRH secretion (Levine & Ramirez 1982) and result from increased gene transcription. β -subunit mRNA synthesis, as measured by nuclear run-on assays, was found to be greatest during proestrus for LH β , and during late proestrus and estrus for FSH β (Shupnik *et al.* 1989a). Similarly, FSH β promoter activity was increased on estrus (versus metestrus) in transgenic mice harboring an ovine FSH β promoter-luciferase (LUC) reporter gene (Huang *et al.* 2001a,b).

In addition to the changes in subunit mRNAs that occur around the preovulatory gonadotropin surge, FSH β mRNA expression increased at metestrus, and both α and LH β mRNAs increased in parallel during diestrus, periods when serum LH and FSH levels are low. The mechanism(s) for the these changes are not well understood, but probably reflect the sensitivity of the subunit genes to differences in GnRH pulse frequencies and steroid milieu; and for FSH β , changes in serum and/or intrapituitary inhibin, activin or follistatin (FS).

Gonadectomy

Several studies have shown that gonadotropin subunit gene expression is differentially regulated after gonadectomy. The loss of negative feedback by sex steroids at the hypothalamus results in increased GnRH pulse amplitude and frequency (Levine & Ramirez 1982). This increase in GnRH drives expression of all three subunit genes, but there are significant differences in the magnitudes of change and timing, both among the subunits and between the sexes.

In male rats, castration (CAST) results in a rapid increase in serum LH, reflecting an increase in GnRH secretion. Coincident with the increase in GnRH, all three subunit mRNAs are elevated 24 h after CAST (Papavasiliou *et al.* 1986, Dalkin *et al.* 2001, Burger *et al.* 2004). Whereas α and LH β mRNA concentrations continue to increase steadily after CAST (Papavasiliou *et al.* 1986, Dalkin *et al.*

1990), increases in FSH β mRNA are more modest and begin to decline by day 7 (Gharib *et al.* 1987, Dalkin *et al.* 1990).

The increases in subunit mRNA after CAST are regulated in part at the level of transcription. We have recently investigated the changes in subunit gene transcription after CAST by measuring subunit primary transcript (PT) concentrations (Fig. 1). These transcripts are newly formed RNA that include both exon and intron sequences, prior to RNA splicing, and thus are closely linked to gene transcription and mRNA formation (Dalkin *et al.* 2001). After CAST, LH β PT increases within 8 h and remains elevated (Dalkin *et al.* 2001, Burger *et al.* 2004). Similarly, LH β promoter activity has been reported to increase after CAST in transgenic mice harboring either a rat LH β promoter-LUC construct (Fallest *et al.* 1995) or an ovine LH β -chloramphenicol acetyltransferase (CAT) reporter (McNeilly *et al.* 1996). Although FSH β mRNA was elevated within 24 h of CAST, we did not observe an acute increase in FSH β PT (Burger *et al.* 2004). However, FSH β transcription was increased 5–7 days after CAST, as measured by either nuclear run-on assays (Paul *et al.* 1990), PT (Dalkin *et al.* 2001) or FSH β promoter activity in transgenic mice carrying an ovine FSH β -LUC reporter gene (Huang *et al.* 2001a,b). In contrast, changes in endogenous α gene transcription after CAST are not consistent; we have reported that α PT was unchanged at day 7 (Dalkin *et al.* 2001), whereas α mRNA synthesis was increased on day 5 (Paul *et al.* 1990). Additionally, α promoter activity increased after CAST in transgenic mice carrying either a human or bovine α promoter-CAT reporter (Clay *et al.* 1993); *in vitro*, the activity of a human α promoter-LUC reporter was greater when transfected into pituitary cells from male rats that had been CAST for 7–21 days (versus intact male rats) (Colin *et al.* 1996).

The utilization of GnRH antagonists or androgens to suppress endogenous GnRH demonstrates that the increased subunit transcription after CAST is GnRH dependent. The synthesis rates of all three subunit mRNAs were no different between intact rats and 4-day CAST rats treated with a GnRH antagonist (Paul *et al.* 1990), and we have also shown that a GnRH antagonist suppresses both LH β and FSH β PT below intact levels within hours after CAST (Fig. 1) (Burger *et al.* 2004). Similarly, in transgenic mice, either GnRH

Figure 1 The effects of GnRH antagonist (A) and testosterone (T) on pituitary β -subunit primary transcripts (PT) in CAST male rats. Rats ($n=5-10$ /group) were CAST only or CAST and treated with GnRH antagonist LRF-147 (200 μ g, s.c.) every 12 h with and without silastic T implants (designed to achieve T levels of 3.5 ng/ml). Rats were killed 0, 8 and 24 h later. All data are presented as percent 0-h ($\pm$ s.e.) controls. Bars with different letters are significantly different ($P < 0.05$). Reproduced with permission from Burger *et al.* 2004.

antagonist or testosterone suppressed the post-CAST increases in rat LH β -LUC (Fallest *et al.* 1995), ovine LH β -CAT (McNeilly *et al.* 1996), and both human and bovine α -CAT promoter constructs (Clay *et al.* 1993). The elevated transcription rates seen after CAST require sustained GnRH input. Administration of a GnRH antagonist to 7-day CAST rats rapidly reduced LH β and FSH β PT, with half-disappearance times of 2.7 and 0.75 h respectively (Fig. 2) (Dalkin *et al.* 2001).

Gonadotropin subunit gene expression is also differentially regulated after ovariectomy (OVX). In female rats, serum LH rises more slowly after OVX and is not significantly elevated until days 2 or 3 (Dalkin *et al.* 1993, Burger *et al.* 2001). Coincident with the increase in GnRH, both α and LH β mRNA expression begins to rise about day 3, and continues to rise through day 21 (Dalkin *et al.* 1990, 1993, Burger *et al.* 2001). In contrast, both serum FSH and FSH β mRNA increase rapidly after OVX; serum FSH doubles by 8 h and FSH β

mRNA by 30–60 min (Dalkin *et al.* 1993), and they continue to rise through day 7 (Dalkin *et al.* 1990, 1993, Burger *et al.* 2001). As in males, increased subunit mRNA levels result from elevated transcription rates; mRNA synthesis rates were increased for all three subunits in 30-day OVX rats (Shupnik *et al.* 1988). Recently, we have examined the changes in subunit transcription in the 7 days after OVX during the dynamic period after the loss of gonadal feedback (Fig. 3) (Burger *et al.* 2001). We found that LH β PT concentrations were increased after day 3, paralleling changes in serum LH and LH β mRNA. In contrast, there was a biphasic change in serum FSH, FSH β mRNA and FSH β PT after OVX, with an acute increase at 12–24 h, followed by an additional increase after 72 h. Although α mRNA was elevated after OVX, as in our earlier studies in males, α -PT did not change.

As in males, increased GnRH secretion after OVX is an important regulator of subunit

Figure 2 Disappearance rates for the gonadotropin subunit primary transcripts (PT) in 7-day CAST rats. PT concentration (fmol PT/100 μ g RNA) is shown on the vertical axis, and time after GnRH antagonist treatment (h) is shown along the horizontal axis. The shaded areas represent the range observed in intact animals. The calculated $t_{1/2}$ for the LH β and FSH β subunits is displayed on the respective curves. Reproduced with permission from Dalkin *et al.* 2001.

transcription. Suppression/blockade of GnRH by administration of either a GnRH antagonist or estradiol (E_2), *in vivo*, reduced α and LH β mRNA synthesis rates significantly, but had either no effect or a modest suppression on FSH β in long-term OVX rats (Shupnik *et al.* 1988, 1990, Fallest & Shupnik 1994). Similarly, we found that GnRH blockade prevented the post-OVX increase in LH β -PT, but had no effect on the acute (12–24-h) increase in FSH β -PT and only partially suppressed the increase in FSH β PT after day 3 (Fig. 4). Sustained GnRH secretion is also required to maintain post-OVX increases in β -subunit transcription; giving a single dose of GnRH antagonist to 7-day OVX rats rapidly reduced both LH β and FSH β PT, with half-disappearance times of 13 and 17 min respectively (Fig. 5).

While α and LH β transcription after OVX appear to be largely regulated by GnRH, control of FSH β transcription is more complex. The modest effect of either GnRH antagonist or E_2 in suppressing FSH β transcription *in vivo*, hinted at a role for inhibin, activin and/or FS. We previously showed that administration of inhibin- α antisera to intact female rats mimics the GnRH-independent increases in FSH and FSH β mRNA that occur within hours after OVX (Dalkin *et al.* 1993, 1998). Similarly, we found that FSH β PT levels increased rapidly after inhibin immunoneutralization and were suppressed in OVX rats treated with recombinant human inhibin, suggesting that inhibin suppresses FSH β transcription (Burger *et al.* 2001). However, we also found that inhibin suppressed FSH β mRNA levels much faster than after a GnRH antagonist, suggesting that inhibin may also affect FSH β mRNA stability.

Several transgenic mouse models have been used to investigate the effects of OVX on subunit promoter activity. Hamernik *et al.* (1992) reported that E_2 suppressed activity of either human α CAT or bovine α CAT constructs in OVX mice and that OVX levels could be restored with pulsatile GnRH. Activity of rat, ovine and bovine LH β promoter-reporter constructs increased after OVX in transgenic mice, and were suppressed by either GnRH antagonist (Fallest *et al.* 1995, Quirk *et al.* 2001) or E_2 (Fallest *et al.* 1995, McNeilly *et al.* 1996). Huang *et al.* (2001a,b) reported that FSH β promoter activity is increased after OVX in transgenic mice harboring an ovine FSH β -LUC construct.

Primary Transcript (fmol PT/100µg RNA)

Gonadotropin subunit regulation by GnRH

Physiologic changes in subunit transcription by pulsatile GnRH

A central question in studying the physiology of the gonadotropins is how a single hormone (GnRH) acting on a single cell type (gonadotrope) can differentially regulate two hormones (LH and FSH). The answer is that GnRH differentially regulates LH and FSH synthesis via changes in the pattern of GnRH pulse secretion. Our laboratory and others have investigated the effects of GnRH pulse amplitude and frequency on subunit mRNA expression both *in vivo* and *in vitro*. In GnRH-deficient male rats, fast-frequency GnRH pulses (every 8 min) favored expression of α and, to a lesser extent, LH β mRNA, fast-physiologic GnRH pulses (every 30 min, the approximate frequency of GnRH pulses found in CAST male rats or intact female rats on the afternoon of proestrus (Levine & Duffy 1988, Levin & Ramirez 1982)) increased all three subunit mRNAs, and slow-frequency pulses (>120-min intervals) selectively increased FSH β mRNA (Haisenleder *et al.* 1988, Dalkin *et al.* 1989, Kirk *et al.* 1994). GnRH pulse amplitude also differentially regulates subunit mRNA, though to a lesser extent than pulse frequency; only LH β mRNA expression was sensitive to GnRH pulse amplitude and was maximally stimulated by GnRH pulse doses of 25 ng or less (Haisenleder *et al.* 1988, Iliff-Sizemore *et al.* 1990).

GnRH regulation of subunit mRNA is somewhat different in female rats. In initial studies, we reported that GnRH pulses increased α and FSH β mRNA levels, but not LH β in GnRH-deficient OVX rats (Kerrigan *et al.* 1993), a finding which was consistent with the lack of LH β mRNA response to GnRH reported in cultured pituitary cells from OVX rats (Weiss *et al.* 1990). Subsequently, we found that testosterone was required for GnRH pulses to increase LH β mRNA in OVX rats, and that the optimal dose of

Figure 3 The changes in gonadotropin subunit primary transcript (PT) concentrations following OVX. Pituitaries were collected from intact rats or rats OVX for the indicated times. $n=5-9$ /group. Each bar represents the mean $\pm$ S.E. Bars with different letters are significantly ($P<0.05$) different. Reproduced with permission from Burger *et al.* 2001.

Figure 4 The effects of GnRH antagonist on gonadotropin subunit primary transcript (PT) concentrations after OVX. Pituitaries were collected from intact rats, and 12- and 72-h OVX rats treated either with the GnRH antagonist LRF-147 (+) or vehicle (-, BSA-saline) $n=5-7$ /group. Each bar represents the mean $\pm$ S.E. * Indicates means are significantly different ($P<0.05$) from intact rats. ** Indicates antagonist-treated group is significantly different ($P<0.05$) from OVX (vehicle, -) at the same time point. Reproduced with permission from Burger *et al.* 2001.

testosterone was similar to levels seen at proestrus (Yasin *et al.* 1996). Although testosterone is required in females, it is not acutely required in males, as GnRH increased LH β mRNA in short-term CAST males with or without T (Yasin *et al.* 1996). On a background of proestrus T levels, we have found

Figure 5 The effects of GnRH antagonist on β -subunit primary transcript (PT) half-disappearance times ($t_{1/2}$). Pituitaries were collected from 7-day OVX rats treated with the GnRH antagonist LRF-147 (30 μ g i.v.) and then killed 0, 30, 60 or 120 min later ($n=5-6$ /time). Each point represents the mean $\pm$ S.E. The shaded areas represent the mean $\pm$ S.E. observed in intact rats. The calculated $t_{1/2}$ for LH β and FSH β primary transcripts is displayed on the respective curves. Reproduced with permission from Burger *et al.* 2001.

that GnRH pulse amplitude and frequency differentially regulate subunit mRNA expression in females similar to males. Alpha and FSH β mRNAs were increased by a wider range of GnRH pulse amplitudes, while LH β mRNA was maximally stimulated by lower GnRH pulse doses (Dalkin *et al.* 1999). Fast-physiologic GnRH frequencies (8–120-min intervals) stimulated all three subunit mRNAs, but only slow-frequency GnRH pulses (every 240 min) increased FSH β (Dalkin *et al.* 1999). The effects of GnRH pulse amplitude and frequency have also been examined in cultured pituitary cells. In female rat pituitary cells, only α mRNA increased with high-amplitude GnRH pulses, whereas LH β and FSH β mRNAs responded to lower doses of GnRH (Haisenleder *et al.* 1993a). In male pituitary cells, α mRNA was increased by all GnRH pulse frequencies examined, LH β mRNA was maximally stimulated by pulses every 30 min, and FSH β mRNA was greatest after 120-min pulses (Kaiser *et al.* 1997b).

Although there is some evidence that GnRH may regulate subunit mRNA concentrations via mRNA stability (Salton *et al.* 1988, Weiss *et al.* 1992), GnRH appears to exert its main action at the transcriptional level. While continuous GnRH stimulated α transcription *in vitro* (Shupnik 1990), a pulsatile GnRH input was required to increase β -subunit transcription both *in vivo* (Haisenleder *et al.* 1991) and *in vitro* (Shupnik 1990, Shupnik & Falset 1994). Furthermore, pulsatile GnRH increased β -subunit transcription in a frequency-dependent manner. Faster GnRH pulse frequencies (≥ 60 -min interpulse interval) preferentially increased LH β mRNA synthesis or PT levels both *in vitro* (Shupnik 1996) and *in vivo* (Haisenleder *et al.* 1991, Burger *et al.* 2002). In contrast, slower GnRH pulse frequencies (≥ 60 -min intervals) preferentially increased FSH β transcription. Alpha-subunit transcription does not appear to be tightly regulated by GnRH pulse frequency, some studies indicating that α transcription is favored by fast-frequency GnRH pulses (Haisenleder *et al.* 1991, Shupnik *et al.* 1996), and others showing that fast and slow frequencies are equally effective (Haisenleder *et al.* 2001, 2003a, Burger *et al.* 2002).

The effects of a GnRH pulse on subunit transcription are both rapid and transient. In male rats, LH β and FSH β PTs increased six- and fourfold 5 min after a GnRH pulse, and declined to basal levels by 30 min, with FSH β PT decreasing

faster than LH β PT (Dalkin *et al.* 2001). Although α PT tended to increase 5–15 min after a GnRH pulse, the rise was not statistically significant. The duration of GnRH pulsatile treatment also appears to be important in regulating subunit transcription rates. In earlier studies, we found that GnRH pulses given every 30 min to male rats increased α , LH β and FSH β mRNA synthesis rates, but that the increases in LH β and FSH β returned to basal at 4–24 h (Haisenleder *et al.* 1991). In a more recent study, we found that only fast-frequency GnRH pulses (30 min) increased LH β PT levels, and that the increase in transcription was sustained after 1–24 h of pulses (Fig. 6) (Burger *et al.* 2003). Unexpectedly, we found that both fast- and slow-(240-min) frequency GnRH pulses increased FSH β PT, but with significantly different time courses. Fast-frequency GnRH pulses transiently increased FSH β PT after 1–6 h, but returned to control levels by 24 h. In contrast, slow-frequency GnRH pulses resulted in a delayed but sustained increase in FSH β PT at 8–24 h, and only 240-min pulses increased both FSH β PT and mRNA expression.

A recent study investigated the effects of GnRH pulse frequency on subunit promoter activities in gonadotrope-derived L β T2 cells and found similar results to those seen *in vivo* or in cultured pituitary cells (Bedecarrats & Kaiser 2003). Fast-frequency GnRH pulses (≥ 60 min) stimulated expression of a rat LH β promoter-reporter to a greater degree than slower frequencies. In contrast, a rat FSH β promoter-reporter was preferentially stimulated by slower GnRH pulse frequencies (≥ 60 min), but a clear preference for a slower GnRH frequency was not observed until after 20 h of pulses, before which fast GnRH frequencies were as effective if not better in stimulating FSH β promoter activity (Bedecarrats & Kaiser 2003). Alpha promoter activity was also stimulated by GnRH in L β T2 cells but was less frequency dependent than the β -subunits; human α -LUC reporter activity increased with all GnRH frequencies but was greatest with more frequent GnRH pulses (Bedecarrats & Kaiser 2003).

Intracellular mechanisms of subunit gene transcription by GnRH

The GnRH receptor (GnRH-R) is a member of the seven-transmembrane receptor family, with receptor binding activating two specific

GTP-binding proteins (G_q , G_{11}) (Hsieh & Martin 1992, Stojilkovic & Catt 1995b, Kaiser *et al.* 1997a, Liu *et al.* 2002b). GnRH-R activation stimulates increased phosphoinositide turnover and a rise in

intracellular diacylglycerol levels, resulting in the activation of protein kinase C (PKC) (Andrews & Conn 1986, Stojilkovic & Catt 1995a). Activation of the GnRH-R also stimulates a transient rise in intracellular calcium, derived from inositol 1,4,5-triphosphate-induced release from intracellular storage pools and from influx via voltage-sensitive(L-type) channels (Naor 1990, Ando *et al.* 2001). GnRH also stimulates an increase in cAMP (Bourne 1988, Garrel *et al.* 1997) and activates members of the mitogen-activated protein kinase (MAPK) family, including extracellular signal-regulated kinase (ERK) (Mitchell *et al.* 1994, Roberson *et al.* 1995, Sundaresan *et al.* 1996, Reiss *et al.* 1997), c-Jun NH₂-terminal kinase (JNK) (Levi *et al.* 1998, Roberson *et al.* 1999, Yokoi *et al.* 2000) and p38 (Roberson *et al.* 1999, Liu *et al.* 2002a) in several species.

As discussed previously, gonadotropin subunit gene transcription is regulated in a differential manner by alterations in GnRH pulse frequency. Recent data suggest that various mammalian species express a second GnRH isoform (GnRH II) and GnRH-R (GnRH-R II) in the hypothalamus and pituitary (Neill 2002). Although effects on sexual behavior and preferential actions on FSH secretion have been described, the physiologic roles of these isoforms remain to be determined. The evidence to date suggests that differential synthesis and secretion of LH and FSH is the product of altered patterns of pulsatile GnRH I secretion by activation of pituitary GnRH-R I. Although the intracellular messengers responsible for transmitting frequency-dependent signals from the plasma membrane to the nucleus within the gonadotrope have yet to be fully characterized, recent findings have provided insights into critical sites in the signal transduction pathways involved.

Figure 6 The effects of fast- and slow-frequency GnRH pulses over 24 h on gonadotropin subunit primary transcripts (PT). CAST plus testosterone-replaced male rats were i.v. pulsed with 25 ng GnRH every 30 (fast) or 240 (slow) min for 1–24 h ($n=4-8$ rats/observation). Data are expressed as fold change versus controls (0 h). * Indicates significant differences ($P<0.05$) versus untreated, CAST plus testosterone (T) controls (0 h). ** Indicates significant differences between GnRH pulse regimens at 24 h. Reproduced with permission from Burger *et al.* 2002.

cAMP

Activation of the cAMP pathway stimulates α (mouse, rat and human) promoter activity (Maurer *et al.* 1999), and cAMP may also have a general stimulatory response to GnRH signaling. The mouse GnRH-R promoter contains two cAMP responsive element-binding (CREB) sites, and increased cAMP/protein kinase A (PKA) activity enhanced mouse GnRH-R promoter activity in lactotropic GH3 cells stably transfected with rat GnRH-R cDNA (GGH3 cell lines) (Han & Conn 1999, Lin & Conn 1999). However, studies conducted in either gonadotrope-derived α T3 cells or transgenic mice models reveal that PKC, MAPK and activator protein-1 (AP-1) play a more prominent role in GnRH regulation of the GnRH promoter (Norwitz *et al.* 1999, White *et al.* 1999, Ellsworth *et al.* 2003). In the human model, CREB plays an essential role in GnRH activation of the α promoter, and GnRH stimulates the phosphorylation of CREB (Delegeane *et al.* 1987, Duan *et al.* 1999). The porcine FSH β promoter also contains a putative CREB site (Kato *et al.* 1999). We have conducted *in vitro* studies in rat pituitary cells to determine whether cAMP stimulates gonadotropin subunit mRNA (Haisenleder *et al.* 1992). The results demonstrated that a diffusible cAMP analog stimulated a rise in α , but not LH β or FSH β mRNA. Of interest, we showed that the pattern of cAMP input was critical, as pulsatile, but not continuous, cAMP enhanced α mRNA expression. More recent studies suggest that the cAMP/PKA pathway plays a role in cross-talk between specific intracellular messenger systems (that is, PKC and ERK) in response to GnRH stimulation (Garrel *et al.* 1997, Han & Conn 1999, Lin & Conn 1999, Fowkes *et al.* 2002).

Calcium

The GnRH-induced increases in intracellular calcium play an essential role in gonadotropin secretion (Naor 1990, Stojilkovic & Catt 1995a,b). Studies in various species and experimental models have also shown that α , LH β and FSH β gene expression are regulated by increases in intracellular calcium (Ben Menahem & Naor 1994, Saunders *et al.* 1998, Weck *et al.* 1998). In the rat, calcium stimulates a rise in α , LH β and FSH β mRNA levels (Ben Menahem & Naor 1994, Haisenleder *et al.* 1993b, 1997). Other reports reveal that calcium

stimulates gonadotropin subunit transcriptional activation, including increases in α and LH β transcription (as measured by nuclear run-on assay or promoter-luciferase construct assay) (Weck *et al.* 2000) and α , LH β and FSH β PT (Haisenleder *et al.* 2001). In contrast, other investigations using GH3 cells that express GnRH-R (GGH3-1 cells) have shown that the response to calcium is selective, stimulating α but not LH β and FSH β (Saunders *et al.* 1998). Another report found that calcium suppresses GnRH- or PKC-induced rat LH β promoter activity in L β T4 cells (Vasilyev *et al.* 2002a). The reasons for these different gonadotropin subunit responses to calcium remain to be determined. However, it is likely that factors such as differences in cell model, promoter constructs used, experimental paradigms and the end products measured play a role in the outcomes seen.

Gonadotrope cells express spikes and oscillations in intracellular calcium, the patterns of which are altered by GnRH and linked to LH secretion (Holl *et al.* 1988, Tse & Hille 1992, Stojilkovic & Catt 1995b). These observations are of interest, as in other cell types, alterations in intracellular calcium can play a role in the regulation of gene expression and activation of downstream mediators of signal transduction pathways (that is, ERK and calcium/calmodulin-dependent kinase II (Ca/CaMK II)) (Villalobos *et al.* 1988, Gu & Spitzer 1995, Dolmetsch *et al.* 1997, De Koninck *et al.* 1998, Durham & Russo 2000). We have investigated whether alterations in intracellular calcium are involved in transmission of frequency-dependent signals from the plasma membrane to the nucleus, and whether these signals mediate the α , LH β and FSH β transcriptional responses to pulsatile GnRH (Haisenleder *et al.* 2001). The model used cultured rat pituitary cells and pulses of the calcium channel activator, Bay K 8644, plus potassium chloride (BK+KCl) given at intervals of 15, 60 or 180 min for 6 h. Gonadotropin subunit PT was measured to determine transcriptional responses to treatment. BK+KCl pulses stimulated release of both LH and FSH with a similar pattern and magnitude to those seen after pulsatile GnRH. Alterations in the frequency of calcium pulse signals revealed that α was selectively stimulated by faster (every 15 min) pulses of BK+KCl, with slower pulses being ineffective (Fig. 7). LH β PT was stimulated by 15- or 60-min pulses, but not 180-min pulses, of

Figure 7 Effect of the calcium channel activator Bay K 8644 (BK) pulse interval (15, 60 or 180 min) on LH β , and FSH β primary transcript (PT) levels. Pulse treatments were given for 6 h, and a GnRH pulse group (60-min interval) was included for comparison. Results are expressed as the percent change versus vehicle-pulsed controls (Con). Means $\pm$ S.E.M. are shown; note the different scale for each panel. The number of chambers per group was 10–12, derived from four or five separate experiments. * $P < 0.05$ versus control, # $P < 0.05$ versus 15' pulse BK group; + $P < 0.05$ versus peak BK treatment group. Reproduced with permission from Haisenleder *et al.* 2001.

BK+KCl. In contrast, FSH β PT was maximally stimulated by the slower (180-min) pulse interval. This pattern of divergent gonadotropin subunit

responses to pulse frequency is similar to previous findings for GnRH (Haisenleder *et al.* 1997). Thus, intermittent changes in intracellular calcium appear to be important in the transmission of GnRH signals, and may mediate the differential actions of pulse frequency on gonadotropin subunit gene expression.

The downstream mediators of calcium action within the gonadotrope have yet to be characterized. Among the putative candidates is Ca/CaMK II, which is an important mediator of calcium signaling in several cell types, including the pituitary (Miller & Kennedy 1986, Hanson & Schulman 1992, Cui *et al.* 1994, 1996, Schulman *et al.* 1995, Nowakowski *et al.* 1997). We have examined this issue in both primary pituitary and L β T2 cells (Haisenleder *et al.* 2003a,b). GnRH stimulated Ca/CaMK II activity, and activation was mediated via increases in intracellular calcium derived from cellular storage pools and influx from plasma membrane calcium channels. Moreover, inhibition of Ca/CaMK II activation by administering a specific blocker, KN-93, partially suppressed GnRH-induced increases in α , LH β and FSH β PT (Fig. 8) or rat α and LH β promoter activity. These findings support the hypothesis that Ca/CaMK II is a downstream mediator of GnRH/calcium signaling within the gonadotrope.

Protein kinase C (PKC)

Several investigations have shown that GnRH activates PKC, and that cross-talk between PKC, calcium and other intracellular pathways regulates gonadotropin secretion and mRNA expression (Naor 1990, Stojilkovic & Catt 1995b, Kaiser *et al.* 1997a, Ando *et al.* 2001). Studies in rat pituitary cells reveal that PKC stimulates a rise α , LH β and FSH β mRNAs and α transcription (Ben Menahem & Naor 1994, Weck *et al.* 1998, Halvorson *et al.* 1999, Vasilyev *et al.* 2002a). We have shown that PKC stimulates α and LH β gene expression in rat pituitary cells *in vitro*; however, unlike results seen for cAMP and calcium, a pulsatile signal pattern does not play a significant role (Haisenleder *et al.* 1995). Data from other investigations in gonadotrope-derived L β T2, L β T4 or α T3 cells, or lactotrope-derived GGH3–1 cells, demonstrate that PKC mediates the α , LH β and FSH β transcriptional response to GnRH (Sundaresan *et al.* 1996, Weck *et al.* 1998, Vasilyev *et al.* 2002a,b). Kaiser

Figure 8 α , LH β and FSH β primary transcript (PT) responses to pulsatile GnRH with and without the Ca/CAMK II-specific inhibitor, KN-93 (10 μ M). Rat pituitary cells received pulses of GnRH (200 pM, 60-min intervals) for 24 h. Results are expressed as the percent change versus medium-pulsed controls (Con). Note the different scale for each panel. Groups marked with different letters (a, b or c) are statistically different ($P < 0.05$). Reproduced with permission from Haisenleder *et al.* 2003a.

et al. (2000) reported that PKC stimulation of the rat LH β gene is mediated via actions on Egr-1 binding within the proximal GnRH responsive region of the LH β promoter. An investigation of the bovine LH β promoter showed that interactions between Egr-1, SF-1 and Ptx-1 mediate the response to PKC (Tremblay & Drouin 1999). PKC has also been shown to stimulate the expression of the Egr-1 gene (Wolfe & Call 1999). In contrast, α -subunit

transcriptional responses to PKC reflect activation of the MAPK pathway (as discussed below). Characterization of the ovine FSH β promoter showed that PKC activation is mediated through multiple responsive regions, including 2 AP-1 sites (Strahl *et al.* 1998). However, these sites do not appear to play an important role in regulating the ovine FSH β promoter in transgenic mice (Huang *et al.* 2001b). Of interest, responses of individual gonadotropin subunits to PKC stimulation have been shown to differ between published reports, possibly reflecting differences in cell model or experimental paradigm (Saunders *et al.* 1998, Weck *et al.* 1998, Vasilyev *et al.* 2002a,b).

Mitogen-activated protein kinase (MAPK)

A number of investigators have shown that GnRH stimulates activation of various members of the MAPK family, including ERK 1 and 2 (Mitchell *et al.* 1994, Roberson *et al.* 1995, Sundaresan *et al.* 1996, Reiss *et al.* 1997), JNK (Levi *et al.* 1998, Roberson *et al.* 1999, Yokoi *et al.* 2000) and p38 (Roberson *et al.* 1999, Liu *et al.* 2002a). GnRH-induced activation of ERK is mediated through both PKC-dependent and PKC-independent pathways, including calcium (Roberson *et al.* 1995, Sundaresan *et al.* 1996, Reiss *et al.* 1997, Mulvaney *et al.* 1999). Moreover, ERK phosphorylates various transcription factors that may play roles in the regulation of gonadotropin subunit gene expression (such as c-Fos, c-Jun and the Ets protein – ELK-1 (Segar & Krebs 1995, Maurer *et al.* 1999, Liu *et al.* 2002a).

We conducted studies to determine whether ERK plays a role in the differential gonadotrope responses to physiologic GnRH stimulation (Haisenleder *et al.* 1998). Initial work examined whether a pulsatile signal pattern is required to maintain ERK responsiveness to GnRH over longer durations. Groups of adult GnRH-deficient rats were given pulses of GnRH every 60 min for 1–8 h *in vivo*, and were compared with animals that received a continuous GnRH infusion for the same duration. Pulsatile GnRH stimulated a two- to fourfold increase in ERK activity, which was maintained over the 8-h experimental duration. However, continuous GnRH stimulated a transient rise in ERK only for the initial 2 h (Fig. 9). Follow-up studies showed that ERK activity is sensitive to GnRH pulse frequency, with slower

Figure 9 ERK responses to pulsatile or continuous GnRH after 1, 2, 4 or 8 h. GnRH was administered to adult male rats either in a pulsatile (50 ng/pulse, 60-min interval) or continuous manner (25 ng/min; this dose was selected to provide sustained levels of circulating GnRH similar to peak levels obtained after a single 50-ng pulse (that is, 200 pg/ml)). Controls (C) received BSA-saline pulses or a continuous infusion for 8 h. The means $\pm$ S.E.M. are shown ($n=3$ /group, except in the pulsatile control group, in which $n=4$). * $P < 0.05$ versus controls. Reproduced with permission from Haisenleder *et al.* 1998.

(120-min) interval pulses being more effective than faster (30- or 60-min) interval pulses. To determine the role of ERK in mediating GnRH-induced stimulation of gonadotropin subunit gene expression, GnRH pulses were administered to rat pituitary cells *in vitro*, with or without the ERK inhibitor, PD-098059 (PD). As shown in Fig. 10, PD blocked the GnRH-induced rise in α , FSH β and GnRH-R mRNAs, but not LH β . This suggests that GnRH-induced stimulation of LH β utilizes a distinct and different intracellular pathway or pathways. It is interesting to note that ERK is maximally stimulated by slower GnRH pulse intervals, and plays an important role in regulating the expression of two gonadotrope genes that are

also maximally stimulated by slower GnRH pulse intervals (FSH β and GnRH-R).

GnRH activation of the JNK pathway is mediated through PKC activation of the protein tyrosine kinase – cSrc (Levi *et al.* 1998). Recent findings suggest that JNK plays a role in the regulation of the rat LH β promoter, via actions on the proximal GnRH-responsive region of the gene (Yokoi *et al.* 2000). The p38 pathway has also been implicated in the activation of various transcription factors, including c-Jun, c-Fos and Elk-1 (Ham *et al.* 1997, Whitmarsh *et al.* 1997, Roberson *et al.* 1999). Experiments using α T3 cells revealed that PKC mediates the GnRH-induced activation of p38 (Roberson *et al.* 1999). However, while p38 activates downstream pathways known to stimulate α gene expression (Maurer *et al.* 1999), published reports suggest that the signal transduction protein does not stimulate α promoter activity (Roberson *et al.* 1999). Whether p38 regulates the expression of the LH β and FSH β genes remains to be determined.

In summary, present information suggests that differential regulation of gonadotropin subunit gene expression by GnRH involves several mechanisms. GnRH may exert selective actions on specific signal transduction pathways (such as ERK regulation of α and FSH β , but not LH β). Alternatively, the responses may reflect differential sensitivity to intracellular messenger systems (for example, specific gonadotropin subunit genes may be more sensitive to calcium or PKC pathways than other subunit genes). Furthermore, it is likely that the role played by GnRH pulse frequency is selectively to activate one or more intracellular signal transduction pathways that are optimal for a specific gonadotropin subunit gene.

Regulation of subunit gene transcription by steroids

Steroids regulate gonadotropin subunit gene expression by acting either at the hypothalamus to alter GnRH pulsatility or directly on the pituitary gonadotropes. Differential regulation of the subunit genes by steroids was first reported in gonadectomized (GDX) animals. Both E_2 and testosterone suppressed the post-GDX increases in pituitary α and LH β mRNAs. In contrast, the post-OVX increase in FSH β mRNA was only

Figure 10 Gonadotrope mRNA responses to pulsatile GnRH (peak chamber concentration, 100 pg/ml) given every 60 min for 24 h in the presence of PD-098059 (PD) or vehicle in perfused male pituitary cells ($n=4$ /group). The data are presented as the percent change versus medium-pulsed controls (Con). Reproduced with permission from Haisenleder *et al.* 1998.

partially reduced by E_2 or E_2 plus progesterone (P_4), reflecting the importance of ovarian inhibin in suppressing FSH β expression (Gharib *et al.* 1987, Dalkin *et al.* 1990, 1993); in CAST rats, testosterone either had no effect or enhanced FSH β responses in a dose-dependent manner (Gharib *et al.* 1987, 1990a, Wierman *et al.* 1988, 1990, Iliff-Sizemore *et al.* 1990). Experiments in GDX plus GnRH antagonist-treated rats, to eliminate the effects of steroids on GnRH secretion, largely show that *in vivo* steroids inhibit both α and LH β gene expression by suppressing GnRH. In contrast, the

regulation of FSH β by steroids appears to be more complex.

α -subunit

In vivo, E_2 markedly suppresses α -subunit gene transcription. It inhibited the post-OVX increase in α -subunit mRNA synthesis (Shupnik *et al.* 1988), and suppressed the post-OVX or -CAST increases in α promoter activity in transgenic mice harboring either a human or bovine α promoter-reporter construct (Keri *et al.* 1991, Hamernik *et al.* 1992,

Clay *et al.* 1993). However, there is evidence that the suppression of α subunit transcription by E_2 is largely indirect. Shupnik *et al.* determined that E_2 reduces α transcription *in vivo* largely by suppressing hypothalamic GnRH; E_2 had no effect on α -subunit mRNA synthesis in rat pituitary cells *in vitro* (Shupnik *et al.* 1989a, Shupnik 1996) or in OVX rats treated with GnRH antagonist (Shupnik & Fallest 1994). Additionally, a functional estrogen response element (ERE) in the human α promoter has not been identified (Keri *et al.* 1991), and E_2 does not affect human α promoter-reporter activity in α T3 cells, even in the presence of an exogenous human estrogen-receptor (ER) (Clay *et al.* 1993). Although it appears that E_2 regulates α transcription largely via hypothalamic GnRH, it may also have a direct effect on the pituitary. Colin *et al.* (1996) reported that E_2 suppressed basal activity of a human α -promoter-LUC construct in the pituitaries of OVX transgenic mice, but enhanced GnRH responsiveness. We and others have also observed that E_2 potentiates GnRH stimulation of α gene expression, but it is unknown whether the effect is direct or indirect (that is, increasing GnRH receptor numbers) (Mercer *et al.* 1989, Dalkin *et al.* 1990, Kerrigan *et al.* 1993, Turgeon *et al.* 1996, Kawakami & Winters 1999).

In vivo, progesterone (P_4) potentiates the suppressive effects of E_2 on α -subunit mRNA, largely via decreased GnRH secretion (Simard *et al.* 1988, Dalkin *et al.* 1990). The effects of P_4 directly on the pituitary are mixed; P_4 (+ E_2) reduces α mRNA in OVX, hypothalamically disconnected ewes (Di Gregorio & Nett 1995), and either reduces (Dalkin *et al.* 1990) or has no effect in the rat (Kerrigan *et al.* 1993). Little is known about P_4 actions on α -subunit transcription; however, neither P_4 nor the P_4 receptor antagonist RU486 modulated E_2 $\pm$ GnRH-induced changes in human α -LUC activity in pituitary cells from transgenic mice (Colin *et al.* 1996).

Androgens, like estrogens, suppress α subunit expression either by reducing hypothalamic GnRH or by direct action on the pituitary. *In vivo*, testosterone suppressed α mRNA and mRNA synthesis rates in male rats even in the presence of GnRH antagonist (Paul *et al.* 1990). Similarly, testosterone or dihydrotestosterone (DHT) suppressed the post-CAST increases in α promoter activity in transgenic mice harboring either a human or bovine α promoter-CAT reporter gene

(Clay *et al.* 1993). The human α -CAT reporter gene was also repressed by DHT when transiently transfected into α T3 cells along with androgen receptor (AR), with suppression requiring both AR and ligand (Clay *et al.* 1993). A putative androgen response element (ARE) has been identified in the proximal promoter of the human α gene between tandem cAMP response elements (CRE) and a CCAAT element (Clay *et al.* 1993). However, this binding site does not mediate the suppressive effect of androgen on α transcription; instead, AR interferes with the proteins that bind an upstream α -basal element and the CRE (Heckert *et al.* 1997). Specifically, AR suppresses α promoter activity by protein-protein interactions with the two CRE-binding transcription factors cJun and ATF2 (Jorgensen & Nilson 2001a).

The effects of glucocorticoids on α gene expression are inconclusive. *In vivo*, corticosterone (B) either increases (Ringstrom *et al.* 1991), decreases (Kilen *et al.* 1996), or had no effect (McAndrews *et al.* 1994) on α subunit mRNA in rats. In pituitary-derived cell lines, dexamethasone suppressed α -subunit mRNA in α T3 cells (Akerblom *et al.* 1990), had no effect on α mRNA in L β T2 cells (Turgeon *et al.* 1996) or increased α promoter activity in GH3 cells transiently transfected with a human α -CAT construct (Gurr & Kourides 1989).

LH β

Estrogens rapidly suppress the post-OVX increases in LH β mRNA synthesis (Shupnik *et al.* 1988). This reflects a hypothalamic action, as the post-OVX increases in LH β transcription were abolished by a GnRH antagonist (Dalkin *et al.* 1993, Shupnik & Fallest 1994, Fallest *et al.* 1995, Burger *et al.* 2001), and additional treatment with E_2 was no more effective than antagonist only (Shupnik & Fallest 1994). However, estrogens also exert direct action on the pituitary, and E_2 rapidly increased LH β mRNA synthesis rates in pituitary cells from OVX female rats (Shupnik *et al.* 1989a, Shupnik 1996), and in pituitary cells from cycling rats, with the greatest effect in cells from rats in proestrus (Shupnik *et al.* 1989a). Shupnik and coworkers found that ER bound the rat LH β promoter at -1388 to -1105 bp, an area containing a 15 bp imperfect ERE; moreover, promoter-reporter constructs of the rat LH β gene containing the 284 bp

estrogen-responsive region, transiently transfected into GH3 cells, were estrogen responsive in an ER-dependent manner (Shupnik *et al.* 1989b, Shupnik & Rosenzweig 1991). However, the estrogen responsiveness of the LH β promoter may not be conserved across the species. While E₂ suppressed LH β promoter activity in OVX transgenic mice harboring a -776 to +10 bp bovine LH β promoter CAT reporter, a high-affinity binding site for the ER was not found in this portion of the promoter (Keri *et al.* 1994). In addition, E₂ had no effect on bovine LH β CAT activity when transiently transfected into L β T2 cells, even when cotransfected with the human ER (Jorgensen & Nilson 2001b). It remains to be determined whether an estrogen-responsive region of the bovine LH β promoter may be more 5' than the area previously investigated. Alternatively, the ER may modulate promoter activity by interacting with other *cis*-acting elements, as described for androgens on the α and LH β promoters.

There is little information on the regulation of LH β gene expression by either P₄ or glucocorticoids. Progesterone alone had no effect on LH β mRNA levels in OVX rats and when combined with E₂ was no more effective than E₂ alone (Dalkin *et al.* 1990, Kerrigan *et al.* 1993). Similarly, while E₂ blocked bovine LH β CAT promoter activity in OVX transgenic mice, the combination of E₂ and P₄ was no more effective than E₂ alone (McNeilly *et al.* 1996). *In vitro*, neither P₄ nor E₂+P₄ altered LH β mRNA levels in female rat pituitary cells, but P₄ alone or in combination with E₂ augmented the GnRH-induced increase in LH β mRNA, perhaps by blocking LH β mRNA degradation (Park *et al.* 1996). Glucocorticoids do not appear to regulate LH β mRNA; B had no effect on LH β mRNA levels in cultured rat pituitary cells (Kilen *et al.* 1996) and dexamethasone did not alter the activity of the rat LH β promoter (Shupnik & Rosenzweig 1991).

Androgens rapidly suppress the post-CAST rise in LH β transcription. Testosterone inhibited LH β mRNA synthesis in CAST rats (Paul *et al.* 1990) and suppressed the post-CAST increases in LH β promoter activity in transgenic mice (Keri *et al.* 1994, Fallest *et al.* 1995). We examined the effects of testosterone on LH β transcription *in vivo*; testosterone rapidly suppressed LH β PT below basal values in GnRH-deficient CAST rats (Fig. 11) (Burger *et al.* 2004). The suppression of LH β PT

in GnRH-deficient rats *in vivo* suggests that testosterone acts directly at the pituitary, but we found no effect of testosterone on LH β PT in cultured rat pituitary cells (Burger *et al.* 2004). Similarly, DHT had little effect on rat LH β promoter LUC reporter activity in L β T2 cells, but did suppress GnRH-induced LH β promoter activity (Curtin *et al.* 2001). This action required the AR even though a high-affinity binding site for the AR was not found in this construct. Curtin *et al.* (2001) determined that the AR suppressed rat LH β promoter activity by protein-protein interactions with the specificity protein-1 (Sp1) transcription factor, and, to a lesser extent, with the early growth response protein (Erg-1), which are required for GnRH stimulation of LH β transcription (Kaiser *et al.* 2000, Weck *et al.* 2000). In contrast to the rat, the bovine LH β promoter was directly responsive to testosterone (Jorgensen & Nilson 2001b). The AR, in a ligand-dependent manner, suppressed the activity of bovine LH β CAT when transfected into L β T2 cells. The bovine LH β promoter also lacks an AR binding site, and the AR suppressed bovine LH β transcription by protein-protein interactions with steroidogenic factor 1 (SF-1), blocking its interaction with Pitx1 and the transcriptional initiation complex (Jorgenson & Nilson 2001b).

FSH β

The effects of E₂ on FSH β transcription have not been extensively studied, and there appear to be differences among species. *In vivo*, E₂ suppressed the post-OVX increase in FSH β mRNA synthesis in the rat (Shupnik *et al.* 1988). The site of E₂ action in the rat is the hypothalamus, as E₂ did not suppress FSH β transcription in OVX rats treated with a GnRH antagonist (Shupnik *et al.* 1989a) and had no effect on FSH β mRNA synthesis in female rat pituitary fragments (Shupnik & Fallest 1994). In contrast to the rat, E₂ suppressed both steady-state FSH β mRNA and mRNA synthesis rates in cultured ovine pituitary cells (Phillips *et al.* 1988, Baratta *et al.* 2001), and suppressed the activity of an ovine FSH β promoter-LUC construct transfected into ovine pituitary cells (Miller & Miller 1996). The estrogen-responsive region of the ovine FSH β promoter is located at -105 to -84 bp, but does not contain an estrogen-response element or bind ER (Miller & Miller 1996). Instead, this estrogen-responsive region contains two AP-1 sites

Figure 11 The time course of testosterone (T) action on pituitary β -subunit primary transcripts (PT) and mRNAs in GnRH antagonist (GnRH-A)-treated CAST male rats. Castrated rats ($n=4-7$ /group) were treated with GnRH-A LRF-147 (100 μ g, s.c.) every 12 h. Four days after CAST, rats received T implants and were killed 0, 3, 8, 24 and 48 later. All data are presented as percent 0-h T ($\pm$ S.E.) controls. Intact rat ($\pm$ S.E.) control values are represented by shading. Points with different letters are significantly different ($P<0.05$).

and is a GnRH enhancer region of the ovine FSH β promoter (Strahl *et al.* 1998). Although E_2 inhibits FSH β transcription in sheep, it had no effect on an ovine FSH β promoter-LUC construct in female transgenic mice, and the authors suggest that mouse gonadotropes lack some factor making them less responsive to negative feedback by estrogen (Huang *et al.* 2001a). We recently investigated the effects of steroids on FSH β transcription in male rats. Contrary to previous results in females, we found that E_2 markedly suppressed FSH β PT, but not FSH β mRNA, and this may suggest that E_2 differentially regulates FSH β transcription between the sexes in rodents (Burger *et al.* 2004).

FSH β transcription is also regulated by P_4 , and, like estrogens, there appear to be differences between species. Progesterone, in combination with E_2 , increased FSH β mRNA expression in immature rats (Attardi *et al.* 1990) and increased the stimulatory effects of GnRH on FSH β mRNA (versus E_2 alone) (Kerrigan *et al.* 1993). Moreover, the preovulatory FSH surge and the accompanying increase in FSH β mRNA can be suppressed by antiprogestins (Ringstrom *et al.* 1997). Three progesterone-response element

(PRE)-like sequences have been identified in the proximal promoter of the rat FSH β gene; these sites bind P_4 receptor (PR) and render a heterologous promoter-reporter construct P_4 responsive (O'Connor *et al.* 1997, 1999). In contrast, P_4 suppressed endogenous FSH β mRNA synthesis in ovine pituitary cell cultures (Phillips *et al.* 1988). The ovine FSH β promoter contains six PRE-like sequences that bind PR and render a heterologous promoter reporter construct P_4 responsive (Webster *et al.* 1995). However, contrary to the suppressive effects of P_4 on endogenous FSH β transcription, P_4 stimulated an ovine FSH β promoter-LUC reporter construct transfected into ovine pituitary cells (Webster *et al.* 1995).

Glucocorticoids affect FSH β gene expression, and B selectively upregulated FSH β mRNA levels (Ringstrom *et al.* 1991, McAndrews *et al.* 1994). These effects are at the level of the gonadotrope, as B or dexamethasone increased FSH β mRNA in rat pituitary cells (Kilen *et al.* 1996, Bohnsack *et al.* 2000, Leal *et al.* 2003) and in rats treated with a GnRH antagonist (McAndrews *et al.* 1994). Moreover, the effects of B appear to be at the level of transcription, as it increased FSH β mRNA in

female rat pituitary cells, without changing post-transcriptional mRNA decay rates (Kilen *et al.* 1996).

The effects of testosterone on FSH β gene expression in the rat are intriguing. As mentioned previously, testosterone does not suppress the post-GDX increase in FSH β mRNA; instead, it either has no effect or further increases FSH β mRNA expression (Gharib *et al.* 1987, 1990a, Wierman *et al.* 1988, 1990, Iliff-Sizemore *et al.* 1990). Studies in GnRH-deficient rats or in pituitary cell cultures demonstrated that the stimulatory effects of testosterone on FSH β gene expression are at the level of the pituitary (Paul *et al.* 1990, Wierman & Wang 1990, Dalkin *et al.* 1992, Winters *et al.* 1992). Earlier we proposed that testosterone regulated FSH β mRNA stability, since testosterone increased the half-disappearance time of FSH β mRNA from 20 to 50 h, but did not significantly increase FSH β mRNA synthesis (Paul *et al.* 1990). However, we recently reinvestigated the effects of testosterone on FSH β transcription, and found that it rapidly and specifically increases FSH β PT levels at 3–48 h in male rats (Fig. 11) and that the stimulatory effects on FSH β transcription are androgen specific (Burger *et al.* 2004). Testosterone also suppresses FS mRNA, which suggests that testosterone's actions on FSH β transcription may be indirect via activin/FS. Others have reported that the effects of testosterone on FSH β mRNA either require activin (Leal *et al.* 2003) or are blunted by FS (Bohnsack *et al.* 2000). To determine whether testosterone stimulation of FSH β PT reflected the fall in pituitary FS, we treated male rat pituitary cells with testosterone with or without exogenous FS. FS alone suppressed FSH β PT, but did not reduce the testosterone-stimulated increase in FSH β PT, suggesting that testosterone increases transcription by direct action on the FSH β gene. Recently, testosterone (or DHT) was reported to increase the activity of an ovine FSH β promoter-reporter transfected into L β T2 cells (Spady *et al.* 2004). Spady *et al.* (2004) identified three candidate androgen-response elements (AREs) at –245/–231, –212/–198 and –153/–139 bp of the ovine FSH β promoter, which are the same steroid-binding elements identified by Webster *et al.* (1995) as PREs. Only the –245/–231 bp putative ARE bound the AR, although mutations in either the –245/–231 or –153/–139 ARE disrupted the stimulation of the

promoter by DHT (Spady *et al.* 2004). In contrast to the rat, the effects of testosterone on the ovine FSH β promoter are activin dependent. The ability of DHT to stimulate the ovine FSH β promoter is abolished by FS treatment, is increased synergistically by activin, and requires an activin-responsive Smad-binding element (SBE) at –138/–124 bp (Spady *et al.* 2004). Although the AREs identified in the ovine FSH β promoter are well conserved across mammalian species (Spady *et al.* 2004), the stimulatory effects of testosterone on FSH β expression are not. Testosterone suppressed FSH β mRNA in male rhesus pituitary cells (Kawakami *et al.* 2002) and a human FSH β promoter construct in transgenic mice (Kumar *et al.* 1992, Kumar & Low 1993, 1995).

Regulation of FSH β gene transcription by activin, FS and inhibin

Differential synthesis and secretion of LH and FSH, while in part dependent on the hypothalamic GnRH signal pattern, also appears to result from the actions of multiple local and gonadal peptide hormones. The hormones inhibin, activin and FS are produced both in the gonads and, at least for activin and FS, in the pituitary gland. Alterations in the balance of activin and inhibin concentrations, either directly or via changes in FS (for activin), selectively alter FSH secretion by regulating FSH β gene expression (Mather *et al.* 1997).

The inhibins and activins are members of the transforming growth factor- β (TGF β) superfamily. The inhibins, heterodimers of an inhibin α -subunit and one of two β -subunits, β_A (inhibin A) or β_B (inhibin B), are produced primarily by the gonads and act in an endocrine manner to suppress pituitary FSH secretion without affecting LH (Burger *et al.* 1997). The activins are dimers of two inhibin β -subunits. There are at least four forms of the β subunit, β_A – β_D , although the β_A and β_B subunits are important in the regulation of FSH. The activins were discovered because of their ability to stimulate FSH synthesis and release it from pituitary cell cultures (Ling *et al.* 1986, Vale *et al.* 1986), but it is now known that the activins are produced in a wide variety of tissues and act largely in a paracrine/autocrine manner to regulate functions ranging from development to cellular homeostasis. The pituitary expresses β_B , and

activin-B ($\beta_B\beta_B$) is synthesized and secreted in the pituitary, whereas most other tissues produce activin-A (Meunier *et al.* 1988). In addition, the activin receptors types I, IIA and IIB are also present in the rat pituitary (Dalkin *et al.* 1996). FS, a glycoprotein hormone structurally unrelated to the activins and inhibins, binds to and neutralizes the bioactivity of activin. The affinity of FS for activin is high, and the dissociation rate after binding is extremely slow, essentially making the binding event a permanent one (Nakamura *et al.* 1990). FS was discovered in ovarian follicular fluid by its ability to suppress FSH (Esch *et al.* 1987), but, like activin, it is also synthesized in a wide array of tissues, including the pituitary gonadotropes and folliculostellate cells (Kogawa *et al.* 1991a,b, Kaiser *et al.* 1992). Although FS can be detected in the circulation, its ability to suppress FSH is largely due to paracrine and/or autocrine regulation of bioactive activin at the level of the gonadotrope (for review, see Phillips & de Kretser 1998).

Activin is an important regulator of FSH secretion and FSH β gene expression; it increased FSH secretion and FSH β mRNA in a dose-dependent manner in primary rat pituitary cells (Carroll *et al.* 1989, Weiss *et al.* 1993), and increased FSH secretion *in vivo* (Rivier & Vale 1991, Carroll *et al.* 1991, Woodruff *et al.* 1993, Lee & Rivier 1997). The intrapituitary regulation of activin action is important in control of FSH β gene expression; after OVX, pituitary β_B and ActRII mRNAs increase in parallel with FSH β mRNA (Dalkin *et al.* 1994, 1998, Prendergast *et al.* 2004), and GnRH pulse frequencies that maximally stimulate FSH β mRNA also stimulate β_B mRNA (Dalkin *et al.* 1999). Activin increases FSH β mRNA expression by increasing transcription. Activin increased FSH β PT in rat pituitary cells, an effect which could be blocked by the transcription inhibitor actinomycin D (Weiss *et al.* 1995, Bernard 2004). Activin has also been reported to stimulate ovine, rat and mouse FSH β promoter-reporter constructs (Huang *et al.* 2001b, Pernasetti *et al.* 2001, Dupont *et al.* 2003, Suszko *et al.* 2003, Bailey *et al.* 2004, Bernard 2004). In light of these data, recent studies have begun to focus on the mechanism(s) whereby activin signaling regulates promoter activity for the FSH β gene.

Activin signaling requires a series of events, including the activation of intracellular messengers (for review, see Attisano & Wrana 1996, Pangus &

Woodruff 2000, Derynck & Zhang 2003). Briefly, activin binds to its type II receptor subunit, either ActRII or IIB, which then pairs with a type I receptor subunit (either ActRI or IB), forming a heteromeric complex at the cell surface. Then serine/threonine kinase activity of the type II subunit phosphorylates the type I subunit, initiating post-receptor signaling/phosphorylation. Although a number of signal cascades may be activated as the result of activin/activin receptor association, the predominant activin-signaling pathway includes the mothers against dpp-related (Smad) proteins.

Eight Smad proteins (Smads 1–8) conduct the intracellular signals for the TGF β super family from receptors to the nucleus (for review, see Derynck & Zhang 2003). For the activin signaling system, receptor-activated Smads2 and 3 are phosphorylated by ActRI and then partner with co-Smad4, which then binds to DNA to regulate gene activity. In pituitary cells, activin-induced increases in FSH β transcription were correlated with increased Smad2 and 3 phosphorylation (Dupont *et al.* 2003, Bernhard 2004). Additionally, rat or mouse FSH β promoter activity was increased by overexpression of Smad3 and attenuated when Smad2 or 3 protein were suppressed (Dupont *et al.* 2003, Suszko *et al.* 2003, Bernhard 2004).

The association of Smad2/3 with Smad4 and the formation of dimers is an important step in signal transduction from the cell surface (Lagna *et al.* 1996). Smad4 appears essential for activin signaling, as transfection of a constitutively active Smad4 construct alone can induce activin-like effects, whereas cellular expression of Smad2/3 alone does not confer responsiveness (Lagna *et al.* 1996), and in pituitary cells expression of Smad4 greatly enhances the ability of Smad3 to increase rat FSH β promoter activity (Suszko *et al.* 2003). Additionally, Smad2/3 activity is regulated by 'inhibitory' Smad7, which binds the type I receptor and prevents Smad2/3 phosphorylation. Smad7 gene expression is detected in both normal pituitary cells and gonadotrope cell lines, and overexpression of Smad7 in L β T2 cells disrupted activin-induced increases in FSH β gene expression (Dupont *et al.* 2003, Bernhard 2004).

To date, data suggest that there is a Smad-binding element (SBE) comprising CAGA- or GTCT-like sequences that are required, but are not sufficient, for Smad binding (Shi & Massague 2003). The affinity of Smads for SBEs is low, and

gene activation requires the presence of other cofactors. Some of these transcriptional regulators are specific to the FSH β gene, as recent reports suggest the importance of the nuclear factor Pitx2, a member of the Pitx subfamily of bicoid-related homeodomain factors (Suszko *et al.* 2003). In that report, critical regions within the rat FSH β promoter were identified for Smad3/4 binding (–281 to –253 bp) and Pitx2 (–230 to –199 bp); more importantly, both Smads and Pitx2 may physically interact in the regulation of the FSH β promoter activation. However, the critical SBE in the rat promoter is conserved only in rodent species. In the ovine FSH β promoter, there are three regions (–973/–962, –167 and –134) required for full activin responsiveness (Bailey *et al.* 2004). The distal –973/–962 site bound Smad4 protein, and the critical –134 site bound Smad4 in association with the TALE homeodomain proteins Pbx1 and Prepl, and the two proximal activin responsive regions (–167 and –134) are conserved across species and also bind Pbx1 and Prepl in the mouse gene (Bailey *et al.* 2004).

Limitation of activin action appears to be an essential component of physiologic gonadotrope function. In addition to the intracellular inhibitor Smad7 described above, the extracellular inhibitors FS and inhibin play key roles in limiting activin action. In the rat pituitary, FS is upregulated by activin, GnRH and PACAP, and is suppressed by testosterone and by FS itself, probably through binding to activin (Simonaka *et al.* 1991, Kirk *et al.* 1994, Winters *et al.* 1997, Burger *et al.* 2003, 2004). Conversely, in primate pituitary cultures, GnRH is ineffective, and testosterone (as well as activin) increases FS expression (Kawakami *et al.* 2002). Current thoughts regarding the physiologic role of pituitary-derived FS remain speculative, as a FS-deficient cell model is lacking and knockout of the FS gene is lethal (Matzuk *et al.* 1995). However, it is widely regarded that pituitary FS influences the FSH response to hypothalamic and gonadal stimuli. FS expression increases after CAST in adult male rats (Kaiser & Chin 1993) and during the reproductive cycle (Halvorson *et al.* 1994). Additionally, there is a reciprocal relationship over time between FS and FSH β transcription after GnRH pulses; fast-frequency GnRH pulses stimulated FSH β only transiently, and this was followed by an increase in FS mRNA, whereas slow-frequency pulses suppressed FS and increased

FSH β , implying that FS attenuates the FSH response (Burger *et al.* 2002). In male primates, by contrast, FS mRNA is unaffected by CAST, and FSH β mRNA increases by about 50-fold (Winters *et al.* 2001). Thus, FS appears to function as a brake on FSH production in rodents, but its role in other mammalian species is less certain.

Circulating inhibin is also important in antagonizing the action of activin on pituitary FSH. Serum inhibin levels in the rat are regulated across the estrous cycle and are greatest early in the cycle, peaking at proestrus and declining to their lowest levels at estrus. Low inhibin levels at estrus are coincident with increased FSH secretion, steady-state FSH β mRNA, and FSH β transcription associated with the secondary FSH surge (Shupnik *et al.* 1989a, Haisenleder *et al.* 1990, Woodruff *et al.* 1996). As mentioned previously, after OVX and the loss of circulating inhibin, there is a rapid and GnRH-independent increase in both FSH β mRNA and FSH β PT, which can be mimicked by giving inhibin- α antisera to intact rats (Burger *et al.* 2001). Inhibin administration also decreased ovine FSH β promoter-LUC activity in pituitary cells from transgenic mice and rapidly suppressed FSH β transcription in OVX rats and ewes (Burger *et al.* 2001, Huang *et al.* 2001a, Clarke *et al.* 1993). In addition to suppressing FSH β transcription, inhibin may also regulate the stability of the FSH β mRNA. In rats, inhibin suppressed FSH β mRNA levels with a half-life of 94 min, much shorter than the 40-h half-life of FSH β mRNA *in vivo* (Burger *et al.* 2001). In sheep, inhibin suppressed FSH β transcription by only 50% but mRNA levels by 100% (Clarke *et al.* 1993). Suppression of FSH β mRNA by inhibin in rat pituitary cells could also be blunted by inhibitors of translation, suggesting that inhibin stimulates transcription of a protein that reduces FSH β mRNA stability (Attardi *et al.* 1991, Attardi & Winters 1993).

The mechanism for the effects of inhibin on FSH β transcription are not clear; the inhibins do bind the activin type II receptors, although with much lower affinity than activin, but this binding does not promote association with the type I receptor, initiate intracellular Smad signaling, or appear to have its own signaling pathway (Mathews & Vale 1991, Attisano *et al.* 1992, dePaolo 1997), suggesting that inhibin may work as a functional antagonist for activin at the level of the activin receptor. However, in view of inhibin's low affinity

for the type II receptors, this does not explain inhibin action even in the presence of excess activin. Recently, two potential receptors/coreceptors for inhibin have been identified: betaglycan and inhibin-binding protein/p120 (InhBP/p120) (Chong *et al.* 2000, Lewis *et al.* 2000). However, new data suggest that betaglycan may be of greater physiologic importance (Chapman *et al.* 2002). Transfection of COS-7 or HEK293 cells with an expression vector encoding the full-length InhBP/p120 cDNA (alone or in addition to either of the type II activin receptors) failed to induce specific inhibin binding. In contrast, transfection with constructs containing full-length betaglycan conferred high-affinity inhibin binding with formation of an inhibin/betaglycan/type II activin receptor complex. Furthermore, betaglycan immunoreactivity localizes with gonadotrope cells in the pituitaries of both male and female rats and is correlated with high-serum inhibin, low-serum FSH, and low-pituitary FSH β immunoreactivity just before and after the FSH surge in cycling rats (MacConell *et al.* 2002, Chapman & Woodruff 2003).

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