Winter 2000

Human Gonadotropin-Releasing Hormone (hGnRH) Gene Expression and Hormone Regulation in Human Placental JEG-3 Cells

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by

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A Dissertation Submitted to the Faculty of Old Dominion University and Eastern Virginia Medical School in Partial Fulfillment of the Requirement for the Degree of

DOCTOR OF PHILOSOPHY

BIOMEDICAL SCIENCE

OLD DOMINION UNIVERSITY AND EASTERN VIRGINIA MEDICAL SCHOOL
December 2000

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ABSTRACT

HUMAN GONADOTROPIN-RELEASING HORMONE (hGnRH) GENE EXPRESSION AND HORMONE REGULATION IN HUMAN PLACENTAL JEG-3 CELLS

Heming Zheng
Eastern Virginia Medical School And Old Dominion University, 2000
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Using the human placental choriocarcinoma JEG-3 cell line as an *in vitro* human placental model, I studied the mechanisms of the tissue-specific expression and steroid hormone regulation of the hGnRH gene in the human placenta. The results showed that all of the previously identified four elements are required for the full activity of the hGnRH upstream promoter in JEG-3 cells, while the element 4 (FP4, -987/-968) is the most important. Studies performed with 5' end deletion of this region confirmed these observations. Further, supershift assay using Oct-1 antibody demonstrated the involvement of Oct-1 in the FP4 DNA-protein interaction in JEG-3 cells.

Transient transfection studies showed that hERα and hERβ mediate inhibitory effects of estradiol on the hGnRH upstream promoter in JEG-3 cells in a receptor-mediated and dose-dependent manner, while hERβ acts to a lesser extent than hERα. Also, hERα and hERβ exhibit distinctive actions in directing the effects of estrone, estradiol, and estriol on the upstream promoter.

Furthermore, mutagenesis studies confirmed the negative (-991 to –935) and positive (-827 to –730) estrogen responsive elements in the hGnRH upstream promoter. However, gel shift assay using hERα protein did not cause any

shifting, suggesting that ER may not be directly involved in the DNA-protein binding.

In addition, progesterone exhibited a stimulatory effect on the hGnRH upstream promoter activity in JEG-3 in a receptor-mediated and dose-dependent manner. PR-B mediated a more potent stimulatory effect than PR-A. Moreover, exogenous expressions of coactivators SRC-1 and CBP, independently and synergistically, upregulated the PR-A and PR-B mediated progesterone effects on the hGnRH upstream promoter in JEG-3 cells.

Taken together, multiple cis-regulatory elements and trans-acting factors involved in the regulation of the hGnRH upstream promoter activity in JEG-3 cells. Different steroid hormone receptor isoforms mediate distinctive effects on the hGnRH upstream promoter activities. Although several hormone responsive elements have been confirmed, there is no evidence for direct binding of hormone receptors with the upstream promoter. Further studies are needed to identify the trans-regulatory proteins important for the expression and regulation of the hGnRH gene in the placental cells.

ACKNOWLEDGMENTS

I would like to appreciate my advisor, Dr. Ke-Wen Dong, for his guidance, support, as well as patience in education and research during my Ph.D. studies. Also, I thank my committee members, Dr. Frank Castora, Dr. Xi Jiang, and Dr. Sergio Oehninger for their advice, patience, and help, as well as Dr. Christopher Osgood and Dr. Kenneth Sommers's advice in my Ph.D. studies.

Special thanks are extended to Dr. Zhenguang Chen, Dr. Tingfung Chi, Susan Gilben, Usa Kullaprawithaya, Zhiyong Lin, Shelly, Zhaoyang Wen, Susan Wilson, Hua Zhao for their support, cooperation, and friendship during my Ph.D. studies.

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INTRODUCTION

Gonadotropin-releasing hormone (GnRH) is the hypothalamic-releasing factor that controls the biosynthesis of the pituitary gonadotropin subunits and, ultimately, reproductive competence. Generally, GnRH is produced in the hypothalamic neurons and secreted into the hypophysioportal circulation. At the level of the pituitary, GnRH acts through its receptor to stimulate the synthesis of the α subunit, LH β subunit, FSH β subunit genes in the gonadotrophs. The biologically active dimmers LH and FSH are then secreted in a pulsatile manner into the peripheral circulation and control both gametogenesis and steroidogenesis in the gonad. In addition, GnRH may serve an important role in mediating sexual behavior directly through GnRH neurons in the brain and/or indirectly through the stimulation of gonadotrophins that subsequently affect target organs. During the past decades, the studies in understanding the function and regulation of GnRH have been primarily concentrated in the hypothalamus. It has been demonstrated that GnRH is regulated by gonadal steroids and peptides, neurotransmitters, other hypothalamic-releasing factors, and growth factors in the hypothalamus.

However, the expressions of GnRH gene and GnRH receptor gene have also been demonstrated in non-neural tissues, especially in reproductive tissues. The immunoreactive GnRH has been found in ovary (Aten et al., 1987), testes

The model journal used for this dissertation was Molecular and Cellular Endocrinology.

(Bhasin et al., 1983), mammary gland (Seppala et al., 1980), and placenta (Petraglia et al., 1990). The GnRH receptor-binding activity has also been demonstrated in the ovary (Latouche et al., 1989; Koves et al., 1989), mammary gland (Eiden et al., 1987), and placenta (Currie et al., 1989). These data together with the demonstration of the presence of GnRH mRNA (Dong et al., 1993; Ikeda et al., 1996 and 1997) and GnRH receptor mRNA (Peng et al., 1994; Lin et al., 1995; Minaretzis et al., 1995) in reproductive tissues strongly indicate that GnRH and GnRH receptor are produced locally in reproductive tissues and act as a local regulatory factor. Therefore, in addition to the classic endocrine function, GnRH may act as an autocrine and/or paracrine factor in local regulation of reproductive functions. It has been found that GnRH exerts multiple extrapituitary actions (Hsueh and Jones, 1981) on reproductive tissues and directly modulate reproductive functions (Hsueh and Erichson, 1979; Hilensjo and Lemaire, 1980; Huseh et al., 1988) through GnRH receptor present in these tissues (Jones et al., 1980; Piper et al., 1981). In the hypophysectomized rat, the action of GnRH in the ovary has been suggested to exert in an autocrine or paracrine manner, and treatment with high concentration of GnRH induces ovarian oocyte maturation (Aaron et al., 1985). GnRH also acts directly at the ovary to alter basal, as well as LH- and FSH-induced, steroidogenesis (Olsson et al., 1990; Bussenot et al., 1993; Peng et al., 1994). Some studies on reproductive cancer cell lines have demonstrated that GnRH analogs can exert direct antiproliferative effects *in vitro* (Emons et al., 1993; Keri et al., 1992; Baumann et al., 1993). All these data

support the role of GnRH as an autocrine and/or paracrine factor in local regulation function in reproductive tissues.

Human placenta is a unique organ that serves to transmit nutrients to the fetus and waste products from the fetus to the maternal circulation. The placenta also exerts hormonal modulation of maternal metabolism at different stages of gestation. The functional unit of the placenta is the chorionic villus, which has a central core of capillary network for the fetal circulation surrounded by the inner syncytiotrophoblast and outer cytotrophoblast. A plethora of data indicate that in addition to the pituitary trophic hormone (such as the placental proteins – human chorionic gonadotropin [hCG], human chorionic somatomammotropin [hCS], and human chorionic corticotropin [hCC]), hypothalamic peptides, including GnRH, corticotropin-releasing hormone (CRH), and somatostatin, are also synthesized by trophoblastic cells. Furthermore, the presence of many of the growth factors and their receptors, as well as the inhibin family of gonadal peptides has been demonstrated as well. Taken together, the endogenous placental regulation of its hormonal products simulates a miniature hypothalamic-pituitary-target hormone unit. Among many of the analogues of the hypothalamic hormones produced in the placenta, GnRH exerts important effects on the autocrine/paracrine hormonal regulation within the placenta.

1. Important roles of GnRH in the physiological regulation of pregnancy

GnRH was first demonstrated in the human placenta in 1975 (Gibbons et al., 1975). Since then, the expression of both GnRH gene (Dong et al., 1993) and its

receptor gene (Peng et al., 1994) in the placenta has been well established. The findings that the contents of bioactive GnRH (Gibbons et al., 1975) and/or the GnRH receptor (Carrie et al., 1989) in the human placenta vary with gestational age suggested the important roles for GnRH during pregnancy. Further studies demonstrated that the placental GnRH is involved in the physiological regulation of production of hCG (Butzow et al., 1982), steroids and prostaglandins (Siler-Khodr et al., 1986). The placental GnRH also exhibits effect on interacting systems, such as CRH, and may be involved in the physiological regulation of pregnancy. Using in situ RT-PCR and immunocytochemistry, GnRH and GnRH receptor gene expression have been co-localized both in the cytotrophoblast and syncytiotrophoblast in first and third trimester placenta (Wolfahrt et al., 1998), further indicating autocrine/paracrine regulation by GnRH in the human placenta.

1.1. Placental GnRH regulates hCG release during the course of human pregnancy

Physiological evidence has shown that maternal serum hCG levels vary in accordance with changes in the serum level of bioactive GnRH. The placental GnRH has also been demonstrated to actively stimulate the release of hCG from cultured trophoblasts (Khodr et al., 1978; Siler-Khodr et al., 1981; Butzow et al., 1982; Petraglia et al., 1987). The effect of GnRH on hCG production varies with gestational age and can be blocked by GnRH antagonist. Further evidence showed that placental GnRH mediated *in vitro* opioids regulation of hCG release from term trophoblast tissue (Cemerikic et al., 1994). On the other hand, the

changes of placental GnRH receptor mRNA expression paralleled the time course of hCG secretion during pregnancy, suggesting that the paracrine/autocrine regulation of hCG secretion by placental GnRH may be mediated by a decline in GnRH receptor gene expression from the first trimester to term placenta (Lin et al., 1995).

1.2. Placental GnRH affects the placental steroidogenesis competence within different gestational ages

Siler-Khodr et al. (1983) showed that GnRH analogs could affect placental hormone production and the outcome of pregnancy. Further, GnRH modulated the placental production of progesterone and estradiol in human placental cells of different gestational ages (Siler-Khodr et al., 1986; Brnachaud et al., 1983; Ringler et al., 1989). Since estrogen is responsible for the developmental regulation of placental 11β-HSD/corticosteroid metabolism and activation of the fetal HPAA, it is most likely that GnRH is involved in maturation of the fetus via estrogen. However, recent studies in the placental JEG-3 choriocarcinoma cells, which more closely resemble term placenta, showed that GnRH does not affect steroidogenesis (Bauer et al., 1998). Thus, depending on physiological circumstances, GnRH affects steroid hormone synthesis in pre-term placenta, but might have no effect on near term placenta.

1.3. GnRH modulates prostaglandin release during gestation

The production of human placental prostaglandin varies during gestation and its release *in vitro* is affected by GnRH, which related to the gestational age of placenta (Haning et al., 1982). Siler-Khodr et al. (1986) further confirmed these results, showing the dependence of first trimester placental prostaglandin production on endogenous GnRH. Since prostaglandins play an important role during pregnancy and labor, GnRH may be an important factor in the normal physiology of pregnancy.

1.4. In vivo evidence of GnRH effects on placental tissue

In vivo studies on primate animal models and humans also support the notion that GnRH plays an important role in the physiology of pregnancy. For example, addition of GnRH antagonists to pregnant baboons in early pregnancy reduced the production of hCG and progesterone (Das et al., 1985). By measuring maternal circulating GnRH levels during pregnancy, Siler-Khodr et al. (1984) have found the women with abnormally low levels of circulating GnRH underwent premature labor or premature delivery.

1.5. The role of GnRH in preimplantation embryonic development

Recent studies (Raga et al., 1999) on pre-implantation murine embryos at various developmental stages showed that GnRH agonist increased, whereas GnRH antagonist decreased, preimplantation embryonic development. Further, GnRH antagonist was able to completely block embryo development and this effect was reversed by increasing concentrations of the agonist. This indicated

that GnRH might have an important role in preimplantation embryonic development as well.

2. Expression of the GnRH and GnRH receptor genes in the placenta

The human cDNA for GnRH was first cloned by Seeburg and Adelman in 1984 (Seeburg et al., 1984). It is a single-copy gene that located on chromosome 8, consisting of four exons and three introns (Fig. 1A). The GnRH gene transcribes an approximate 600 bp pro-GnRH mRNA, which encodes a precursor protein for the GnRH decapeptide. The GnRH precursor protein is well conserved across species in the region of the signal peptide and the decapeptide GnRH but diverges in the sequences encoding GAP (GnRH-associated peptide). In addition to the hypothalamus where the GnRH gene primarily expressed, increasing evidence has demonstrated the expression of GnRH gene in non-hypothalamic tissues, particularly in reproductive tissues. The non-hypothalamus expression of GnRH gene was further supported by the identification of an upstream GnRH promoter especially used in reproductive tissues, while the downstream promoter is mainly utilized by hypothalamic neurons (Fig. 1B) (Dong et al., 1993).

2.1. The existence of bioactive and/or immunoreactive GnRH-like activity in the placenta

The presence of GnRH-like peptide was first reported in the human placenta in 1975 (Gibbons et al., 1975). Using carboxylmethyl-cellulose ion-exchange

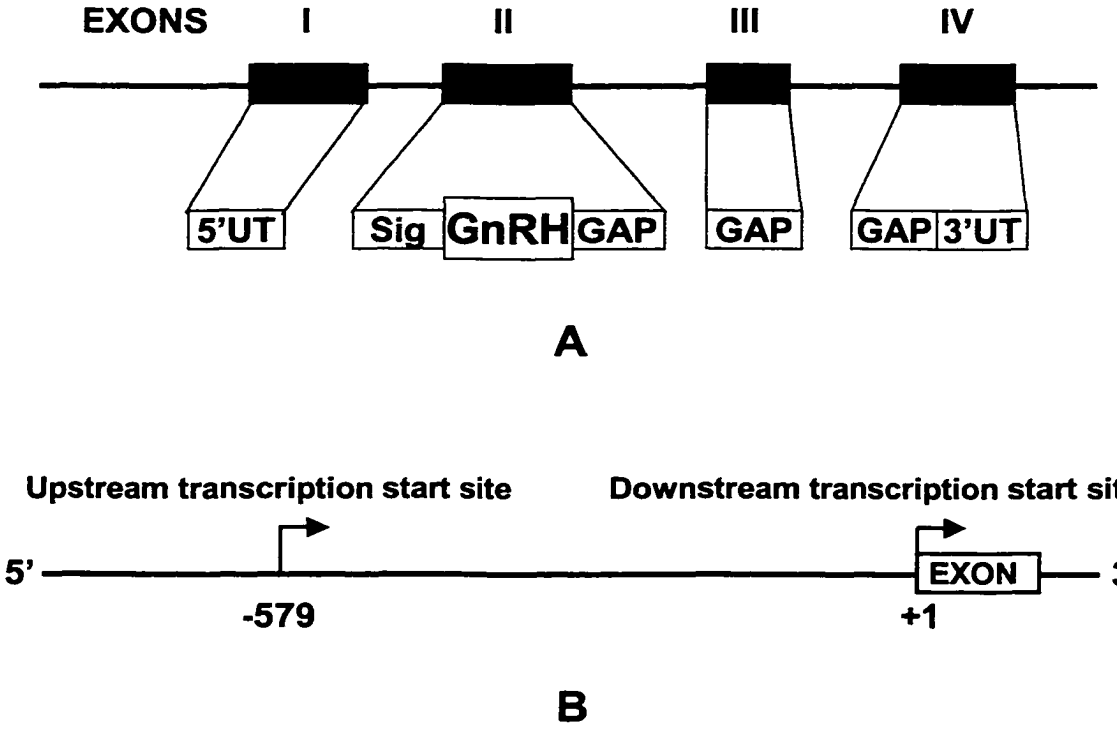


Fig. 1. The GnRH gene. A: The GnRH gene is coded for by four exons and three introns. Exon 1 encodes the 5'-untranslated region (5'UT) and exon 2 the signal peptide (Sig), the decapeptide GnRH, and GnRH-associated peptide (GAP). Exon 3 also codes for GAP, and exon 4 codes for the carboxyl region of GAP and the 3'-untranslated region (3'UT). B: Two transcriptional start sites of the human GnRH gene.

chromatography, Gibbons' group demonstrated that human chorionic membrane and placental tissue contain GnRH-like substances. Homogenates prepared from fresh human term placenta caused release of luteinizing hormone when bioassayed in rats (Gibbons et al., 1975). Further, immunoreactive GnRH-like peptide was found in the human placenta by using radioimmunoassay and immunofluorescence microscopy (Siler-Khodr et al., 1978). Khodr et al. (1980) subsequently demonstrated that GnRH is synthesized by the placenta and can stimulate LH/hCG *in vitro* and *in vivo*. Moreover, GnRH antagonists prevent the GnRH-induced increase in hCG release as well as decrease basal hCG production in the human placenta (Siler-Khodr et al., 1983). In addition to the human placenta, the placentas of other animals were also found to contain GnRH-like peptide. Using GnRH radioimmunoassay, Nowark et al. (1984) demonstrated the presence of immunological GnRH-like activity in rabbit placenta collected in day 18 of gestation. The presence of bioactive and higher molecular forms of GnRH in the rat placenta was also reported (Sarkar, 1986). Thus, bioactive and/or immunoreactive GnRH-like peptide exists in the placenta.

Within the placenta, the presence of immunoreactive GnRH has been localized in the cytotrophoblast, villus stroma, and syncytiotrophoblast. Using immunofluorescent localization, Khodr et al. (1978) demonstrated that the GnRH activity could be localized predominantly in the cytotrophoblast of the 10-12 week human placenta. GnRH has also been found in the syncytiotrophoblast in very early gestation (Seppala et al., 1980). Immunocytochemical studies further confirmed that in placental villi at term, immunoreactive inhibin α and β A

subunits and GnRH had a distribution that was superimposable in several areas. Both the outer layer and the inner trophoblasts contained immunoreactive hormonal products (Petraglia et al., 1992). These findings provide an anatomical basis for a paracrine/autocrine modulation of GnRH in placental hormonogenesis.

The GnRH not only presents in the placenta, its level varies in accordance with different gestation periods as well. Radioimmunoassay studies in the human placenta indicated that the concentration of GnRH varies according to the duration of pregnancy but not the sex of the fetus. The human placenta of 15-40 weeks gestation contains large amounts of GnRH-like activity with the highest concentration in early gestation (Siler-Khodr et al., 1978). The total immunoreactive GnRH content in the placenta, as measured by radioimmunoassay, progressively increases during the first 24 weeks of gestation and remains relatively constant in the third trimester. Using a quantitative immunohistochemical method, Miyake et al. (1982) detected the most intense staining for GnRH in the placenta during the 8^{th} week of gestation with low staining during the remaining gestation period. The variable content of GnRH in the placenta has also been demonstrated in the rat (Sarkar, 1986). These data support the important role of placental GnRH during gestation.

2.2. Expression of GnRH gene in the placenta

Immunocytochemical studies have demonstrated the presence of the GnRH peptide, but do not address whether the GnRH is maternal, fetal, or placental in

origin. To confirm the local production of the placental GnRH, many studies have been conducted to demonstrate the GnRH gene expression in the placenta. In 1984, Seeburg and Adelman first reported the cloned genomic and cDNA sequences encoding the precursor form of GnRH in the human placenta. These DNA sequences code for a protein of 92 amino acids in which the LHRH decapeptide is preceded by a signal peptide of 23 amino acids and followed by a Gly-Lys-Arg sequence, as expected for enzymatic cleavage of the decapeptide from its precursor and amidation of the carboxy-terminal of LHRH (Seeburg et al., 1984). Furthermore, using an avidin-biotin-Cy.5 detection system in conjunction with laser scanning confocal microscopy, the presence of pro-GnRH was shown to be present in both cytotrophoblasts and syncytiotrophoblasts of placental villi. Southern blot and DNA sequence analysis showed that the hybridized transcript is identical to the hypothalamic GnRH. Immunocytochemical staining using an antiserum to amino acids 6-16 of pro-GnRH demonstrated the presence of translated pro-GnRH in both the cytotrophoblast and syncytiotrophoblast epithelia (Duello et al., 1993).

Regarding the gestational-related changes of the placental GnRH mRNA content, Kelly et al. (1991) using solution hybridization/ribonuclease protection assay demonstrated that the levels of GnRH mRNA remain constant throughout gestation, in contrast to the observation that immunoreactive GnRH varies with the gestational age. This is supported by the findings that the changes of GnRH receptor mRNA parallel the time course of hCG secretion during pregnancy (Lin et al., 1995). It is most likely that the changes of GnRH receptor level, rather than

the GnRH level, is responsible for the dynamic changes in GnRH mediated hCG secretion.

Because the expression of hGnRH gene in the human placenta primarily uses the upstream transcription start site, the upstream promoter may direct the tissue-specific expression of hGnRH in the human placenta. Dong et al. (1993) demonstrated that the upstream transcription start site (-579 bp from downstream transcription start site) lacks the TATA and CAAT elements often present in RNA polymerase II promoters, but contains the sequence GGTCTTGCT located 84 bp 5' to the upstream transcriptional start site similar to other genes that lack TATA/CAAT boxes. Further, human and monkey are highly homologous in the upstream promoter region of GnRH gene with a similar gene expression pattern but different from those of rat and mouse (Dong et al., 1996), suggesting that the dual promoters of GnRH gene are conserved within primates. Subsequent studies also demonstrated that the mRNA transcribed from upstream promoter is capable of directing translation into GnRH precursor *in vitro*, and identified the region between –1048 bp and –723 bp important for the upstream promoter activity in the human placenta (Dong et al., 1997). Thus, the upstream promoter of GnRH gene directs the tissue-specific expression of GnRH gene in the placenta.

2.3. Expression of GnRH receptor gene in the placenta

GnRH stimulates the release of hCG from cultured placental cells and placental explants in a dose-dependent manner (Khodr et al., 1978; Butzow et

al., 1982). This stimulatory action of GnRH can be blocked by GnRH antagonists (Currie et al., 1992; Barnea et al., 1991). These findings indicate that the role of GnRH in the placenta is receptor mediated. Further studies demonstrated the presence of highly specific, moderate affinity GnRH binding sites in the human placental membrane (Currie et al., 1989; Belisle et al., 1984; Iwashita et al., 1986; Bramley et al., 1992). However, placental GnRH binding sites differ from those of pituitary GnRH receptors. First, placental GnRH binding sites do not distinguish between GnRH and agonist analogues with super-activity in the pituitary. Hence, rat pituitary and human placental binding sites have similar potencies for GnRH, but different potencies for GnRH agonists. Second, pituitary GnRH receptors do not bind non-mammalian isoforms of GnRH, whereas human placental GnRH binding sites bind chGnRH II, sGnRH. Third, a number of GnRH antagonists with high affinity for pituitary GnRH receptors bind poorly to human placental binding sites (Bramley et al., 1994).

Using *in situ* hybridization, Lin et al. (1995) demonstrated that GnRH receptor mRNA is expressed in both cytotrophoblasts and syncytiotrophoblasts and exhibits changes paralleling the time course of hCG secretion during pregnancy, further supporting autocrine/paracrine modes of action for GnRH in regulating hCG secretion. Thus, it is most likely that placental GnRH is a primary autocrine/paracrine regulator of hCG secretion, and that the functionality of the GnRH-hCG system is controlled by the changes in GnRH receptor gene expression during the course of human pregnancy.

3. Regulation of the GnRH secretion in the placenta

A plenty of data indicate that the placenta produces a variety of pituitary and hypothalamic-like hormones and peptide, growth factors and their receptors, as well as the inhibin family of gonadal peptides. Further evidence reveals that the hormonal regulation within the placenta may be paracrine, autocrine, or endocrine, simulating a miniature hypothalamic-pituitary-target hormone unit, in which GnRH has an important central role.

3.1. Steroid hormone regulation of placental GnRH secretion

Steroid hormones play important roles in the physiology of pregnancy. For example, estrogen and progesterone are involved in initiating and maintaining pregnancy, while glucocorticoids affect parturition. Increasing evidence has indicated that steroid hormones can modulate GnRH release in the placenta. Studies conducted in placental cultures showed that both estriol and estradiol increased, while progesterone decreased, the immunoreactive GnRH (irGnRH) release induced by 8-bromo-cAMP. The stimulatory effect of estriol or estradiol was reversed by the concomitant addition of progesterone. Estriol increased, while progesterone decreased, the secretagogue effect of activin on irGnRH release from cultured placental cells. The action of estriol was counteracted by both tamoxifen, an estrogen antagonist, and progesterone. The inhibitory effect of progesterone was completely reversed by RU486, a specific progesterone receptor antagonist. Thus, steroid hormones and opiate receptor agonists influence irGnRH release from human cultured cells, suggesting that local

interaction between steroids and peptides modulates irGnRH release from human placenta (Petraglia et al. 1990). These observations are consistent with previous studies of estrogen's increasing and progesterone's decreasing basal and GnRH-stimulated hCG release and hCG mRNA levels (Ringler et al., 1989; Wilson et al., 1984; Maruo et al., 1986). In human placenta, the presence of estrogen receptor and progesterone receptor has been demonstrated (Younges et al., 1981; Chibbar et al., 1995; Shanker et al., 1997 and 1998; Rossmanith et al., 1997), further supporting the concept that steroid hormones modulate placental GnRH secretion.

3.2. Activin and inhibin regulation of placental GnRH secretion

Since activin and inhibin, which are gonadal glycoproteins but possess opposing activities, are also produced in the placenta (Meunier et al., 1988; Petraglia et al., 1989), they may modulate placental hCG production by regulating GnRH activity as well. The addition of inhibin antiserum to placental cell cultures increased GnRH release and a parallel rise in hCG secretion. This effect was reduced by addition of a GnRH antagonist, suggesting the interaction of inhibin and GnRH on hCG secretion. Furthermore, addition of purified inhibin had no effect on hCG or GnRH production but reversed GnRH induced hCG secretion from cultured placental cells. In contrast, activin increased the GnRH induced secretion of hCG in cultured trophoblast cells, an effect that was reduced by the addition of inhibin (Petraglia et al., 1989). These results suggest that

inhibin and activin may play a role in regulating the release of GnRH, hCG, and progesterone from placenta.

3.3. Regulation of placental GnRH secretion by other biomolecules

Evidence has shown that cell membrane depolarization stimulates the release of placental GnRH by promoting the influx of calcium into the cell as occurs in the hypothalamus. Prostaglandins (PGE2 and PGF2) and epinephrine stimulate the release of GnRH from placental cells, probably acting through cAMP. The epinephrine-induced GnRH release can be reversed by propranolol (a β-adrenergic receptor antagonist), suggesting the involvement of β-adrenergic receptors in regulating placental GnRH release (Petraglia et al., 1987). Insulin and vasoactive intestinal peptide can also stimulate placental GnRH release in a dose-dependent manner (Petraglia et al., 1990), as can EGF (Barnea et al., 1990).

4. Regulation of the GnRH gene expression in the placenta

Complex mechanisms have evolved to ensure proper spatial and temporal expression of genes in differentiated tissues. In part, this control is achieved at the transcriptional level, so that regulatory regions containing multiple cis-acting sequence elements control both the level of gene expression and its restriction to appropriate cell types. Simply, tissue-specific gene expression is achieved through DNA binding of a transcription factor restricted to a particular cell type (Johnson et al., 1989; Mitchell et al., 1989). However, more commonly, tissue-

specific expression is the result of a unique combination of sequence elements binding more broadly expressed proteins.

4.1. Cis-regulatory elements involved in GnRH promoter activity

To better understand the regulation of placental GnRH production, especially at the pre-translational level, the human placental choriocarcinoma cell line JEG-3 has been widely used as an *in vitro* placenta model to study the hGnRH promoter activity in the placenta (Radovick et al., 1990, 1991; Wierman et al., 1992; Dong et al., 1993). Radovick and his colleague (1991) observed a stimulatory effect of estradiol on the hGnRH promoter activity in JEG-3 cells. The investigators demonstrated a 32 bp sequence between –534 and –521 bp mediated the stimulatory response to estrogen. In contrast, Wierman et al. (1992) observed no stimulatory by estradiol in similar constructs using the rat GnRH (rGnRH) promoter in JEG-3 cells. Estrogen consistently negatively regulated rGnRH promoter activity via an ER dependent mechanism. The sequences important for placental cell expression and estrogen regulation of rGnRH locate in the region from –73 to –16. Further, potential repressor sequences active in placental cells are present between –903 to +424. It is of interest that the sequences identified as the positive estrogen responsive element (ERE) in the hGnRH promoter are not conserved in the rat (Adelman et al., 1986; Bond et al., 1989) or mouse (Mason et al., 1986) GnRH genes. Thus, the opposing effect of estrogen on GnRH may represent the species-specific difference between the human and rat GnRH genes (Dong et al., 1997). In addition, Dong et al. (1996)

demonstrated that estrogen directly down-regulates the hGnRH upstream promoter activity in JEG-3 cells via estrogen receptor α (ERα). The negative regulatory effect of estrogen on the GnRH upstream promoter has also been observed in the Chinese hamster ovary-derived CHO-K1 tumor cells (Chen et al., 1999). Further studies showed that cortisol and its synthetic analogue, dexamethasone, can stimulate the hGnRH upstream promoter activity in transfected JEG-3 cells in a glucocorticoid receptor (GR)-dependent manner, suggesting that cortisol is physiologically involved in the regulation of GnRH gene expression in the human placenta (Chen et al., 1998). These data strongly suggest that steroid hormones are directly involved in the regulation of placental GnRH gene expression through specific cis-regulatory elements and their different effects on the GnRH gene expression might be dependent on the variations of species, tissues, receptor contents, and DNA sequences.

4.2. Trans-acting factors involved in GnRH promoter activity

Regarding the trans-acting proteins involved in the regulation of GnRH gene expression, the POU-homeodomain protein Oct-1 has been demonstrated to be an important transcriptional factor mediating GnRH gene expression. Oct-1 is known to be expressed in a variety of tissues and cell types (Sturm et al., 1987) including JEG-3 cells and GT1-7 cells, it is believed that Oct-1 participates in tissue-specific gene expression by interaction with either other transcription factors (Voss et al., 1991;Poellinger et al., 1992) or tissue-specific co-activators (Luo et al., 1992; Strubin et al., 1995). The role of Oct-1 in tissue-specific

expression has been demonstrated in GT1-7 cells for rat GnRH and mouse GnRH (Clark et al., 1995; Eraly et al., 1998; Chandran et al., 1999) and in JEG-3 cells for leukemia inhibitory factor receptor (LIFR) gene (Wang et al., 1998). Another POU-homeodomain protein SCIP/Oct-6/Tst-1 has also been reported to be able to regulate rat GnRH promoter activity in GT1-7 cells (Wierman et al., 1997). Also, GATA factors have been shown to be essential for activity of the neuron-specific enhancer of rat GnRH gene (Lawson et al., 1996). In addition, other studies demonstrate that Fos and Jun transcription factors mediate down-regulation of both human and rodent GnRH promoter activity in neuronal cell lines (Zhen et al., 1997;Bruder et al., 1996). However, the transcription factors involved in GnRH gene expression in the placenta are still unknown.

5. Interactions of placental GnRH as well as other biomolecules in the placenta

In summary (Fig. 2), GnRH stimulates the release of hCG, which in turn stimulates the release of steroid hormones. Estrogen negatively, and progesterone positively, exert feedbacks to the GnRH production. The hCG also negatively feedback to the GnRH production. Furthermore, activin positively, and inhibin negatively, regulate the release of the placental GnRH. Therefore, placental GnRH interacts with hCG, steroid hormones, as well as other biomolecules in the placenta to regulate placental steroidogenesis, indicating an important physiological role of placental GnRH in the maintenance of the function of placental-ovarian axis and pregnancy.

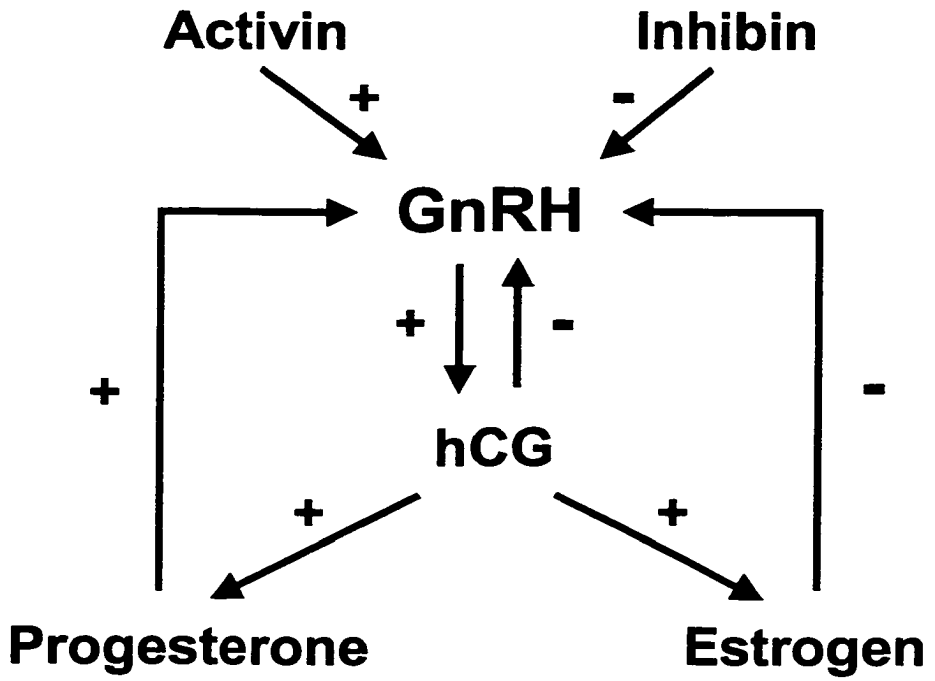


Fig. 2. Paracrine/autocrine regulation of GnRH in the placenta.

SPECIFIC AIMS OF THE STUDY

Specific Aim 1: Characterize the hGnRH upstream promoter cis-regulatory elements and trans-acting factors that are responsible for tissue-specific expression of the GnRH gene in the human placental JEG-3 cells.

1.1. Analyze the hGnRH upstream promoter cis-regulatory elements for their contributions to the tissue-specific expression of hGnRH gene in JEG-3 cells.

1.2. Identify trans-acting factors that interact with the hGnRH upstream promoter elements for tissue-specific expression in JEG-3 cells.

Specific Aim 2: Investigate the roles of estrogen, estrogen receptor α and β in the regulation of the hGnRH upstream promoter activity in JEG-3 cells.

2.1. Determine the expression of human estrogen receptor α (hERα) and β (hERβ) genes in JEG-3 cells.

2.2. Study the roles of estradiol, hERα and hERβ in the regulation of the hGnRH upstream promoter activity in JEG-3 cells.

2.3. Compare hERα and hERβ mediated effects of estrone, estradiol, and estriol on the upstream promoter activity of the hGnRH gene in JEG-3 cells.

2.4. Investigate the interactive effects of hERα and hERβ on the hGnRH upstream promoter activity in JEG-3 cells.

2.5. Analyze the negative and positive estrogen responsive elements in the hGnRH upstream promoter region.

Specific Aim 3: Investigate the roles of progesterone, progesterone receptor A and B forms in the regulation of the hGnRH upstream promoter activity in JEG-3 cells.

3.1. Determine the expression of human progesterone receptor A (hPR-A) and B (hPR-B) genes in JEG-3 cells.

3.2. Study the roles of progesterone, hPR-A and hPR-B in the regulation of the hGnRH upstream promoter activity in JEG-3 cells.

3.3. Examine the effects of coactivators, SRC-1 and CBP, on hPR-A and hPR-B mediated progesterone regulation of the hGnRH upstream promoter activity in JEG-3 cells.

EXPERIMENTAL DESIGN

Specific Aim 1: Characterize the hGnRH upstream promoter cis-regulatory elements and trans-acting factors that are responsible for tissue-specific expression of the GnRH gene in the human placental JEG-3 cells.

Rationale: The human GnRH gene consists of two transcription start sites: the upstream transcription start site is primarily utilized in human reproductive tissues, while the downstream transcription start site is mainly used in the hypothalamus (Dong et al., 1993). Further studies demonstrated a 325-bp region (-1048 to –723) mediates the upstream promoter activity of the hGnRH gene in the human placental JEG-3 cells (Dong et al., 1997). DNase I footprinting analysis of the upstream promoter region revealed four specific footprinted elements (FP1, -876 to –851; FP2, -919 to –896; FP3, -960 to –940; FP4, -987 to –968) (Fig. 3) that are capable of binding to the nuclear extract from the human placental JEG-3 cells but not from the hypothalamic neuronal GT1-7 cells. The interaction of nuclear proteins from JEG-3 cells with these four elements was further confirmed by gel mobility shift assay (Dong et al., 1997). These results suggest that the four footprinted elements may be involved in the tissue-specific expression of the hGnRH gene in the placenta. This is supported by the observation that by constructing transgenic mice with various fragments of the hGnRH gene 5'-flanking region, a fragment between –1131 and –484 of the hGnRH gene is essential for tissue-specific expression of the hGnRH gene

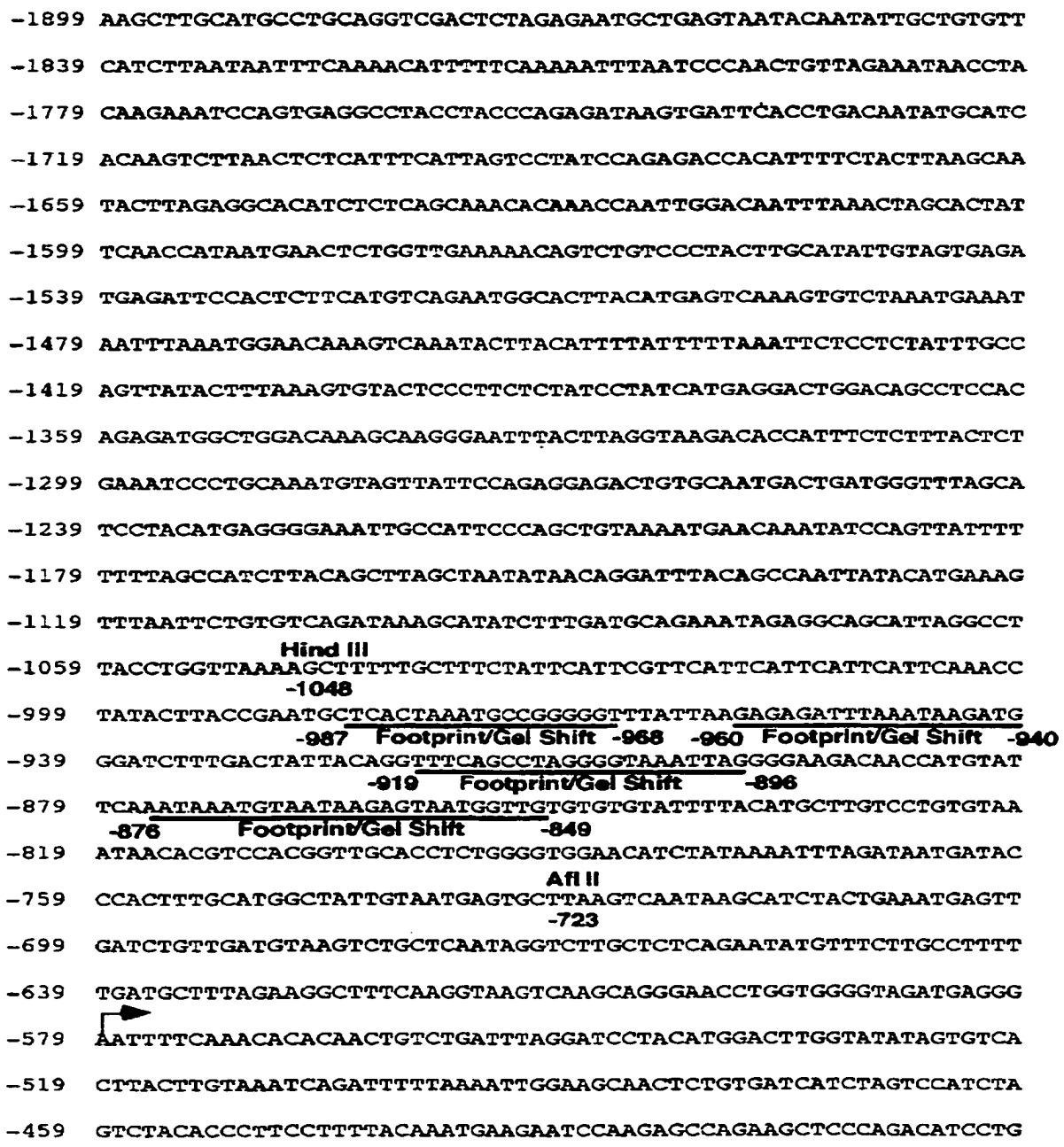


Fig. 3. DNA sequence of the hGnRH 5'-flanking region. The numbers are relative to the downstream transcription start site of the hGnRH gene. The upstream transcription start site is shown as a bent arrow at –579. The DNAse footprinting regions are underlined.

(Radovick et al., 1990). The present study is designed to analyze in detail the contributions of the four footprinted elements, individually or in combination, to the tissue-specific hGnRH upstream promoter activity in the human placental JEG-3 cells. To achieve this goal, PCR and restriction enzyme digestion will be applied to generate individual or combined four element(s). These upstream promoter elements are subsequently cloned into TK/luciferase reporter vector for transient transfection studies in JEG-3 cells.

In addition, detailed sequence analysis revealed that the four footprinted elements are short AT-rich regions with octamer motif (consensus sequence ATGCAAAT) that are possible bound by the POU-homeodomain transcription factors. The importance of the POU-homeodomain protein Oct-1 in the regulation of gene expression has been demonstrated in the neuronal GT1-7 cells for rat and mouse GnRH (Eraly et al., 1998; Chandran et al., 1999), and in the placental JEG-3 cells for leukemia inhibitory factor receptor (LIFR) gene (Wang et al., 1998). It is believed that Oct-1 is involved in the tissue-specific gene expression by interacting with other transcription factors (Voss et al., 1991; Poellinger et al., 1992) or tissue-specific coactivators (Luo et al., 1992; Strubin et al., 1995). Thus, Oct-1 may be involved in the tissue-specific expression of the hGnRH in the placenta. In the present study, gel mobility shift and super-shift assays will be conducted by using nuclear extract from JEG-3 cells and specific antibody against Oct-1 protein to determine whether Oct-1 protein is involved in the DNA-protein interaction of the footprinted upstream promoter elements.

Experimental design:

1.1. *Analyze the hGnRH upstream promoter cis-regulatory elements for their contributions to the tissue-specific expression of hGnRH gene in JEG-3 cells.*

To determine the contributions of the individual or combined footprinted elements for the hGnRH upstream promoter activity in the placenta, the human placental choriocarcinoma cell line JEG-3 was utilized as *in vitro* human placental model. Functional transient transfections were performed in JEG-3 cells using calcium phosphate coprecipitation assay with co-transfected pCMV β-galactosidase plasmid as control for transfection efficiency. Using PCR with specific primers, the individual or combined DNA sequences of the four footprinted elements were generated. Each of the amplified DNA fragments was inserted into a herpes simplex thymidine kinase (TK) minimal promoter/luciferase reporter vector, pT109luc, to produce hGnRH upstream promoter element(s)/TK-luciferase constructs (TKU432, TKU43, TKU4, TKU321, TKU21, TKU1, TKU421, and TKU431). A 272-bp fragment (-994 to –723) containing the four footprinted elements derived from the full-length 5'-flanking region of the hGnRH gene was cloned into the pT109luc to produce TKU (Fig. 4).

The constructs of TK, TKU, and hU that contains the full length 5'-flanking region of the hGnRH upstream promoter were transfected into the human placental JEG-3 cells, neuron GT1-7 cells, and non reproductive tissue human kidney 293 cells, respectively, to examine the four elements in directing tissue-specific hGnRH upstream promoter activity. Subsequently, the constructs containing individual or combined element(s) were transfected into JEG-3 cells to

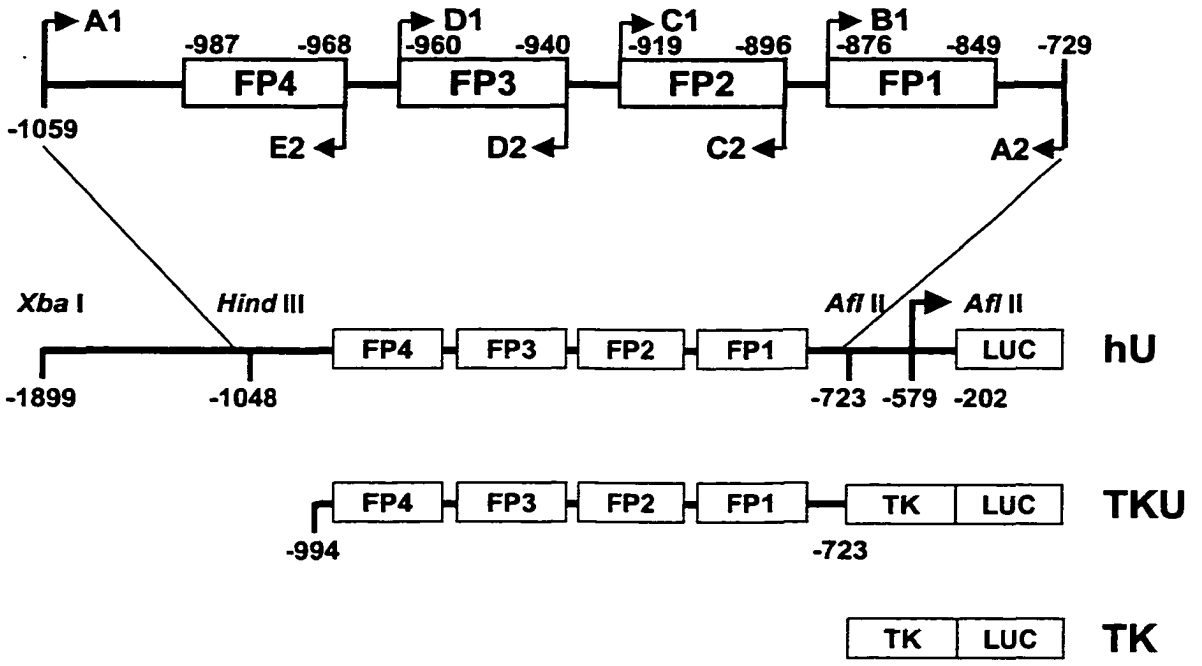


Fig. 4. Schematic structures of plasmid constructs. The four footprinted elements are represented as FP1-FP4. A1, A2, B1, C1, C2, D1, D2, and E2 are oligonucleotides used for PCR to generate individual or combined four footprinted element(s)/thymidine kinase (TK)-luciferase constructs (TKU1, TKU21, TKU321, TKU432, TKU43, TKU4). The hU luciferase construct contains the 5' full-length hGnRH upstream promoter region inserted into promoter-less pxp2-luciferase vector. The upstream transcription start site is shown as bent arrow. TKU was made by inserting the fragment (-994 to –723) containing the four footprinted elements into pT109luc vector. TK is the pT109luc itself.

determine the contributions of different combinations of the four elements in mediating the hGnRH upstream promoter activity in JEG-3 cells. Also, a set of 5' end deletion in the hGnRH upstream promoter/luciferase constructs that retain the original hGnRH upstream promoter was transfected into JEG-3 cells to further confirm the roles of the four elements in the tissue-specific upstream promoter activity in JEG-3 cells. In addition, mutational studies on the element(s) that may direct major upstream promoter activity were conducted to support the functional roles of these cis-regulatory elements in the hGnRH upstream promoter activity.

1.2. *Identify trans-acting factors that interact with hGnRH upstream promoter elements for the tissue-specific expression in JEG-3 cells.*

Sequence analysis of the four footprinted elements indicated the presence of possible binding sites for the POU-homeodomain protein Oct-1. EMSA and supershift assays were, therefore, performed with nuclear extract from JEG-3 cells and specific antibody against Oct-1 to determine whether Oct-1 protein involved in the DNA-protein interaction of the four footprinted elements. The probes were prepared by annealing wild-type oligonucleotides of the four elements and labeled with [γ-^{32}P]ATP. Also, western blot assay was conducted with specific antibody against Oct-1 to determine the presence of Oct-1 protein in our JEG-3 cells.

Specific Aim 2: Investigate the roles of estrogen, estrogen receptor α and β in the regulation of the hGnRH upstream promoter activity in JEG-3 cells.

Rationale: Estrogen is one of the major hormones produced in the placenta and its plasma level varies greatly with gestation. The placenta also produces GnRH that plays an important role in reproductive tissues as well as pregnancy. It is known that estrogen through estrogen receptor affects the GnRH production in the placenta (Petraglia et al. 1990). Further studies demonstrated the direct effect of estrogen on the hGnRH promoter activity in the placenta (Radovick et al., 1991; Dong et al., 1996). Recently, a negative and a positive estrogen responsive element have been identified in the hGnRH upstream promoter region (Chen et al., 1999 and 2000). Thus, it is of interesting to understand the mechanisms behind the estrogen actions on the upstream promoter.

The physiological response to steroid hormones is generally mediated by specific intracellular receptors. It is known that steroid hormone receptors exist as multi-forms with distinctive tissue distributions and functions. The human estrogen receptor has two major subtypes, estrogen receptor α (hERα) and β (hERβ) (Fig. 5. A) (Kuiper et al., 1997; Paech et al., 1997). ERβ has the DNA binding domain that is almost identical to the homologous ERα, implying that both ERα and ERβ might share the same DNA response element. They can also form heterodimer complex, suggesting their possible cross-talking (Sumito et al., 1998). However, ERα and ERβ have different activation function region and ligand binding domain that might result in the distinctive transactivational mechanisms. Also, ERβ has been shown to distribute in tissues differently as

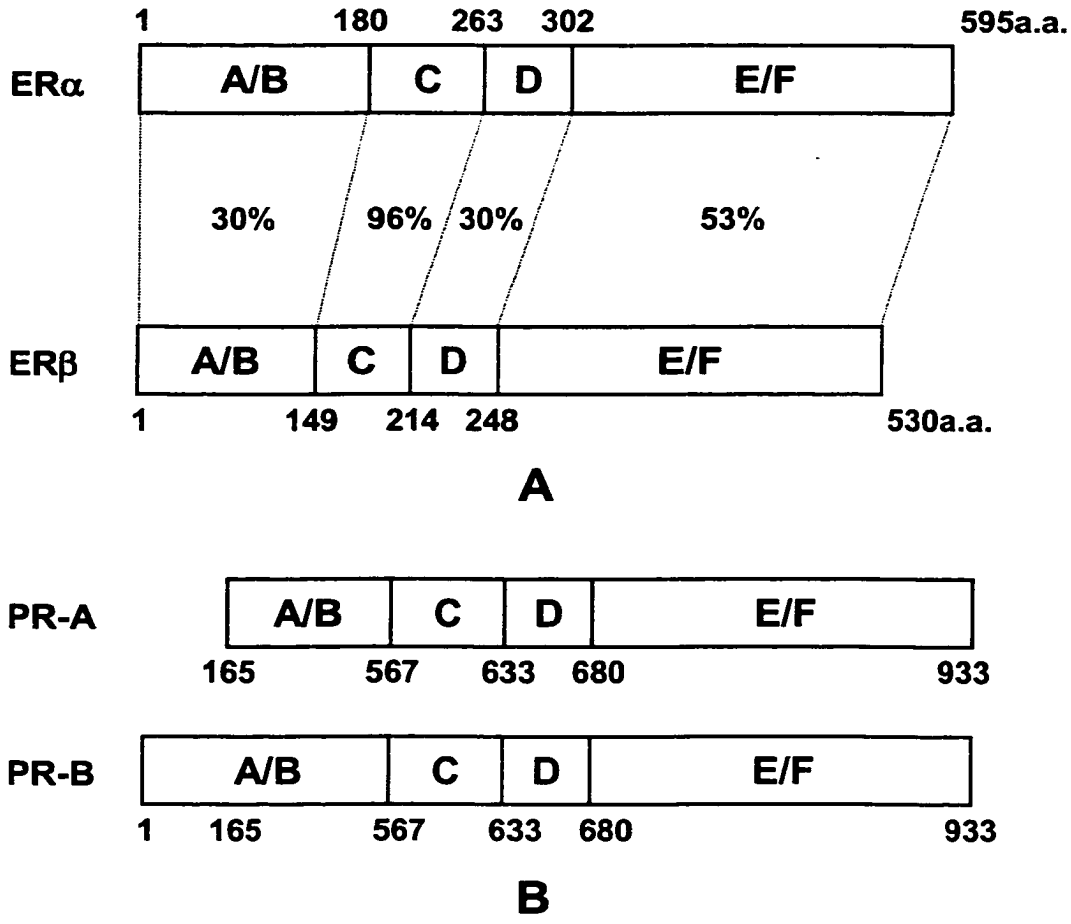


Fig. 5. Schematic representation of human estrogen receptor (hER) and progesterone receptor (hPR). A/B: activation function-1 (AF-1) domain; C: DNA binding domain; D: hinge domain; E/F: ligand binding and activation function-2 (AF-2) domain. A. hERα and hERβ. The well-conserved domains are DNA binding domain (96%) and ligand binding domain (60%). B. hPR-A and hPR-B. hPR-A lacks 164 amino acids at the N-terminus that are present in the full-length 966-amino acid hPR-B.

compared to ERα (Paech et al., 1997). Some tissues contain exclusively ERα (such as kidney) or predominant ERα (i.e. uterus, pituitary, epididymis), while other tissues show equal or greater levels of ERβ RNA (i.e. ovary, prostate), implying distinctive physiological roles for ERβ. In the human placenta, the presence of estrogen receptor has been demonstrated (Younges et., 1981; Chibbar et al., 1995). Further studies showed both ERα and ERβ are present in the placenta (Brandenberger et al., 1997; Moore et al., 1998). However, the human placental JEG-3 cells have been reported to be deficient in ER (Chatterjee et al., 1989). As an *in vitro* human placenta model, JEG-3 cells have been utilized in studying estrogen regulation of GnRH gene expression in the placenta by exogenous expression of ER in the cells (Wierman et al., 1992; Dong et al., 1996). In the present studies, the presence of hERα and hERβ in our JEG-3 cells will be first examined. Further, hERα and hERβ expression plasmids will be transfected into JEG-3 cells to investigate the receptor-mediated estrogen regulation of the hGnRH upstream promoter activity.

Experimental design:

2.1. *Determine the expression of human estrogen receptor α (hERα) and β (hERβ) genes in JEG-3 cells.*

To determine whether hERα and/or hERβ are expressed in our JEG-3 cells, RT-PCR was conducted on cytoplasmic RNA from JEG-3 cells by using specific primers for hERα and hERβ. MCF-7 cells, which express hERα and hERβ proteins (Dotzlaw et al., 1997), were used as positive control. If the expressions

were low or no expression, hERα and hERβ expression vectors would be transfected into JEG-3 cells to mediate the effect of estrogen.

2.2. *Study the roles of estradiol, hERα and hERβ in the regulation of the hGnRH upstream promoter activity in JEG-3 cells.*

To investigate the effect of estradiol on the hGnRH upstream promoter activity in JEG-3 cells, transient transfections were performed in JEG-3 cells with the hGnRH upstream promoter/luciferase construct (hU) and pCMV β-galactosidase plasmid, with or without hERα or hERβ expression vectors. The cells were then treated with 10 nM of estradiol with or without 1 μM of ICI164,384 for 20 h. The ICI164,384, a potent antiestrogen for both hERα and hERβ, was used to determine whether the effect of estrogen was ER-mediated. The cell lysates were assayed for luciferase activity and β-galactosidase activity as a control for transfection efficiency. To confirm the actions of hERα and hERβ in mediating estrogen effect on the hGnRH upstream promoter, various amounts of hERα or hERβ (from 0.25 μg to 4 μg) were co-transfected with hU into JEG-3 cells and treated with 10 nM of estradiol to study the dose effect of exogenous ER expression on the promoter activity.

To determine whether the estradiol effect was dose-dependent, JEG-3 cells were transfected with hU plasmid and hERα or hERβ expression vectors, and treated with various concentrations of estradiol (from 0.01 nM to 100 nM) for 20 h. The lysates were assayed for luciferase activities. The doses of estradiol applied were within its physiological range during gestation. The pCMV β-

galactosidase plasmid was also co-transfected into JEG-3 cells with test construct(s) to correct for variations in transfection efficiency.

2.3. *Compare hERα and hERβ mediated effects of estrone, estradiol, and estriol on the upstream promoter activity of the hGnRH gene in JEG-3 cells.*

To determine the effects of different estrogen isoforms on the hGnRH upstream promoter activity in the placenta, JEG-3 cells were transfected with the hGnRH upstream promoter/luciferase plasmid (hU) and hERα or hERβ expression vectors. The cells were then treated with 10 nM of estrone, estradiol, or estriol, respectively, and harvested after 20 h incubation. The cell lysates were assayed for luciferase activities. The pCMV β-galactosidase plasmid was also co-transfected into JEG-3 cells with test construct(s) to correct for variations in transfection efficiency.

2.4. *Investigate the interactive effects of hERα and hERβ on the hGnRH upstream promoter activity in JEG-3 cells.*

The hERα and hERβ have distinctive transactivational functions and can form heterodimer to cross-talk to each other. To see if hERα and hERβ could modulate each other in mediating the estrogen effect on the hGnRH upstream promoter, various amounts of hERα and hERβ expression vectors were transfected into JEG-3 cells with the hGnRH upstream promoter/luciferase construct (hU). In one experiment, 1 μg of hERα was introduced with various amounts of hERβ (from 0.25 μg to 4 μg). In another experiment, 1 μg of hERβ

was transfected with various amounts of hERα (from 0.25 μg to 4 μg). Thus, the ratio of transfected hERα and hERβ (hERα/hERβ or hERβ/hERα) was kept at 4:1, 2:1, 1:1, 1:2, and 1:4. The cells were then treated with 10 nM of estradiol for 20 h, and lysates were assayed for luciferase activities. Transfection efficiency was corrected by co-transfection of pCMV β-galactosidase plasmid with test construct(s). In addition, RT-PCR was conducted on the cytoplasmic RNAs prepared from hERα and/or hERβ transfected JEG-3 cells with specific primers for hERα and hERβ to confirm the transfection of hERα and hERβ in JEG-3 cells.

2.5. *Analyze the negative and positive estrogen responsive elements in the hGnRH upstream promoter region.*

Previous studies in our laboratory have identified a negative and a positive estrogen responsive element in the hGnRH upstream promoter. To further verify these findings, functional mutation studies were performed on these two elements. Using PCR with specific primers, the negative estrogen responsive element was replaced by two unrelated DNA sequences, while the positive element was replaced by one unrelated DNA sequence, and cloned into promoter-less luciferase reporter, pxp2-luc. These constructs were transfected into JEG-3 cells, respectively, with hERα or hERβ and pCMV β-galactosidase plasmid as control for transfection efficiency. The cells were then treated with or without 10 nM of estradiol for 20 h and assayed for luciferase activities.

Specific Aim 3: Investigate the roles of progesterone, progesterone receptor A and B forms in the regulation of the human GnRH upstream promoter activity in JEG-3 cells.

Rationale: It is well known that progesterone plays an important role in pregnancy and involves in the regulation of hypothalamic-pituitary-gonad axis. This regulation has been shown to be mediated by progesterone receptor (PR) (Kim et al., 1985). It is further supported by *in vitro* study in GT1-7 cells that direct DNA binding of PR to nonconsensus elements in the proximal rat GnRH promoter inhibits rat GnRH gene expression (Kepa et al., 1996). In the human placenta, progesterone decreases the release of immunoreactive GnRH and this action is mediated by its receptor (Petraglia et al., 1990). Using ribonuclease protection assay, PR mRNA has been detected in the human placenta (Chibbar et al., 1995). Further, Shanker et al. (1997) using RT-PCR demonstrated the expression of PR mRNA in the first trimester human placenta. Subsequently, the presence of PR was reported at all stages of placental development (Rossmanith et al., 1997). In addition, human PR exists as two isoforms, hPR-A and hPR-B (Fig. 5B), that are produced from a single gene by alternative translation (Kastner et al., 1990). They have similar DNA and ligand binding affinities but different functions (Giangrande et al., 1997; Bethea et at., 1998). The hPR-B functions as a transcriptional activator in most contexts, whereas in most cells hPR-A does not activate transcription but functions as a strong trans-dominant repressor of hPR-B (Vegeto et al., 1993). Recently, the presence of only PR-A but not PR-B in the first trimester and term placenta has been reported (Shanker et al., 1998).

Therefore, the different receptor isoforms of PR may have distinctive roles in mediating progesterone actions on the regulation of the hGnRH gene expression.

Co-regulators, steroid receptor coactivator-1 (SRC-1) and CREB binding protein (CBP), have been reported to exert important roles in nuclear receptor mediated gene expression. It has been shown that SRC-1 and CBP, independently or synergistically, enhance PR dependent transcription in a variety of cell lines (Onate et al., 1995; Lundblad et al., 1995; Smith et al., 1996). Since these coactivators have been demonstrated to be expressed ubiquitously (Misiti et al., 1998), SRC-1 and CBP may be also involved the hPR-A and hPR-B mediated regulation of the hGnRH gene expression in the placenta.

Experimental design:

3.1. *Determine the expression of human progesterone receptor A (hPR-A) and B (hPR-B) genes in JEG-3 cells.*

RT-PCR was performed on the cytoplasmic RNAs from JEG-3 cells and MCF-7 cells with specific primers for PR. MCF-7 cells, which express both PR isoforms, as well as hPR-A and hPR-B expression vectors were used as positive control. Because hPR-A and hPR-B are produced by alternative translation, western blot assay with specific antibodies against hPR-A/B and hPR-B was performed on the proteins from JEG-3 cells to determine what kind of PR expressed in our JEG-3 cells, hPR-A or hPR-B, or both.

3.2. *Study the roles of progesterone, human progesterone receptor A and B in the regulation of the hGnRH upstream promoter activity in JEG-3 cells.*

The stimulatory effect of progesterone on the hGnRH upstream promoter activity has been demonstrated previously in our laboratory. In the present study, I focused on the difference between hPR-A and hPR-B in mediating the progesterone effect on the upstream promoter. Using calcium phosphate coprecipitation method, the hGnRH upstream promoter/luciferase plasmid (hU) was transfected into JEG-3 cells with hPR-A or hPR-B expression vectors. The cells were treated with 0.1 nM of progesterone for 20 h, and lysates were assayed for luciferase activity. Also, a progesterone antagonist (RU486) was used to block the receptor-mediated pathway to further confirm the progesterone action was receptor-mediated. Moreover, various amounts of hPR-A or hPR-B expression vectors (from 0.25 μg to 4 μg) were co-transfected into JEG-cells with the hU plasmid and treated with 0.1 nM of progesterone to see the effects of exogenous expression of PRs on the hGnRH upstream promoter activity.

To determine whether the receptor-mediated progesterone action was dose-related, 3 μg of hU and 1 μg of hPR-A or hPR-B co-transfected JEG-3 cells were treated with various concentrations of progesterone (from 0.01 nM to 100 nM) for 20 h. The lysates were assayed for luciferase activities. The pCMV β-galactosidase plasmid was also co-transfected into JEG-3 cells with test construct(s) in each experiment to correct for variations in transfection efficiency.

3.3. *Examine the effects of coactivators SRC-1 and CBP on hPR-A and hPR-B mediated progesterone regulation of the hGnRH upstream promoter activity in JEG-3 cells.*

To determine the effects of exogenously expressed coactivators SRC-1 or CBP on the hPR-A and hPR-B mediated progesterone action, 1 μg of SRC-1 or CBP were transfected into JEG-3 cells with the hU plasmid and hPR-A or hPR-B, respectively. The cells were then treated with progesterone and harvested for luciferase activity assay. Transfection efficiency was corrected by co-transfection of pCMV β-galactosidase plasmid with test constructs in each experiment.

MATERIALS AND METHODS

Cell culture

Human placental choriocarcinoma cells (JEG-3), mouse neuronal cells (GT1-7), and human kidney cells (293) were purchased from American Type Culture Collection (ATCC, Rockville, MD). JEG-3 and GT1-7 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS). The 293 cells were grown in Modified Eagle Medium (MEM; Sigma, St. Louis, MO) with 10% FBS. The cells were passaged or frozen when 80% confluence. For hormone treatment studies, the cells were cultured in serum and phenol red free DMEM or MEM (Life Technologies, Gaithersburg, MD) during treatment.

Construction of plasmids

TKU was made by inserting a 272-bp fragment (-994 to –723) containing all the four footprinted elements derived from the full length 5'-flanking region of the hGnRH gene into a herpes simplex thymidine kinase (TK) minimal promoter/luciferase reporter vector, pT109luc (Fig. 4). The individual or combined sequences of the four elements were generated by PCR using the following specific primers:

A2: 5'-TATCTCGAGAAGCACTCATTACAATAG-3',

B1: 5'-GTCGACAATAAATGTAATAAGAGTAATGGT-3',

C1: 5'-GTCGACTTTCAGCCTAGGGGTAAATTAG-3';

D1: 5'-CAGTCGACGAGAGATTTAAATAAG-3',

C2: 5'-TATCTCGAGCTAATTTACCCCTAGGCT-3',

D2: 5'-TATCTCGAGCCATCTTATTTAAATCTC-3',

A1: 5'-GGTCGACCTACCTGGTTAAAAGCT-3',

E2: 5'-TATCTCGAGGCATTTAGTGAGCATTCG-3',

and cloned into the pT109luc vector to produce hGnRH upstream promoter element/TK-luciferase constructs (TKU432, TKU43, TKU4, TKU321, TKU32, and TKU1. Kindly prepared by Kei-Li Yu, University of Hong Kong, Hong Kong, China) (Fig.4). For element knockout studies (deletion of FP3 or FP2), the construct TKU421 and TKU431 were generated by annealing the oligonucleotides 5'-CCAAGCTTCACTAAATGCCGGGGGGATGGGATCTTTGACTATT-3' and A2, 5'-CCCTCGAGCAACCATTACTCTTATTACATTTATTCCTGTAATAGTCAAAGATC-3' and A1, and then cloned into pT109luc vector, respectively. For block mutation of FP4 or FP3 and estrogen responsive element studies, the plasmid hU5a-m4, hU5a-m3, and mERE were made by annealing the sense oligonucleotides 5'-CCAAGCTTAATGGCAGGACTGCCCGAATATAATGGCATTAAGAGAGATTTAAATAA-3', 5'-CCAAGCTTGCCGCTCTGTCGGGCCCGCGCTCGAAACCTTTGACTATTACAGGTTTC-3', 5'-CCAAGCTTTGTAAATAACCATAGTTGAATTGCACCTCTGGGGTGGAAC-3', with antisense oligonucleotide 5'-CCCTCGAGCTTAAGTGCAGCCATTAAAACC-

3', and cloned into promoter-less pxp2-luc vector, respectively. All constructs were transformed into DH5 *E.coli* competent cells and positive clones were confirmed by restriction mapping, Southern blot analysis, and DNA sequencing. For serial deletion study, a set of 5' end deletion in the hGnRH upstream promoter/pxp2-luc constructs (hU5, hU5a, hU5b, hU5c, hU5d, and hU5e) was generated by ExonucleaseIII/Mung Bean Nuclease deletion (Kindly prepared by Kei-Li Yu, University of Hong Kong, Hong Kong, China).

Preparation of plasmid DNAs

Plasmid DNAs were transformed into DH1 *E.coli* bacteria host and amplified in 500 ml Luria-Bertani medium (5 g Yeast, 10 g Trypton, 5 g NaCl, pH7.5, and 50 μg/ml of Ampicilin) in shaking incubator at 37°C, 200-rpm overnight. Bacteria cells were harvested by centrifugation at 4°C, 6,000 rpm for 10 min. Plasmid DNAs were prepared by CONCERT High Purity Plasmid Maxiprep System (Life Technologies, Gaithersburg, MD) according to the manufacture's instruction with minor modification. DNA concentration was determined spectrophotometrically by absorbance at 260 nm and verified by comparison with known mass lambda DNA standards on agarose gel.

Transient transfection assay and luciferase activity assay

Transfection assays were carried out in triplicate by the calcium phosphate coprecipitation method without glycerol shock (Sambrook et al., 1989). The amounts of DNA in each experiment are given in the figure legends. All

transfections included a pCMV β-galactosidase construct (Clontech, San Francisco, CA) as an internal control for transfection efficiencies. A constant amount of transfected DNA was maintained by the addition of nonspecific plasmid DNA. Cells were plated at a density of 2-5 x 10^5 cells per 60-mm dish one day before transfection. Transfected plates were washed three times with 1 x PBS and fresh serum and phenol red free medium was applied with or without treatment. Cells were further incubated for 20 h before harvesting by scraping the cells into 200 μl of lysis buffer (1% Triton-X 100; 25 mM glycylglycine, pH7.8; 15 mM MgSO4; 4 mM EGTA; and 1 mM dithiothreitol). After centrifugation at 4°C, 14,000 rpm for 5 min, luciferase activity (de Wet et al., 1987) was measured on a Lumat LB9501 luminometer (EG&G Berthold) by mixing 100 μl of the cell extracts, 360 μl of luciferase assay buffer (25 mM glycylglycine, pH 7.8; 15 mM MgSO4; 4 mM EGTA; 15 mM KPB, pH 7.8; 1 mM dithiothreitol; and 2 mM ATP), and 100 μl of luciferin buffer (2 mM D-luciferin [Sigma, St. Louis, MO] and 25 mM glycylglycine, pH7.8) (Wondisford et al., 1989). To correct protein amount for luciferase activity, protein concentrations of the lysates were determined by BCA Protein Assay kit (PIERCE, Rockford, IL). Portion of the harvested cell extract was used to quantify β-galactosidase synthesis by colorimetric assay at 405 nm.

Isolation of nuclear extract

To prepare JEG-3 cell nuclear extract for EMSA, the JEG-3 cell pellets were resuspended in five packed cell volumes of ice-cold buffer A (10 mM HEPES-NaOH, pH 7.9; 10 mM KCl; 1.5 mM $MgCl_2$; 1 mM dithiothreitol; 50 μg/ml

phenymethylsulfonylfluoride [PMSF]) and incubated on ice for 10 min. The cell nuclei were separated from cytoplasmic supernatant by centrifugation at 4°C, 12,500 rpm for 25 min, and resuspended in 0.25 packed cell volume of ice-cold buffer C (20 mm HEPES-NaOH, pH 7.9; 10 mM KCl; 1.5 mM $MgCl_2$; 1 mM dithiothreitol, 50 μg/ml PMSF, 0.25 mM EDTA, and 25% glycerol) and homogenized in Dounce homogenizer. After rocking at 4°C for 30 min, the nuclei extract was harvested and the supernatant was dialyzed against 250 ml buffer D (20 mM HEPES-NaOH, pH 7.9; 100 mM KCl; 1 mM dithiothreitol, 50 μg/ml PMSF, 0.2 mM EDTA, and 20% glycerol). Then, the nuclei extract solution was collected and saved at -80°C for subsequent EMSA. Protein concentration was measured by BCA Protein Assay kit (PIERCE, Rockford, IL).

Electrophoretic mobility shift assay (EMSA) and supershift assay

For the EMSA, annealed wild-type oligonucleotides containing sequences of the four footprinted elements (FP1: -876 to –851, 5'-AATAAATGTAATAAGAGTAATGGTTG-3'; FP2: -919 to –896, 5'-TTTCAGCCTAGGGGTAAATTAG-3'; FP3: -960 to –940, 5'-GAGAGATTTAAATAAGATG-3'; FP4: -987 to –968, 5'-CTCACTAAATGCCGGGGG-3'), as well as the putative ERE of the hGnRH upstream promoter (pERE: -824 to –784, 5'-TGTGTAAATAACACGTCCACGGTTGCACCTCTGGGGTGGAACAT-3') were labeled with [γ-^{32}P]ATP (6,000 Ci/mmol; NEN, Boston, MA) by using T4 polynucleotide kinase. Binding reactions were carried out in 10 mM Tris-HCl (pH

8.0), 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 4 μg of poly (dI:dC), 5% glycerol, 3 μg of nuclear extract or 7.3 pmol of recombinant ERα protein (Panvera, Madison WI), and 20,000 cpm of labeled probe in total volume of 25 μl. Reaction mixtures were incubated at room temperature for 10 min, loaded onto 8% polyacrylamide gel (acrylamide-bisacrylamide [37.5:1], 0.25x TBE), and electrophoresed for 2 h at 200 V in cold room. Gels were pre-run for 30 min in 0.25x TBE. Then, the gels were dried and subjected to autoradiography. For competition reactions, the reactions were preincubated with the specified amount of excess unlabeled oligonucleotide at room temperature for 20 min before the addition of probe. Supershift assays were performed by incubating the reactions with 1 μl of Oct-1 antibody (sc-232x, Santa Cruz Biotechnology Inc., Santa Cruz, CA) or rabbit IgG control at room temperature for 1 h before the addition of probe.

RNA isolation

To isolate cytoplasmic RNA for RT-PCR, cells were rinsed twice in cold 1x PBS and scraped into 0.5 ml of ice-cold lysis buffer (10 mM Tris-HCl, pH7.4; 3 mM $CaCl_2$; 2 mM $MgCl_2$; 0.5 mM dithiothreitol; 0.3 M Sucrose; 0.15% Triton X-100). The cell lysis was then layered over 0.35 ml of cushion buffer (10 mM Tris-HCl, pH7.4; 1.5 mM $MgCl_2$; 0.4 M Sucrose) in 1.5 ml microcentrifuge tube on ice. After centrifugation for 10 min at 5000 g, 4°C, 0.5 ml of the supernatant was transferred to a fresh microcentrifuge tube and mixed with 58 μl of 10x SET (10% SDS; 50 mM EDTA; and 100 mM Tris-HCl, pH7.4), 12 μl of proteinase K (10

mg/ml), and 17 μl of 5 M NaCl. The mixture was incubated at 45°C for 1 hour, followed by extraction with phenol/chloroform and precipitation with 100% ethanol at -20°C overnight. RNA was recovered by centrifugation for 20 min at 14,000 rpm, 4°C, air-dried and resuspended in 20 μl RNase free water. The quantity of RNA was determined by measuring the absorbance of 260 and 280 nm. The integrity of RNA obtained and absence of chromosomal DNA were verified by running 2% agarose gel.

RT-PCR

RT-PCR was used to determine the presence of hER α and hERβ, as well as PR in JEG-3 cells. For first-strand cDNA synthesis, approximate 10 μg of cytoplasmic RNA were reverse transcribed in a final volume of 30 μl. The reaction mixture included 1.0 x RT buffer (50 mM Tris-HCl, pH 8.3; 75 mM KCl; and 3 mM $MgCl_2$), 0.1 mg/ml BSA, 1.0 mM dithiothreitol, 0.05 μg/μl oligo-dT-(12-18), 0.5 mM dNTP, 1 U/μl RNAsin, and 200 U M-MLV reverse transcriptase (Life Technologies, Gaithersburg, MD). The reaction mixture was incubated at 42°C for 2 min, followed by incubation at 37°C for 1.5 hours, and then quenched on ice.

For PCR amplification, 5 μl of the first-strand cDNA was amplified in a final volume of 25 μl. The reaction mixture included 1 x PCR buffer (20 mM Tris-HCl, pH 8.4; 50 mM KCl; and 5 mM $MgCl_2$), 0.25 mM dNTP, 1.5 mM $MgCl_2$, 1.25 U *Taq* polymerase (Life Technologies, Gaithersburg, MD), and the following primers: hERα sense, 5'-GAGACATGAGAGCTGCCAAC-3' and antisense, 5'-

CCAAGAGCAAGTTAGGAGCA-3'; hERβ sense 5'-AGTATGTACCCTCTGGTCACAGCG-3' and antisense 5'-CCAAATGAGGGACCACACAGCAG-3'; hPR sense, 5'-GATTCAGAAGCCAGCCAGCCAG-3' and antisense 5'-AGTTGCCTCTCGCCTAGT-3'. Amplification conditions were 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 30 sec, and extension at 72°C for 2 min, followed by a final extension at 72°C for 10 min. The PCR products were run on 2% agarose gels containing ethidium bromide, and visualized using a UV illuminator.

Western blot

Total cell protein extracts were prepared by lysis of cell monolayers with protein lysis buffer (1% Triton X-100; 25 mM glycylglycine, pH 7.8; 15 mM $MgSO_4$; 4 mM EGTA; and 1 mM dithiothreitol). Protein concentration was measured by BCA Protein Assay kit (PIERCE, Rockford, IL). Equal amounts of total cellular protein extracts (10-20 μg) were separated by 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and blotted onto a cellulose nitrate membrane (Amersham LIFE SCIENCE, Arlington Heights, IL). Proteins were visualized using ECL detection system (Amersham LIFE SCIENCE, Arlington Heights, IL) according to manufacture's instruction with modification. Briefly, the protein-blotted membrane was incubated in 10% BSA blocking buffer at 4°C overnight, followed by incubation with 1st antibody (Oct-1 [sc-8024x], PR-A/B [sc-810], and PR-A [sc-811] mouse monoclonal antibody

from Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 4°C for 1 h. The membrane was then washed three times by 0.4% Tween20/PBS for 15 min and detected by 2nd antibody (mouse Ig, horseradish peroxidase-linked whole antibody). After washing three times with 0.3% and 0.1% Tween20/PBS for 15 min, the membrane was developed and exposed to XAR-5 film (Kodak).

Data analysis

All transient transfection assays were carried out in triplicate and repeated at least three times on independent experiments. Data were standardized for protein concentration and β-galactosidase activity, and represented as relative light unit (x1,000) and mean values ± SEM. Student's *t* test was used to compare differences between mean values of two different treatments. Data for dose response studies were analyzed for statistical significance using one-way ANOVA. When the F test for the ANOVA reached statistical significance, differences between specific mean values were assessed by least-significant-difference (LSD) test. Differences of $P < 0.05$ were accepted as statistically significant.

RESULTS

Part I: Multiple cis-regulatory elements mediate hGnRH upstream promoter activity in the tissue-specific expression of hGnRH gene in JEG-3 cells.

1. Four cis-regulatory elements confer the tissue-specific expression of the full hGnRH upstream promoter in JEG-3 cells.

To determine whether the four footprinted elements can act as cis-regulatory elements to confer tissue-specific expression of the hGnRH upstream promoter in human placenta, the sequence containing these four elements (-994 to –723) was placed upstream to the TK/luciferase reporter (TKU in Fig. 6). The human placental JEG-3 cells were used as *in vitro* placenta model, while GT1-7 cells as hypothalamic neurons and human kidney 293 cells as non-reproductive tissue. The TKU construct, TK vector and the 5' full-length hGnRH upstream promoter/luciferase construct (hU in Fig. 6) were transfected into JEG-3, GT1-7, and 293 cells, respectively. As shown in Fig. 6, the promoter activity conferred by these four elements alone (TKU) was similar to that of the full-length hGnRH upstream promoter. In contrast, the TK promoter itself had very low activity in JEG-3 cells. All tested constructs were not active in GT1-7 and 293 cells. These results indicate that the four footprinted elements, but not the TK promoter, confer tissue-specific expression of the TKU in JEG-3 cells.

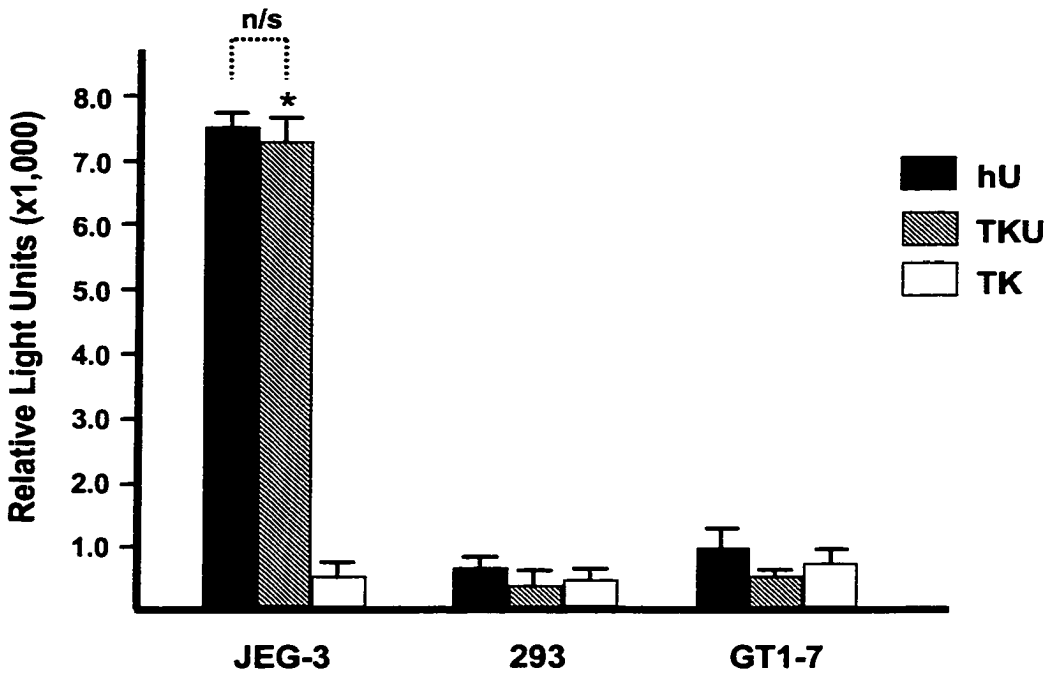


Fig. 6. The four footprinted elements in the hGnRH upstream promoter are responsible for the tissue-specific expression in JEG-3 cells. Three micrograms of hU, TKU, or TK were co-transfected with 2 μg of pCMV β-galactosidase into JEG-3, 293, and GT1-7 cells, respectively. The cells were harvested after 20 h and lysates were assayed for β-galactosidase and luciferase activities. Data were standardized to β-galactosidase activities as a control for transfection efficiency. Each bar represents the mean ± SEM of luciferase activity, which is shown as relative light units. *, $P < 0.05$ as compared to TK; n/s, not significant (by Student's *t* test).

2. Footprinted element 4 (FP4) is the most important for the tissue-specific expression of the hGnRH gene in JEG-3 cells.

To determine how these four elements contribute to the tissue-specific expression of the hGnRH upstream promoter in JEG-3 cells, different combinations of these four elements were generated by PCR with specific primers (Fig. 4, as described in Materials and Methods) and then cloned into TK/luciferase reporter. These deletion constructs were transfected into JEG-3 cells for luciferase activity assay. As showed in Fig. 7, deletion of FP4 dramatically decreased the luciferase activity by nearly four-fold ($P < 0.05$), while deletion of FP1, FP2, or FP3 only caused about two-fold decrease of luciferase activity, respectively ($P < 0.05$). Further deletion of FP3 or FP2 only caused slightly additional decrease of the luciferase activity (TKU21 and TKU43 in Fig. 8). Deletion of three of the four elements from either side (FP4, FP3, FP2, or FP3, FP2, FP1) resulted in an additional decrease of less than 10% (TKU1 and TKU4 in Fig. 9). Taken together, deletion of FP4 significantly decreased the luciferase activity (TKU321 in Fig. 10). Further deletion of the other elements (FP3 or FP3 and FP2) only caused slightly additional decrease of the luciferase activity (TKU21 and TKU1 in Fig. 10). On the other hand, deletion of FP1 caused a 50%, FP2 45%, and FP3 35 % loss of the luciferase activity (TKU432, TKU431, and TKU421 in Fig. 10,), respectively. These results clearly indicated that all four elements are required to confer the full hGnRH upstream promoter activity in JEG-3 cells. However, the FP4 is the most important for the hGnRH upstream promoter activity in JEG-3 cells.

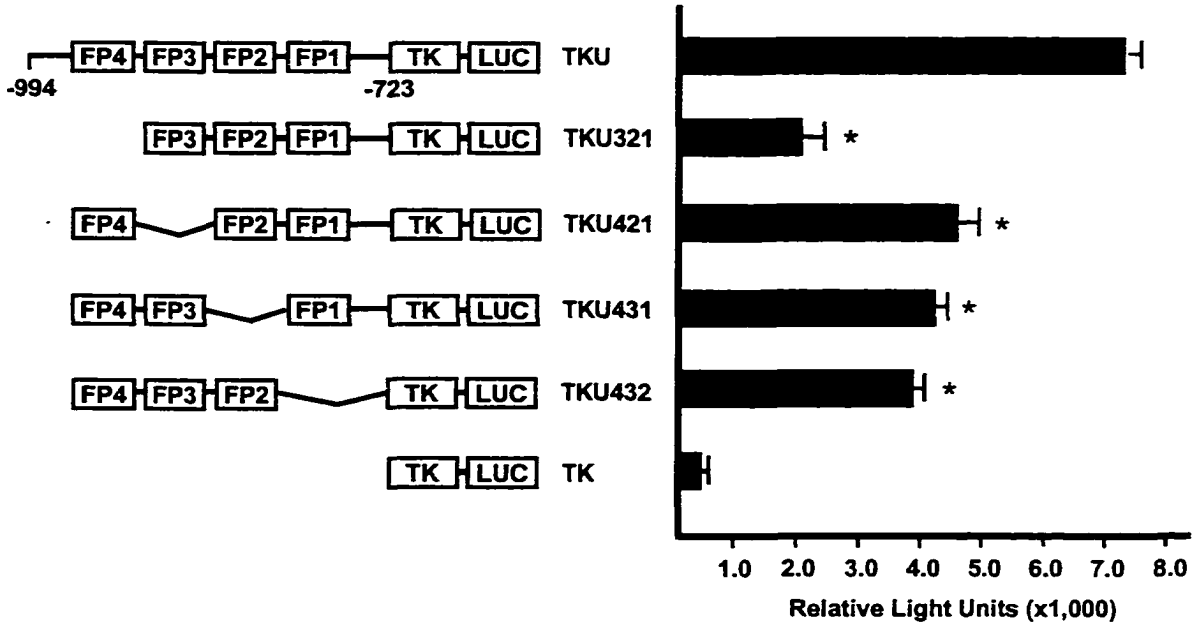


Fig. 7. Single-element deletion analysis of the four footprinted elements in the tissue-specific expression of hGnRH gene in JEG-3 cells. Three micrograms of hGnRH deletion constructs (TKU321, TKU421, TKU431, and TKU432), four-element construct (TKU), or TK and 2 μg of pCMV β-galactosidase were co-transfected into JEG-3 cells, respectively. After 20 h, the cells were harvested and lysates were assayed for β-galactosidase and luciferase activities. Data were standardized to β-galactosidase activities and shown as relative light units. Each bar represents the mean ± SEM. *, $P < 0.05$ as compared to TKU (by one-way ANOVA and LSD).

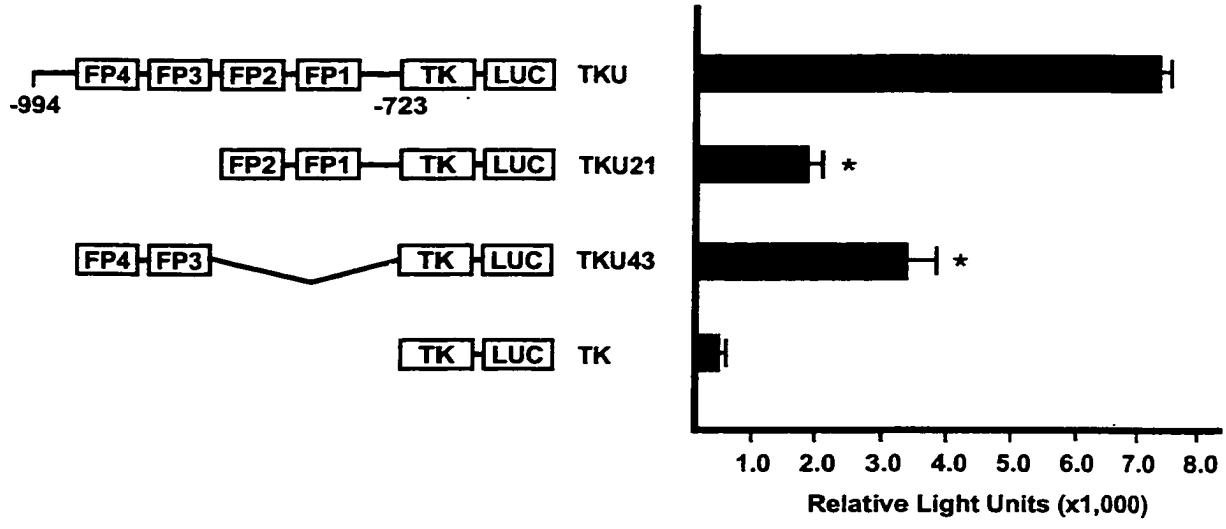


Fig. 8. Two-element deletion analysis of the four elements in the tissue-specific expression of hGnRH gene in JEG-3 cells. Three micrograms of hGnRH deletion constructs (TKU21 and TKU 43), TKU, or TK and 2 μg of pCMV β-galactosidase were co-transfected into JEG-3 cells, respectively. After 20 h, cell lysates were assayed for β-galactosidase and luciferase activities. Data were standardized to β-galactosidase activities and shown as relative light units. Each bar represents the mean ± SEM. *, $P < 0.05$ as compared to TKU (by one-way ANOVA and LSD).

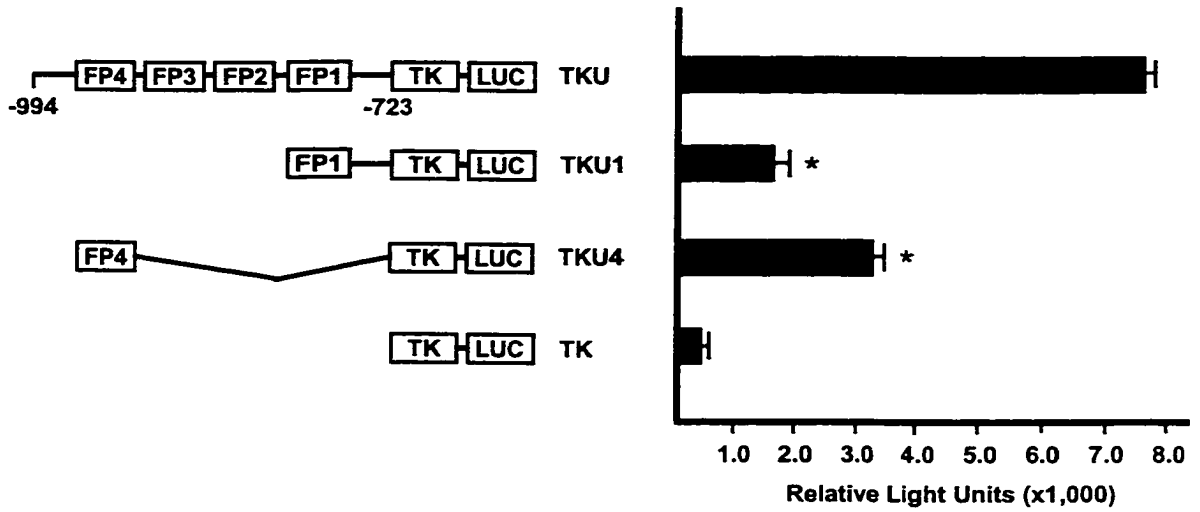


Fig. 9. Three-element deletion analysis of the four footprinted elements in the tissue-specific expression of hGnRH gene in JEG-3 cells. Three micrograms of hGnRH deletion constructs (TKU1 and TKU 4), TKU, or TK and 2 μg of pCMV β-galactosidase were co-transfected into JEG-3 cells, respectively. After 20 h, cell lysates were assayed for β-galactosidase and luciferase activities. Data were standardized to β-galactosidase activities and shown as relative light units. Each bar represents the mean ± SEM. *, $P < 0.05$ as compared to TKU (by one-way ANOVA and LSD).

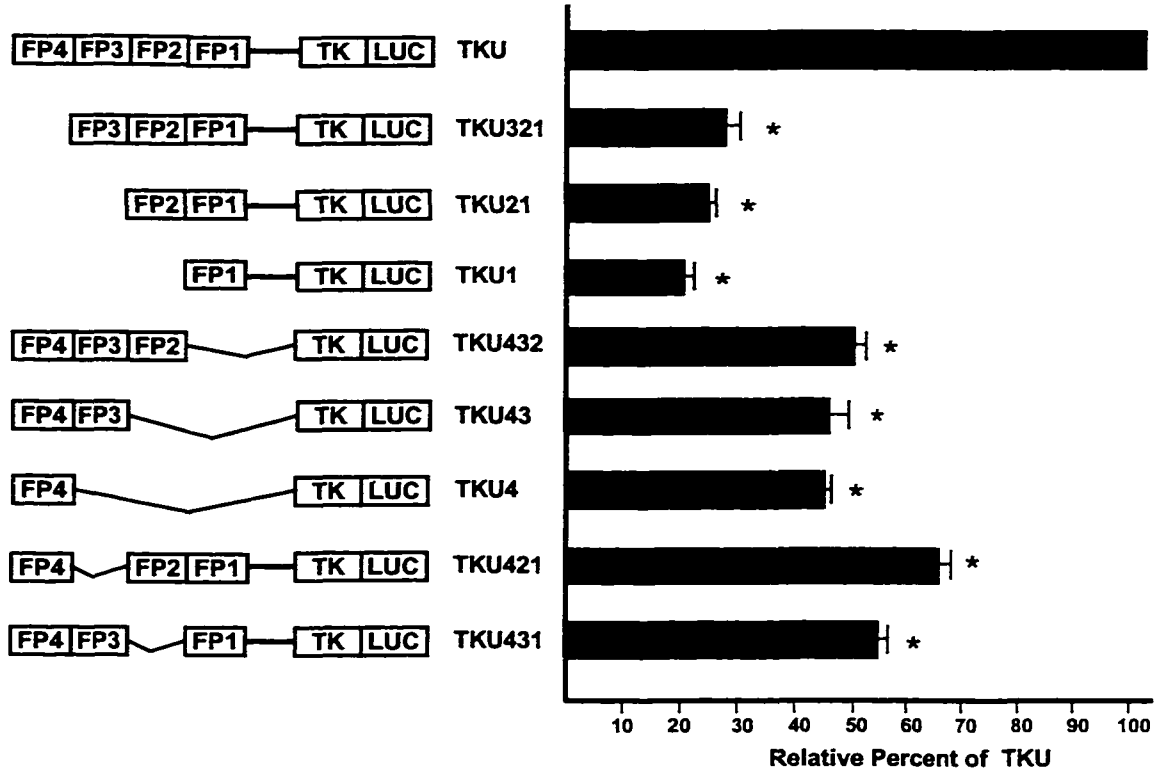


Fig. 10. Comparison of luciferase activities between various hGnRH upstream promoter element(s)/TK-luciferase constructs. Relative luciferase activities from various hGnRH deletion constructs and TKU transfected JEG-3 cells were assayed and represented as the mean ± SEM of relative percent of TKU's luciferase activity. *, $P < 0.05$ as compared to TKU (by one-way ANOVA and LSD).

3. Serial deletion analysis supports the roles of the four elements in the tissue-specific expression of the hGnRH gene in JEG-3 cells.

To confirm the interactive roles of the four elements in the tissue-specific expression of the hGnRH upstream promoter in JEG-3 cells, transient transfections were performed in JEG-3 cells with a set of 5' end deletion of the hGnRH upstream promoter/luciferase constructs. As shown in Fig. 11, deletion of the hGnRH upstream promoter to –991 bp retained most of the full upstream promoter activity. Deletion to –935 bp (FP4 and FP3 were removed) resulted in significant decrease of the full upstream promoter activity. However, further deletion to –880 bp (FP4, FP3, and FP2 were removed) only resulted in a slightly additional decrease in the upstream promoter activity. When deletion was up to –827 bp (all four elements were removed), the hGnRH upstream promoter activity was dramatically decreased to a level more than six-fold below that of the four-element construct (hU5a). Additional deletion to –775 bp made no further significant change of the luciferase activity. Thus, the results of serial deletion analysis further confirmed that the four cis-regulatory elements are essential for the hGnRH upstream promoter activity in JEG-3 cells.

In addition, mutational analysis was performed to provide further evidence for the importance of FP4 in the hGnRH upstream promoter activity in JEG-3 cells. Using PCR with specific primers (as described in Materials and Methods), FP4 and FP3 were replaced by unrelated sequences and cloned into promoter-less luciferase reporter. This two mutant constructs were then transfected into JEG-3 cells for luciferase activity analysis. As shown in Fig. 12, mutation of FP4

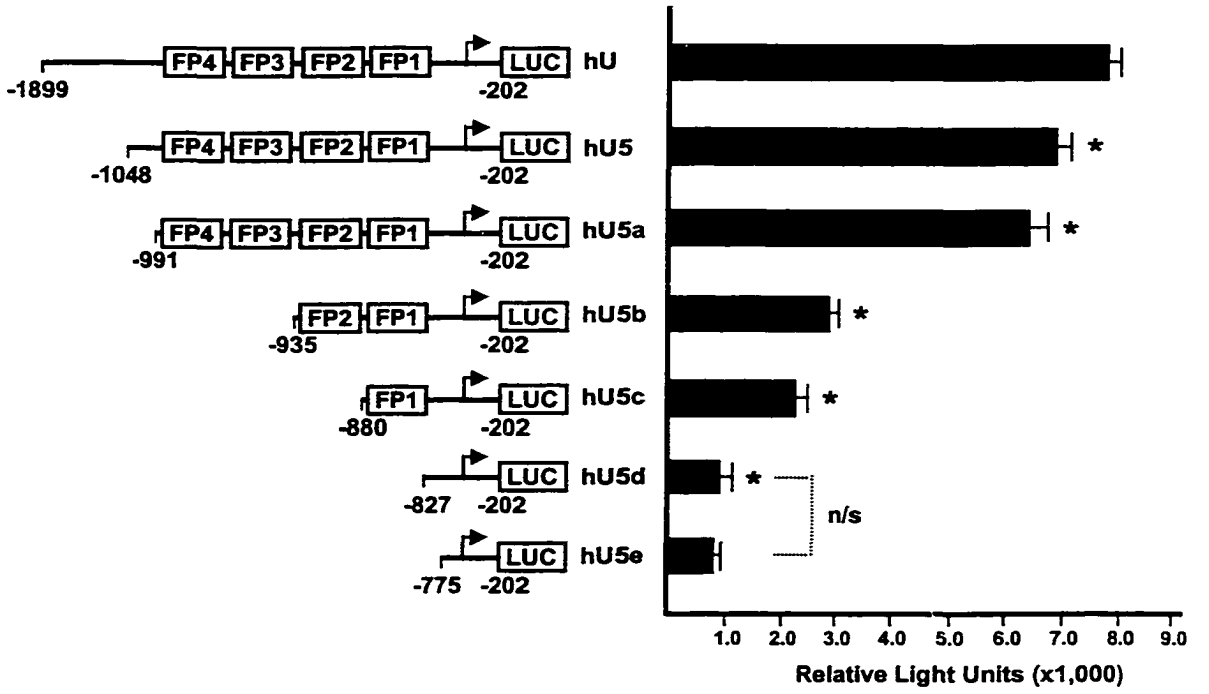


Fig. 11. Serial 5' deletion analysis of hGnRH upstream promoter activity in JEG-3 cells. All 5' deletion constructs shown extend to –202 bp in their 3' extent. The hGnRH upstream transcription start site is shown as bent arrow. Three micrograms of test plasmids and 2 μg of pCMV β-galactosidase were co-transfected into JEG-3 cells and cells harvested after 20 h. Lysates were assayed for β-galactosidase and luciferase activities. Data were standardized to β-galactosidase activities as a control for transfection efficiency. Each bar represents the mean ± SEM of luciferase activity, which is shown as relative light units. *, $P < 0.05$ as compared to hU (by one-way ANOVA and LSD). n/s, not significant (by Student's *t* test).

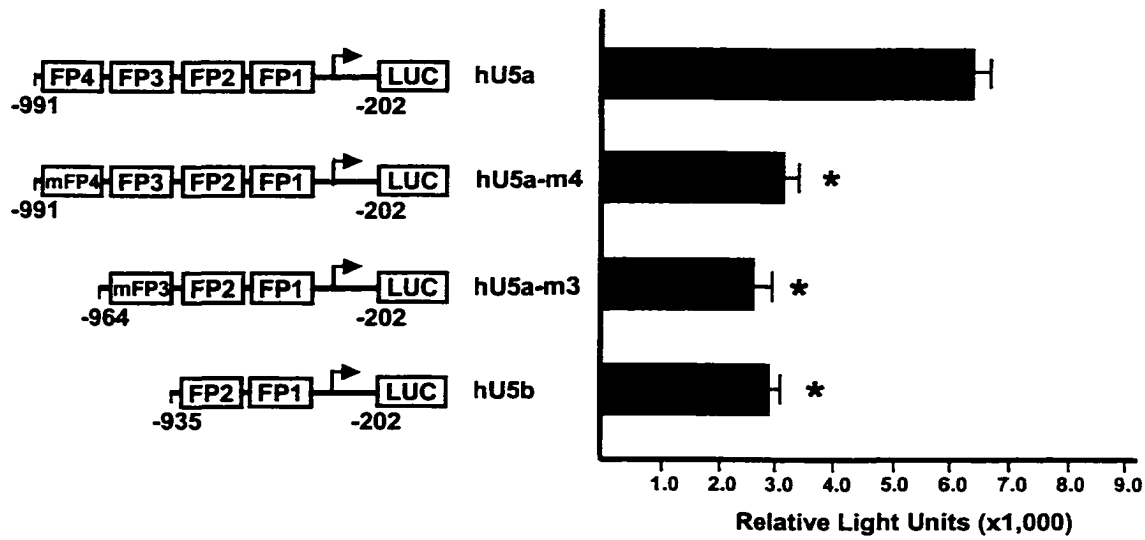


Fig. 12. Mutational analysis of FP4 and FP3 elements. FP4 and FP3 block mutation constructs (hU5a-m4 and hU5a-m3) were made by PCR as described in Materials and Methods. Three micrograms of test plasmids and 2 μg of pCMV β-galactosidase were co-transfected into JEG-3 cells. After 20 h incubation, cell lysates were assayed for β-galactosidase and luciferase activities. Data were standardized for β-galactosidase activities as a control for transfection efficiency. Each bar represents the mean ± SEM of luciferase activity, which is shown as relative light units. *, $P < 0.05$ as compared to TKU (by one-way ANOVA and LSD).

resulted in two-fold decrease, while mutation of FP3 only resulted in slightly additional decrease of the luciferase activities similar as that of FP1/FP2 construct (hU5b). These results further support the important role of FP4 in the hGnRH upstream promoter activity in JEG-3 cells. Taken together, all the four elements are involved in the tissue-specific expression of the hGnRH gene in JEG-3 cells, while the FP4 element is the most important.

4. POU-homeodomain protein Oct-1 is involved in the DNA-protein interaction of FP4 in the hGnRH upstream promoter.

Sequence analysis revealed that the four elements are short AT-rich, suggesting the possible binding of transcriptional factor POU-homeodomain protein Oct-1. To determine whether Oct-1 is expressed in our JEG-3 cells, western blot assay was performed with specific antibody against Oct-1 protein. As shown in Fig. 13, the expression of Oct-1 was detected in our JEG-3 cells with higher concentration in nuclear extract. Subsequently, gel mobility shift assay was conducted to examine the DNA-protein interaction between the four elements and nuclear extract from JEG-3 cells. As expected, all of the four elements bound with JEG-3 nuclear extract (Fig. 14, lane 2, 7, 12, and 17). Also, the bound protein(s) can be competed out with 500-fold of excess unlabeled corresponding oligonucleotides (Fig. 14, lane 3, 8, 13, 18), indicating the specific DNA-protein interaction. Furthermore, supershift assay using Oct-1 antibody was carried out to see if Oct-1 protein is directly involved in the DNA-protein interaction. As shown in Fig. 14 (lane 19), only FP4 DNA-protein complex was

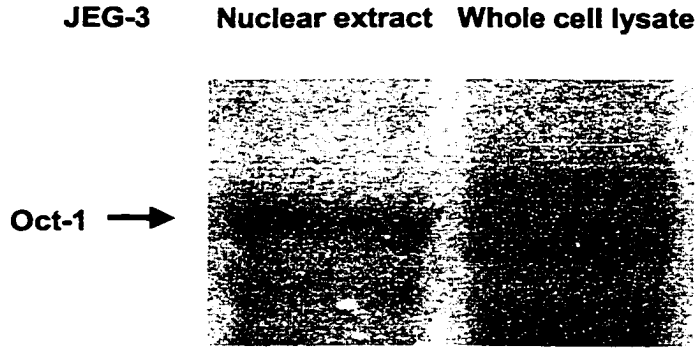


Fig. 13. Expression of POU homeodoamin protein Oct-1 in JEG-3 cells. Western blot was performed to determine the presence of Oct-1 in JEG-3 cells. Ten micrograms of nuclear extract or whole cell lysate from JEG-3 cells were loaded on to 8% SDS-PAGE gel and detected with specific antibody against Oct-1 protein.

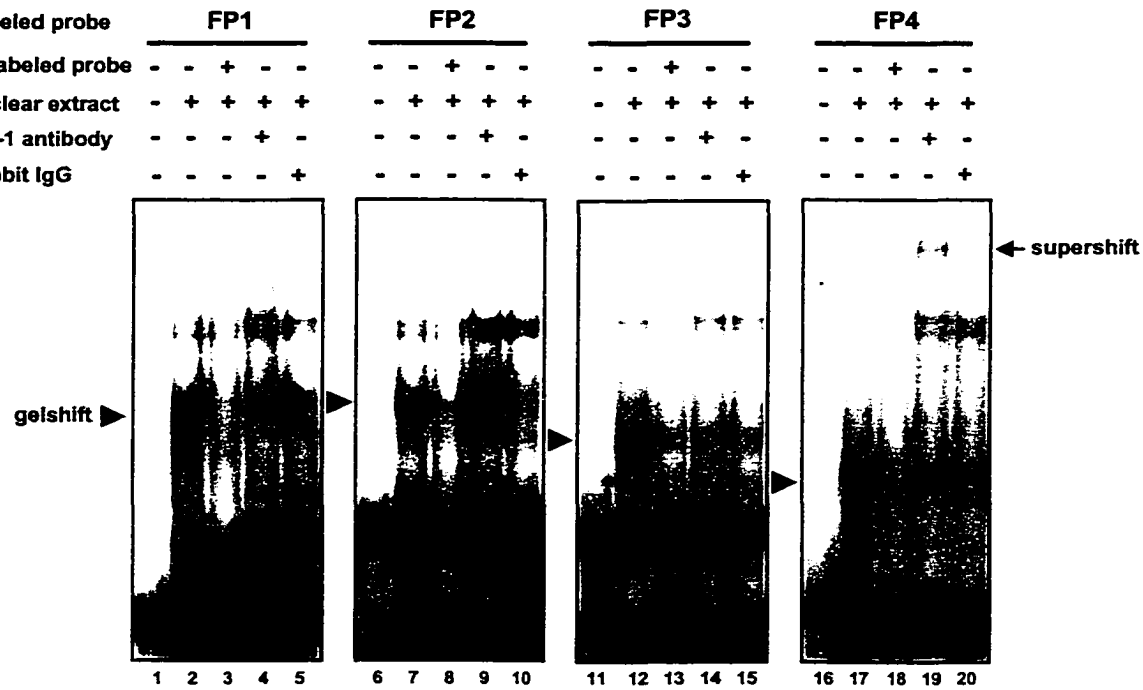


Fig. 14. Oct-1 binding within FP4. EMSAs were performed with [γ-^{32}P]ATP labeled oligonucleotides corresponding to footprints 1 to 4 (FP1, lanes 1-5; FP2, lanes 6-10; FP3, lanes 11-15; FP4, lanes 16-20) and 3 μg of nuclear extract from JEG-3 cells (lanes 2-5, 7-10, 12-15, 17-20). Competition assays were conducted with 500-fold of excess unlabeled corresponding oligonucleotides (lanes 3, 8, 13, 18). Supershift assays were conducted in the presence of either Oct-1 specific antibody (lanes 4, 9, 14, 19) or nonspecific rabbit IgG (lanes 5, 10, 15, 20). The arrows on the left show the gelshift bands, while on the right is supershift band.

supershifted by Oct-1 antibody but not by rabbit IgG (Fig. 14, lane 20). Other elements (FP1, FP2, FP3) were not supershifted by Oct-1 antibody (Fig. 14, lane 4, 9, 14). These results indicated that Oct-1 binds to FP4 element and may function as a transcriptional regulatory protein in the hGnRH upstream promoter in JEG-3 cells.

Part II: Estrogen receptor α and β mediated estrogen regulation of the hGnRH upstream promoter activity in JEG-3 cells.

1. Detection of human estrogen receptor α (hERα) and β (hERβ) mRNAs in JEG-3 cells.

Although the presence of both ERα and ERβ in human placenta has been demonstrated (Brandenberger et al., 1997; Moore et al., 1998), the human placental JEG-3 cells have been reported to be deficient in ER (Chatterjee et al., 1989). To determine whether our JEG-3 cell line expresses hERα and hERβ, RT-PCR was performed on cytoplasmic RNAs from JEG-3 cells with specific primers for hERα and hERβ (as described in Materials and Methods). The RT from MCF-7 cells, which are known for hERα and hERβ expression, and ER cDNA plasmids were used as positive control. Also, cyclophilin primers were applied as internal control. As shown in Fig. 15, hERα and hERβ transcripts were amplified from hERα and hERβ cDNAs as well as MCF-7 cells, but not from our JEG-3 cells. These results indicated that our JEG-3 cells do not express hERα and hERβ. Thus, hERα and hERβ expression vectors will be transfected into JEG-3 cells in later estrogen treatment studies.

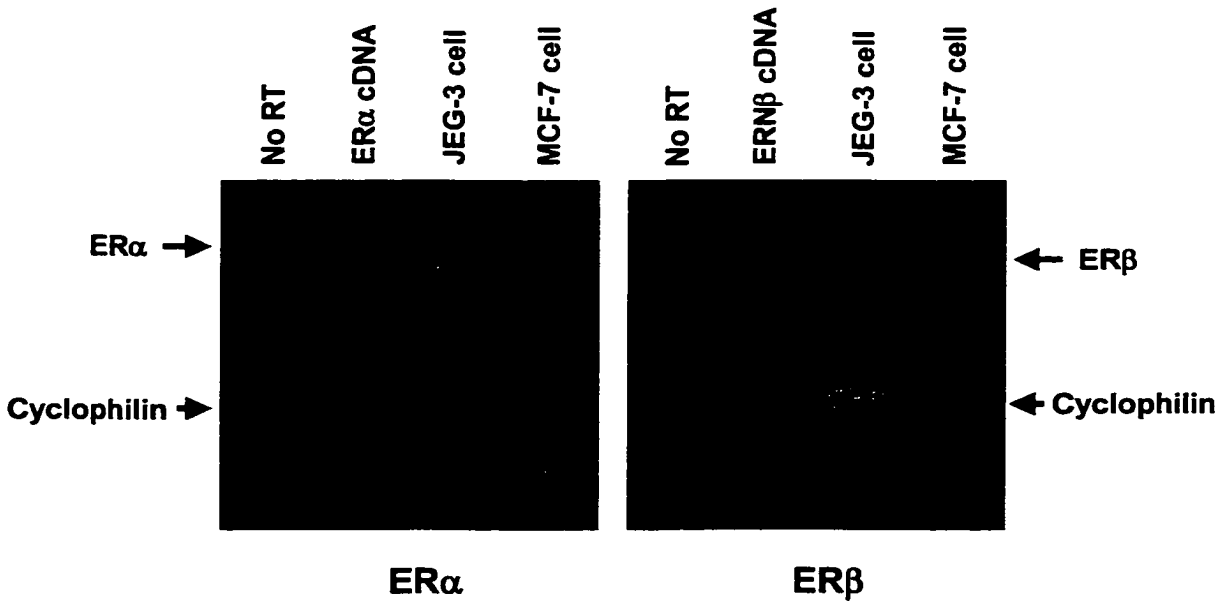


Fig. 15. Detection of hERα and hERβ mRNAs in JEG-3 cells by RT-PCR. Cytoplasmic RNAs were prepared from JEG-3 and MCF-7 cells. RT-PCR was then performed with specific primers for hERα and hERβ. Cyclophilin was used as internal control. The hERα and hERβ cDNA plasmids, MCF-7 sample were used as positive control. The amplified cyclophilin, hERα and hERβ products are shown as arrows.

2. Both hERα and hERβ mediate inhibitory effects of estradiol on the hGnRH upstream promoter activity in JEG-3 cells.

The ERα mediated estradiol down-regulation of the hGnRH upstream promoter activity has been reported previously (Dong et al., 1996). Because hERα and hERβ possess distinctive transactivational domains, I co-transfected hERα or hERβ expression vectors with the hGnRH upstream promoter/luciferase construct (hU) into JEG-3 cells to determine the differential effect of hERα and hERβ mediated estradiol regulation of the hGnRH upstream promoter activity in JEG-3 cells. The cells were then treated with 10 nM of 17β-estradiol (E_2) with or without 1 μM of antiestrogen ICI164,384 (ICI). As shown in Fig. 16, the luciferase activity had little change with the treatment of estradiol and/or ICI164,384 when without co-transfection of hERα and hERβ, supporting the deficiency of ER in JEG-3 cells. When hERα was co-transfected into JEG-3 cells, as expected estradiol treatment significantly decreased the luciferase activity. Further, addition of ICI164,384 abolished the inhibitory effect of estradiol on the hGnRH upstream promoter activity, while ICI164,384 alone made little change on the luciferase activity. Co-transfection of hERβ displayed the similar inhibitory effect as hERα did, but in a less extent. These results indicate that both hERα and hERβ mediate inhibitory effects of 17β-estradiol on the hGnRH upstream promoter activity in JEG-3, while hERβ acts less potent than hERα does.

3. The hERα- and hERβ-mediated inhibitory effects of estradiol on the hGnRH upstream promoter activity are dose-dependent.

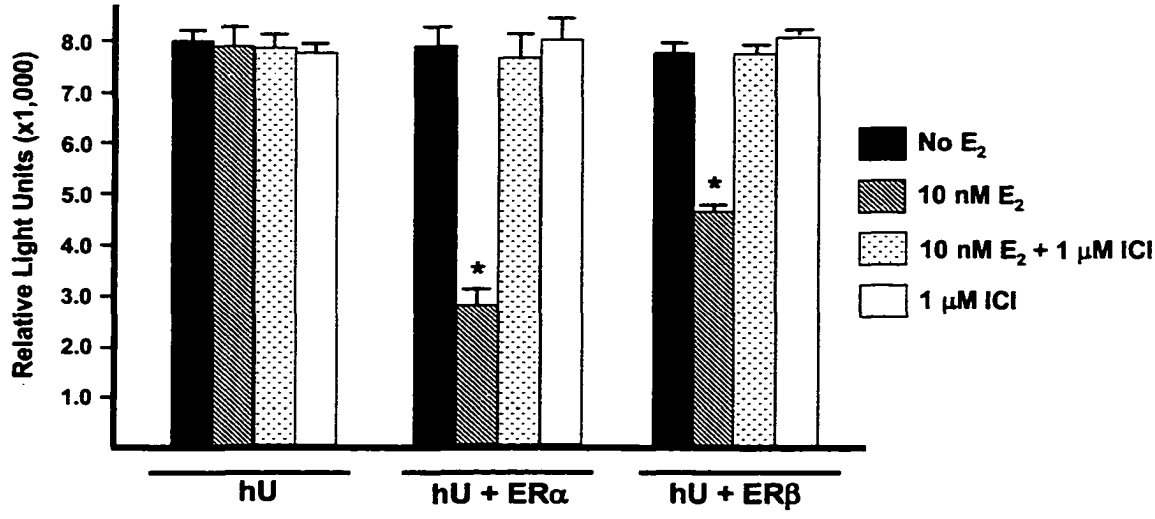


Fig. 16. ER-mediated estradiol regulation of hGnRH upstream promoter activity. The cells were transfected with 3 μg of hU and 2 μg of pCMV β-galactosidase, with or without 1 μg of hERα or hERβ. Then, the cells were treated for 24 h with various combinations of vehicles, E_2 (10 nM) and ICI (1 μM) as indicated. Lysates were assayed for β-galactosidase activity and luciferase activity as described in Materials and Methods. Each bar represents the mean ± SEM and standardized luciferase activities are shown as relative light units. *, $P < 0.05$ as compared to control (no E_2) (by Student's *t* test).

To confirm that hERα and hERβ mediate the estradiol effects on the hGnRH upstream promoter, 3 μg of hU and various amounts of hERα or hERβ were co-transfected into JEG-3 cells in the presence of 10 nM of 17β-estradiol. As expected (Fig. 17, hERα), increasing exogenous expression of hERα resulted in decreasing luciferase activity, with maximal estradiol action was obtained at 1 μg of transfected hERα. Transfection of additional more hERα (2 or 4 μg) did not result in further decrease. On the other hand, hERβ displayed maximal inhibitory effect at 0.25 μg transfected (Fig. 17, hERβ). Increasing transfected hERβ (up to 4 μg) resulted in less inhibitory effect, but still mediated the down-regulation of 17β-estradiol on the hGnRH upstream promoter activity in JEG-3 cells. These different effects may be due to the differential transactivation of hERα and hERβ.

To determine whether the estradiol effect on the hGnRH upstream promoter is dose-dependent or not, JEG-3 cells were co-transfected with 3 μg of hU construct and 1 μg of hERα or hERβ expression vector, and then treated with various concentrations of 17β-estradiol. As shown in Fig. 18, estradiol down-regulated the hGnRH upstream promoter activity in a dose-dependent manner. Compared to that of hERα, hERβ mediated the effect in a less extent. For both hERα and hERβ, 17β-estradiol produced a significant decrease in the luciferase activity at concentration as low as 0.01 nM, with a maximal effect at concentration of 100 nM. Thus, both hERα and hERβ mediate dose-dependent response of the hGnRH upstream promoter to 17β-estradiol, while hERβ in a less extent.

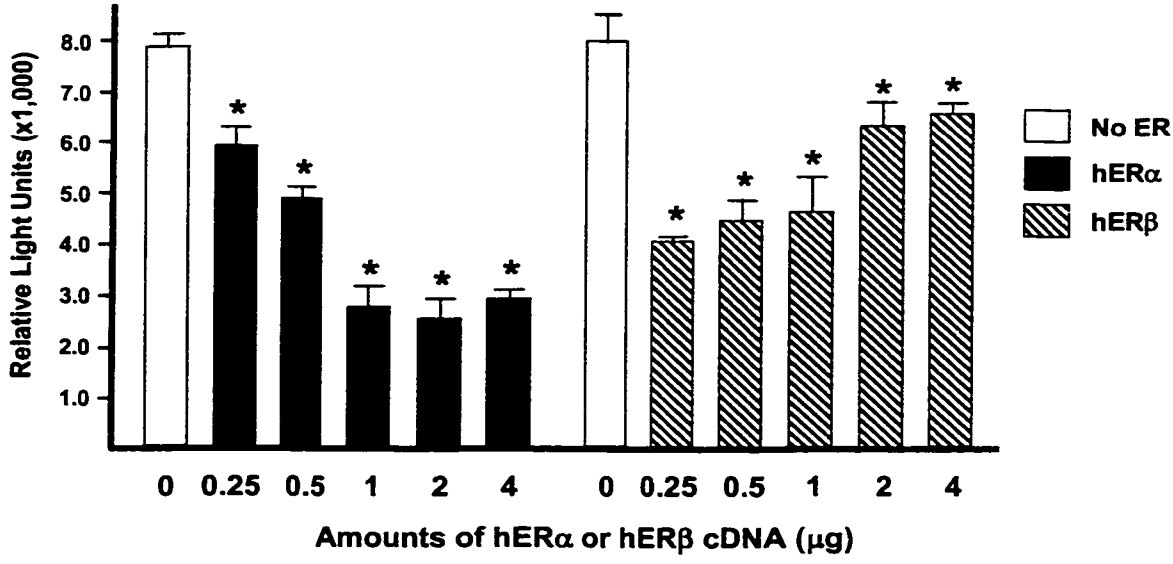


Fig. 17. Dose-dependent effect of exogenous hERα or hERβ expression on hGnRH upstream promoter activity in JEG-3 cells. JEG-3 cells were transfected with 3 μg of hU plus various amounts of hERα or hERβ expression vector as indicated. After treatment with 10 nM E_2 for 24 h, cell lysates were assayed for luciferase activities as described in Materials and Methods. Each bar represents the mean ± SEM and luciferase activities are shown as relative light units. *, $P < 0.05$ as compared to control (No ER) (by one-way ANOVA and LSD).

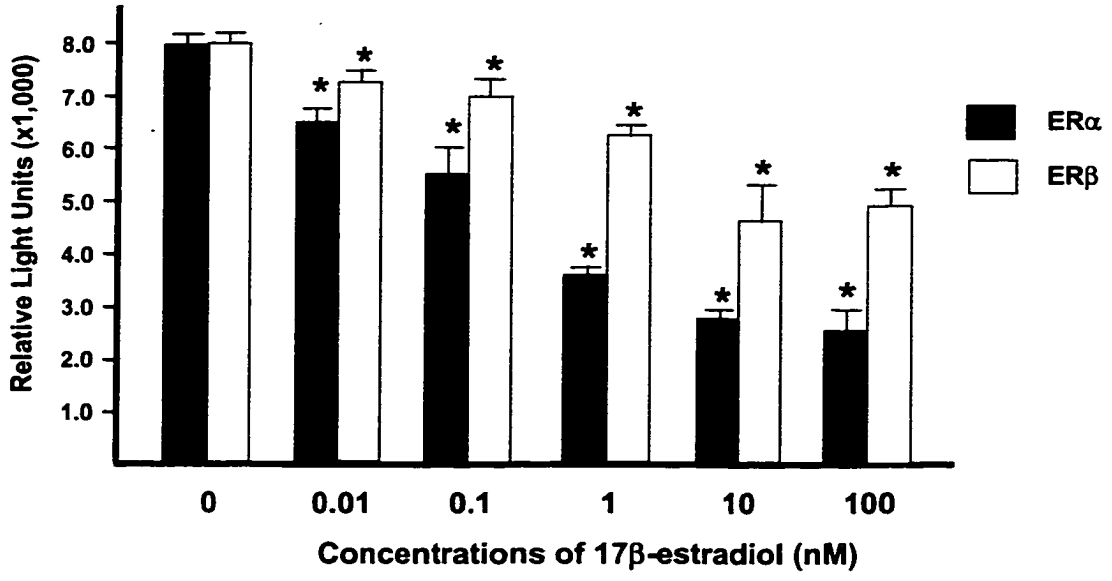


Fig. 18. Dose-dependent response of hGnRH upstream promoter to estradiol treatment. Transient transfections were performed in JEG-3 cells with 3 μg of hU plus 1 μg of hERα or hERβ. After 24 h treatment with various concentrations of estradiol as indicated, lysates were assayed for luciferase activities as described in Materials and Methods. Each bar represents the mean ± SEM and luciferase activities are shown as relative light units. *, $P < 0.05$ as compared to control (no 17β-estradiol) (by one-way ANOVA and LSD).

4. The hERα- and hERβ-mediated regulatory effects of estrogen isoforms on the hGnRH upstream promoter activity in JEG-3 cells.

Estrogen has multiple isoforms, such as estrone, estradiol, and estriol that have been found to be present in the human placenta. It is of interesting to see the effects of these estrogen isoforms on the hGnRH upstream promoter activity. For this purpose, JEG-3 cells were co-transfected with the hGnRH upstream promoter/luciferase construct (hU) and hERα or hERβ expression vector, and then treated with estrone, estradiol, or estriol for 24 h, respectively. The cell lysates were analyzed for luciferase activity. As shown in Fig. 19, without ER co-transfected, these estrogen isoforms made little change of the luciferase activity. When hERα co-transfected, estradiol and estriol, but not estrone significantly decreased the luciferase activities ($P < 0.05$). While co-transfection of hERβ, all these estrogens resulted in significant loss of luciferase activity ($P < 0.05$). However, estradiol displayed more potent inhibitory effect via hERα than hERβ, while estriol showed similar effect via hERα or hERβ.

5. Interactive effects of hERα and hERβ on the hGnRH upstream promoter activity in JEG-3 cells.

It has been reported that ERα and ERβ can interact each other to form heterodimer and thus modulate transactivational activities each other (Paech et al., 1997). To determine whether hERα and hERβ modulate each other in

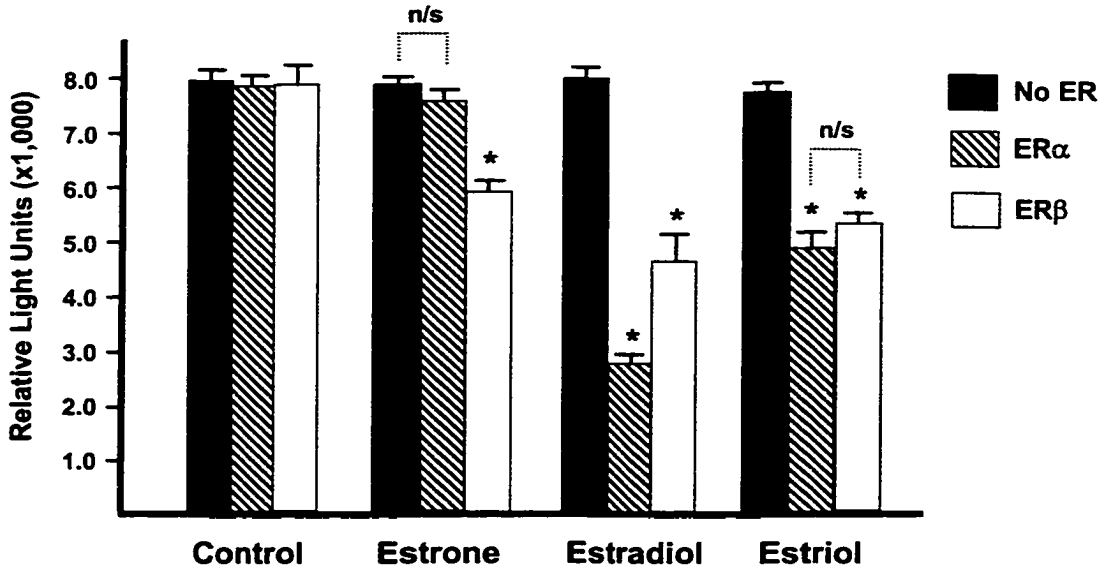


Fig. 19. Effects of different estrogens on hGnRH upstream promoter activity in JEG-3 cells. JEG-3 cells were transfected with 3 μg of hU and 1 μg of hERα or hERβ. The cells were then treated for 24 h with 10 nM of various vehicles, estrone, estradiol, or estriol. Lysates were assayed for luciferase activities as described in Materials and Methods. Control without any treatment. Each bar represents the mean ± SEM and luciferase activities are shown as relative light units. *, $P < 0.05$ as compared to no ER; n/s, not significant (by Student's *t* test).

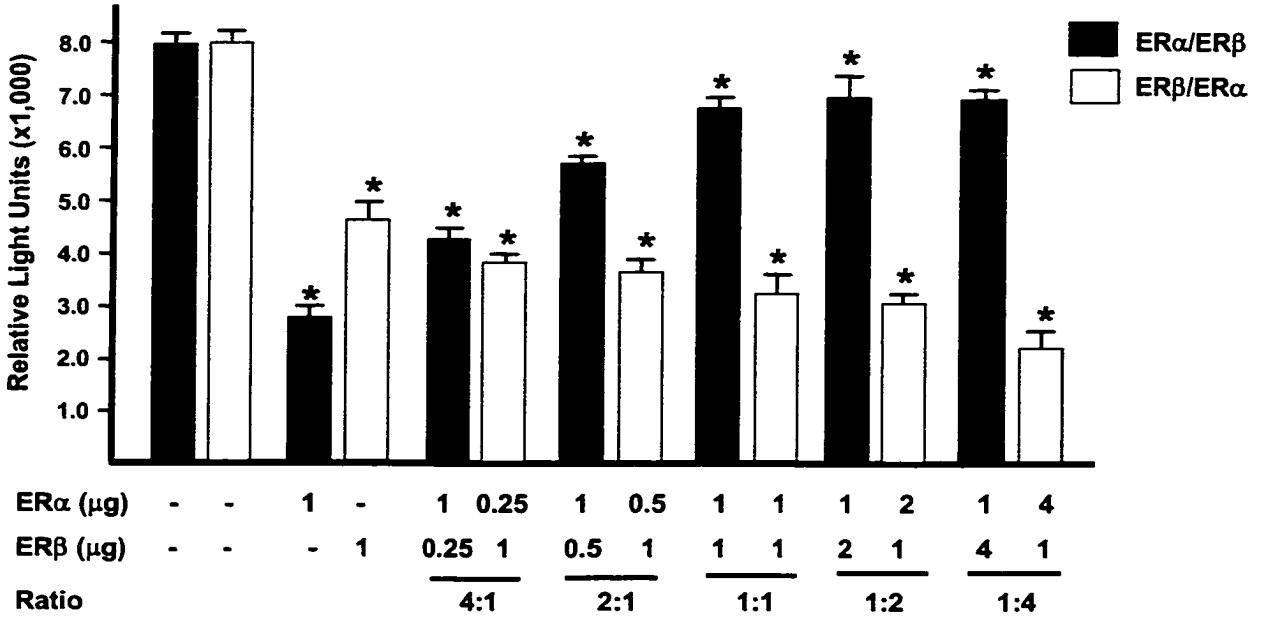


Fig. 20. Interactive effects of hERα and hERβ on hGnRH upstream promoter activity in JEG-3 cells. JEG-3 cells were transfected with 3 μg of hU and various amount of hERα and hERβ as indicated. The cells were then treated with 10 nM of 17β-estradiol for 24 h. Lysates were assayed for luciferase activities as described in Materials and Methods. Each bar represents the mean ± SEM for luciferase activities shown as relative light units. *, $P < 0.05$ as compared to control (no ER transfected) (by one-way ANOVA and LSD).

mediating the estradiol effect on the hGnRH upstream promoter, various amounts of hERα and/or hERβ as indicated in Fig. 20 were co-transfected with hU into JEG-3 cells. The cells were then treated with 10 nM of 17β-estradiol for 24 h before luciferase assay. When 1 μg of hERα was transfected with increasing amount of hERβ (from 0.25 to 4 μg), the inhibitory effect of 17β-estradiol on the promoter activity was lessened to the maximal at 2 μg of hERβ co-transfected. On the other hand, the down-regulation of estradiol on the hGnRH upstream promoter was enhanced with increasing amount of hERα (from 0.25 to 4 μg) and 1 μg of hERβ co-transfected. These results indicate that hERα and hERβ can modulate each other in mediating the estradiol effect on the hGnRH upstream promoter activity in JEG-3 cells.

To confirm the co-transfection of hERα and/or hERβ into JEG-3 cells, RT-PCR was performed on the cytoplasmic RNAs prepared from hERα and/or hERβ transfected JEG-3 cells. As shown in Fig. 21, hERα was detected only in hERα transfected cells, but not in hERβ as well as no ER transfected cells. Also, hERβ was only found in hERβ transfected but not in hERα and no ER transfected cells.

6. Analysis of negative and positive estrogen responsive elements in the hGnRH upstream promoter.

Previous studies in our laboratory have demonstrated a negative estrogen responsive element (-991 to –935) and a positive (-827 to –730) estrogen responsive element in the hGnRH upstream promoter region. To further confirm

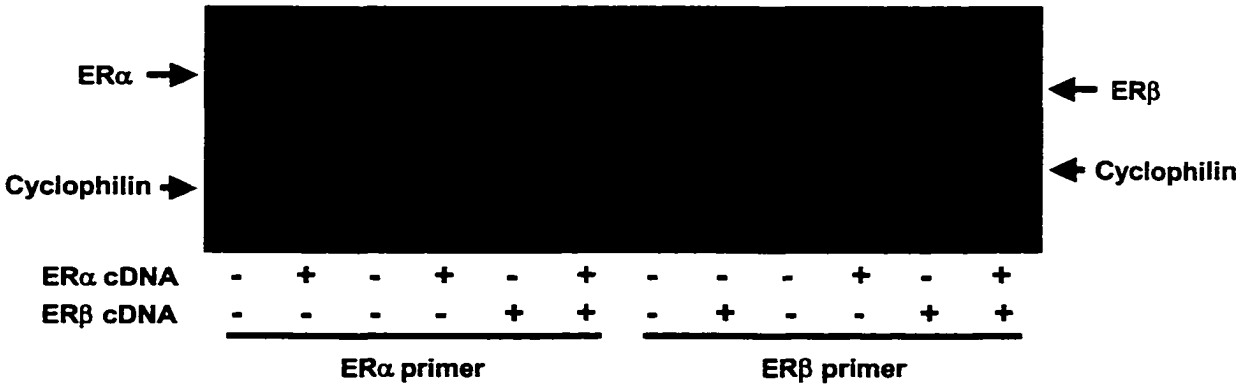


Fig. 21. Determination of hERα and hERβ transfection in JEG-3 cells by RT-PCR. Cytoplasmic mRNAs were prepared from 3 μg of hU, 1 μg of hERα and/or hERβ, co-transfected JEG-3 cells. RT-PCR was then conducted with specific primers for hERα and hERβ. Cyclophilin was used as internal control.

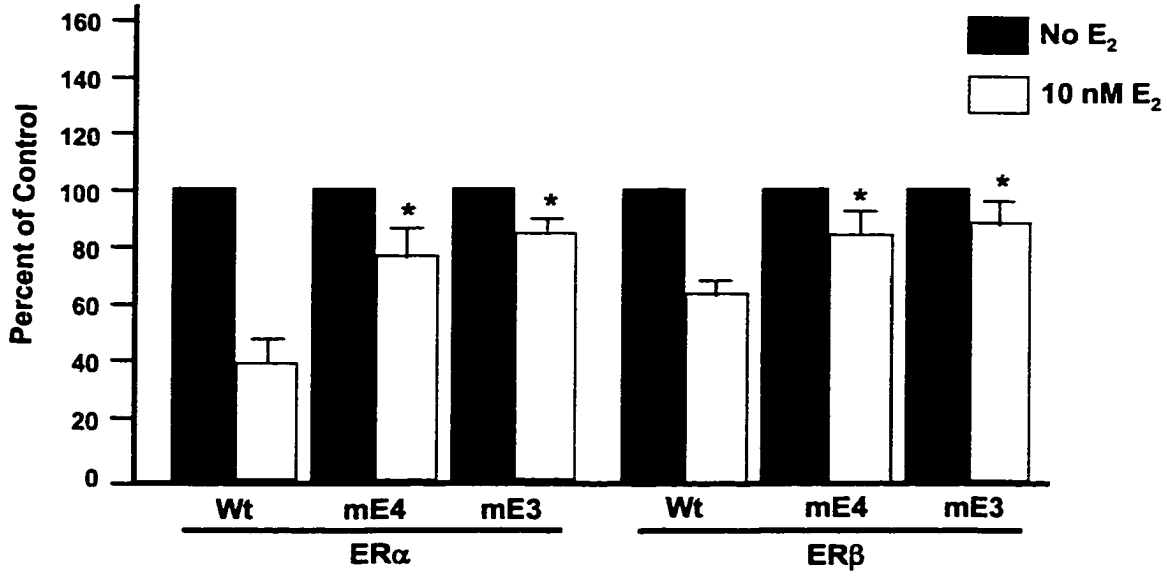
these findings, mutational studies on the negative and positive estrogen responsive elements were conducted. Using PCR, the negative estrogen responsive element was replaced by two unrelated sequences (Fig. 22A) and then cloned into the promoter-less luciferase reporter, pxp2luc. The mutant constructs and wild type plasmid were transfected into JEG-3 cells with hERα or hERβ. As shown in Fig. 22B, replacement of the negative estrogen responsive element with unrelated sequence partially abolished ERα- or ERβ-mediated responsiveness of hGnRH upstream promoter to 17β-estradiol treatment. The positive estrogen responsive element containing putative ERE was also replaced by unrelated sequence using PCR (Fig. 23A). The mutant element was subsequently cloned into pxp2luc and transfected with hERα or hERβ into JEG-3 cells, followed by 17β-estradiol treatment. As shown in Figure 23B, mutation of the positive estrogen responsive element partially abolished ERα-mediated stimulatory effect, but no significant change for ERβ.

To explore the protein(s) bound to the putative ERE which located within the positive estrogen responsive element, EMSA and supershift with anti-ERα antibody have been performed previously, and showing no supershift of the element-protein complex. In the present study, EMSA was conducted with [γ-^{32}P]ATP labeled the putative ERE and recombinant hERα protein to further determine whether ER protein directly binds to this putative ERE. As shown in Fig. 24, hERα protein did not bind to the putative ERE, indicating that ER protein does not directly involve in the DNA-protein interaction.

wE4: 5'-CCAAGCTTCCGAATGCTCACTAAATGCCGGGGGTTTATTAAGAGAGATTTAAATAA-3'
mE4: 5'-CCAAGCTTAATGGCAGGACTGCCCGAATATAATGGCATTAAGAGAGATTTAAATAA-3'

wE3: 5'-CCAAGCTTTATTAAGAGAGATTTAAATAAGATGGGATCTTTGACTATTACAGGTTTC-3'
mE3: 5'-CCAAGCTTGCCGCTCTGTCGGGCCCGCGCTCGAAACCTTTGACTATTACAGGTTTC-3'

A



B

Fig. 22. Mutational analysis of negative estrogen response element in the hGnRH upstream promoter. A. The sequences of wild type negative estrogen response element and corresponding mutations. B. JEG-3 cells were co-transfected with 1 μg of hERα or hERβ and 3 μg of wild type negative estrogen response element construct Wt (hU5a, -991/-202), mutational construct mE4 and mE3, respectively. The cells were then treated with or without 10 nM of 17β-estradiol for 24 h. Lysates were assayed for luciferase activities. Data are standardized to β-galactosidase activity (co-transfected as control for transfection efficiency) and expressed as percent of control (no E_2 treatment). *, $P < 0.05$ as compared to E_2 treated Wt (by Student's *t* test).

Putative ERE: 5'-CCAAGCTTTGTAAATAACACGTCCACGGTTGCACCTCTGGGGTGGAAC-3'

Mutant ERE: 5'-CCAAGCTTTGTAAATAACCATAGTTGAATTGCACCTCTGGGGTGGAAC-3'

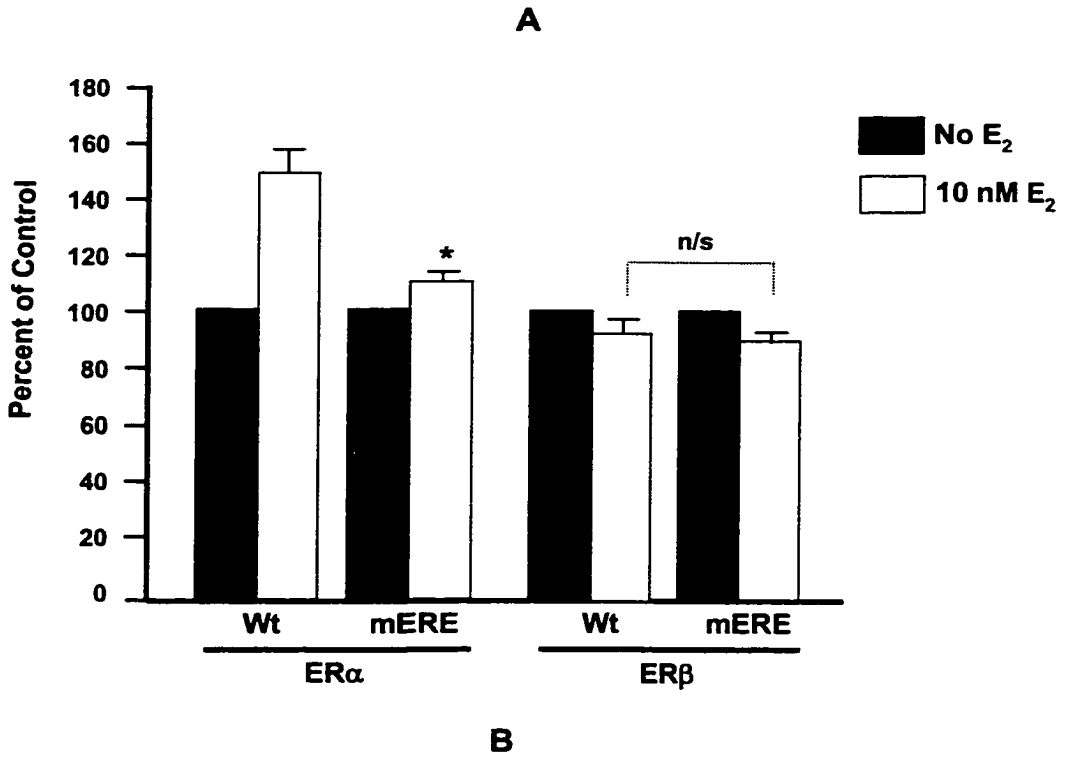


Fig. 23. Mutational analysis of positive estrogen response element in the hGnRH upstream promoter. A. Sequences for wild type putative ERE and mutated ERE. B. Transient transfections were performed in JEG-3 cells with 1 μg of hERα or hERβ and 3 μg of wild type putative ERE construct Wt (hU5d, -827/-202) or mutant ERE construct mERE. After 24 h treatment with 10 nM of 17β-estradiol, lysates were assayed for luciferase activities. Data are shown as percent of the control (no E_2 treatment). *, $P < 0.05$ as compared to E_2 treated Wt; n/s, no significance (by Student's *t* test).

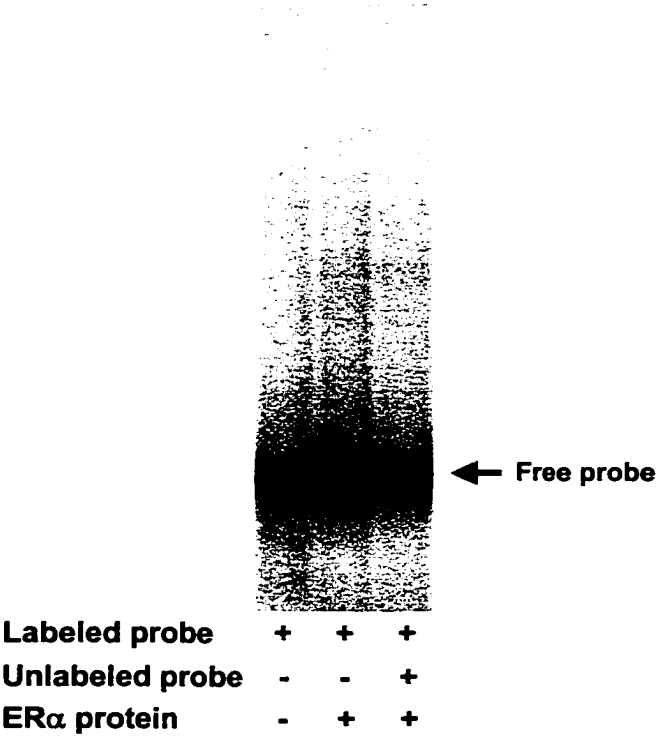


Fig. 24. The hERα protein does not bind to the putative ERE in the hGnRH upstream promoter. EMSA was performed with [γ-^{32}P]ATP labeled oligonucleotides corresponding to the putative ERE (-824 to –784) in the hGnRH upstream promoter and 7.3 pmol of hERα protein. Competition assays were conducted with 500-folk excess of the unlabeled probe.

Part III: Progesterone receptor A and B mediated progesterone regulation of the hGnRH upstream promoter activity in JEG-3 cells.

1. Detection of human progesterone receptor A (hPR-A) and B (hPR-B) in JEG-3 cells.

The expression of hPR has been reported in the human placenta (Chibbar et al., 1995; Shanker et al., 1997). To determine whether our JEG-3 cell line expresses hPR, RT-PCR was performed on the cytoplasmic RNAs from JEG-3 cells with specific primers for hPR (as described in Materials and Methods). The RT from MCF-7 cells, which are known for hPR expression, and hPR-A and hPR-B expression vectors were used as positive control. As shown in Fig. 25A, hPR transcript was amplified from our JEG-3 cells. Since hPR-A and hPR-B are differentially translated from the same mRNA transcript, western blot assay with specific antibodies against hPR-A/B and hPR-B was conducted. As shown in Fig. 25B, both hPR-A and hPR-B were detected from our JEG-3 cells. These results indicate that our JEG-3 cells express hPR-A and hPR-B protein.

2. The hPR-A mediates stimulatory effect of progesterone on the hGnRH upstream promoter in JEG-3 cells.

The stimulatory effect of progesterone (P4) on the hGnRH upstream promoter has been demonstrated in JEG-3 cells with or without hPR-B expression vector transfected (unpublished data in our lab). To determine the action of hPR-A in mediating the effect of progesterone on the hGnRH upstream promoter, 1 μg of hPR-A was co-transfected with the hGnRH upstream promoter/luciferase reporter (hU) into JEG-3 cells and treated with various vehicles, 0.1 nM P4 and 5

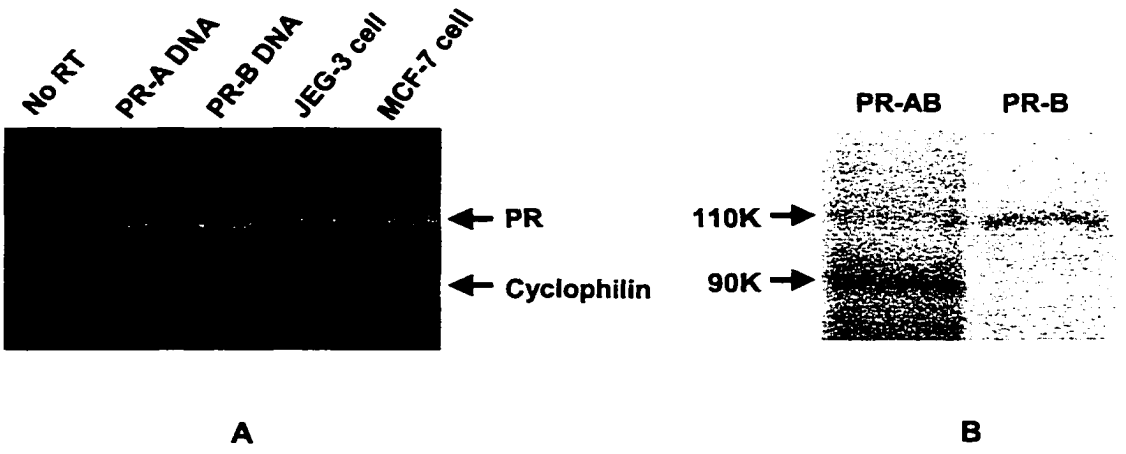


Fig. 25. Detection of hPR expression in JEG-3 cells by RT-PCR and Western blot. **A.** Cytoplasmic mRNAs were prepared from JEG-3 and MCF-7 cells. RT-PCR was then performed with specific primers for hPRs and cyclophilin (internal control). The hPR-A and hPR-B expression plasmids as well as MCF-7 sample were used as positive control. **B.** Western blot was conducted with specific antibodies against hPR-A/B and hPR-B as described in Materials and Methods.

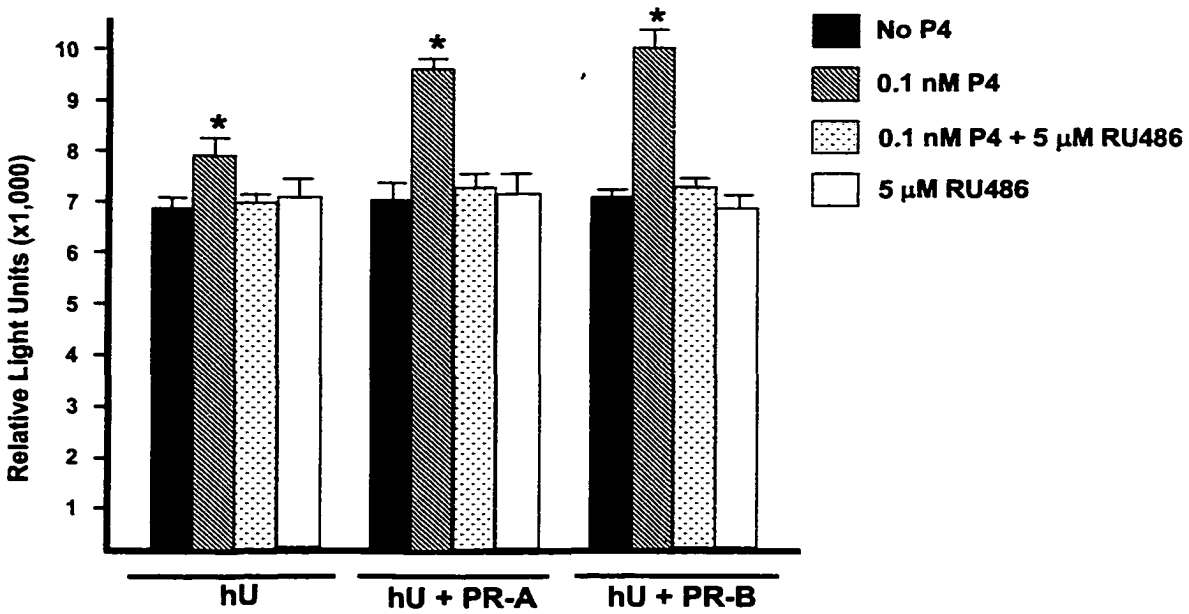


Fig. 26. PR-mediated progesterone stimulatory effect on hGnRH upstream promoter. JEG-3 cells were transfected with 3 μg of hU and 2 μg of pCMV βgal, with or without 1 μg of hPR-A or hPR-B. The cells were then treated for 24 h with various combinations of vehicle, P4 (0.1 nM) and RU486 (5 μM) as indicated. Lysates were assayed for luciferase activity as described in Materials and Methods. Each bar represents the mean ± SEM and luciferase activities are shown as relative light units. *, $P < 0.05$ as compared to control (no treatment) (by Student's *t* test).

μM RU486 as indicated in Fig. 26. As expected, P4 treatment increase the luciferase activity, while exogenous expression of hPR-B further enhanced the stimulatory effect of P4. This stimulatory effect was abolished by addition of RU486, a progesterone antagonist, which had no significant effect on the hU. Co-transfection of hPR-A exhibited similar stimulatory effect as hPR-B did but with a less potent. Thus, hPR-A also mediates a stimulatory effect of progesterone on the hGnRH upstream promoter in JEG-3 cells.

To confirm the hPR-A mediated stimulatory effect of P4 on the hGnRH upstream promoter, various amounts of hPR-A expression vector and 3 μg of hU were co-transfected into JEG-3 cells and treated with 0.1 nM of P4. As shown in Fig. 27, increasing amount of exogenous hPR-A transfection resulted in augment of luciferase activity. The maximal luciferase activity reached was at 2 μg of hPR-A. Further addition of hPR-A could not increase the luciferase activity any more. As a comparison, various amounts of hPR-B were co-transfected into JEG-3 cells with hU and treated with 0.1 nM of P4. The results showed that hPR-A mediated the stimulatory effect of P4 in a less potent than that of hPR-B at all amount of DNA transfected. Taken together, hPR-A mediates stimulatory effect of progesterone on the hGnRH upstream promoter activity but with a less potent as compared to that of hPR-B.

3. Dose-dependent response of the hGnRH upstream promoter to exogenous hPR-A and hPR-B mediated progesterone treatment.

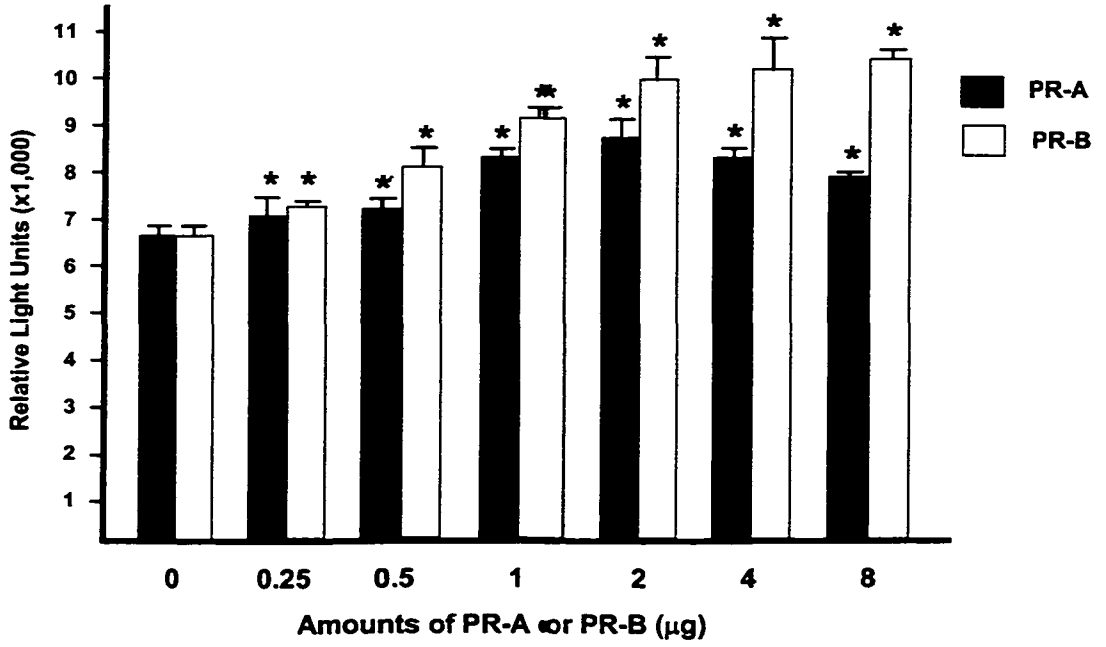


Fig. 27. Dose-dependent effect of exogenous hPR-A and hPR-B expression on the hGnRH upstream promoter activity in JEG-3 cells. JEG-3 cells were transfected with 3 μg of hU plus various amounts of hPR-A or hPR-B expression vector as indicated. The cells were then treated for 24 h with 0.1 nM P4. Lysates were assayed for luciferase activity. Two microgram of pCMV β-galactosidase was co-transfected to control transfection efficiency. Results are represented as relative light units with the mean ± SEM. *, $P < 0.05$ as compared to control (no PR) (by one-way ANOVA and LSD).

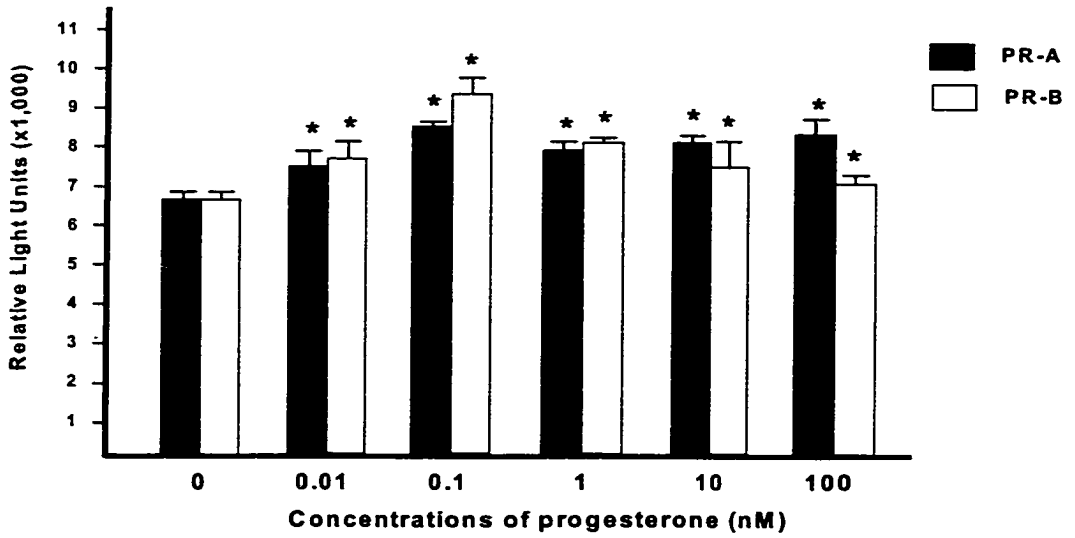


Fig. 28. Dose-dependent response of hGnRH upstream promoter to progesterone treatment. Transient transfections were performed in JEG-3 cells with 3 μg of hU and 1 μg of hPR-A or hPR-B. After 24 h treatment with various concentrations of P4 as indicated, lysates were assayed for luciferase activity. Each bar represents the mean ± SEM for luciferase activity which is shown as relative light units. *, $P < 0.05$ as compared to control (no progesterone) (by one-way ANOVA and LSD).

To determine whether the responsiveness of the hGnRH upstream promoter to hPR-A and hPR-B mediated progesterone treatment is dose-dependent, hPR-A or hPR-B and hU co-transfected JEG-3 cells were treated with various concentrations of progesterone (P4, from 0.01 nM to 100 nM). As shown in Fig. 28, progesterone at the concentration of 0.1 nM produced maximal effect on the promoter activity in both hPR-A and hPR-B transfected cells. However, when increasing the concentration of progesterone, little change in luciferase activity was observed in hPR-A transfected cells, while decreasing luciferase activity was displayed in hPR-B transfected cells. Therefore, hPR-A and hPR-B mediated stimulatory effects of progesterone on the hGnRH upstream promoter are dose-dependent, but also is biphasic in hPR-B transfected cells.

4. Coactivators SRC-1 and CBP upregulate hPR-A and hPR-B mediated stimulatory effect of progesterone on the hGnRH upstream promoter.

It has been reported that steroid receptor coactivator-1 (SRC-1) and CREB binding protein (CBP), independently or synergistically, enhance PR dependent transcription in a variety of cell lines (Onate ert al., 1995; Lundblad et al., 1995; Smith et al., 1996). In this study, SRC-1 and CBP expression vectors were co-transfected with the hU plasmid and hPR-A or hPR-B into JEG-3 cells to see whether exogenous expression of coactivator(s) could modulate hPR-mediated hGnRH upstream promoter activity. As shown in Fig. 29, co-transfection of SRC-1 or CBP significantly increased the luciferase activity in hPR-A or hPR-B transfected cells, while SRC-1 showed stronger effect than that of CBP. When

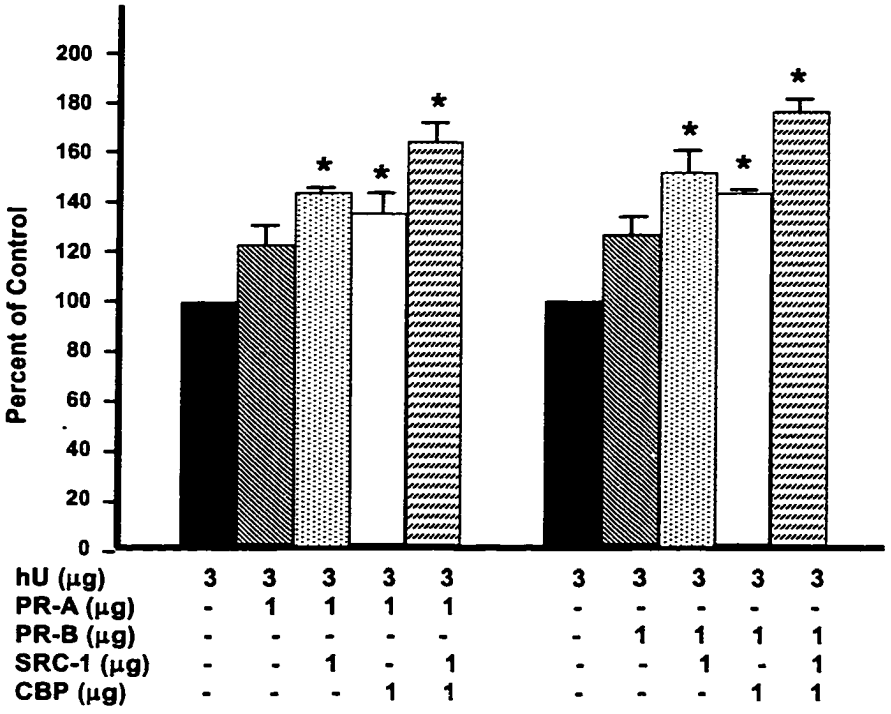


Fig. 29. Coactivator SRC-1 and CBP upregulate PR-mediated action on hGnRH upstream promoter activity in JEG-3 cells. The cells were co-transfected with different constructs as indicated and treated with 0.1 nM of progesterone for 24 h. The lysates were assayed for luciferase activity. Data are represented as percent of control (hU). *, $P < 0.05$ as compared to no coactivator transfected (by one-way ANOVA and LSD).

both SRC-1 and CBP were simultaneously co-transfected, the PR-mediated progesterone stimulatory effect on the upstream promoter was further increased. These results indicated that SRC-1 and CBP, independently or synergistically, upregulate hPR-A and hPR-B mediated stimulatory effect of progesterone on the hGnRH upstream promoter in JEG-3 cells.

DISCUSSION

1. The application of the human placental JEG-3 cell line as *in vitro* human placenta model.

To study the hGnRH gene expression and regulation in the human placenta, *in vivo* placenta, primary placental cell culture, and placental derived cell lines could be used as the study models. Due to the extremely low level of the GnRH produced in the placenta, it is not applicable to detect the changes in the GnRH level in the *in vivo* placenta. Also, the *in vivo* study of the hGnRH promoter activity could only be conducted in transgenic animals (Wolfe et al., 1996). Furthermore, the gene expression is under complex control, and therefore it is unlikely to be able to investigate single factor in the *in vivo* circumstances. Primary cell culture of the human placenta provides an alternative source for studying the hGnRH gene expression and regulation in the placenta. The advantage is that the primary placental cell culture might be more closely resemble to the *in vivo* placenta. The disadvantage, however, is that once removed from the *in vivo* placenta, the cells face a constantly changing environment which is difficult to control for gene expression study. The difficulty to obtain large amount of placentas also limits its application in the present study.

The available immortal cell lines have made gene expression and regulation studies practical. For GnRH promoter activity studies, the human placental JEG-3 cells and mouse GT1-7 cells have been widely used as *in vitro* models for placenta and neuron (Radovick et al., 1991; Wierman et al., 1992; Weiner et al.,

1993; Wetsel et al., 1993; Kepa et al., 1996; Dong et al., 1993). JEG-3 cell line is a human choriocarcinoma cell cloned from the Woods strain of the Erwin-Turner tumor in its 387th passage in hamster check pouch (Kohler et al., 1971). Similarities between normal and tumor placental cells have also been reported (Elston, 1979). This cell line can release human chorionic gonadotropin (hCG), human chorionic somatomammotrophin (hCS), and progesterone. It is also be able to transform steroid precursors to estrone and estradiol. Furthermore, the presence of the hGnRH and hGnRH receptor mRNAs in this cell line has been demonstrated (Dong et al., 1993; Yin et al., 1998). Although the presence of estrogen receptor and progesterone receptor in the human placenta has been reported (Younges et la., 1981; Kim et al., 1985; Chibbar et al., 1995; Rossmanith et al., 1997; Shanker et al., 1997 and 1998), only PR has been detected in our JEG-3 cells.

The mouse GT1-7 cell line was developed by targeting the SV-40 large T antigen to the hGnRH expressing hypothalamic neurons with the rat GnRH promoter (Mellon et al., 1990). This cell line can express prepro-GnRH mRNA and biosynthesize, process, and release pro-GnRH-derived peptides into the medium (Wetsel et al., 1991). GT1-7 cells also maintain the pulsatile pattern of the hGnRH secretion that is critical for successful reproduction (Wetsel et al., 1992). Thus, GT1-7 cell line is a suitable hypothalamic neuron model for GnRH studies.

2. The application of luciferase reporter gene in promoter activity studies.

The luciferase reporter gene has provided a sensitive, convenient, and cost-effective approach to study the transcriptional activity of cloned DNA sequences after introducing these elements into appropriate target cells. The mechanism of the luciferase catalysis reaction is: luciferase + luciferin + ATP^{Mg2+} <-> luciferase*luciferyl-AMP + PPi, luciferase*luciferyl-AMP + O_2 -> luciferase + oxyluciferin + AMP + CO_2 + $h\gamma$ (de Wet et al., 1987). Since mammalian cells do not contain any kind of luciferase, this reporter gene, in theory, is only limited by the "background noise" of the instrument. Light emission is directly proportional to the amount of luciferase produced in the transfected cells.

In the present study, two luciferase reporter genes were applied. One is promoter-less pxp2-luc, in which the hGnRH upstream promoter was cloned upstream luciferase gene to drive the expression of luciferase. Another is herpes simplex thymidine kinase (TK) minimal promoter/luciferase reporter pT109-luc, in which the hGnRH upstream cis-regulatory elements were cloned upstream the truncated TK promoter to regulate its activity.

3. The application of transient transfection assay in promoter activity studies.

In this study, calcium phosphate co-precipitation method was used for transient transfection assay because of the simplicity and low cost of this method. Also, its transfection efficiency of 10-20% in JEG-3 cells was high enough for the present study. Blank plasmid DNA, such as pCDNA, was used to

keep the total transfected DNA equal for each transfection. To correct the variation in transfection efficiency, a pCMV β-galactosidase plasmid was co-transfected into the cells with test plasmids. Further, the protein concentrations of the cell lysates were measured to normalize the protein amount for luciferase assay.

For steroid hormone treatment studies, it is essential to use phenol red free and fetal bovine serum (FBS) free medium to limit the influence of other stimulants and endogenous steroid hormones within cells. Since 10% FBS was critical for transfection in our JEG-3 cells, the regular medium supplemented with 10% FBS was used after transfection. The cells were then culture in phenol red free and FBS free medium with or without treatment for additional 20-24 h. Thus, the cells may still contain endogenous steroids at effective level during the study, resulting slightly higher background luciferase activities for non-treated cells.

4. The tissue-specific hGnRH upstream promoter activity is directed by multiple cis-regulatory elements.

Complex mechanisms have been evolved to ensure proper spatial and temporal expression of genes in differentiated tissues. In part, this control is achieved at the transcriptional level, so that regulatory regions containing multiple cis-acting sequence elements control both the level of gene expression and its restriction to appropriate cell types. The expression of the hGnRH gene has been found only in the hypothalamic neurons and a few non-hypothalamic tissues such as reproductive tissue, indicating the tissue-specific expression of

this gene. The identification of a second promoter (upstream promoter) that mainly used in reproductive tissue but not in neural cells further supports this concept (Dong et al., 1993). This observation has also been confirmed by the other group (Kepa et al., 1996). Recently, a 325-bp region between –1048 and –723 has been demonstrated to be involved in directing the activity of the hGnRH upstream promoter in the human placental JEG-3 cells. DNase I footprinting and gel mobility assays indicated four specific elements in this region that bind to nuclear extracts only from the human placental JEG-3 cells but not from the hypothalamic neural GT1-7 cells (Dong et al., 1997). These results strongly suggest that these four footprinted elements may be involved in the tissue-specific expression of the hGnRH gene in the human placental JEG-3 cells. In the present study, we demonstrated that the element 4 (FP4, -987/-968) is the most important for the hGnRH upstream promoter activity in JEG-3 cells. However, FP4 alone only produced about 40% of TKU activity (Figure 10). No single element can confer the full promoter activity. Therefore, all four elements are required to confer the tissue-specific expression of the hGnRH gene in JEG-3 cells, indicating that multiple components may be involved in the regulation of tissue-specific expression of the hGnRH gene in the placenta.

In the rat GnRH gene promoter, several groups (Whyte et al., 1995; Kepa et al., 1992 and 1996; Clark et al., 1995; Eraly et al., 1995) have reported the presence of potent neural-specific enhancer as well as cis-acting regulatory elements. This neural-specific enhancer is located in the distal promoter region between –1863 and –1571 in the rat GnRH promoter, while the cis-acting

regulatory elements are crowded in the proximal region of 173 bp 5' of the transcriptional start site (Whyte et al., 1995; Eraly et al., 1995). Results of studies by using GT1-7 neural cell line have demonstrated that the hGnRH gene also possesses a neural-specific enhancer (Kepa et al., 1996) but its location in the proximal promoter region (–535 to –47) is different from that of the rat neural-specific enhancer. Further studies have indicated that the sequences of the proximal region of GnRH genes are conserved across species and consist of multiple cis-acting elements that are necessary for basal and regulated GnRH gene transcription (Eraly et al., 1995). A nucleotide sequence comparison of the GnRH promoter between rat and human revealed that there is high homology in the proximal promoter region between –1 to –551 of hGnRH and –1 to -424 of rat GnRH (Kepa et al., 1996). In contrast, the hGnRH promoter region (–1131 to -551) has little similarity in the distal promoter region when compared to that of rat (–1031 to -424). It is of interesting that the hGnRH gene upstream promoter is located in this region. Thus, the results of the present study also suggest a species-specific difference in the structural organization of cis-acting promoter elements used to confer the tissue-specific expression of the hGnRH gene in the human placenta.

The presence of putative DNA-binding sites for transcription factors on the 5' flanking region of the hGnRH gene have been reported (Kepa et al., 1996). Since POU homeodomain protein Oct-1 is expressed in a variety of tissues and cell types (Sturm et al., 1987) including placental JEG-3 cells and neuronal GT1-7 cells, it is believed that Oct-1 participates in tissue-specific gene expression by

interaction with either other transcription factors (Voss et al., 1991; Poellinger et al., 1992) or tissue-specific co-activators (Luo et al., 1992; Strubin et al., 1995). In the rat GnRH promoter, it has been reported that binding of Oct-1 to the promoter elements is required for the tissue-specific expression in the hypothalamic cell-line, GT1-7 (Eraly et al., 1998). Close examination of the four footprinted elements from the hGnRH upstream promoter revealed short AT-rich sequences, all containing the octamer-like sequence TAAAT, which is most likely the target of POU homeodomain proteins. Although the octamer-like motifs within these four elements are somewhat deviated from the octamer consensus sequence ATGCAAAT, Oct family proteins are known to interact with AT-rich sequences with relaxed specificity (Bendall et al., 1993). In the present study, we demonstrated that Oct-1 only binds to the FP4 (5'-CTCACTAAATGCCGGGGG-3') which shows a four of eight match to the octamer consensus sequence, but not the FP3 (5'-GAGATTTAAATAAG-3') which represents a six of eight match, as well as other elements, suggesting other POU homeodomain transcription factors may interact with these elements. Furthermore, western blot assay has demonstrated the expression of Oct-1 protein in our JEG-3 cells. Therefore, Oct-1 may participate in the placenta-specific expression of the hGnRH gene by direct binding to FP4 and interacting with the other tissue-specific proteins and/or transcriptional factors.

5. Estrogen receptor α and β mediated estrogen regulation of the hGnRH upstream promoter activity in JEG-3 cells.

It is known that estradiol and progesterone are among the most important steroid hormones in regulating the hypothalamic GnRH production. The finding that GnRH-secreting GT1 neuronal cells contain ER and PR has indicated that the actions of steroid hormones are mediated by their receptors (Roy et al., 1999; Kepa et al., 1996). The human placenta also contains both ER and PR and steroid hormones exert their effects in a receptor-mediated manner in the placenta (Younges et al., 1981; Chibbar et al., 1995; Shanker et al., 1997 and 1998; Rossmanith et al., 1997). Furthermore, the placenta is a major source to produce estrogen and progesterone during gestation. Since the hGnRH upstream promoter is the major one functioning in the placenta, the present study was focused on the regulation of the hGnRH upstream promoter activity in JEG-3 cells. A luciferase reporter-transient transfection system was used in the human placental JEG-3 cells to investigate the receptor-mediated effects of estradiol and progesterone on the hGnRH upstream promoter activity.

Recent studies have shown that human estrogen receptor has two major subtypes, estrogen receptor α and β, with similarities and differences (Kuiper et al., 1997; Paech et al., 1997). ERβ has the DNA binding domain that is almost identical to the homologous ERα, implying that both ERα and ERβ might share the same DNA response element. They can also form heterodimer complex, suggesting their possible cross-talking (Sumito et al., 1998). However, ERα and ERβ have different activation function region and ligand binding domain that might result in the distinctive transactivational mechanisms (Paech et al., 1997). Evidence has indicated that each receptor subtype may perform specific

biological functions. In tissues where both receptors are co-expressed, however, the two receptors could play redundant roles: physically interact to form heterodimers or act as targets of independent signaling pathways. Thus, the presence of two receptor isoforms increases the complexity of ER-mediated effects on estrogen responsive genes. Using RT-PCR, the expression of hERα and hERβ in the human placenta has been reported (Brandenberger et al., 1997; Moore et al., 1998). JEG-3 cells are known to be deficient in ERα. I herein using RT-PCR demonstrated that our JEG-3 cells are also deficient in ERβ. Thus, ERα and ERβ expression vectors were transfected into JEG-3 cells to create ER-positive cells for estrogen regulation studies.

Previous studies in our laboratory have shown an ERα-mediated inhibitory effect of estradiol on the hGnRH upstream promoter activity in JEG-3 cells (Dong et al., 1996; Chen et al., 2000). The present studies demonstrated that ERβ also mediated a negative effect of estradiol on the upstream promoter, but to a lesser extent and decreased inhibitory effect when increasing exogenous expression of ERβ. In contrast, ERα mediates a more potent repressive effect and increased inhibition when exogenously overexpressed in JEG-3 cells. This was further supported by the result from co-transfection of both ERα and ERβ with various ratios (Figure 20), suggesting ERα and ERβ could modulate each other. Because the placenta contains both ERα and ERβ proteins, it is possible they co-express in the same kind of cell and interact each other. However, it is not clear how exactly ERα and ERβ interact inside the JEG-3 cells, forming heterodimer or acting as targets of independent signaling pathways. Treatment with various

estrogen isoforms also resulted in different responses between ERα and ERβ transfected cells. These differences may be due to the distinctive transactivational mechanisms of ERα and ERβ (Paech et al., 1997), especially in the AF-1 and AF-2 activation function regions. In addition, other groups demonstrated negative regulation of rat and human GnRH promoter activity (Wierman et al., 1992) and positive regulation of hGnRH promoter activity (Radovick et al., 1991) by estrogen. Such controversial results may be due to different promoter regions to be studied as well as tissue and species differences.

Furthermore, a negative (-991 to –935) and a positive (-827 to –730) estrogen responsive element in the hGnRH upstream promoter functioning in JEG-3 cells have been demonstrated in our laboratory (Chen et al., 2000). The positive element also contains a putative ERE, but its DNA-protein complex in supershift assay could not be super-shifted by anti-ER antibody. In the present study, mutations of the two elements abolished the responses to estradiol, further confirmed their roles in the estradiol regulation of the hGnRH upstream promoter in JEG-3 cells. However, gel-shift assay showed no binding of the putative ERE with recombinant ERα protein, suggesting that ER does not directly interact with the putative estrogen responsive element. This is further supported by other studies on the transcriptional repression of the GnRH gene by estrogen (Wierman et al., 1992; Kepa et al., 1994). They were also unable to detect ER binding to their negative response element even though the DNA-binding domain of the ER was required for estrogen-mediated repression.

The mechanism of ER-mediated regulation of gene transcription is generally thought to be direct interaction of ER protein with its palindromic consensus DNA sequences (ERE). However, increasing evidence has shown that steroid hormone receptor-mediated negative regulation, unlike the positive regulation, does not usually occur through direct interaction with consensus DNA sequence. For example, protein-protein interactions are thought to be important for inhibition of AP-2 stimulated activity by the SV40 large tumor antigen (Mitchell et al., 1989), inhibition of prolactin promoter activity by the estrogen receptor binding to Pit 1 (Adler et al., 1988), ligand-dependent repression of the erythroid transcription factor GATA-1 by ER (Blobel et al., 1995), and repression of IL-6 gene expression by ER binding to NF-IL6 and NF-κB (Ray et al., 1997). The similar mechanism of repression is also postulated for functional antagonism between Fos/Jun and estrogen via the interference at the non-receptor binding AP-1 site that involves formation of heterodimeric complexes between ER and Fos/Jun (Doucas et al., 1991; Paech et al., 1997). Alternatively, different trans-acting factors may compete for binding at the same DNA sequences as described for glucocorticoid receptor interference at cAMP element binding sites in the human α-subunit gene (Akerblom et al., 1988). In addition, trans-acting factors may interfere directly with normal transcriptional initiation apparatus as suggested by studies of negative regulation by thyroid hormone of the glycoprotein hormone α-subunit gene (Chatterjee et al., 1989) and rat growth hormone gene (Crone et al., 1990) by binding to region adjacent to the TATA box, as well as transcriptional repression of the ovine FSHβ gene by estrogen (Miller et al., 1996). Also,

negative regulation may occur via protein interaction with DNA sequences within the gene itself thus resulting in premature termination of the primary transcript elongation as in regulation of the human thyroid stimulating hormone β-subunit gene (Wondisford et al., 1989). Finally, recent evidence have demonstrated the possible presence of plasma membrane ER that mediate the nongenomic action of estrogen through signal transduction pathways (such as MAP kinase) even though it usually mediates acute effect (Levin, E.R., 1999; Shaul, P.W., 2000). Therefore, all these examples demonstrate that the classical concept of ER regulating genes following an obligatory step of DNA binding is no longer generally applicable. Results from the present studies and other groups (Wierman et al., 1992; Kepa et al., 1994) indicated that ER most likely binds to other transcription factors that are in contact with the negative and positive response elements to regulate hGnRH gene transcription. Future studies into the transcription factor(s) that bind to the response element(s) and interact with ER will aid in the elucidation of transcriptional repression of the hGnRH gene by estrogen.

6. Progesterone receptor A and B mediated progesterone regulation of the hGnRH upstream promoter activity in JEG-3 cells.

Progesterone can either stimulate or inhibit GnRH production in the hypothalamus depending on different physiological circumstances through direct or indirect actions on the GnRH neurons. The demonstration of the presence of progesterone receptor in the GnRH neurons suggests that progesterone may

directly exert its actions on the GnRH gene expression through its specific receptor. In the placenta, Petraglia et al. (1990) showed that progesterone decreased the release of immunoreactive GnRH from cultured human placental cells and this inhibitory effect was completely reversed by RU486, a specific receptor antagonist, suggesting a receptor-mediated action. Recent studies in our laboratory demonstrated a progesterone receptor-mediated positive effect of progesterone on the hGnRH upstream promoter activity in the human placental JEG-3 cells. Because the human PR exists as two isoforms, hPR-B and hPR-A, with distinctive transactional domains, the different effects of progesterone on the GnRH gene expression may be due to the receptor-mediation by different isoforms. Using RT-PCR, the presence of PR mRNA has been detected in the human placenta as well as in our JEG-3 cells. However, due to PR-A and PR-B forms are resulted from mRNA alternative splicing, RT-PCR cannot distinguish between PR-A and PR-B. Shanker et al. (1998) using EMSA demonstrated the presence of only PR-A but not PR-B in the first trimester and term placenta. Using western blot assay with specific antibodies against hPR-A/B or hPR-B, both forms of PR were detected in our JEG-3 cells in the present study.

Although in most cells hPR-A does not activate transcription but functions as a strong trans-dominant repressor of hPR-B (Vegeto er al., 1993), the result in the present study showed that both forms of PR mediate stimulatory effect of progesterone on the hGnRH upstream promoter activity in JEG-3 cells. To study the roles of PR isoforms in directing progesterone effect on the upstream promoter, hPR-A and hPR-B expression vectors were introduced into JEG-3 cells

in this study. However, JEG-3 cells contain endogenous PR-A and PR-B although their concentrations are not determined yet. Thus, endogenous PRs may have somewhat influence on the luciferase activity. Furthermore, PRs generally exert their actions through binding to the consensus PRE (progesterone responsive element) on the cis-regulatory region of target genes. Most of the studies on the PR regulated transcription are based on the reporter construct that containing PRE. Although the direct DNA binding of PR to nonconsensus elements has been demonstrated in the proximal rat GnRH promoter (Kepa et al., 1996), the progesterone responsive element(s) have not been fully clarified in the hGnRH upstream promoter.

Coativators, SRC-1 and CBP, have been demonstrated to increase PR dependent transcription in a variety of cell lines by using PRE-containing reporter constructs (Onate et al., 1995; Lundblad et al., 1995; Smith et al., 1996). Our functional transfection studies showed that SRC-1 and CBP, independently and synergistically, enhance both hPR-A and hPR-B mediated hGnRH upstream promoter activity in JEG-3 cells, indicating SRC-1 and CBP may be also involved in the PR dependent transcription of hGnRH gene in the placenta.

CONCLUSIONS

Multiple cis-regulatory elements are involved in the tissue-specific expression of hGnRH gene in JEG-3 cells. No single element is sufficient to confer full transcriptional activity. However, the sequence between -987 and –968 (FP4) is the most important for the hGnRH upstream promoter activity in JEG-3 cells, and the POU homeodomain protein Oct-1 involves in its DNA-protein interaction, indicating that Oct-1 may be an important factor in regulating the tissue-specific expression of hGnRH in the placenta.

Both ERα and ERβ mediate inhibitory effect of estrogen on the hGnRH upstream promoter activity in JEG-3 cell with a dose-dependent manner, and can modulate each other's regulation of transcription. However, ERα and ERβ exhibit different ability to direct the effects of estrone, estradiol, and estriol on the hGnRH upstream promoter activity in JEG-3 cells. Moreover, the sequence between -991 and –935 mediates negative estrogen response, while the sequence between -827 and –730 mediates positive estrogen response in the hGnRH upstream promoter. However, ER protein does not directly involve in DNA binding.

Progesterone increases the hGnRH upstream promoter activity in JEG-3 cells in a PR-A and PR-B dependent manner. This stimulatory effect can be enhanced by coactivators, SRC-1 and CBP, independently and synergistically. However, PR-A and PR-B exert their actions in mediating the hGnRH upstream promoter activity in JEG-3 cells with different extent.

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