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Positive Regulation of PKA on Human Gonadotropin-Releasing Hormone (hGnRH) Gene Expression in Human Placental JEG-3 Cells

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by

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ABSTRACT

POSITIVE REGULATION OF PKA ON HUMAN GONADOTROPIN-RELEASING HORMONE (hGnRH) GENE EXPRESSION IN HUMAN PLACENTAL JEG-3 CELLS

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Using the human placental choriocarcinoma JEG-3 cell line as an *in vitro* human placental model, we studied the mechanisms of the PKA positive regulation of the hGnRH gene expression in the human placenta. Studies in JEG-3 cells showed that through the PKA catalytic subunit α, human GnRH upstream promoter activity was stimulated by PKA signaling pathway in a cAMP dependent mechanism. The sequence between –202 (Afl II) and –554 (BamH I) base pair in the human GnRH upstream promoter region appeared to be responsible for the PKA positive regulation of the gene expression. Furthermore, Western blot analysis demonstrated the involvement of phospho-CREB in the PKA regulation of hGnRH gene expression in JEG-3 cells, and CREB-binding protein (CBP) could further enhance the PKA stimulatory effect on the hGnRH upstream promoter activities.

Transient transfection studies showed that 10 μM forskolin, an activator of adenylate cyclase, stimulated the hGnRH upstream promoter activities in JEG-3 cells in a dose dependent fashion. The cAMP analogue, eight-bromo-cAMP(8-CPT-cAMP) also increased hGnRH gene expression in a dose- and time – dependent fashion. The 8-CPT-cAMP stimulatory effect on hGnRH gene

expression was abolished by 10 μM of H-89, an anagonist of PKA. The role of PKA catalytic subunits in hGnRH gene expression was also determined: The α subunit stimulated the hGnRH promoter activity by two fold, while the γ subunit did not significantly change the activity.

When the fragment between sequences –202 and –554 bp was deleted away, the PKA positive stimulatory effect disappeared, suggesting that both cis- and trans- elements were involved. Western blot results showed an increased amount of phospho-CREB protein after treatment with 8-CPT-cAMP or co-transfection with PKA catalytic subunit α compared to the control cells without any treatment. Furthermore, transient transfection studies demonstrated that CREB-binding protein (CBP) enhanced the PKA stimulatory effect on hGnRH upstream promoter activities, suggesting that CBP may be also involved in the PKA regulation of hGnRH gene expression.

While PKA signaling pathway has been involved in many placental functions, we have demonstrated, for the first time, the PKA positive regulation on hGnRH gene expression which should help to further elucidate the mechanism of local hGnRH functions in the placenta.

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At last, I would like to contribute my thesis work to my parents in China for their love, guidance, and support in my whole life. Also I would share this achievement with my husband, Bin. We are always together.

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INTRODUCTION

Gonadotropin-releasing hormone (GnRH) is the hypothalamic-releasing factor that controls the biosynthesis and releasing 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 pituitary level, GnRH acts through GnRH receptor to stimulate the synthesis and release of FSH and LH. Then biologically active follicle stimulatory hormone (FSH) and luteinizing hormone (LH) dimers are secreted into the peripheral circulation and act on ovary to regulate the estrogen and progesterone synthesis and secretion. Recent studies in the last decade have shown GnRH not only acts as an endocrine factor, but also plays as an important paracrine/autocrine factor in the non-neuronal tissues.

The expressions of GnRH and GnRH receptor have been demonstrated in non-neural tissues, especially in reproductive tissues. Immunoreactive GnRH has been found in ovary (Aten et al., 1987), testes (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). Furthermore, GnRH mRNA (Dong et al., 1993; Ikeda et al., 1996, 1997) and GnRH receptor mRNA (Peng et al., 1994; Lin et al., 1995) have been found in reproductive tissues indicating that GnRH and GnRH receptor

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were produced locally in reproductive tissues and may function as an autocrine/paracrine factor.

Human placenta is a unique organ that serves to transit nutrients to the fetus and waste products from the fetus to the maternal circulation. Both GnRH and GnRH receptor mRNA was found in the placenta. GnRH plays an important role in the regulation of pregnancy. Placental GnRH regulates hCG release during the course of human pregnancy, affects the placental steroidogenesis competence within different gestation ages. GnRH also modulates prostaglandin release during gestation, and plays as a critical role in preimplantation embryonic development. Seeburg and Adelman first cloned the human cDNA for GnRH in 1984 (Seeburg et al., 1984). It was identical to hypothalamic GnRH, consisting of four exons and three introns (Fig. 1A). In addition to the hypothalamus where GnRH gene primarily was expressed, increasing evidence has demonstrated the expression of GnRH gene in non-hypothalamic tissues, particularly in reproductive tissues. The non-hypothalamic expression of GnRH gene was further supported by the identification of an upstream GnRH promoter particularly used in placenta, while the downstream promoter is mainly utilized by hypothalamic neurons (Fig. 1B) (Dong et al., 1993).

Cyclic adenosine monophosphate (cAMP) is a ubiquitous second messenger produced in cells in response to hormones and nutrients. An important function of cAMP is to activate the phosphorylating enzyme, protein kinase A (PKA). The key roles of cAMP and protein kinase A in the phosphorylation and regulation of enzyme substrates involved in intermediary

metabolism are well known. A newly discovered role for protein kinase A is a phosphorylation and activation of transcription factors such as CREB (cAMP response element binding protein) that are critical for the control of the transcription of genes in response to elevated levels of cAMP. The cAMP signaling pathway included: G-couple protein activation of adenylate cyclase, increase of intracellular cAMP level, activation of PKA, phosphorylation of transcription factors, and eventually activation of gene transcription. Both PKA and PKC pathways have been demonstrated to effect GnRH synthesis and secretion. Forskolin, activators of adenylate cyclase to raise cAMP levels, had shown stimulated GnRH secretion but no effect on GnRH mRNA in GT1-7 cell line (Wetsel et al., 1993). Rao and his colleagues have found that PKA signaling and transacting factors such CREB, Fos, and Jun were probably involved in transcription of GnRH gene induced by hCG in GT1-7 neurons (Lei et al., 1995). Our previous studies have shown that the steroid hormone, estrogen, negatively regulates the hGnRH gene expression in the placental JEG-3 cells. Furthermore, JEG-3 cells also accumulate progesterone and aromatize androgen precursors to estradiol by a cAMP- and phorbol 12-myristate 13-acetate (PMA)-responsitive mechanisms (Ritvos et al., 1988). Estrogen receptor could be activated by increased level of cAMP (Sharma et al., 1999). It will be very interesting to study the direct effect of cAMP-dependent signaling pathway on hGnRH gene expression in JEG-3 cells.

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). From then on, the expression of both GnRH gene (Dong, et al., 1993) and GnRH 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 the GnRH receptor (Currie et al., 1989) in the human placenta vary with gestational age suggested the important roles for GnRH during pregnancy. Further studies demonstrated that placental GnRH is involved in the physiological regulation of production of hCG (Butzow et al., 1982), steroids and prostaglandins. Placental GnRH also exhibits effects 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 the third trimester placenta (Wolfahrt et al., 1998), further indicating autocrine/paracrine regulation by GnRH in the human placenta.

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. Placental GnRH has also been demonstrated to actively stimulate the release of hCG from cultured trophoblasts (Khodr et al., 1978; Siler-Khodr et al., 1980; Butzow et al., 1982; Petraglia et al., 1987). The effect of GnRH on hCG production varies with gestation age and can be blocked by GnRH antagonist. Further evidence showed that placental GnRH mediated *in vitro* opioids regulation of hCG release

from term trophoblastic 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).

Placental GnRH may affect the placental steroidogenesis competence within different gestation ages. Siler-Khodr et al. (1983) have 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 difference gestational ages (Siler-Khodr et al., 1986; Ringler et al., 1989). Since estrogen is responsible for the developmental regulation of placental 11β -HSD/corticosteroid metabolisms, it is most likely that GnRH is involved in maturation of the fetus via estrogen. However, recent studies in the placental JEG-3 choriocarcinoma cels, which more closely resemble term placenta, have shown that GnRH does not affect steroidogenesis (Bauer et al., 1998). Thus, depending on physiological circumstances, GnRH may affect steroid hormones synthesis in pre-term placenta, but might have no effect on near term placenta.

GnRH may also modulates prostaglandin release during gestation. The production of human placental prostaglandin varies during gestation and its release *in vitro* is affected by GnRH, which is 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.

There is *in vivo* evidence that 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.

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 increasing concentrations of the agonist reversed this effect. 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

Seebug and Adelman first cloned the human cDNA for GnRH in 1984 (Seeburg et al., 1984). It was shown to be a single-copy gene located on chromosome 8, consisting of four exons and three introns (Fig.1). 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 GnRH-associated peptide (GAP). In addition to the hypothalamus where the GnRH gene primarily expressed, increasing evidence was demonstrated that the expression of GnRH gene in non-hypothalamic tissues, particularly in reproductive tissues. The non-hypothalamic expression of GnRH 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 (Dong et al., 1993).

The existence of bioactive and immunoreactive GnRH-like activity in the placenta has been demonstrated. The expression of GnRH –like peptide was first reported in the human placenta in 1975 (Gibbons et al., 1975). Using carboxymethyl-cellulose ion-exchange chromatography, Gibbons' group demonstrated that human chorionic membrane and placental tissue contained 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 was synthesized by the placenta and could stimulate LH/hCG *in vitro* and *in vivo*. Moreover, GnRH antagonist prevented the GnRH-induced increase in

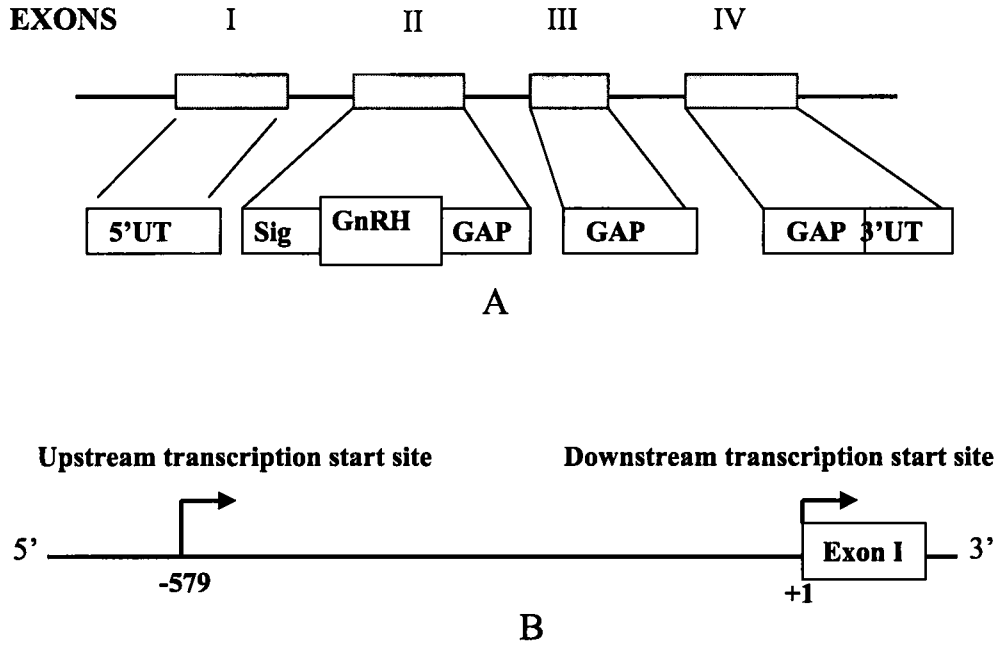

Fig.1. The GnRH gene. A: The GnRH gene is coded for by four exons and three introns. Exon I encodes the 5'-untranslated region (5'UT) while exon II contains the signal peptide (Sig), the decapeptide, GnRH, and GnRH-associated peptide (GAP). Exon III also codes for GAP, and exon IV codes for the carboxyl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

hCG release as well as decrease basal hCG production in the human placenta (Siler-Khodr et al., 1983). In addition to human placenta, the placentae 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 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. These finding provide an anatomical basis for a paracrine/autocrine modulation of GnRH in placental hormonogenesis.

The GnRH not only is produced in the placenta, but its level varies with different gestation periods as well. Radioimmunoassay studies in the human placenta were 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 immunoreactive GnRH content in the placeta, 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 8th 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. These data support the important role of placenta GnRH during gestation.

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 then 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 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).

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 finding 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 change of GnRH receptor concentration, rather than the GnRH level, is responsible for the dynamic changes in GnRH mediated hCG secretion.

GnRH stimulates the release of hCG from cultured placenta 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). These findings indicate that the role of GnRH in the placenta is receptor mediated. Further studies have demonstrated that presence of highly specific, moderate affinity GnRH bindings sites in the human placental membrane (Currie et al., 1989; Belisle et al., 1984). 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 chicken GnRH (ch GnRH) II, salmon GnRH (s GnRH). 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 secreting during pregnancy, further supporting autocrine/paracrine modes of action for GnRH in regulating hCG secretion. Thus, it is most likely that placental GnRH is 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.

Since 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 GnRH 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 –723bp 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.

3. Regulation of the GnRH gene expression in the placenta

Many studies have indicated that the placenta produces a variety of pituitary and hypothalamic-like hormones and peptides; 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, stimulating a miniature hypothalamic-pituitary-target hormone unit, in which GnRH has an important central role.

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 (Mitchell et al., 1989). However, more commonly, tissue-specific expression is the result of a unique combination of sequence elements binding to more broadly

expressed proteins. 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 GnRH 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.

Evidence has indicated that steroid hormones can regulate GnRH release in the placenta. cAMP-induced release of immunoreactive GnRH (irGnRH) was increased by estriol and estrone and decreased by progesterone. Tamoxifen, an estrogen antagonist, and progesterone counteracted the action of estradiol. The inhibitory effect of progesterone was completely reversed by RU486, a specific progesterone receptor antagonist. This suggests that local interaction between steroids and peptides modulates irGnRH release from human placenta (Petraglia et al., 1990). 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), further supporting the concept that steroid hormones modulate placental GnRH secretion.

4. Second messenger signaling pathways are involved in the GnRH gene expressions

Cyclic adenosine monophosphate (cAMP) is a ubiquitous second messenger produced in cells in response to hormones and nutrients. An important function of cAMP is to activate the phosphorylating enzyme, protein kinase A. The key roles of cAMP and protein kinase A in the phosphorylation and regulation of enzyme substrates involved in intermediary metabolism are well known. A newly discovered role for protein kinase A is a phosphorylation and activation of transcription factors such as CREB that are critical for the control of the transcription of genes in response to elevated levels of cAMP.

Using cell culture as a model, studies indicated various second messenger pathways could affect GnRH release (Kim et al., 1985, Ojeda et al., 1985, Valenca et al., 1985). Protein Kinase-C signaling pathway has been studied intensively and the results clearly demonstrated that the PKC decreased the GnRH mRNAs and repressed the GnRH expression at the transcription level in the neuron GT1-7 cell line. The PKA pathway has also been studied. Forskolin, an activator of adenylate cyclase, has been shown to stimulate GnRH secretion but no effect on GnRH mRNAs in GT1-7 cell line (Wetsel et al., 1993). cAMP could directly initiate GnRH secretion (Vitalis et al., 2000) in hypothalamic tumor cell line. Rao and his colleagues also demonstrated that PKA signaling positively regulates the GnRH gene expression induced by hCG in GT1-7 neurons. It will be of interest to study the PKA regulation on the hGnRH gene expression in placental cells JEG-3 cell.

Eukaryotes have developed elaborate signal transduction systems by which information provided externally in the form of hormones, ions, and other signaling molecules is converted into intracellular information that regulates the internal working of the cell, such as the transcription of specific genes. From events occurring at the cell membrane to changes in gene transcription the cyclic AMP (cAMP)-dependent signaling pathway is a paradigm of signal transduction. Most components of the cAMP-dependent signaling pathway are well characterized. The binding of ligands (hormones, neurotransmitters, growth factors) to their specific receptors located in the plasma membranes of cells activates GTP-binding proteins (G-proteins) that are coupled to the receptors. The G_s protein stimulates the activation of the enzymes adenylyl cyclase, which converts ATP to cAMP. The cAMP activates protein kinase A (PKA), which phosphorylates numerous proteins. Activation of the cAMP response element binding protein (CREB) is one of the best-studied links between the activation of PKA and gene expression. Activation of CREB leads to specific residue Serine 133 phosphorylation (Gonzalez et al., 1989); CREB binding protein (CBP) could associate with Phosphorylated CREB to augment cAMP-induced transcription (Kwok et al. 1994). Co expression of CBP increases stimulus-induced CREB transcription of a CRE reporter gene. Evidence suggested that CBP serves as a molecular bridge that allows upstream transcription factors, such as CREB, to recruit and stabilize the RNA polymerase II transcription complex at the TATA box (Berger et al, 1996, Barlev et al., 1995).

The cAMP-dependent signal transduction pathway involves several enzymatic reactions. The properties of the key enzymes, adenylyl cyclase, phosphodiesterases, PKA and phosphatases are unique.

Adenylyl cyclase is the first component in the cascade signaling pathway. Nine isoforms of adenylate cyclase are known; and all are activated by $G_{\alpha s}$ subunits, which are released from inactive heterotrimeric G-protein complexes after the agonist binds to the receptor.

It is well known PKA holoenzyme consists of two catalytic subunits and two regulatory subunits. The PKA catalytic subunits are responsible for the phosphorylation of intracellular proteins including the transcription factors which in turn regulate the gene expression (Nigg et al., 1985, Habener et al., 1995). Three isoforms of the catalytic subunits (Cα, Cβ, Cγ) have been found (Uhler et al., 1986a, 1986b, Beebe et al., 1992). The various isoforms are different in their tissue distribution and function specificities. In particular, Cγ is expressed specifically in the testis (Beebe et al., 1992) and does not interact with PKA-specific inhibitor proteins. There is a difference in the tissue distribution of the subunits while implies a specificity of function, and some differences are seen in the range of the cAMP sensitivity shown by combinations of R and C subunits (Cadd et al., 1990, Gamm et al., 1996). Tamanini et al. first purified PKA catalytic subunits in the placenta; however, attempts to isolate the catalytic subunit failed. Furthermore, both the PKA and PKC pathways were active in placenta JEG-3 cells. It would be of interest to determine which PKA catalytic subunit is responsible for the positive regulation of human GnRH gene in placental cells.

Regulatory II subunits differ from RI subunits in that they associate with A-kinase anchoring proteins (AKAP). AKAP are a large family of proteins, the full extent of which has not been defined. These proteins influence subcellular localization of PKA by interacting with RII subunits. AKAPs may facilitate activation of PKA by bringing it near the site of stimulation, as is the case in dendritic spines (Carr, et al., 1992, Glantz, et al., 1992), or they may limit activation by co-localizing with enzymes that degrade cAMP (Coghlan et al., 1995). Localization of PKA within the nucleus by AKAP-95 may contribute to rapid cAMP-driven changes in transcription (Coghlan et al., 1994).

Despite a number of functional differences, PKA subunits show redundancy in gene knockout models. Mice lacking either RIβ or the Cβ splice variant $C\beta_1$ are viable and fertile, with no obvious behavioral abnormalities (Brandon et al., 1995; Huang et al., 1995; Qi, et al., 1996). However, detailed investigations of hippocampal synaptic pathways reveal deficiencies associated with cAMP-dependent processes as long-term potentiation of the mossy fiber system, long-term depression, de-potentiation, and late-phase long-term potentiation. Remarkably, these deficiencies occur in Cβ partial knockouts, which still express two splice variants of Cβ at significant levels. Mice lacking RIIβ have markedly reduced deposits of white fat and are resistant to diet-induced obesity (Summings et al., 1996). The underlying cause for this phenotype appears to be a compensatory increase in RIα in the brown adipose tissue. This increase in RIα was previously noted in experiments involving over expression of the Cα and Cβ subunits (Uhler et al., 1987) and results from increased subunit stability within

the holoenzyme rather than from the increase in levels of RIα mRNA. Protein kinase inhibitor (PKI) is a class of small proteins (71-76 amino acids) that inhibit the function of C. Three different genes give rise to the α, β, γ isoforms of PKI (Collins et al., 1997). The three different PKIS share a homologous region near their amino terminus. This region contains a pseudo substrate motif through which PKI with C promotes nuclear export of C (Fantozzi et al., 1994). This is an active transport phenomenon, apparently triggered by a nuclear export signal located in the amino acid sequence of PKI. (Wen, et al., 1994). The inhibitory and nuclear export functions of the PKIs are distinct; a sub fragment of PKI inhibits the kinase activity of PKA without preventing nuclear localization. The exact physiological role of the PKIs in different tissues, and the control of specific genes, has not been thoroughly investigated. However, high endogenous levels of PKI expression in an insulinoma cell line renders CREB unresponsitive to stimulation by cAMP even when additional PKA activity is introduced by co-transfection and expression of the C-subunit (Vallejo et al., 1995).

Cyclic AMP (cAMP) stimulates target gene expression via a conserved cAMP-responsive element (CRE), which consists of an eight-base pair palindrome (TGACGTCA) and is typically found within 100 nucleotides of the TATA box (Comb et al., 1986; Montminy et al., 1986). The palindromic CRE can be separated into two CTTCA motifs, which may be configured on the same or on opposite strands to function cooperatively in response to cAMP stimulation (Fink et al., 1988). Indeed, multimerization of the CRE strongly enhances cAMP inducibility, as revealed by the cooperative actions of two tandem CREs located

on the α chorionic gonadotropin gene promoter (Delegeane et al., 1987). When evaluated in the context of the heterologous promoter, the CRE confers inducibility at a promoter proximal position (<100bp from the TATA box) but is less active at distal positions (>500bp from the TATA box). The CRE has subsequently been shown to bind CRE binding protein (CREB), present in nuclear extracts. CREB has been purified from nuclear extracts of the pheochromocytoma cell line PC12 using CRE affinity chromatography and shown to be a 43-kDa phosphoprotein (Montminy et al., 1987). A human CREB cDNA was cloned by screening the human cDNA expression library with a double-stranded ^{32}P-labeled CRE (Hoeffler et al., 1988). The human clone encodes a 327-residue protein, whereas the rat clone encodes a 341-residue protein. The two forms differ only by the presence of a 14-residue insert, termed the α-peptide, in the longer form (Hoeffler et al., 1990). Subsequent to characterization of CREB, two other highly related gene products were characterized: activating transcription factor 1 (ATF-1) and cAMP-response element modulator (CREM). ATF-1 was identified originally as one of several factors that bind to CRE-like elements present in adenovirus promoters (Lee et al., 1987; Hurst et al., 1987; Hurst et al., 1990). Subsequent screening of a Hela cDNA library with CRE probes, identified a large family of ATFs of which one, ATF-1 is 65% identical to CREB in primary structure. Examination of ATF-1 mRNA in different cell lines derived from a variety of tissue types suggests that ATF-1 is widely expressed (Rehfuss et al., 1991). Screening of a mouse pituitary cDNA library with a probe corresponding to the DNA-binding and dimerization

domain of CREB led to isolation of a series of novel cDNAs all derived from a single gene. This gene, CREM (Foulkes et al., 1991), has extensive sequence identity with CREB (Ruppert et al., 1992). One particularly interesting characteristic of CREM is that by alternative splicing, the same gene generates either activator or repressor forms of CREM. The α,β, and γ isoforms of CREM all bind to CREs, but function as inhibitors of CREB and cAMP-mediated transcription (Foulkes et al., 1991). Unlike CREB, the various isoforms of CREM are not expressed uniformly across different tissues. $CREM_{\Gamma}$ mRNA is particularly enriched in testis, with some expressing in brain (Foulkes et al., 1992); Whereas expression of the truncated CREM isoforms appears to be confined to tissues of the neuroendocrine axis (Delmas et al., 1992).

CREB binds to its DNA target sequence as a dimer (Yamamoto et al. 1988). Dimerization occurs through a conserved structural motif at the C-terminus of the protein formed by a heptad repeat of leucine residues, referred to as the leucine zipper (Yun et al., 1990; Dwarki et al., 1990). A basic domain, a lysine- and arginine-rich stretch of amino acids just amino-terminal to the leucine zipper, mediates DNA binding. Presence of both the basic and leucine zipper (bZIP) domains places CREB within a larger family of bZIP transcription factors, including mammalian c-Fos, c-Jun, c-Myc, and C/EBP, as well as yeast Gcn4 (Vinson et al., 1989). Stimulus-induced activation of CREB is mediated by phosphorylation. Exposure of cells to forskolin (Seamon et al., 1981), an activator of adenylyl cyclase, leads to CREB phosphorylation at a specific residue, serine 133 (Ser 133). This site is phosphorylated *in vitro* by cAMP-

dependent protein kinase A (PKA). Phosphorylation of Ser 133 is required for signal-induced transcription *in vivo*, as mutation of Ser 133 to a nonphosphorylatable residue (alanine) abolishes transcriptional response to elevated cAMP (Gonzalez et al., 1989). Phosphorylation at Ser 133 could affect the stability of CREB, so that it is less labile when phosphorylated. Since CREB functions in the nucleus, phosphorylation at Ser 133 might induce translocation of cytoplasmic CREB to the nucleus. Within the nucleus, phosphorylation at Ser 133 might affect the ability of CREB to dimerize with different bZIP partners, or phosphorylation at Ser 133 might promote CREB binding to the CRE. Finally, phosphorylation of Ser 133 might lead to transcriptional activation by promoting interaction with components of the basal transcription machinery, such as TFIID and RNA polymerase II (Pol II), or other factors. There is evidence for and against each of these regulatory mechanisms and these findings suggest which particular mechanisms are most likely to contribute to phosphorylation-dependent activation of CREB.

As discussed as above, CREB can activate transcription in response to a stimulus when recruited to a promoter through a heterologous DNA-binding domain (Sheng et al., 1991). Thus, processes other than DNA-binding regulate CREB activity. Ser 133 is located within a 60-residue region of CREB, called a kinase-inducible domain (KID). The KID (residues 100-160) element encompasses multiple potential phosphorylation sites for various protein kinases (Gonzalez et al., 1991). The KID is both necessary and sufficient for signal-induced activation of CREB. How does phosphorylation of the KID result in

transcriptional activation? To search for factors that associate with CREB in a phosphorylation-dependent manner, a human thyroid cDNA expression library was screened with ^{32}P-labeled CREB and a protein was isolated that specifically bound to Ser 133-phosphorylated CREB (Chrivia et al., 1993). This factor, CREB-binding protein (CBP), is a 265-kDa nuclear protein that associates with phosphorylated CREB through a region at the N-terminus of CBP known as the KID interaction (KIX) domain (Parker et al., 1996). The core KIX domain is a 94-residue sequence (positions 586-679). The same residues of KID most critical for transcriptional activation by CREB (positions 140-160) are also required for interaction of CREB with the KIX domain of CBP, suggesting that CBP binding is important for CREB activity. CBP is critical for stimulus-induced activation of CREB: co-expression of CBP increases stimulus-induced CREB transcription of a CRE reporter gene, an effect is lost when Ser 133 is mutated to an alanine (Kwok et al., 1994), microinjection of cells with neutralizing anti-CBP antibodies inhibits cAMP-induced activation of a CRE reporter gene. Microinjection of a KIX peptide into cells inhibits stimulus-induced activation of a CRE reporter gene. Structure of Ser133- phosphorylated CREB KID complexed to the CBP KIX domain has been investigated using NMR spectroscopy and these studies suggested that phosphorylation promotes association (Radhakrishnan et al., 1997). In the absence of phosphorylation or of binding to the CBP KIX domain, the KID of CREB is not highly ordered. However, when phosphorylated at Ser 133 and bound to the KIX domain of CBP, the KID assumes a structure consisting of two α helices that link closely to the phosphorylation site at Ser 133.

The helix C terminal to the link is bound tightly within a hydrophobic pocket created by a palisade of three α helices of the KIX domain. This hydrophobic interaction presumably contributes significantly to the KID/KIX domain interaction because functional data show that the corresponding segment of CREB (residues 140-144) is critical for transcriptional activation *in vivo* (Gonzalez et al., 1991; Shih et al., 1996). CBP shares extensive similarity throughout its length with another protein, termed p300, which was initially characterized as an adenovirus E1A-associated protein (Lundblad et al., 1995). Although CBP and p300 have very similar actions in mediating CREB function, the use of ribozymes to inhibit production of p300 or CBP in vivo has revealed functional distinctions between these two co-activators at the level of target gene expression (Kawasaki et al., 1998). Moreover, the association of p300 with Ser133-phosphorylated CREB is not required for CREB activity in every cell type (Yao et al., 1999). The idea that CBP acts as a transcriptional adaptor linking Ser133-phosphorylated CREB to the basal transcription machinery is supported by biochemical evidence. CBP co fractionates with Pol II during ion exchange and gel-filtration chromatography of HeLa cell nuclear extracts, and both CBP and Pol II co-immunoprecipitate with Ser133-phosphorylated CREB (Kee et al., 1996). Pol II recruitment to the CREB-CBP complex requires that the KID be phosphorylated, and in vitro experiments suggest that the Ser133- phosphorylated KID is both necessary and sufficient for Pol II recruitment *in vitro* (Nakajima et al., 1997). In addition to recruiting Pol II, CBP also contributes to CREB-mediated transcription by affecting chromatin structure. CBP possesses an intrinsic histone

acetyltransferase (HAT) activity and associates with another HAT-containing factor termed p/CAF (Bannister et al., 1996). By catalyzing acetylation of lysine residues in the N-termini of histones, CBP and p/CAF alter chromatin structure in a fashion believed to make the DNA template more accessible to the transcriptional machinery (Struhl et al., 1998). The mechanism through which this occurs has not yet been clearly elucidated.

Studies indicated various second messenger systems have been reported to affect GnRH release (Kim et al., 1985, Ojeda et al., 1985, Valenca et al., 1985). The protein Kinase-A and –C (PKA and PKC) pathways have been implicated in GnRH biosynthesis and secretion (Wetsel et al., 1989, Lee et al., 1990). Especially in the hypothalamus, Bruder et al., 1992 have shown that treatment of the GT1-7 neuronal cell line with 12-O-tetrade-canoylphorbol 13-acetate (TPA), a PKA agonist, increases PKC activity and decreases rGnRH mRNA levels in a dose- and time-dependent fashion. These effects are blocked by staurosporine, a PKC antagonist. TPA represses rGnRH expression at the level of transcription through DNA sequence in the proximal rGnRH promoter (Bruder et al., 1993). Wetzel et al., observed similar decreases in GnRH mRNA and a reduction in rat GnRH promoter-CAT construct activity in GT1-7 cells treated with TPA (Wetsel et al., 1993). In contrast, Radovick and colleagues have reported that TPA stimulates rather inhibits the human (h) GnRH promoter using stable transfectants in a different transgenic ally-derived GnRH-producing cell line, Gn-10 (Radovick and Yuefen, 1992). At the same time, the PKA signaling pathway has also been studied. Forskolin, activators of adenylate cyclase to

raise cAMP levels, had shown stimulated GnRH secretion but no effect on GnRH mRNA in GT1-7 cell line (Wetsel et al., 1993). Rao and his colleagues have found that PKA signaling and transacting factors such CREB, Fos, and Jun are probably involved in transcription of GnRH gene by hCG in GT1-7 neurons (Lei et al., 1995). Furthermore, a 95-kDa trans-acting protein, which binds to AT-rich cis-acting elements in the 5' -flanking region of the human GnRH gene, has been found responsible for the hCG-induced inhibition of the gene transcription. However recent studies implicated that cAMP could directly to initiate GnRH secretion by opening cAMP-gated cation channel, but not by activation of PKA (Vitalis et al., 2000) in hypothalamic tumor cell line. So how the second messenger pathway, especially cAMP signaling pathway regulates the hGnRH gene expression needs to be elucidated. In the placenta cells, both cAMP-mediated and PKC-medicated pathways appeared to be active. JEG-3, human choriocarcinoma cells, like normal trophoblasts, convert cholesterol to pregnenolone in a cAMP-responsive fashion via induction of mRNAs for steroidogenic enzymes (Ringler et al., 1989, Golos et al., 1987). JEG-3 cells also accumulate progesterone and aromatize androgen precursors to estradiol by a cAMP- and phorbol 12-myristate 13-acetate (PMA)-responsitive mechanisms (Ritvos et al., 1988). Leung and his colleagues found both the PKC and PKA pathway were involved in regulating GnRHR gene expression in JEG cells. However how the second messenger signaling pathway affects the hGnRH gene expression in the placenta still needs to be studied.

SPECIFIC AIMS OF THE STUDY

1. To test the hypothesis that PKA signaling pathway stimulates the hGnRH upstream promoter in the human placental JEG-3 cells

Forskolin, an activator of adenylate cyclase, will be used to treat JEG cells transfected with human GnRH gene upstream promoter construct to determine whether PKA can positively regulate the GnRH gene expression. Then Eight-bromo-cyclic AMP (8-CPT-cAMP) will be used to further confirm the PKA positive regulation of GnRH gene in JEG cells. Dose response of 8-CPT-cAMP as well as time course will be also investigated. Specific PKA inhibitor H-89 will be carried out to finally verify the involvement of PKA pathway in GnRH gene regulation. Involvement of PKA catalytic subunit in GnRH gene regulation will be determined by transfection of PKA α-subunit and γ-subunit expression vectors into JEG cells.

2. Characterization of the involvement of trans- and cis-element, which were induced by PKA pathway, on GnRH gene regulation in JEG cells

Serial deletion analysis will be carried out to localize DNA sequences respond to positive regulation of PKA. The phosphorylation of CREB induced by PKA will also be determined in the JEG cells by immunoblotting. Furthermore, co-activators CBP/P300 and PKA will be co-transfected with GnRH upstream promoter gene into JEG cells to determine the function of co-activators in PKA positive regulation of GnRH gene.

EXPERIMENTAL DESIGN

1. To test the hypothesis that PKA signaling pathway stimulates human GnRH upstream promoter activity in the human placental JEG-3 cells

Rationale: Our previous studies showed that through estrogen receptor, estrogen negatively regulated the hGnRH gene expression in JEG-3 cells. Studies from other laboratory have demonstrated that estrogen receptor could be activated by increased level of cAMP (Sharma et al., 1999). It has also been reported that estrogen receptor alpha/beta are activated by elevated levels of intracellular cAMP in ovary (Richards, et al., 2001). Thus, it will be important to understand the whether cAMP signal pathway is directly mediate the hGnRH upstream promoter activity in the placenta. Using cell culture as a model, previous studies indicated various second messenger pathways could affect GnRH release (Kim et al., 1985, Ojeda et al., 1985, Valenca et al., 1985). Protein Kinase-C signaling pathway has been studied intensively and the results clearly demonstrated that the PKC decreased the GnRH mRNAs and repressed the GnRH expression at the transcription level in the neuron GT1-7 cell line. PKA pathway has also been studied. Forskolin, an activator of adenylate cyclase, has shown to stimulate GnRH secretion but has been shown to have no effect on GnRH mRNAs in the GT1-7 cell line (Wetsel et al., 1993). cAMP could directly initiate GnRH secretion in hypothalamic tumor cell line (Vitalis et al., 2000). Rao and his colleagues also demonstrated that PKA signaling positively regulate the GnRH gene expression induced by hCG in GT1-7 neurons. Our preliminary studies have shown similar results on the PKA positively regulation of hGnRH full-length upstream promoter

activity in the JEG-3 cells. The present study is designed to further confirm the PKA positive regulation of hGnRH upstream promoter activity in JEG-3 cells treated by 8-CPT-cAMP. PKA catalytic subunits are responsible for the phosphorylation of intracellular proteins including the transcription factors, which in turn regulate the gene expression (Nigg et al., 1985; Habener et al., 1995). The various isoforms displayed difference in their tissue distribution and function specificities; especially the Cγ is expressed specifically in the testis (Beebe et al., 1992) and does not interact with PKA-Specific inhibitor proteins (PKI). In this proposal, we will determine which PKA catalytic subunits are responsible for the positive regulation of hGnRH upstream promoter activity in the JEG-3 cells.

Experimental Design:

Results of our preliminary studies have shown that ten micromole of forskolin can significantly stimulate GnRH gene upstream promoter activity. Since forskolin is an activator of adenylate cyclase, the effect on the GnRH upstream promoter activity could possibly also come from the signaling pathways besides the PKA. Therefore, it is necessary to use a cAMP analogue, 8-CPT-cAMP to further confirm the positive effect of PKA pathway on GnRH gene regulation. Our preliminary studies have demonstrated that fifty micromole of 8-CPT-cAMP could stimulate GnRH gene upstream promoter activity almost two fold compared to the control of non-stimulation. Two sets of experiments were designed for this study.

First, a dose response study was performed to determine whether there is a dose-dependent response of 8-CPT-cAMP in human JEG-3 cells as well as also

demonstrate the optimal concentration of 8-CPT-cAMP for further study. Since our preliminary studies have demonstrated that fifty micromoles of 8-CPT-cAMP could significantly stimulate GnRH gene upstream promoter activity, the concentrations of 8-CPT-cAMP on this study were 0.01, 0.1, 1, 10, 100, 1000 uM. The JEG-3 cells were cultured for one or two days to reach 60-70% confluence and transient transfected with 3 ug of GnRH full-length upstream construct. After 18 hours of transfection, the cells were treated without or with the above-stated different concentrations of 8-CPT-cAMP for 24 hours in triplicate. The cells were harvested for testing the Luciferase activity. To correct for the different transfection efficiencies, a pCMV Beta-galactosidase construct (GUS) was co transfected into the cells with GnRH promoter-luciferase construct.

After the optimal concentration of 8-CPT-cAMP was found, a second study, a time course study was performed to determine whether acute or chronic response of GnRH gene expression to 8-CPT -cAMP. The time course was 0.5, 1, 2, 4, 8, 16, 24-hours and was used to study both an acute or chronic response of GnRH. JEG-3 cells was transfected with the full length GnRH upstream promoter construct and then was cultured with an optimal dose of 8-CPT-cAMP in triplicate at different time points (the times points described above are counted from the harvest of the cells). Untreated cells at each point were harvested as a control. Finally the cells were harvested for testing the Luciferase activity.

Results of previous studies have shown that H89 is a specific inhibitor of PKA pathway. Transient transfection assay were performed to confirm the PKA pathway was involved in the regulation of the full length GnRH upstream

promoter activity. The cells were transfected with the full-length GnRH upstream promoter construct and were treated with 10uM H89 for 30 min and with 10uM 8-CPT-cAMP for 24 hours in triplicate. Then the cells were harvested for testing the luciferase activity. Untreated cells were also harvested as a control.

PKA is composed of two catalytic subunits bound to a homo dimer of two regulatory subunits. The activity of PKA is dependent on catalytic subunits that can phosphorylate other proteins which, in turn, trigger other signaling pathways. Studies have reported that PKA catalytic subunits are varied in different human tissue. Our preliminary studies have demonstrated that catalytic subunit alpha significantly stimulated the GnRH upstream promoter activity while gamma-subunit had only a slight increase. Therefore in this study, we focused our investigation on the effect of PKA alpha subunit on GnRH upstream promoter activity. Our preliminary studies have shown that transfection of 3ug of PKA alpha subunit expression vector could significantly stimulate GnRH upstream promoter activity. Further studies were performed to transfect 0.5,1,3,6, and 9 μg of PKA alpha subunit expression vector into the JEG-3 cells together with GnRH upstream promoter construct. Such experiments determined whether there is a dose dependent response of PKA alpha subunit expression vector as well as the optimal amount of PKA expression vector required for the positively stimulation. To further confirm such stimulatory effect is specifically coming from PKA alpha subunit, a mutant PKA alpha subunit expression vector (PCα K42 M, generously provided by Dr. Beebe from our school), which Lys in 72 position has been

changed to Met and lose the PKA catalytic activity, was transfected in the same experiment as a negative control.

2. Characterization of the involvement of trans- and cis-element, which were induced by PKA pathway, on GnRH gene regulation in JEG cells

Rationale: To locate the specific upstream promoter region that is response to PKA stimulation, serial deletion studies were conducted. A series of constructs containing 5'- or 3' deletion of the human GnRH promoter were fused to the luciferase reporter gene and tested for their promoter activities in response to cAMP stimulation. It is known that PKA regulates gene expression by phosphorylating nuclear protein CREB, which in turn binds to CRE DNA sequences to activate gene transcription. Since our GnRH upstream promoter region contains 5'TGACTTCA-3' sequence similar to the classic CRE 5'-TGACGTCA-3', it was of interest to investigate the effects of cAMP and PKA catalytic subunit on both total and phosphorylated CREB protein levels in JEG-3 cells by immunoblotting. The major effect of the phosphorylation of CREB is on the association of transcription co-activators, nuclear factor CBP (CREB binding protein); CBP could augment cAMP-induced transcription (Kwok et al., 1994). In this proposal, the roles of CBP and P300 (a closely related to CBP but distinct nuclear factor) on the human GnRH upstream promoter activity will be investigated by transfecting CBP/P300 together with GnRH and PKA in JEG-3 cells.

Experimental Design:

To detail locate the exact sequences which response to the PKA stimulation, 5' and 3' deletion studies was carried out. A set of 5' and 3' deletion in the human GnRH upstream promoter was generated by Exonuclease III/Mung Bean Nuclease Deletion (Stratagene) and ligated to a promoterless luciferase vector (pxp2 Luc) as described in Materials and Methods. Each of these GnRH promoter/Luc constructs was transfected in triplicate into JEG-3 cells by calcium-phosphate precipitation method and treated with 8-CPT-cAMP. Luciferase activity was measured to analyze the promoter activity as previous experiments.

To investigate the cAMP and PKA stimulation effect on specific transcription factors which might possibly be involved in the regulation of hGnRH upstream promoter expression. JEG-3 cells were cultured with 100 μM 8-CPT-cAMP for 24 h, or transfected with 3μg PKA catalytic subunit for 24h. Then cells were homogenized in lysis buffer and protein concentration was measured by BCA protein assay. Furthermore, 20μg of proteins from JEG cells treated with cAMP, or co-transfected with PKA α subunit or from JEG-3 cells without treatment as control was separated by discontinuous 10% SDS PAGE under reducing conditions and electro-blotted to cellulose nitrate membranes. Antibody dilutions of 1:2000 for phosphorylated CREB and total CREB were used for detection of the corresponding proteins. The molecular size of the proteins was determined by running standard molecular weight marker proteins in an adjacent lane. Proteins were visualized using ECL detection system according to manufacture's instruction with modification.

As CREB binding proteins, the roles of CBP and P300 (a closely related to CBP but distinct nuclear factor) on the human GnRH upstream promoter activity were investigated by transfecting CBP/P300 together with GnRH and PKA in JEG-3 cells. Both time course and dose response were demonstrated by transfected CBP or P300 expression vector with or without exogenous PKA α catalytic subunit in JEG-3 cells for 24h, then the cells were harvested for testing the luciferase activity.

MATERIALS AND METHODS

1. Cell culture

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

2. Preparation of plasmid DNAs

Plasmid DNAs were transformed into DH1 E.coli bacterial host and amplified in 500 ml Luria-Bertani medium (5g Yeast, 10g Trypton, 5g NaCl, pH. 7.5, and 50μg/ml of Ampicillin) in shaking incubator at 37°C, 200-rpm overnight. Bacterial cells were harvested by centrifugation at 4 °C, 6000 rpm for 10 min. Plasmid DNAS were prepared by CONCERT High Purity Plasmid Maxipre System (Life Technologies, Gaithersburg, MD) according to the manufacture's instruction with minor modification. DNA concentration was determined spectrophotometrically by absorbance at 260nm and verified by comparison with known mass lambda DNA standards on agarose gel.

3. 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 2-5X10^5 cells per 60-mm dish one day before transfection. Transfected plates were washed three times with 1XPBS 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-X100; 25 mM glycylglycine, pH 7.8; 15mM $MgSO_4$; 4 mM EGTA; and 1mM dithiothreitol). After centrifugation at 4 °C, 14000rPm for 5min, luciferase activity (De Wet et al., 1987) was measured on a Lumat LB 9501 Luminometer (EG&G Berthold) by mixing 100μl of the cell extracts, 360 μl of luciferase assay buffer (25 mM glycylglycine, pH 7.8; 15 mM $MgSO_4$; 4 mM EGTA; 15 mM KPB; pH 7.8; 1mM dithiothreitol and 2mM ATP), and 100μl of luciferin buffer (2mM D-Luciferin [Sigma, ST. Louis, MO] and 25 mM glycylglycine, pH 7.8) (Wondisford et al., 1989). To correct for protein interference on luciferase activity, protein concentrations of the lysates were determined by BCA Protein Assay kit (PIERCE, Rockford, IL). A portion of the harvested cell extract was used to quantify β-galactosidase synthesis by colorimetric assay at 405nm.

4. Western blot

Total cell protein extracts were prepared by lysis of cell monolayers with protein lysis buffer (1% Triton X-100, 25mM glycylglycine, pH 7.8; 15mM $MgSO_4$; 4mM EGTA; and 1mM dithiothreitol). Protein concentration was measured by

BCA protein Assay kit (PIERCE, Rockford, IL). Equal amounts of total cellular protein extracts (10-20μg) 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 anti-CREB and Anti-phospho-CREB at 44 °C for 1h. The membrane was then washed three times by 0.4% Tween 20/ PBS for 15 min and detected by 2nd antibody (rabbit Ig, horseradish peroxidase-linked whole antibody). After washing three times with 0.3% and 0.1 % Tween 20/PBS for 15 min, the membrane was developed and exposed to XAR-5 film (Kodak).

5. Data analysis

All transient transfection assays were carried out in triplicate and repeated at lease three times on separate occasions. Data were standardized for protein concentration and β-galactosidase activity. And represented as relative light unit (X1000) and mean±SEM, Statistical analysis was evaluated by Student's t test.

RESULTS

1. Analysis of the regulation of PKA signaling pathway on Human GnRH upstream promoter in JEG cells

To determine whether the PKA signaling pathway is involved in the regulation of hGnRH upstream promoter activity in JEG-3 cells, 10 μM forskolin, an activator of adenylate cyclase, had been used to treat the JEG-3 cells transfected with 3μg upstream hGnRH promoter construct. As shown in Fig.2, Forskolin produced very modest stimulatory effects on luciferase activity of full-length upstream hGnRH promoter construct. Furthermore, 50 μM 8-CPT-cAMP, an analogue of cAMP, was used to treat the JEG-3 cells transfected with 3μg upstream hGnRH promoter construct for 24 h, a significant increase of promoter activity was observed compared to the control cells without treatment. These results indicated that both forskolin, activator of adenyl cyclase, and 8-CPT-cAMP, an analogue of cAMP stimulated the hGnRH gene expression in JEG-3 cells.

To determine whether the forskolin effect on the hGnRH upstream promoter was dose-dependent or not, JEG-3 cells were transfected with 3μg hGnRH upstream promoter expression vector. And then treated with various concentrations of forskolin. As shown in Fig.3, a clear dose-response relationship was established when JEG-3 cells were treated with forskolin from 5 μM to 80 μM for 24 h. Forskolin produced a significant increase in the luciferase activity at concentration as low as 5 μM, with a maximal effect at concentration of

80μM. Thus, forskolin stimulated the hGnRH upstream promoter activity in a dose-dependent fashion in JEG-3 cells.

Although forskolin had been proved to stimulate the hGnRH gene expression in JEG-3 cells, forskolin, as an activator of adenylyl cyclase, may also stimulate other signaling pathways, which in turn to increase the hGnRH upstream promoter activity. To better confirm PKA signaling pathway is response for the increase of hGnRH gene expression by forskolin, 8-CPT-cAMP, an analogue of cAMP was used in the following experiments. Triplicate plates of JEG-3 cells were treated for 24 h with increasing concentrations of 8CPT (0.01μM-1mM). Cells were harvested for luciferase measurements (Fig.4.). The luciferase activities were increased in a dose-dependent way of 8-CPT compared to the control without 8CPT treatment. Initial studies demonstrated that 8CPT-cAMP Increased the hGnRH luciferase activities maximally at the concentration of 100μM. However 1mM 8-CPT-cAMP showed decreased luciferase activity

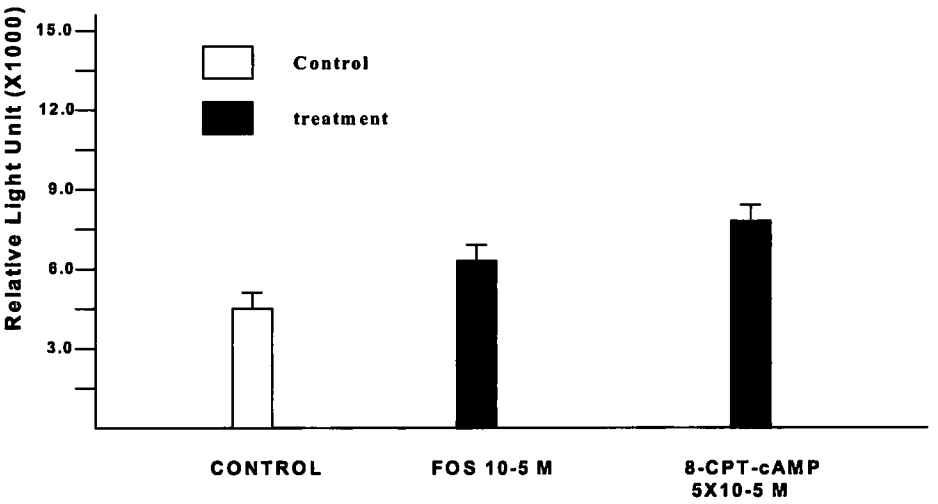

Fig. 2. PKA positive regulation of hGnRH upstream promoter activity in JEG-3 cells. JEG-3 cells were transfected with 3 μg of hGnRH upstream promoter construct and 2 μg of pCMV β-galactosidase. Then the cells were treated for 24h with 10 μ M Forskolin (Fos) or 50 μM 8-CPT-cAMP, cells without any treatment was used as control. Lysates were assayed for both luciferase activities and β-galactosidase activities as described in Materials and Methods. Each bar represents the mean±SEM and standardized luciferase activities are shown as relative light units.

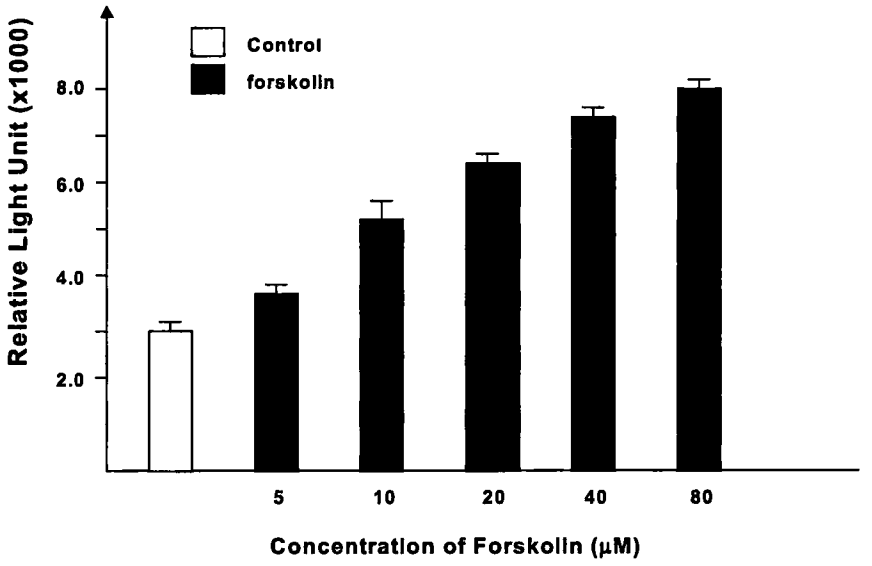

Fig. 3. Dose-response effect of Forskolin, activator of adenylate cyclase, on full-length GnRH upstream promoter activity. JEG-3 cells transfected with 3μg hGnRH upstream promoter construct and 2 μg of pCMV β-galactosidase in triplicate dishes were treated with Forskolin (5μM-100μM), or without Forskolin (control) as indicated. Cells were harvested after 24 h incubation and the cells lysate were tested for luciferase activity and galactosidase activity as described resulted in the Materials and Methods. 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.

compared to the maximal stimulatory effect at 100μM 8-CPT-cAMP, which may indicate feedback effect of 8-CPT-cAMP on the full-length hGnRH upstream promoter expression. These data are representative of three experiments showing similar stimulatory effect of the full-length hGnRH upstream promoter activity.

The time-dependent effects of cAMP analogue, 8-CPT-cAMP on hGnRH upstream promoter activities were studied using 100μM 8-CPT-cAMP in JEG-3 cells. For up to 24h (Fig.5), 8-CPT-cAMP induced a time-dependent increase in the luciferase activity of full-length hGnRH upstream promoter construct. Stimulatory effects were evident within 1h-8h, and were maximal after approximately 24h of treatment. The increase in luciferase activity after treatment of cells with 8-CPT-cAMP was as high as 5-fold. These results suggested that the 8-CPT-cAMP stimulated the hGnRH upstream promoter activity, in turn approved the involvement of PKA signaling pathway in the regulation of hGnRH upstream promoter activity in JEG-3 cells. As an analogue of cAMP, 8-CPT-cAMP has been approved positively regulate full-length hGnRH upstream promoter activity, to further confirm the conclusion, we treated the JEG-3 cells with the specific PKA inhibitor H89 before increasing 8-CPT-cAMP levels. Treatment with 10μM H-89 was sufficient to block 8-CPT-cAMP induced hGnRH upstream promoter activity in JEG-3 cells as shown in Fig.6. H89 alone slightly inhibited the hGnRH upstream promoter activity in JEG-3 cells. Therefore, activation of PKA is necessary for the cAMP-induced stimulation of

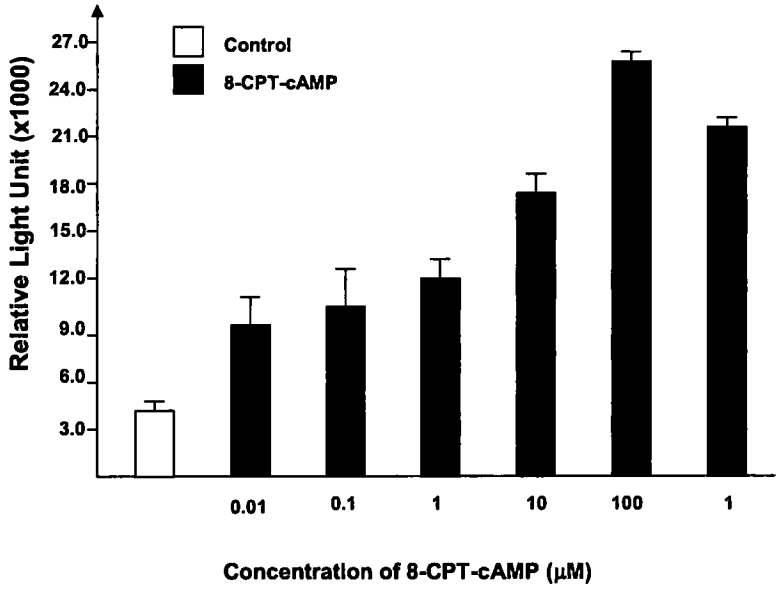

Fig. 4. Dose-response effect of cAMP analogue, 8-CPT-cAMP, on full-length hGnRH upstream promoter activity. JEG-3 cells transfected with 3μg hGnRH upstream promoter construct and 2 μg of pCMV β-galactosidase in triplicate dishes were treated with 8-CPT-cAMP (10nM-1mM), or without 8-CPT-cAMP (control) as indicated. Cells were harvested after 24 h incubation and the cells lysate were tested for luciferase activity and galactosidase activity as described in the Materials and Methods. 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.

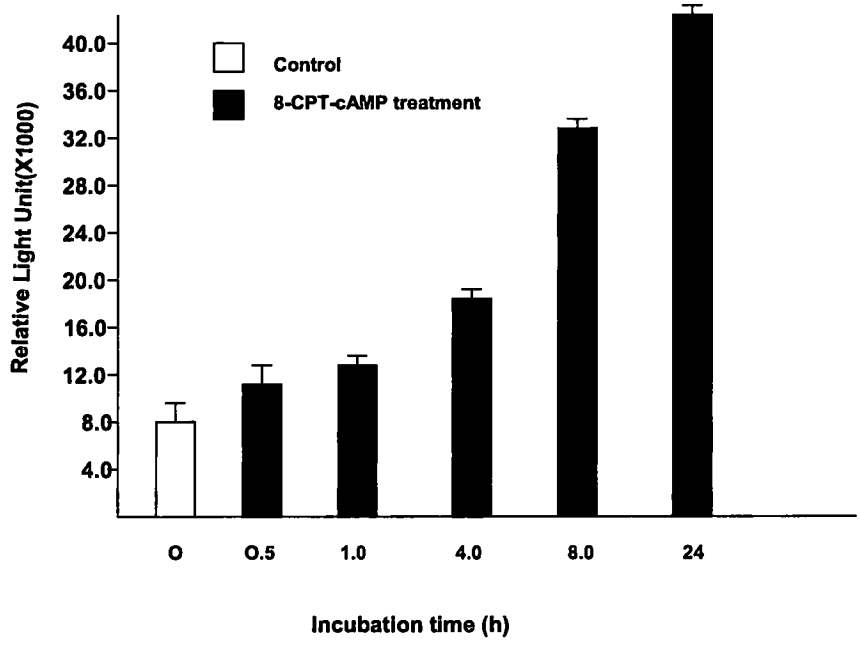

Fig. 5. Time course of 8-CPT-cAMP (100μM) on the full-length hGnRH upstream promoter activity. Triplicate dishes of the JEG-3 cells were treated with 100μM 8CPT-cAMP for different time such as 0.5h, 1h, 4h, 8h, and 24h. Then the cells were harvested and lysates were assayed for β-galactosidase and luciferase 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.

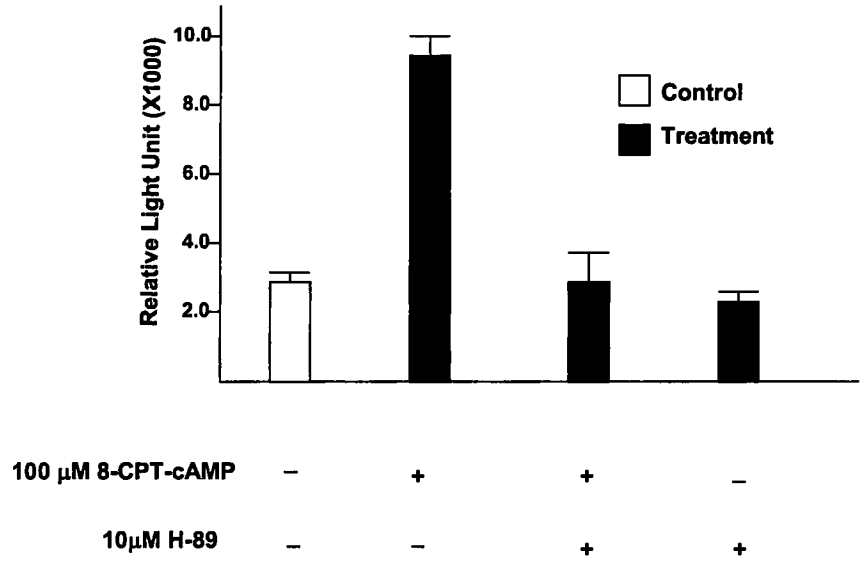

Fig.6. Effect of blockade of PKA activity on GnRH upstream promoter in JEG-3 cells. Treatment with H-89 (10μM) had inhibitory effect on stimulation of GnRH upstream promoter activity by addition of 8-CPT-cAMP (100μM) to JEG-3 cells. Data were standardized to β-galactosidase activities as a control for transfection efficiency. Each bar represents the mean ±SEM of luciferase activity, which is shown as relative light units.

the hGnRH upstream promoter activity in JEG-3 cells, furthermore, the observation that a PKA inhibitor inhibited the basal GnRH promoter activity suggested that H89 could also inhibit the intracellular PKA activity in turn suppress the basal GnRH promoter activity.

PKA is composed of two catalytic subunits bound to a homodimer of two regulatory subunits. The main function of PKA is dependent on catalytic subunits, which can phosphorylate the other proteins in turn to trigger the signaling pathways. Transfect the JEG-3 cells with 3μg of PKA alpha (cPKA α) subunit expression vector together with the upstream hGnRH promoter expression vector significantly stimulated GnRH upstream promoter activity by 3 fold, however the over expression of PKA γ subunit in JEG-3 cells only slightly increased the basal hGnRH promoter activity (Fig.7). To further confirm PKA alpha catalytic subunit positive effect, a PKA alpha mutant expression vector (Cα K42 M, generously provided by Dr. Beebe from Eastern Virginia Medical School) in which Lys in 72 positions has been changed to Met and lose the PKA catalytic activity, was used as a negative control. Shown in Fig. 7, no significant promoter activities changes had been observed compared to the control. Therefore we focused our energy to investigate the effect of PKA alpha subunit on GnRH upstream promoter activity.

To better evaluate this cPKA α regulation, triplicate plates of JEG-3 cells were transfected with increasing amount of cPKA α expression vector from 0.5μg to 9 μg together with 3 μg hGnRH upstream promoter expression vectors (Fig.8). Cells were harvested for Luciferase measurements after 24 h incubation; the

luciferase activities were increased in a dose-dependent way of the amount of cPKA α compared to the control without cPKA α. Our data demonstrated that over expression of cPKA α resulted in dramatic increase of 3 fold of luciferase activity at concentration of 3μg in JEG-3 cells after 24 h incubation. The minimus dose needed for this stimulatory effect was also determined by this dose-response study, however 6 and 9 μg cPKA α showed decrease of luciferase activity compared to the maximal stimulatory effect at 3 μg cPKA α. Which may indicate the feedback effect of cPKA α on its own catalytic activities as phosphorylation.

Furthermore, we treated the JEG-3 cells with the specific PKA inhibitor H89 before increasing levels. Treatment with 10μM H-89 was sufficient to block cPKA α stimulatory effect on the hGnRH upstream promoter activity in JEG-3 cells as shown in Fig.9. And H-89 alone slightly changed the basal hGnRH upstream promoter activities in the JEG-3 cells.

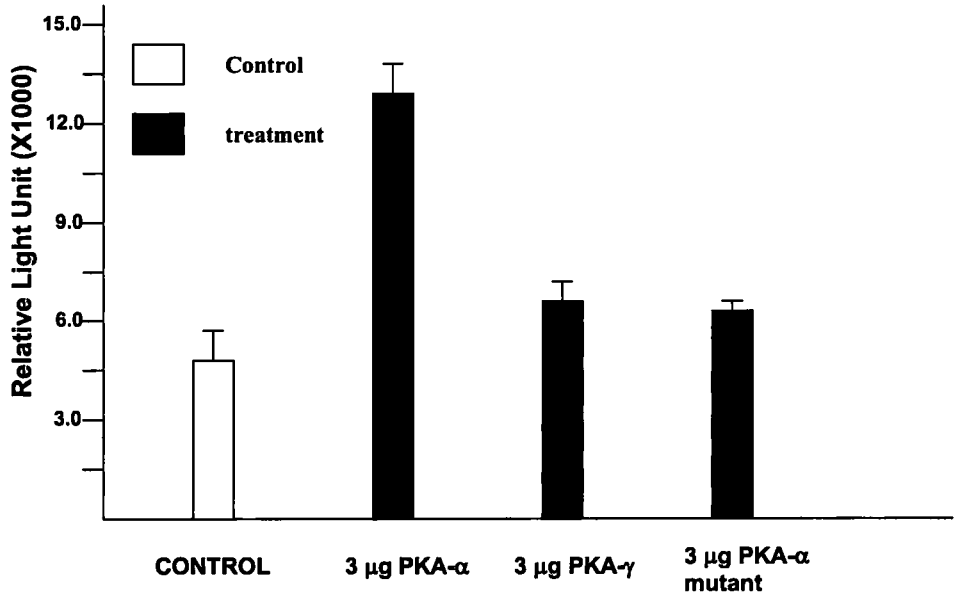

Fig.7. Stimulation of full-length upstream hGnRH promoter by different protein kinase A catalytic subunits. Transient expression assays were performed in JEG-3 cells transfected with the different PKA catalytic subunit expression vectors shown, Open bars, hGnRH Luciferase vectors alone as control; filled bars, hGnRH luciferase vectors plus cPKA expression vectors. Then the cells were harvested and lysates were assayed for luciferase activities. Each bar represents the Mean±SEM of luciferase activity, which is shown as relative light units.

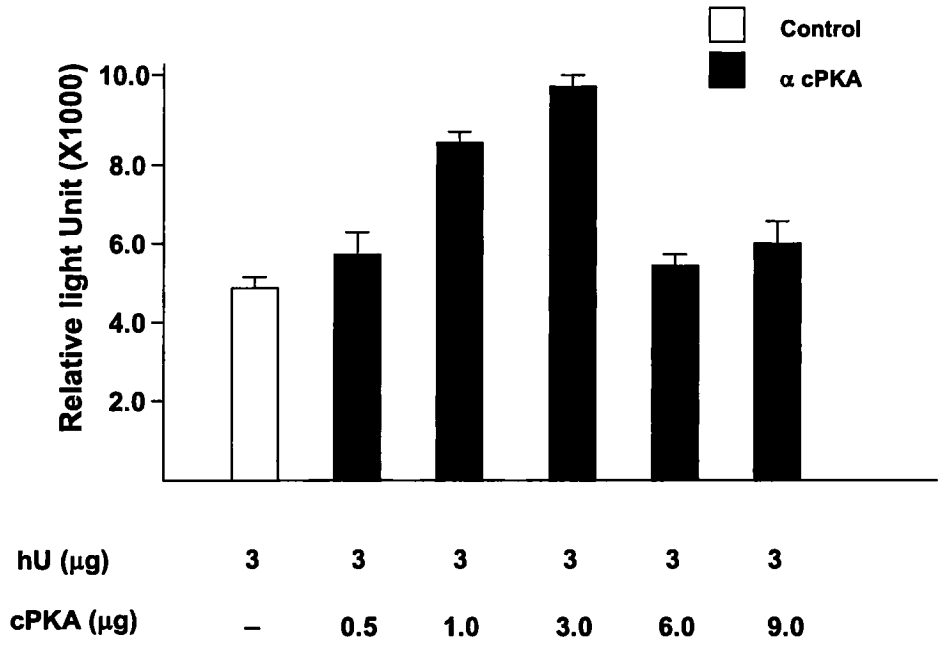

Fig.8. Dose-dependent effect of exogenous PKA catalytic subunit α (cPKA α) expression on hGnRH upstream promoter activity in JEG-3 cells. JEG-3 cells were transfected with 3μg of hGnRH (hU) plus various amounts of cPKA α expression vector (0.5-9μg). After incubate 24 h, cell lysates were assayed for luciferase as described in Materials and Methods. Each bar represents the Mean ±SEM and luciferase activities are shown as relative units.

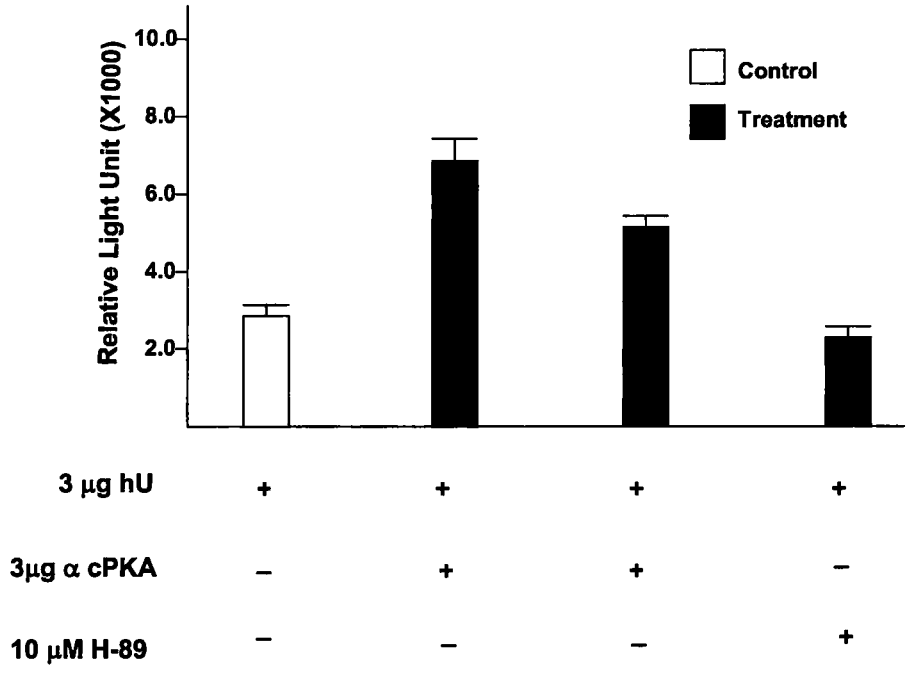

Fig. 9. H-89 inhibitory effect on the PKA positive regulation on the hGnRH upstream promoter activity. JEG-3 cells were transfected with 3μg of hU plus 3 μg cPKA α expression vector. After incubate 24 h, cell lysates were treated with or without 10μM H-89 for 30 min, then assayed for luciferase as described in Materials and Methods. Each bar represents the mean ±SEM and luciferase activities are shown as relative units.

2. Characterization of the involvement of trans- and cis-elements, which were induced by PKA pathway, on GnRH gene regulation in JEG-3 cells

To determine the cis-regulatory elements in the regulation of hGnRH upstream promoter activity by PKA signaling pathway, transient transfection were performed in JEG-3 cells with a set of 5' and 3'end deletion of the hGnRH upstream promoter/luciferase constructs. As shown in Fig.10, after deletion of the hGnRH upstream promoter to –723 bp from 5' end, stimulatory effect of 8-CPT-cAMP on the hGnRH upstream constructs still existed. However if fragment between –554 bp (BamH I) and –202 bp (Afl II) was deleted away, the stimulatory effect of 8-CPT on hGnRH upstream promoter activity disappeared. Sequence between –554bp and –202 bp may be the PKA response element on the hGnRH upstream promoter.

To demonstrate the involvement of CREB in the PKA-mediated transactivation of the hGnRH upstream promoter, we therefore performed the western blot analysis to determine whether CREB-1 was phosphorylated after 8-CPT treatment in JEG-3 cells and after co-transfection of JEG-3 cells with PKA catalytic subunit α (cPKA α) using antiserum specific for the phosphorylated form of CREB. Culturing the JEG-3 cells with 100 μM 8-CPT-cAMP or co-transfected with 3 μg cPKAα expression vector had no significant effect on 43kDa total CREB protein levels. On the other hand, treatment resulted in a significant increase of 43kDa phosphorylated CREB protein levels at 24 h compared to the control without any treatment (Fig. 11).

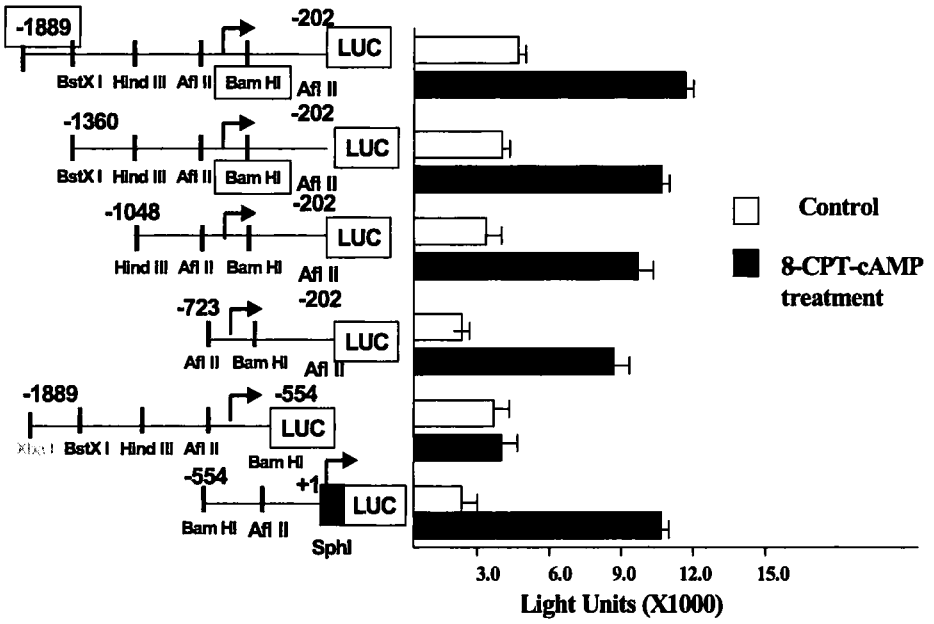

Fig. 10. Serial 5'deletion analysis of hGnRH upstream promoter activity in JEG-3 cells. All 5' deletion constructs shown extend to –202bp in their extent. The hGnRH upstream transcription start site is shown as bent arrow. Three micrograms of test plasmids and 2μg of pCMV β-galactosidase were cotransfected into JEG-3 cells and cells treated with 100 μM 8-CPT-cAMP, 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.

The phospho-CREB antiserum did not detect purified unphosphorylated CREB demonstrating the specificity of the antibody. Both antibodies recognized a high molecular size; perhaps CREB associated protein, which did not change with the treatment. This finding was replicated in three separate transfection experiments. It is likely that the amount of phospho-CREB in the PKA-induced JEG-3 cells when compared to 8-CPT-cAMP treated cells was underestimated since only a fraction of the cells in the dish were transfected with cPKA construct, whereas all of the cells in the dish were exposed to the 8-CPT-cAMP.

CREB binds to genes containing CREs as a homodimer, and after phosphorylation by protein kinase A (PKA); CREB-binding protein (CBP) has been demonstrated involved in the regulation of gene expression by CREB. Which is proposed to activate histone acetyltransferase and displace nucleosomes as well as recruit RNA polymerase II to the transcription complex. Here we tested the hypothesis that the CBP is also involved in PKA positive regulation on hGnRH gene expression. After JEG-3 cells were transfected with hGnRH and cPKAα expression vector, various amount of CBP expression vector (0.5-9 μg) potentiated the PKA stimulatory effect on hGnRH upstream promoter activity. Dose response shown (Fig.12) that CBP could enhance the PKA stimulatory effect by 4 fold maximally at the concentration of 6 μg CBP expression vector. Transfected 9 μg of CBP expression vector dropped the luciferase activity compared the maximally enhanced effect at 6 μg, which may due to the transfection saturation.

Fig.11. CREB phosphorylation in response to cPKA and 8-CPT-cAMP. Western blot analysis of nuclear extracts prepared from untreated (control) and 8-CPT-cAMP, or cPKA co-transfected (cPKAα) JEG-3 cells. Blots were probed with antiserum specific for CREB and phosphorylated CREB. Size markers are shown.

These results suggested that CBP was also involved in the PKA positive regulation of hGnRH gene expression in JEG-3 cells. To further confirm this enhancement was due to the PKA activation of CBP protein, another set of dose-dependent study of CBP alone on hGnRH gene expression was conducted. JEG-3 cells were co-transfected with 3 μg of hGnRH promoter construct (hU) as well as increased amount of CBP expression vector (0.5-9 μg), in Fig.13, No significant changes of luciferase activities were observed. This results suggested CBP stimulatory effect on human GnRH upstream promoter activity was dependent on PKA signaling pathway, CBP alone had no significant effect on the hGnRH gene expression in JEG-3 cells.

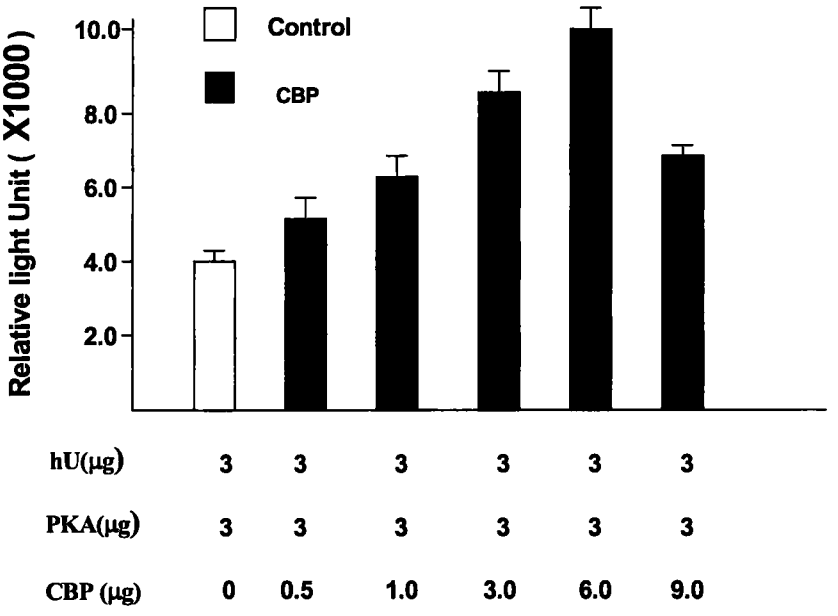

Fig. 12. CREB-binding protein (CBP) potentiates PKA induced hGnRH gene expression in JEG-3 cells. JEG-3 cells were co-transfected with hGnRH upstream promoter construct (hU) and PKA α catalytic subunit expression vector, and various amount of CBP expression vector (0, 0.5,1, 3, 6, 9 μg). Luciferase activities were determined as described in Material and Methods. Each bar represents the Mean±SEM of luciferase activity, which is shown as relative light units.

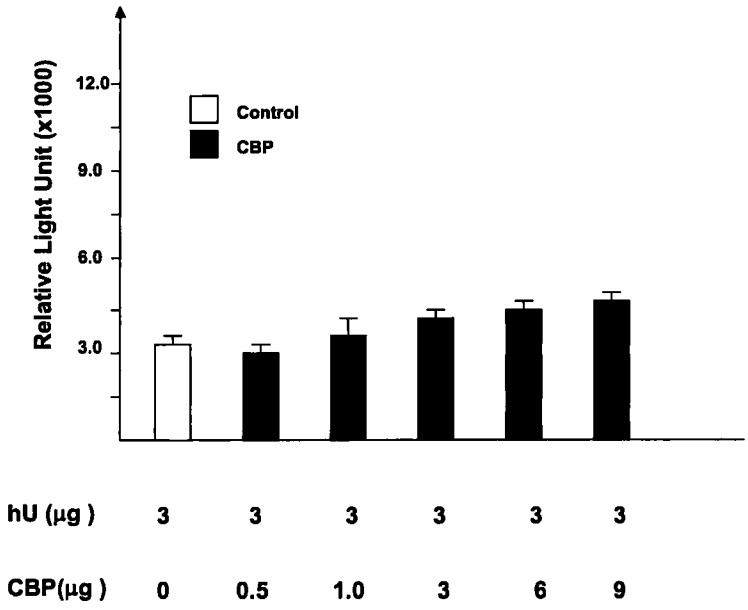

Fig. 13. CREB-binding protein (CBP) alone had no significant effect on hGnRH gene expression in JEG-3 cells. JEG-3 cells were co-transfected with hGnRH upstream promoter construct (hU), and various amount of CBP expression vector (0, 0.5,1, 3, 6, 9 μg). Luciferase activities were determined as described in Material and Methods. Each bar represents the Mean±SEM of luciferase activity, which is shown as relative light units.

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 have been used as the study model. Due to the extremely low level of the GnRH produced in the placenta, it is not possible 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. Furthermore, since the gene expression is under complex control, it is not possible to be able to investigate an isolated factor in the *in vivo* experiment. Primary cell culture of the human placenta provides an alternative mechanism for studying the hGnRH gene expression and regulation in the placenta. The advantage of this approach is that the primary placenta cell culture might 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 a gene expression study. The difficulty in obtaining 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 cell have been widely used as *in vitro* models for placenta and neuron (Radovick et al., 1991; Wierman et al., 1992; Weiner et al., 1993; Wetssel 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 somatomammotropin (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 human placenta has been reported (Younges et al., 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 presence of progesterone in JEG-3 cells might stimulate GnRH gene expression, to eliminate the progesterone effect, an antagonist of progesterone might be used in the future study.

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, *Luciferyl-AMP +O_2 ->luciferase +oxyluciferin +AMP+CO_2 +nγ (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, luciferase reporter gene, promoter-less pxp2-Luc was applied, in which the hGnRH upstream promoter was cloned upstream luciferase gene to drive the expression of luciferase.

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

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 in to the cells with test plasmids. Further, the protein concentration of the cell lysates was measured to normalize the protein amount for luciferase assay.

4. PKA signaling pathway stimulates the GnRH gene expression in the JEG-3 placental cells

Our previous studies showed that steroids hormones estradiol and progesterone effected the hGnRH gene expression in placental JEG-3 cells in a receptor-mediated pattern. Other labs have demonstrated that JEG-3 cell accumulates progesterone and aromatizes androgen precursor to estradiol by cAMP response mechanism (Ringler et al., 1989). Also elevated cAMP could activate estrogen receptor (Shamma et al., 1999). These results suggested that cAMP-dependent signaling pathway might directly effect hGnRH gene

expression in JEG-3 cells. 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 effect of cAMP-dependent signaling pathway on the hGnRH upstream promoter activity.

The results in Fig.2 showed that 10μM forskolin, an activator of adenylate cyclase, stimulated hGnRH upstream promoter activity in JEG-3. Studies showed that forskolin subsequently raised intracellular cAMP level in the cells, then it was reasonable to our hypothesis that PKA signaling pathway, which is induced by elevated level of cAMP in cells will effect the hGnRH gene expression in JEG-3 cells. However forskolin may activate other signaling pathways besides PKA, It will be interesting to further confirm by using 8-CPT-cAMP, a cell permeable analog of cAMP. JEG-3 cells which was transfected with hGnRH upstream promoter construct, were treated with 50μM 8-CPT-cAMP for 24h, the results suggested that 8-CPT increased the hGnRH promoter activity by three fold maximally, dose and time dependent fashion were observed in Fig.3 and Fig.4. PKA specific inhibitor H-89, was used to further confirm the results, in Fig.5, 10 μM H89 abolished the stimulatory effect of 8-CPT-cAMP stimulatory effect on the hGnRH upstream promoter activity. And H89 alone had no significant effect on the promoter activity. Herein, we could conclude that PKA signaling pathway stimulated the hGnRH gene expression in JEG-3 cells.

The PKA signaling pathway has been studied in the regulation of GnRH release and synthesis. Lee et al., showed forskolin, activators of adenylate cyclase to raise cAMP levels, enhanced GnRH mRNA steady state levels in the hypothalamus. However, Wetsel et al, 1993, found forskolin only stimulated GnRH secretion but no effect on GnRH mRNA in GT1-7 cell line (Wetsel et al., 1993). These two different results may due to the different study system. The latter was performed in GnRH neurons GT1-7 cell immortalized by simian virus-40 T-antigen, which may render them unresponsive to forskolin stimulation, while the former study was conducted in hypothalamic tissues slices, which contained many different neurons that can affect the GnRH synthesis and secretion. Our results were similar to Lee's, Forskolin increased the hGnRH upstream promoter activity in the placental JEG-3 cells in a dose dependent fashion. Furthermore, our results showed 8-CPT-cAMP, an analogue of cAMP, stimulated hGnRH upstream promoter activity in a dose and time dependent fashion. Pharmacological blockade of PKA activity by H-89 abolished the 8-CPT-cAMP stimulatory effect. This result further confirmed our hypothesis that PKA signaling pathway may be involved in the hGnRH gene expression in JEG-3 cells. Vitalis et al., 2000, showed cAMP increased GnRH secretion in GT1 neuron cells, however this increase is due to the opening cAMP-gated cation channel in the GT1 cells, but not by activation of PKA. Then it will be interesting to elucidate the mechanisms of the 8-CPT-cAMP stimulatory effect on hGnRH gene expression.

PKA, the holoenzyme, consisting of two identical regulatory (R), and two catalytic (C) subunits, dissociates when each of the R subunits binds two cAMP

molecules. The C subunit phosphorylates serine residues in the substrate proteins involved in mediating the biological response to hormonal stimulation. Multiple genes exist for the catalytic subunits Cα, Cβ, and Cγ. Cα and Cβ are approximately 90% identical in amino acid sequence and have different tissue distribution (Uhler, et al., 1986). Cα is expressed in most mammalian tissues; Cβ is highly expressed in brain. Cγ is functional different from the other two isoforms and is expressed specifically in testes (Beebe et al., 1992). Our results suggested that Cα expression vector significantly stimulated the hGnRH promoter activity while Cγ slight increased the hGnRH gene expression in JEG-3 cells. It is the first time to demonstrate which catalytic subunit is responsible for the positive regulation on the hGnRH promoter activity. Tamanini et al., 1991, first purified the catalytic subunit of PKA from human placenta by DEAE-cellulose and HTP chromatography. However the attempts of separating the isoforms of the C subunit purified from human placenta by analytical IEF on polyacrylamide gel resulted in the aggregation and precipitation of the sample at the application point. So in our results we used different exogenous PKA catalytic subunit to determine which isoform is responsible for the stimulatory effect on the hGnRH gene expression in JEG-3 cells. In order to confirm the PKA positive regulation of hGnRH gene expression is through Cα subunit. A PKA catalytic Cα mutant expression vector (Cα K42 M, generously provided by Dr. Beebe) in which Lys in 72 position has been changed to Met and lose the PKA catalytic activity, was used as a negative control. Results in the Fig.9 showed the partly blockade of PKA Cα stimulatory effect on the hGnRH upstream promoter activity, This may

suggested that PKA pathway is partially active in JEG-3 cells. Although protein kinase A (PKA) inhibitor H-89 blocked the exogenous PKA Cα positive regulation on GnRH gene expression, there may still exist the endogenous active PKA subunit to regulate the gene expression. Results in Fig.9 further confirmed this hypothesis by that H-89 alone slight inhibited basal GnRH gene expression.

Although PKA catalytic subunit is critical for the PKA activity, PKA regulatory subunit also plays an important role in the regulation of PKA holoenzyme activity. Over-expression of R subunits relative to C, or expression of less-sensitive RII β subunits, may dampen cAMP responses, promoting a refractory environment until R/C equilibrium is restored. Such mechanism are likely responsible for the extended refractory period (3-5days) for cAMP signaling observed in FRTL-5 cells following their exposure to thyroid-stimulating hormone (Armstrong et al., 1995). At least three R subunits: RIα, RIIα and RIIβ are transcriptionally stimulated by cAMP in Sertoli cells (Landmark et al., 1991, Oyen et al., 1988). RII subunits differ from RI subunits in that they associate with A-kinase anchoring proteins (AKAPs). AKAPs are a large family of proteins, the full extent of which has not been defined. These proteins influence subcellular localization of PKA by interacting with RII subunits. Localization of PKA within the nucleus by AKAP-95 may contribute to rapid cAMP-driven changes in transcription (Coghlan V.M. et al., 1994). AKAP may facilitate activation of PKA by bringing it near the site of stimulation as is the case in dendritic spines Carr et al., 1992). Or may limit activation of co localizing with enzymes that may contribute to rapid cAMP-driven changes in transcription. Briefly, the various isoforms of the R and C subunits

display difference in their tissue distribution, which implies specificity of their function, and some specialization is seen in the range of cAMP sensitivity shown by combination of R and C subunits.

5. cAMP response element binding protein (CREB) and CREB binding protein (CBP) are involved in the PKA positive regulation on hGnRH gene expression in JEG-3 cells

The expression of hGnRH gene has been found only in the hypothalamic neurons and a few non-hypothalamic tissues such as reproductive tissues, indicating the tissues-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 supported this concept (Dong et al., 1993). In the present study, we are using the JEG-3 placental cell line to demonstrate the PKA positive regulation on hGnRH gene expression. hGnRH upstream promoter has been used. To further determine the cis-element on the hGnRH upstream promoter response to the PKA regulation, a series of deletion experiments have been performed. We demonstrated that the fragment between –202 (Afl II) and –554 bp (BamH I) is most important for the PKA stimulatory effect on the hGnRH upstream promoter activity. Close examination of this fragment, we could find the sequence 5'-TGACTTCA-3' similar to the consensus cAMP response element (CRE) 5'-TGACGTCA-3', which further supported our findings. While in GT1-7 neurons (Lei et al., 1997), transient transfection of GT1-7 neurons with the 5'-flanking region of the rat GnRH gene-luciferase fusion constructs revealed that a 53-base pair (bp) sequence between –126 and –73 is required for the hCG

inhibition through cAMP signaling pathway. Footprinting with GT1-7 cell nuclear extracts protected the –99 and –79 bp region, which contained a so-called imperfect AP-1 site, and two AT-rich palindromic sequences. In summary, AT-rich sequences in the 5'-flanking region of r GnRH is critical for the cAMP signaling regulation. Our response element was different to this rat GnRH region, but similar to CRE, it will be very interesting to investigate the involvement of CRE binding protein (CREB) in the PKA positive regulation of hGnRH gene expression in JEG-3 cells.

CRES are known to form DNA-protein complexes with the CREB/ATP family of transcription factors. In order to demonstrate the involvement of CREB, western blot analysis were performed with both anti-CREB and anti-phospho-CREB. Phosphoration of CREB by PKA is essential for its transactivating functions (Gonzalez et al., 1991). In the present study, we have shown that hGnRH promoter in JEG-3 cells can be transactivated by the PKA pathway and is mediated by phosphorylation of CREB. These results are similar to the Lei's results in 1995: treatment of GT1-7 neurons with hCG through PKA pathway resulted in an increase of 43kDa phosphorylated CREB protein levels. Also in both JEG-3 and Calu cells, phospho-CREB antiserum detected a 47kDa CREB-1 polypeptide in cells either treated with forskolin or co-transfected with cPKA expression vector (Ying et al., 1997), this CREB phosphorylation is responsible for the HERN promoter activities transactivated by the PKA pathway in calu-6 cells. CREB belongs to a group of transcription factors that contain basic region leucine zippers, termed bZIPs. The bZIP family includes Jun, Fos,

CAAT/enhancer binding protein, the activating transcription factors, (ATFs), and yeast GCN4. The cAMP-responsive bZIP transcription factors, including CREM and ATF-1 (Meyer et al., 1993), form a distinct subclass. CREB, CREM and ATF-1 share a conserved structure, with a carboxyl terminal bZIP region, an amino proximal terminal kinase-inducible domain (KID). Central to the role of cAMP-responsive transactivation, the KID contains potential phosphorylation sites for several different kinases. However it is the phosphorylation of serine 133 in CREB 347, or 119 in CREB 327, that is critical for the transactivation properties of CREB (Gonzalez et al., 1989). Unphosphorylated CREB is predominately located in the nucleus in vivo; its translocation is mediated by nuclear translocation sequences located within the bZIP region (Waeber et al., 1991). Whereas CREB forms dimmers and binds DNA in the absence of phosphorylation, phosphorylation of serine may increase the strength of the binding of CREB to CREs found in certain promoters, notably those with imperfect (asymmetric) CREs (Nichols et al., 1992). In addition to residues that function in PKA recognition, CREB KID contains motif that are critical for transactivation. In deletion mutagenesis experiments, removal of a DLSSD motif C-terminal to Ser-133 completely disrupted PKA dependent CREB activity even though this mutant CREB polypeptide was phosphorylated at Ser-133 to the same extent as wild-type CREB (Gonzalez et al., 1991). Within the DLSSD motif, Asp-140 with Asn-140 also abolished PKA responsiveness in transient transfection assays. Another important domain in CREB protein, the glutamine-rich activator Q2 region is also very important for CREB to activate gene

expression. Removal of Q2 domain abolished CREB activity in transient transfection assay (Quinn et al., 1993). Q2 activity required addition of the TFIID fraction, which in addition to the TATA binding protein (TBP), contains several TBP-associated factors or TAFs that function as co activators (Hoey et al., 1993).

Precisely how CREB phosphorylation stimulates CREB function and in turn activates gene expression is still unknown. One model is that phosphorylation may allow recruitment of co activators, which then interact with basal transcription factors. This factor, CREB-binding protein (CBP), is a 265-kDa nuclear protein that associates with CREB phosphorylated CREB. Other data suggests co expression of CBP increases stimulus-induced CREB transcription of a CRE reporter gene; an effect is lost when Ser133 is mutated to an alanine (Kwok et al., 1994). CBP, via an N-terminal domain, binds to CREB and functions to increase transcription through a more C-terminal domain, which is proposed to both activate histone acetyltransferase (Ogryzko et al., 1996) and display nucleosomes as well as recruit RNA polymerase II to the transcription complex (Kee et al., 1996; Nakajima et al., 1997). Recently Zanger et al., 1999 had demonstrated CBP could independently function of its recruitment to the transcription complex in the pituitary cells. CBP through two cysteine-histidine rich domains specifically interacts with Pit-1, a member of a family (POU homeodomain proteins) of transcription factors. Pit-1 and CBP synergistically activates the PRL gene after PKA stimulation (Zanger et al., 1999). Our data showed that CBP enhanced the 8-CPT-cAMP stimulatory effect on human GnRH gene expression in JEG-3 cells, which further supported our hypothesis that CBP

is also involved in the PKA positive regulation of hGnRH upstream promoter activity in JEG-3 cells. In addition to recruiting Pol II, CBP also contributes to CREB-mediated transcription by affecting chromatin structure. CBP possesses an intrinsic histone acetyltransferase (HAT) activity and associates with anoter HAT-containing factor termed p/CAF (Yang et al, Ogryzko, et al, Bannister et al., 1996). By catalyzing acetylation of lysine residues in the N-termini of histones, CBP and p/CAF alter chromatin structure in a fashion believed to make the DNA template more accessible to the transcriptional machinery (Struhl, et al., 1998). The mechanism through which this occurs has not yet been clearly elucidated.

6. Other signaling pathways are involved in hGnRH gene expression

Our data supported the PKA signaling pathway positive regulation on human GnRH gene expression in JEG-3 cells, other signaling pathways are also demonstrated in the regulation of GnRH gene expression. A ligand receptor/G-protein-coupled complex that activates phospholipase-C initiates the protein kinase C pathway. Phospholipase-C cleaves phosphatidylinositol 4, 5, -bisphosphate to diacylglycerol (DAG) and inositol 1,4, 5, -triphosphate. DAG then activates PKA by a mechanism by which the enzyme undergoes a translocation from the cytosol to the plasma membrane. PKC is a family of enzymes, which when activated, phosphorylates many proteins and results in the induction or activation of many nuclear transcription factors such as Fos and Jun (Nishizuka et al., 1986). Fos and Jun family members bind in a dimeric fashion to the AP-1 consensus DNA binding element (Curran and Franzak et al., 1988). These transcription factors then positively or negatively regulate gene expression

following PKA activation. Treatment of the GT1-7 neuronal cell line with the phorbol ester, 12-O-tetradecanoylphorbol 13-acetate (TPA), a PKA agonist, increases PKC activity and decreases rat GnRH mRNA levels (Bruder et al., 1994). Furthermore, phorbol esters repress GnRH expression at the level of transcription through DNA sequences between –73 to –126 bp in the proximal rGnRH promoter. In contrast, Radovick and colleagues have reported that TPA stimulates rather than inhibits the human GnRH promoter using stable transfectants in a different transgenically derived GnRH-producing cell line, Gn-10 (Radovick and Yuefen, et al., 1992). Then it will be interesting to elucidate the PKC regulation of hGnRH gene expression in the placental cells.

In addition to PKA, other signal transduction pathways targeted the CREB protein, in order to either increase or decrease its transcriptional activity. For example, the Ca^{2+}-calmodulin-dependent kinase IV (CamKIV) phosphorylates CREB ate Ser 133 after membrane depolarization in neuronal cells (Bito et al., 1996). Also signal transduction pathways triggered by growth factors and inflammatory cytokines leaded to a phosphorylation of CREB. Furthermore, PKC, glycogen synthase kinase III (GSK-3), and casein kinase II (CKII) can all phosphorylate residues in the CREB KID domain. PKC can also activate MAPK; indeed, T-cell receptor stimulation induces CREB phosphorylation through a PKC-dependent pathway that requires MAPK activation (206).

CONCLUSIONS

Using the human placental choriocarcinoma JEG-3 cell line as an *in vitro* human placental model, the mechanisms of the PKA positive regulation of the hGnRH gene expression in the human placenta has been studied. Through PKA catalytic subunit α, human GnRH upstream promoter activities are stimulated by the PKA signaling pathway by a cAMP-dependent mechanism in JEG-3 cells. In addition, the sequence between –202 (Afl II) and –554 (BamH I) in the human GnRH upstream promoter region is responsible for the PKA positive regulation of the gene expression. Furthermore, Western blot analysis demonstrated the involvement of phospho-CREB in the PKA regulation of hGnRH gene expression in JEG-3 cells, and CREB-binding protein (CBP) could further enhance the PKA stimulatory effect on the hGnRH upstream promoter activities.

We have demonstrated that the PKA signaling pathways positively regulated the human GnRH upstream promoter activities in JEG-3 cells. This will further help to elucidate the mechanisms of hGnRH local regulation in the placenta.

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