TRANSCRIPTION REGULATION OF HUMAN GONADOTROPIN-RELEASING HORMONE RECEPTOR GENE EXPRESSION

By

KWAI WA CHENG

B. Sc. (Hons.), Hong Kong Baptist University, 1992

M.Phil., The University of Hong Kong, 1996

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Department of Obstetrics & Gynecology

The University of British Columbia
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ABSTRACT

Human placental GnRHR cDNA isolated from human choriocarcinoma JEG-3 cells, immortalized human extravillous trophoblasts (IEVT) and primary culture of cytotrophoblasts was identical to the pituitary counterpart. In addition, placental GnRHR was shown coupling to both the protein kinase C (PKC) and protein kinase A (PKA) signaling transduction pathways. Interestingly, homologous down-regulation of GnRHR mRNA level was not observed in placental cells as in pituitary cells, suggesting that a different regulatory mechanism may exist in controlling the expression of this gene in these two tissues. Using JEG-3 and IEVT cells as models, an upstream promoter was shown to confer the placental cell-specific expression of hGnRHR gene both *in vitro* and *in vivo*. Four putative transcription factor binding sites, namely hGR-Oct-1, hGR-CRE, hGR-GATA and hGR-AP-1, were located and confirmed to be essential for the placental expression of this gene. Importantly, hGR-CRE and hGR-GATA motifs were subsequently found to be placenta-specific.

A differential regulation of human GnRHR promoter activity by progesterone (P) in the pituitary and placenta was observed. P treatment decreased the promoter activity at the level of pituitary. In contrast, P stimulated the expression of this gene in the placenta. A progesterone response element, namely hGR-PRE, mediated the P-action. Interestingly, human progesterone receptor (PR)-B exhibits a cell-dependent transcriptional activity, such that it functions as a transcription activator in the placenta but a transcription repressor in the pituitary. In contrast, human PR-A acts as a transcription repressor in both tissues. The increase in hGnRHR promoter activity after cAMP/PKA pathway activation by either

pharmacological agents or by PACAP and hCG in the pituitary and placenta, respectively, implies that any hormones, which activate cAMP/PKA pathway, may increase the hGnRHR gene transcription. Two elements, namely hGR-AP/CRE-1 and –2, were subsequently demonstrated to be responsible for mediating this stimulatory effect. The comparison studies on the transcriptional regulation of hGnRHR gene by P and cAMP/PKA pathway at the level of the pituitary and placenta implicate that the regulation of hGnRHR gene transcription is constantly under fine-tuning by a complex regulatory mechanism through the availability of different transcription factors and the activation of multiple signal transduction pathways.

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LIST OF ABBREVIATIONS

ACI	adenylate cyclase inhibitor (SQ22536)
AGT	DL-aminoglutethimide
AP-1	activating protein-1
bHLH	basic helix-loop-helix
bZIP	basic leucine zipper
cAMP	3',5'-cyclic adenosine monophosphate
cDNA	complementary DNA
CRE	cAMP response element
CREB	cAMP-response element binding protein
CTX	cholera toxin
DAG	diacylglycerol
DIG	digoxigenin
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic disodium salt
ER	estrogen receptor
FBS	fetal bovine serum
FSH	follicle-stimulating hormone
GMSA	gel mobility shift assay
GnRH	gonadotropin-releasing hormone
GnRHa	GnRH agonist
GnRHR	gonadotropin-releasing hormone receptor
GPCR	G-protein coupled receptor
GRAS	GnRHR activating sequence
GRKs	G-protein coupled receptor kinases
GTF	general transcription factor
HBSS	hank balanced salt solution
hCG	human chorionic gonadotropin
HTH	helix-turn-helix
IP_3	inositol 1,4,5-trisphosphate
kb	kilobase

kDa	kilodalton
L	liter
lacZ	β-galactosidase
LH	luteinizing hormone
Luc	luciferase
μ	micro (1×10^{-6})
MAPK	mitogen activated protein kinase
ml	milliliter
mRNA	messenger RNA
P	progesterone
PACAP	pituitary adenylate cyclase activating polypeptide
PBS	phosphate buffered saline
PI-3K	phosphatidylinositol-3 kinase
PKA	protein kinase A
PKAI	protein kinase A inhibitor
PKC	protein kinase C
PKCI	protein kinase C inhibitor (GF109203X)
PLC	phosphoinositidase C
PMSF	phenylmethylsulfonyl fluoride
PR	progesterone receptor
PRE	progesterone response element
RNA pol II	RNA polymerase II
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
RU486	mifepristone
SDS	sodium dodecyl sulfate
SF-1	steroidogenic factor-1
TAFs	TBP-association factors
TBP	TATA-binding protein
TIP	transcription initiation complex
TPA	phorbol ester

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CHAPTER 1. INTRODUCTION

1.1 Gonadotropin-Releasing Hormone

Hypothalamic gonadotropin-releasing hormone (GnRH) plays a major role in controlling reproductive function in animals. It was first isolated and sequenced from mammals (Amoss et al. 1971, Matsuo et al. 1971), and its functions have been well conserved for at least 500 million years of vertebrate evolution (Kasten et al. 1996). To date, there are at least 13 forms of GnRH identified in vertebrates (Carolsfield et al. 2000), and they are distributed in a range of tissues (King and Millar 1995, Kasten et al. 1996, Sherwood et al. 1997). All known GnRH variants are comprised of ten amino acids, with conserved amino acid residues at positions 1, 2, 4, 9, and 10, whereas, positions 5 to 8 are highly variable (Table 1.1). GnRH is synthesized in hypothalamus neurosecretory cells and then released in a pulsatile pattern into the hypothalamo-hypophyseal portal circulation (Conn 1986). After binding to a specific membrane receptor (GnRHR) at the pituitary, it stimulates the biosynthesis and release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary (Gharib et al. 1990). These gonadotropins enter the general circulation and regulate steroidogenesis and gamete maturation in the gonads of both sexes. In addition, GnRH has been suggested to be an important autocrine/paracrine regulator of certain extra-pituitary tissues including the placenta, since both GnRH and its receptor have been detected in these tissues (See Chapter 3, Section 3.1 for review).

Besides of the hypothalmic GnRH system, GnRH secretion and GnRH mRNA have also been detected in the rat anterior pituitary (Pagesy et al. 1992) and mouse αT3-1 cells (Krsmanovic et al. 2000). Similarly, GnRH mRNAs have been detected in normal human

pituitary and human pituitary tumors, including gonadotroph adenomas, null cell adenomas, GH-producing tumors and ACTH-producing adenomas (Miller et al. 1996, Sanno et al. 1997). The presence of GnRH-producing cells and GnRHR in the pituitary gland suggests that the locally produced GnRH could play an autocrine role in the pituitary. In an early attempt to study the potential physiological role of the locally produced GnRH from rat pituitary gonadotrope, GnRH antagonist and anti-GnRH serum has been employed to block endogenous pituitary GnRH action (Krsmonavic et al. 2000). The basal LH release from the perifused rat pituitary was significantly decreased in the presence of GnRH antagonist or anti-GnRH serum. In addition, a spontaneous Ca^{2+} oscillations in the absence of GnRH stimulation was also been observed in primary culture of rat pituitary gonadotropes exhibits, which were comparable to those induced by GnRH treatment, and could be abolished by GnRH antagonist administration (Krsmonavic et al. 2000). These results suggest that the locally produced GnRH may participate in the maintenance of basal LH release through a GnRHR-mediated process. However, the exact role of this pituitary GnRH remains unclear.

TABLE 1.1 Comparison of GnRH primary structure

GnRH	Amino acid sequences 1 2 3 4 5 6 7 8 9 10	Position of variation
Mammal	pGlu His Trp Ser Tyr Gly Leu Arg Pro Gly NH_2	n/a
Chicken I	- - - - - - - Gln - - NH_2	8
Seabream	- - - - - - - Ser - - NH_2	8
Salmon	- - - - - - Trp Leu - - NH_2	7,8
Catfish	- - - - His - - Asn - - NH_2	5,8
Herring	- - - - His - - Ser - - NH_2	5,8
Guinea pig	- Tyr - - - - Val - - - NH_2	2,7
Chicken II	- - - - His - Trp Tyr - - NH_2	5,7,8
Dogfish	- - - - His - Trp Leu - - NH_2	5,7,8
Tunicate I	- - - - Asp Tyr Phe Lys - - NH_2	5-8
Tunicate II	- - - - Leu Cys His Ala - - NH_2	5-8
Lamprey III	- - - - His Asp Trp Lys - - NH_2	5-8
Lamprey I	- - Tyr - Leu Glu Phe Lys - - NH_2	3, 5-8

1.2 Gonadotropin-Releasing Hormone Receptor

1.2.1 Structure of GnRHR cDNA

The GnRHR was first cloned from αT3-1 cells using a polymerase chain reaction-based homology cloning strategy (Tsutsumi et al. 1992). Mouse GnRHR clones have also been identified by the use of Xenopus oocyte (Reinhart et al. 1992) and mammalian cell line expression cloning (Perrin et al. 1993). After elucidating the mouse receptor cDNA sequence, the homologous pituitary GnRHR cDNAs were identified in five addition mammalian species including the human (Kakar et al. 1992, Chi et al. 1993), rat (Eidne et al. 1992, Kaiser et al. 1992, Perrin et al. 1993), sheep (Brooks et al. 1993, Illing et al. 1993), cow (Kakar et al. 1993), and pig (Weesner et al. 1994). Analysis of the primary sequence of the cloned GnRHR revealed several hydrophobic regions that correspond to the seven transmembrane domains, which are characteristic of the G protein-coupled receptor (GPCR) family (Fig.1.1). The predicted amino acid sequence for the GnRHR is more than 85% conserved in the six mammalian species reported (Table 1.2), and is nearly identical within the putative transmembrane domains. However, the GnRHRs in cow, pig, sheep, and human are 328 amino acids long, while the mouse and rat receptors contain only 327 amino acids, due to the absence of a residue in the second extracellular domain. Interestingly, recent cloning of non-mammalian GnRHR from catfish (Tensen et al. 1997), goldfish (Illing et al. 1999), frog and chicken (Troskie et al. 1998) revealed the presence of a C-terminal tail.

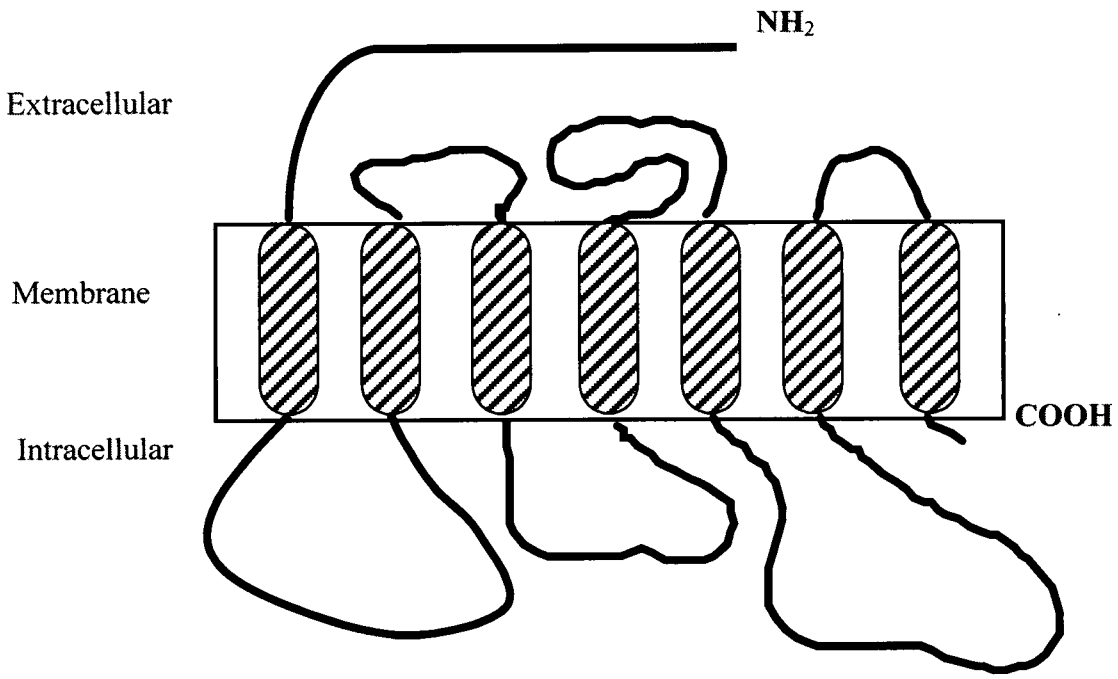


FIG 1.1 Diagrammatic representation of primary structure of human GnRHR cDNA. The hydropathy plot of the deduced amino acid sequence revealed seven hydrophobic regions that correspond to the putative transmembrane domains found in G protein coupled receptor and is notable for the absence of a carboxy-terminal cytoplasmic domain.

TABLE 1.2 Mammalian GnRHR cDNA and amino acid sequences homology comparison

Species	% Identity to Human	
	Coding region	Amino acid
Human	**100 %**	**100 %**
Pig	**88.4%**	**91.8%**
Cow	**89.1%**	**91.2%**
Sheep	**89.5%**	**90.5%**
Mouse	**85.4%**	**89.3%**
Rat	**85.2%**	**87.8%**

1.2.2 Isolation and characterization of the GnRHR gene

Using GnRHR cDNA as a probe, GnRH receptor gene and its 5'flanking region have been isolated from mouse (Albarracin et al. 1994, Clay et al. 1995), human (Fan et al. 1995, Kakar 1997), rat (Reinhart et al. 1997, Pincas et al. 1998), and ovine (Campion et al. 1996). Early characterization of the 5' flanking region of the mouse, rat, sheep and human GnRHR genes revealed a similar gene structure, but with complex organization of the transcriptional start sites (Fig. 1.2). Each of these genes has two introns in the same locations in the reading frame. The first intron is greater in the mouse (15 kb) and rat (12 kb) than in the sheep (>10 kb) and human (4 kb). Sequence homology comparison of the mouse, rat, sheep and human 5' flanking region revealed significant homology. Analysis of the 5' flanking sequence for up to 0.9 kb from the ATG codon showed a 42% similarity among these four genes. The phylogenetic comparison further revealed a major homology region that contained at least 65% identical base pairs and stretched over 300 nucleotides upstream of the ATG codon. One significant different between the mouse, rat, sheep and human genes is the location of their transcriptional start sites. The start sites for the mouse and rat genes are clustered in a region within 110 base pair (bp) from the ATG start codon except the distal site described by Clay and co-workers (1995). Interestingly, there is no consensus TATA or CAAT box sequences in the mouse and rat promoter near the transcriptional initiation sites (Albarracin et al. 1994, Reinhart et al. 1997, Pincas et al. 1998). However, the putative human GnRHR promoter appears to be more complex than in other species. The human GnRHR gene contains multiple TATA sequences (seven compared with none in mouse) and CAAT sequences (six) residing in close proximity to one another in a cluster-like arrangement (Fan et al. 1995, Kakar 1997). In addition, the start sites for the human and sheep genes are at least 600 bp upstream from the ATG start codon. These findings suggest the presence of multiple transcriptional start sites in the human GnRHR gene. We have reported the presence of five transcriptional start sites using human brain RNA (Fan et

al. 1995). In contrast, others identified 18 transcriptional start sites in human pituitary RNA (Kakar 1997). The existence of multiple and different transcriptional start sites in the pituitary and brain raises the possibility that alternative transcriptional initiation sites and/or transcriptional regulatory mechanism are utilized in the pituitary and extrapituitary tissues. Nevertheless, all transcriptional start sites identified in human tissues are closely related to the TATA- and CAAT boxes. Sequence homology analysis of the 5' flanking region of the human GnRHR gene revealed the existence of a number of regulatory sequences for various hormones and transcription regulators (Fan et al. 1995, Kakar 1997); this provides considerable potential for regulating the expression of the human GnRHR gene (Fig. 1.3). These regulatory sequences include a putative cAMP response element (CRE), a glucocorticoid/progesterone response element (GRE/PRE), a thyroid hormone response element (TRE), a PEA-3 binding site, a activating protein-1 (AP-1) binding site, a protein kinase responsiveness AP-2 binding site, pituitary-specific response element (Pit-1) and steroidogenic factor-1 (SF-1) binding site (Fan et al. 1995, Kakar 1997, Ngan et al. 1999).

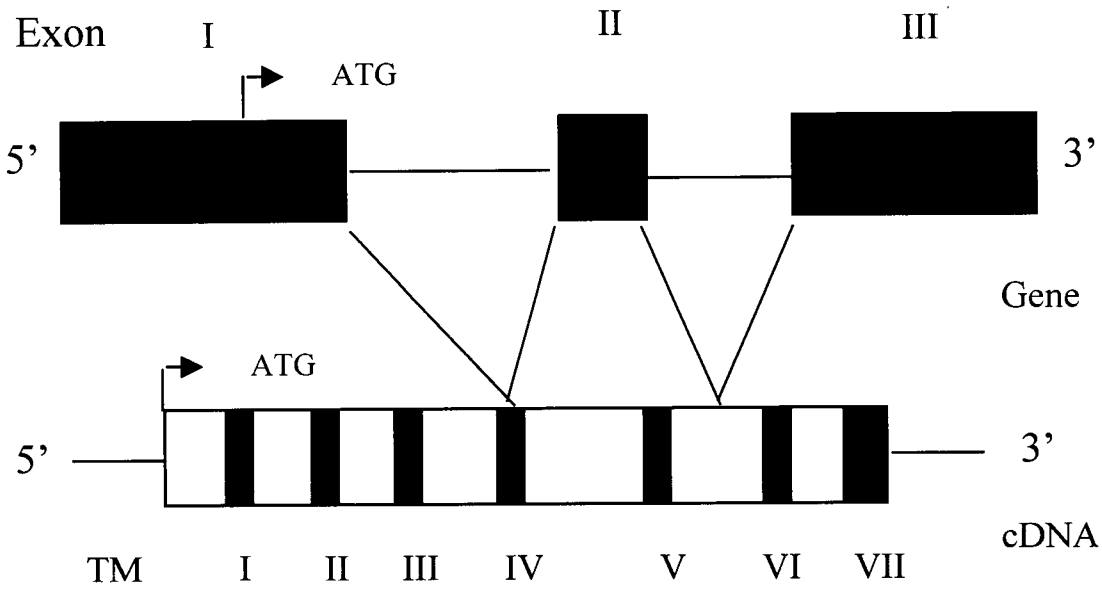


FIG. 1.2 Diagrammatic representation of GnRHR cDNA and gene structure. (*Upper panel*) The Exon-intron structure of GnRHR gene. The shaded boxes represent exons and the intervening lines represent introns. (*Lower panel*) The structure of the GnRHR cDNA. The open box represents the protein-coding regions, and black boxes are the putative transmembrane domains.

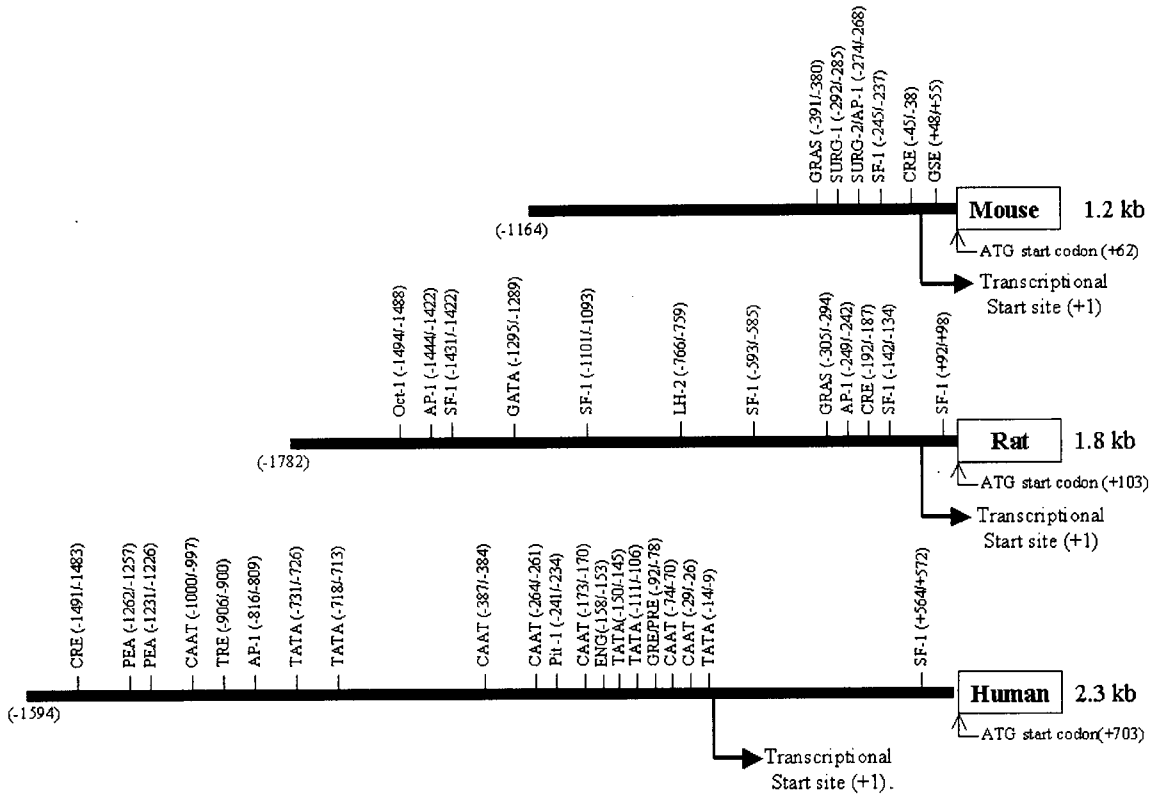


FIG. 1.3 Diagrammatic representations of mouse (1.2 kb), rat (1.8 kb) and human (2.3 kb) GnRHR 5' flanking region. The *cis*-regulatory elements were identified by either experimentally or based on DNA sequence homology comparison.

1.3 Expression of the GnRHR

1.3.1 Pituitary

It is well established that GnRH-binding sites are located mainly on the gonadotropes in the pituitary (Clayton 1989). Northern blot analysis of mouse pituitary and αT3-1 messenger ribonucleic acid (mRNA) revealed two hybridization species of 3.5 and 1.6 kb (Kaiser et al. 1992, Reinhart et al. 1992, Tsutsumi et al. 1992). Solution hybridization protection assay detected GnRHR mRNA in the mouse pituitary and αT3-1 gonadotropes, but not in AtT-20 corticotropes or GH3 somatolactotropes (Tsutsumi et al. 1992). In the rat pituitary gland, use of Northern blot analysis and a mouse GnRHR cDNA probe detected a single 4.5 kb band (Reinhart et al. 1992). In another report, additional bands of 1.8 kb and 5-5.5 kb were detected by a rat GnRHR cDNA probe (Kaiser et al. 1992). In the human pituitary, Northern analysis with a human GnRHR cDNA probe detected a predominant mRNA of 4.7-5.0 kb (Chi et al. 1993, Kakar 1997) and two fainter bands of 2.5 and 1.5 kb (Kakar 1997). In the sheep pituitary, RNA transcripts of 5.5, 3.6, 2.3 and 1.4 kb have been detected (Brooks et al. 1993, Illing et al. 1993).

Characterization of pituitary GnRHR by photoaffinifty labelling studies revealed that the high-affinity GnRH binding is associated with membrane proteins of 63, 53, and 42 kilodaltons (kDa) in the rat, 42 kDa in bovine, 39 kDa in ovine and two 51 kDa proteins in goldfish pituitary (Catt et al. 1985, Iwashita and Catt 1985, Hazum et al. 1986, Habibi et al. 1990). The use of a monoclonal antibody raise against human GnRHR revealed a 62 kDa protein by Western blotting analysis (Karande et al. 1995). The presence of more than one GnRH receptor mRNA and pituitary GnRH binding protein implies the possible presence of multiple GnRHR isoforms. However, only one form of GnRHR cDNA has been isolated from the mammals, and the calculated molecular mass of the GnRHR from the deduced amino acid sequence is 38 kDa.

Thus, the detection of multiple GnRH binding sites may due to the different post-translation modification. Biochemical studies of the GnRHR have suggested that it is a sialic acid residue-containing glycoprotein (Sealfon et al., 1997). Two potential sites for N-linked glycosylation, one at the amino terminus and one in the first extracellular domain, were found in bovine, ovine, and human GnRHR. The rodent species contain an additional potential glycosylation site in the amino terminus. Mutation of these two N-terminus glycosylation sites of the mouse GnRHR did not only result in the appearance of a lower molecular weight protein in gel electrophoresis, but also lower the expression level of GnRHR in transfected cells, when compared with wild-type receptor (Davidson et al. 1995, 1996).

1.3.2 Ovary

There is strong evidence for the presence of specific GnRH binding sites on the gonads of the rat (Huseh and Jones 1981). Functionally, GnRH has been demonstrated to modulate ovarian functions, including steroidogenesis, oocyte maturation, ovarian gene expression, ovulation and ovarian ateria (Huseh and Erickson 1979, Hillensjo and LeMaire 1980, Leung and Steels 1993, Billig et al. 1994, Richards 1994, Srivastava et al. 1995). By the use of the radioligand binding assay, GnRH binding was demonstrated on both granulosa and luteal cells (Clayton et al. 1979, Reeves et al. 1980). Since the isolation of rat GnRHR cDNA (Kaiser et al. 1992), several studies have detected the expression of GnRHR mRNA in the rat ovary (Bauer-Dantion and Jameson 1995, Kogo et al. 1995, Whitelaw et al. 1995). Recent cloning of GnRHR cDNA from the rat ovary revealed that the ovarian GnRHR was identical in nucleotide sequence to pituitary GnRHR (Moumni et al. 1994). Although GnRH functions in the human ovary have not yet been well established, GnRH treatment affects steroidogenesis in human ovarian granulosa cells (Gaetje 1994, Furger et al. 1996, Peng et al. 1994) and induces a luteolytic effect (Casper and Yen 1979, Lemay et al. 1992). In addition, GnRH binding sites were identified in

the human ovarian granulosa cell and in the corpus luteum (Popkin et al. 1983, Bramley et al. 1986, Latouche et al. 1989). By the use of PCR amplification and RNase protection assay, we and others have reported the expression of human GnRHR mRNA in the ovary as well as in granulosa cells (Peng et al. 1994, Minaretzis et al. 1995). It has been further confirmed by *in situ* hybridization (Fraser et al. 1996). Recently, we detected the expression of GnRHR mRNA in the human ovarian surface epithelium (OSE). Molecular cloning of this GnRHR revealed its identity, and sequence analysis showed a 100% homology to the human pituitary counterpart (Kang et al. 2000). Taken together, these results confirmed the expression of GnRHR in different compartments of the human ovary.

1.3.3 Placenta

The human placenta contains specific binding sites for GnRH, which interact with both GnRH agonist and antagonist analogues (Belisle et al. 1984, Iwashita et al. 1986, Bramley et al. 1992). However, the GnRHR in the placenta has a 10 to 100 fold lower affinity than that of pituitary GnRHR. By *in situ* hybridization, GnRHR mRNAs were detected in human placenta and located in both cytotrophoblast and syncytiotrophoblast cell layers (Lin et al. 1995). Using primer specific to the human GnRHR, we have detected a PCR product from choriocarcinoma JEG-3 cells; this was confirmed to be the GnRHR by Southern hybridization with a GnRHR cDNA probe (Yin et al. 1998). Similarly, human placental GnRHR cDNA has also been detected from human placental tissue by RT-PCR (Boyle et al. 1998) and by *in situ* RT-PCR (Wolfahnt et al. 1998). Taken together, these results strongly suggest the expression of GnRHRs in the human placenta.

1.4 Regulation of GnRHR Expression

1.4.1 Homologous Regulation by GnRH

It is well documented that GnRH can both up- and down regulate the numbers of its receptor in the pituitary (Loumaye and Catt 1982, McArdle et al. 1987). Similarly, exposure of αT3-1 cells to low concentrations of GnRH induces a 50% increase in receptor numbers (Tsutsumi et al. 1993), and prolonged exposure to continuous high concentrations of GnRH resulted in a decrease in GnRHR (Tsutsumi et al. 1995). It has also been shown that GnRH can regulate GnRHR mRNA levels in the pituitary cells of the rat (Kaiser et al. 1993, Bauer-Dantoin et al. 1995), sheep (Turzillo et al. 1995), and cow (Vizcarra et al. 1997). Furthermore, in the estrous cycle of both rat and sheep, the changes in GnRHR mRNA levels correlate with the changes in GnRHR numbers (Bauer-Dantoin et al. 1993, Turzillo et al. 1994); this supports the hypothesis that there is regulation of the GnRHR from its mRNA levels. However, studies using mouse gonadotrope-derived αT3-1 cell yielded inconsistent results. A dose- and time-dependent reduction in the levels of GnRHR mRNA by GnRH agonist has been observed (Mason et al. 1994, Kakar et al. 1997). In contrast, others reported no change in GnRHR mRNA after GnRH treatment (Tsutsumi et al. 1993 and 1995, Alarid and Mellon 1995).

1.4.2 Regulation by Gonadal Steroid Hormones

Ovarian steroid hormones have been shown to alter GnRHR expression in pituitary cells. An increase in GnRHR mRNA has been reported in castrated female and male rats, and treatment of female and male rats for several days with estradiol and testosterone, respectively, decreased GnRHR mRNA (Kaiser et al. 1993). In primary cultures of rat pituitary cells, estradiol can both increase (chronic exposure) and decrease (short-term exposure) GnRHRs (Menon et al. 1985, Emons et al. 1988). An induction of GnRHR mRNA levels *in vivo* by

estrogen at the time of the preovulatory gonadotropin surge has been reported recently (Bauer-Dantoin et al. 1995). Similarly, an increase of GnRHR mRNA in the rat pituitary gland *in vivo* was detected after 24 and 48h of estradiol treatment by Northern blot analysis and *in situ* hybridization, when compared to ovariectomized rats (Quinones-Jenab et al. 1996). In the ewe, treatment with exogenous estradiol consistently increased GnRHR numbers both *in vivo* (Gregg and Nett 1989, Kirkpatrick et al. 1998) and *in vitro* (Gregg et al. 1990, Laws et al. 1990). Further studies also demonstrated the increase in GnRHR mRNA level both *in vivo* (Hamernik et al. 1995, Cowley et al. 1998, Kirkpatrick et al. 1998) and *in vitro* (Turzillo et al. 1994, Wu et al. 1994) after estradiol treatment. Recent study has suggested a synergistic interaction between estradiol and GnRH in regulating the ewe GnRHR expression *in vivo* (Kirkpatrick et al. 1998), since a significant increase in both GnRHR numbers and mRNA levels in hypothalamic-pituitary disconnected ovariectomized ewes was only observed in the presence of both estradiol and GnRH.

It has also been showed that progesterone negatively regulated the hypothalamic-pituitary functions through a negative feedback mechanism in animals (Sagrillo et al. 1996, Schumacher et al. 1999) and in human (Schweiger et al. 1990, Poindexter et al. 1993, Alexandris et al. 1997). Recent studies in the ovine have demonstrated that the numbers of GnRHR were relatively lower during the luteal phase of the estrous cycle (Crowder and Nett 1984) and induction of luteolysis by prostaglandin F2α resulted in an increase in both GnRHR numbers and mRNA levels *in vivo* (Turizillo et al. 1994). Likewise, administration of progesterone led to a decrease in GnRHR mRNA levels in sheep pituitary *in vivo* (Bauer-Dantoin et al. 1995) and *in vitro* (Wu et al. 1994). The progesterone-induced inhibition on GnRHR mRNA level was further supported by Northern blot analysis using total RNA isolated from ovine pituitary glands collected at various stages of the ovarian cycle (Cowley et al. 1998). In this study, the basal pituitary GnRHR mRNA level was lower in the luteal phase compared

with the follicular phase. Using primary culture ovine pituitaries, a decrease in GnRHR mRNA levels was observed after progesterone-treatment (Sakurai et al. 1997, Wu et al. 1994, Kirkpatrick et al. 1998) further supporting the negative role of progesterone in regulating the GnRHR gene expression.

1.4.3 Regulation by Gonadotropins

Recent studies have demonstrated ovarian GnRHR mRNA expression is also modulated by gonadotropins. In cultured human granulosa-luteal cells, treatment with human chorionic gonadotropin (hCG) for 24 hours induced a dose-dependent inhibition of GnRHR mRNA levels (Peng et al. 1994). The inhibition of GnRHR gene expression by gonadotropin has also been demonstrated in cultured rat granulosa cells (Olofsson et al. 1995). Treatment of rat granulosa cells with luteinizing hormone resulted in dose- and time-dependent suppression of GnRHR mRNA levels. In pregnant mare serum gonadotropin (PMSG)-primed immature rats, injection of hCG caused a decrease in GnRHR mRNA levels in vivo (Olofsson et al. 1994). Since GnRH has been shown to inhibit LH-induced steroidogenesis (Leung and Steele 1992), the down regulation of GnRHR gene expression by LH/hCG suggests a regulatory feedback loop between LH and GnRHR in ovary. Similarly, a down-regulation of GnRHR gene expression by hCG/LH has also been observed in mouse hypothalamic neural cell-line (GT1-7) [Li et al. 1996]. These findings further support the hypothesis that the feedback loop is not only present in ovary but also in hypothalamus.

1.4.4 Regulation by Second Messenger Activators

GnRH-induced up-regulation of receptors is mediated by Ca^{2+} and activation of protein kinase C (PKC) (Naor et al. 1987, Huckle et al. 1988, Clayton 1989, Braden et al. 1991); However, the phorbol ester (TPA) that activates PKC had no effects on GnRHR mRNA levels

in αT3-1 cells (Alarid and Mellon 1995). On the other hand, activation of adenylyl cyclase by forskolin, which leads to an increase in intracellular cAMP levels and hence activation of protein kinase A (PKA), resulted in a decrease in GnRHR mRNA levels in αT3-1 cells (Alarid and Mellon 1995). In contrast, cAMP and forskolin have been demonstrated to increase the number of GnRHRs in cultured rat pituitary gonadotrope cells (Clayton 1989, Clayton et al. 1985). The mechanisms leading to the difference in response towards phorbol ester and forskolin in primary culture of pituitary cells and αT3-1 gonadotropes is still unclear. Numerous studies have shown that cAMP can enhance the responsiveness of the gonadotrope towards GnRH stimulation (Turgeon and, Waring 1986, Hawes et al. 1993, Abdilnour and Bourne 1995, Cassina et al. 1995). In addition, recent studies have also demonstrated the increase in GnRHR number (Clayton et al. 1985, Young et al. 1984, Clayton 1989) and gene expression (Lin and Conn 1998) by cAMP or forskolin.

1.5 Desensitization

Most G-protein coupled receptors undergo rapid desensitization on exposure to their agonists. This phenomenon was termed homologous desensitization, and this is one of the major regulatory mechanisms for the control of the membrane receptor number and function (Freeman and Lefkowitz 1996). Extensive studies with a number of GPCRs have revealed a general scheme for homologous desensitization. This involves rapid uncoupling of the receptor from the G-protein, subsequent sequestration of the receptor from the plasma membrane, and internalization followed by proteolytic degradation (Chuang et al. 1996, Freeman and Lefkowitz 1996). Studies of Gs-coupled receptors have revealed the importance of agonist-induced receptor phosphorylation in rapid homologous desensitization. The site(s) of receptor phosphorylation was identified primarily within the C-terminal tail (Rodriguez et al. 1992,

Lattion et al. 1994) and/or in regions of the third intracellular loop (Liggett et al. 1992). It is now clear that many Gq/11 (phosphoinositidase C; PLC) coupled receptors, like Gs-coupled receptors, also undergo rapid desensitization (Wojcikiewicz et al. 1993, Tobin 1998), and there are similar regulatory mechanisms (Wojcikiewicz et al. 1993, Tobin 1998). However, cloning of mammalian GnRHRs revealed that they lack any C-terminal tail, and have a comparatively short third intracellular loop; this is unique amongst known GPCRs, and raises the question of how could GnRHRs desensitize.

1.5.1 GnRHR desensitization

Homologous desensitization of GnRHRs in gonadotrope cells has been studied using primary pituitary cell cultures (McArdle et al. 1987, Weiss et al. 1995, Cassina et al. 1999) as well as by the gonadotrope αT3-1 cells (Davidson et al. 1994, Anderson et al. 1995, McArdle et al. 1995, Polin et al. 1998, Willars et al. 1998a). The functional desensitization of PLC-activating GPCRs is most often experimentally demonstrated as the failure to maintain the ligand-stimulated total [H^3]-inositol phosphate (IPx) accumulation in cells in which inositol monophosphatase has been blocked with Li^+ (Wojcikiewicz et al. 1993, Willars et al. 1998a). This desensitization is often reflected by a rapid accumulation of [H^3]IPx followed by either a decrease in, or absence of, further accumulation. However, none of these hallmarks were observed in the αT3-1 cell when GnRH stimulated the endogenous mouse GnRHR (Windle et al. 1990). During experiments with αT3-1 cells, the GnRH-induced [H^3]IPx accumulation was sustained for at least 90s (Davidson et al. 1994), and the accumulation of inositol 1,4,5-triphosphate (IP_3) was maintained for at least 5 min after GnRH-stimulation (Anderson et al. 1995, McArdle et al. 1996, Willars et al. 1998a). These data suggest that the GnRHR does not undergo rapid desensitization in αT3-1 cells. This has been further supported and extended by

similar experiments on these same cells, in which there was a rapid desensitization of phospholipase C-coupled muscarinic M3 receptors, but not of GnRHR; this suggests that the lack of acute desensitization is specific to the GnRHR (Willars et al. 1998). Similarly, there was no rapid desensitization observed in human or mouse GnRHR transfected GH3 cells (Davidson et al. 1994) further supports this idea.

Although short exposure of αT3-1 cells to GnRH did not reveal a clear cut rapid GnRHR desensitization, sustained GnRH pretreatment (60 min) of these cells resulted in a substantial loss of the GnRH-evoked IP response (Gautron et al. 1995, McArdle et al. 1995), a significant decrease in GnRHR number, and a reduction of both the GnRH-induced cytosolic Ca^{2+} spike and plateau phase (Anderson et al. 1995, McArdle et al. 1995). How these effects were mediated is still unclear. Studies have shown that prolonged stimulation of several PLC-coupled GPCRs results in a down-regulation of IP_3-receptors by a Ca^{2+}-induced proteolytic process (Wojcikiewicz and Nahorski 1991). Similarly, a time- and dose-dependent parallel loss of IP_3-receptors was observed in GnRH-treated αT3-1 cells (McArdle et al. 1997). In addition, an alteration in the efficiency of G-protein in activating its effectors could be another possible mechanism for the observed impairment of GnRH-induced PLC activation after GnRH-pretreatment. It has been shown recently in αT3-1 and GGH3 cells that prolonged exposure to GnRH selectively regulated the cellular levels of Gq/11 (Shah et al. 1995, Stanislaus et al. 1997). The effect was agonist specific and PKC activation did not mimic this action.

1.5.2 Receptor phosphorylation

Phosphorylation is the most rapid means of GPCR desensitization; it is achieved rapidly after agonist stimulation by two classes of serine/threonine protein kinases including: 1) cAMP-

dependent protein kinase A and/or protein kinase C; and 2) the G-protein coupled receptor kinases, GRKs (Premont et al. 1995).

1.5.2.1 Serine/threonine protein kinases phosphorylation in GnRHR

Recent studies by Cassina and co-workers (1999) have demonstrated the phosphorylation of a synthetic peptide, corresponding to either the first and third intracellular loop of the GnRHR, by PKC *in vitro*. Furthermore, the consensus PKA phosphorylation in the first loop was also being phosphorylated by PKA *in vitro*. In studies which have used primary rat pituitary cells as a model, pretreatment with TPA and cholera toxin for 3h inhibited GnRH-stimulated LH secretion, whereas treatment with either agent alone had no effect; this suggested the co-operative desensitization of GnRHR by both PKC and PKA pathways in LH secretion, presumably acting on the first and third intracellular loops. However, this type of receptor regulation is not specific; any stimulant that activates PKA or PKC has the potential to cause the phosphorylation at the appropriate PKC and/or PKA consensus phosphorylation sites and led to receptor desensitization.

1.5.2.2 GRKs phosphorylation in GnRHR

It is now accepted that another major cellular mechanism mediating rapid, agonist-specific, homologous desensitization of G protein coupled receptors consists of a two-step process, in which the agonist-occupied receptors are phosphorylated by a GRK. It permits association with an arrestin protein, thereby inhibiting G protein coupling (Lohse et al.1992, Pippig et al. 1993). It has been shown that GRK2, 3 and 6 were expressed in αT3-1 cells and in rat pituitary gonadotropes (Neill et al. 1996, 1998). Furthermore, the GnRHa-induced IP_3 accumulation was inhibited by the overexpression of GRK2, 3 and 6 in the rat pituitary cells

(Neill et al. 1998). In addition, co-transfecting β-arrestin together with GRK2 and rat GnRHR onto COS-1 resulted in a significant decrease in GnRHa-induced IP_3 production, when compared to the responses in GRK2 and GnRHR transfected cells (Neill et al. 1998). These studies demonstrate the potential role of GRK2 and β-arrestin in GnRHR desensitization. This has been further examined by the same group using adenovirus as a means of introducing GRK2-expression clone into primary cultures of rat pituitary gonadotropes (Neill et al. 1999). In this study, the adenoviral-infected gonadotropes showed a 5-fold increase in GRK2, and there was at least an 80% decrease in GnRH-stimulated LH secretion. Taken together, these results suggest that the homologous desensitization in GnRHR may be carried out through both second messenger dependent kinase and the GRK/arrestin system, as reported by others GPCRs (Fig. 1.4).

1.5.3 Role of C-terminal in GnRHR desensitization

The lack of rapid desensitization of the GnRHR may result from the lack of a C-terminal tail and the comparatively short third intracellular loop. To date, all mammalian forms of GnRHR have no C-terminal tail, whereas non-mammalian GnRHRs contain C-terminal tails. Transient transfection studies have demonstrated the importance of the C-terminal tail in rapid desensitization of the GnRHR. Recent studies in HEK-293 cells showed an acute desensitization of catfish GnRHR and rat GnRHR with a C-terminal donated from catfish GnRHR, whereas no such desensitization was observed in HEK-293 and GH3 cells transfected with wild type rat GnRHR (Heding et al. 1998, Lin et al. 1998). Similarly, a 12-fold more rapid internalization was observed in chicken GnRHR as compared to human GnRHR (Pawson et al. 1998). Truncation of the C-terminal tail of the chicken GnRHR resulted in a marked reduction in internalization, similar to that of the wild-type human receptor. *In vivo* phosphorylation studies,

performed by immunoprecipitation of normal and/or hepitope tagged receptors have revealed that in these cells, GnRH does not induce phosphorylation of the rat GnRHR, but rapidly increases the phosphorylation of the catfish GnRHR, and of the rat GnRHR chimeras with catfish GnRHR C-terminal tails (Willars et al. 1998b). Taken together, these data strongly suggest that mammalian GnRHR fails to undergo rapid desensitization because of missing the potential phosphorylation sites at the C-terminals.

1.6 Regulation of Gene Transcription

Gene expression is frequently controlled at the level of transcription through two distinct classes of DNA elements and cognate transcription factors, namely common core promoter and gene specific regulatory elements. Common core promoter elements, (located at or near the transcription start site), interact with RNA polymerase II (RNA pol II) and associated accessory factors, collectively known as general transcriptional machinery (GTM) that mediates basal transcription. Gene-specific regulatory elements, located distal from the core promoter, interact with gene-specific factors, either constitutive or induced, that modulate the functions of the GTM to control accurate transcription from the core promoter. Formation of a transcriptionally competent, sequence-specific, multiprotein complex not only ensures high fidelity of gene expression but also allows for multiple points of regulation.

1.6.1 Core Promoter Architecture

The transcriptional regulation of a functional gene is specified by the interplay of general transcription factors (GTFs; including at least six transcription factor, namely TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH), RNA pol II, DNA sequence elements at the core promoter DNA, which contains the TATA boxes and/or initiator (Inr) elements, and specific regulatory factors (Novina and Roy 1996). Formation of the transcription initiation complex (TIP) in the core promoter takes place in a stepwise fashion in vitro (Fig. 1.5). Binding of the TATA-binding protein (TBP) to the TATA box is the initial step of TIP formation and is critical for the rate and efficiency of this process. TBP is the only GTF making sequence-specific contacts with the DNA. It follows by a binding of a group of proteins known collectively as TBP-associated factors (TAFs) (Burley and Roeder 1996), together making up the complex known as transcription factor IID (TFIID) (Albright and Tjian 2000). The formation of TIP continues with

the recruitment of TFIIA to form TFIIA-TFIID complex. This complex undergoes a conformational change or isomerization to allow binding of TFIIB. Binding of TFIIB facilities association of the pre-assembled TFIIF-RNA pol II (non-phosphorylated form) with the growing complex. TFIIE then interacts directly with RNA pol II and subsequently recruits TFIIH to the C-terminal domain (CTD) of the RNA pol II (Albright and Tjian 2000). Although initiation of transcription is effectively complete at this stage, the CTD of the RNA pol II must be phosphorylated in order for transition from initiation to elongation to take place (O'Brien et al. 1994). Phosphorylation of the CTD allows the removal of TFIIB, TFIIE and TFIIH, which in turn permits the phosphorylated RNA pol II to commence elongation (Maxon et al. 1994).

FIG. 1.5 Pathway for assembly of the transcription initiation complex (TIP) at eukaryotic promoter. A core promoter containing a TATA box allows interaction with TATA-binding protein (TBP) at initial step of TIP formation. It follows by a binding of a group of proteins known collectively as TBP-associated factors (TAFs) and recruitment of TFIIA to form TFIIA-TFIID complex. This complex undergoes a conformational change or isomerization to allow further binding of TFIIB, TFIIF-RNA pol , TFIIE and TFIIH to the C-terminal domain (CTD) of the RNA pol II. Phosphorylation of the CTD allows the removal of TFIIB, TFIIE and TFIIH, which in turn permits the phosphorylated RNA pol II to commence elongation.

1.6. 2 Gene specific transcription regulation

The presence of a core promoter sequence and assembly of the TIP is all that required to initiate transcription of any given gene. However, the level of transcription achieved at such a promoter a minimal, and other transcription factors (either enhancer, silencer or repressor) are required to modify the transcription rate. Hundreds of specific activator and repressors have been identified. These protein binds to a particular DNA sequences. Characterization of these transcription factors revealed that nearly all of them use a helix to make specific contacts with base in the major groove and can be classified into several major families.

1.6.2.1 Helix-Turn-Helix (HTH)

One of the simplest DNA-binding motifs is the HTH. This is the first and by far the best characterized structural motif for DNA binding in prokaryotic activator and repressor including the *Escherichia coli* catabolite activator protein (CAP) and λ repressor (Schultz et al. 1991, Jordan and Pabo 1988). Comparison of HTH protein revealed a conserved recognition motif consists of an α-helix, a turn and a second α-helix (Nelson 1995). By co-crystal structure analysis, the HTH proteins bind as a dimer with each monomer recognizes a half-site with the second α-helix, also known as recognition helix, fits into the major groove of DNA.

1.6.2.2 Homeodomain

The homeodomain is a DNA-binding motif that is present in a large family of eukaryotic regulatory protein (Scott et al. 1989, Gehring et al. 1990). Structural studies confirmed that the homeodomain contains a HTH motif with three α-helices (Tan and Richmond 1998). Helix 1 and helix 2 pack against each other in an antiparallel arragement while helix 3 is roughly perpendicular to the first two helices. Again, the main contacts in the engrailed complex are made by residue in helix 3, which binds to the major groove, as in HTH protein.

1.6.2.3 Zinc Finger

Proteins in this family usually contain tandem repeats of the 25-30 residues zinc finger motif. Each zince finger motif contains two cysteines (C) residues, near the turn in the β-sheet region, and two histidines (H) residues, in the α-helix region, in a sequence pattern C-X_{2or4}-C-X_{12}-H-X_{3or5}-H (Krizek et al. 1991). The C_2-H_2 arrangement coordinates with a central zinc ion and hold these secondary structure together to form a compact globular domain (Pavletich and Pabo 1991). Studies in Zif268-DNA complex revealed that each zinc finger binds to the DNA major groove and wrap around the double helix (Pavletich and Pabo 1991)

1.6.2.4 Steroid receptor

The steroid receptors (SRs) are an important family of regulatory proteins include receptor for androgen, glucocorticoid, mineralocorticoid, progesterone, estrogen, thyroid, vitamin D, retinoic acid and orphan receptors. Molecular cloning of the SR cDNAs greatly advanced our understanding in regulating gene transcription by SRs. The SR contains three major domain including the trans-activation domain, the DNA binding domain (DBD) and the steroid binding domain (SBD) (Beato 1989). The DBD is the most conserved region among the steroid receptor family. It contains about 70 residues that folds into a single globular domain, and have nine conserved cysteine residues, of which eight interact in a coordinated fashion to form two separate tetrahedral zinc binding motif, providing the basis for the protein–DNA interaction (Vijay Kumar and Tindall 1998).

1.6.2.5 Leucine zipper and Helix-loop-helix

Transcription factor in this family employs a coiled-coil structure to facilitate the protein-DNA binding. Basically, two α-helices line up parallel and twist into a left-handed supercoil. The DNA binding domain of the leucine zipper proteins generally contain 60-80

residues with two distinct subdomains including the leucine zipper region, which mediates dimerization, and a amino-terminal basic region, which contacts the DNA (bZIP) (Landschulz et al. 1988). Leucine zipper region is characterized by a heptad repeat of leucines over a region of 30-40 residues (Landschulz et al. 1988). Biochemical studies suggested that the leucine zipper forms a coiled-coil arrangement and the basic amino-terminal domain bows out to straddle the DNA in the major groove (O'Shea et al. 1989).

The basic helix-loop-helix (bHLH) has an addition second coiled-coil at the carboxy-terminal in addition to the bZIP toward the amino-terminal (Anthony-Cahill et al. 1992). These two coiled-coil domains are separated by a Ω loop that allows the flipping of the two coiled-coil structures to form a tetramer (Vinson and Garcia 1992).

1.7. Transcriptional Regulation of GnRHR gene expression

GnRH response is controlled in part by regulating the expression of GnRHRs in pituitary gonadotropes. Since the isolation of the GnRHR, studies have shown that changes in GnRHR mRNA is one of the mechanisms which regulate the expression of the pituitary GnRHR (Bauer-Dantoin et al. 1993, Kaiser et al. 1993, Smith and Reinhart 1993, Mason et al. 1994, Turzillo et al. 1994, Alarid and Mellon 1995, Hamernik et al. 1995, Quinones-Jenab et al. 1996, Cowley et al. 1998, Kirkpatrick et al. 1998). The isolation of the GnRHR gene and its 5' flanking region provides a means to study the possible regulation of this gene at the transcriptional level.

1.7.1 Identification of Putative Promoter for Mouse GnRHR expression

Preliminary studies using the 1.2 kb 5' flanking region of the mouse GnRHR gene reveal high activity in mouse gonadotrope αT3-1 cells but lower activity in rat somatolactotrope (GH3) and non-pituitary cells including human choriocarcinoma JEG-3, monkey kidney fibroblast CV-

1, monkey kidney COS-7, human cervical cancer HeLa and C127 cells (Albarracin et al. 1994, Clay et al. 1995). Further studies have identified a 500 bp region, which mediated the basal and GnRH-stimulated expression of the mouse GnRHR gene (Clay et al. 1995, Duval et al. 1997, White et al. 1999, Norwitz et al. 1999). In agreement with the observation obtained from mouse promoter activity, use of the rat 5' flanking region results in a high activity in gonadotrope αT3-1 cells but not in somatolactotrope GH3B6, chinese hamster ovary CHO, and monkey kidney COS-7 cells (Reinhart et al. 1997, Pincas et al. 1998). Interestingly, as in αT3-1 cells, a high promoter activity was observed in the mouse GnRH neuronal GT1-7 cells, previously reported to express GnRHR (Krsmanovic et al. 1993), after transfected with the rat 5' flanking region. This cell-specific expression of the GnRHR gene indicates the presence of tissue- or cell-specific transcription regulator, which binds to the GnRHR promoter in controlling the expression of this gene. This notion is further confirmed by developing transgenic mice carrying a mouse GnRHR promoter fusion gene. A predominantly pituitary-specific expression of mouse GnRHR promoter-luciferase fusion gene was observed with a detectable luciferase activity in brain and pituitary (McCue et al. 1997). Similarly, target expression of mouse GnRHR promoter driven simian virus 40 (SV40) large T antigen (Tag) in transgenic mice resulted in formation of a pituitary tumor *in vivo*, in which the Tag mRNA was only detected in the pituitary cells but not in any other extra-pituitary tissues (Albarracin et al. 1999). These results indicated that the 5' flanking region of the mouse GnRHR contains regulatory sequences that direct gene expression specifically to the pituitary *in vivo*. Interestingly, this putative promoter region lacks the consensus TATA and CAAT sequences that are important for determining the accurate start site for transcription. Therefore, transcription of this gene may be regulated by alternative initiator mechanism(s); these could include GATA-like motifs, AT-rich elements, CpG islands, and transcriptional initiator elements in conjunction with Sp1 binding sites (Aird et al. 1994, Javahery et al. 1994).

1.7.2 Molecular Regulation of Mouse GnRHR Gene Expression

Progressive 5' deletions in the mouse GnRHR gene promoter revealed that the 500 bp upstream of the ATG site are enough to drive a high luciferase activity in the gonadotrope αT3-1 cell line, while one or more cis-acting elements located between -500 and -400 are necessary for transcriptional activity (Clay et al. 1995). Further deletion and mutational analysis identified a tripartite enhancer that appears to be responsible for regulating cell-specific basal expression of the mouse GnRHR gene (Duval et al. 1997). These elements include binding sites for SF-1, AP-1 and a novel element referred to as the GnRHR activating sequence (GRAS: 5'-CTAGTCACAACA-3'); the latter is located in -391 to -380; related to transcriptional start site (Fig. 1.6). These elements contributed equally to regulate the expression of the mouse GnRHR gene, since mutation of each element alone resulted in a loss of approximately 60% of the promoter activity. Double mutation of any two elements decreased 80% of the promoter activity, while no promoter activity was observed if all three elements were mutated. In addition, the GRAS has subsequently been shown to mediate activin responsiveness in the mouse GnRHR gene (Duval et al. 1999). In human, a putative gonadotropin-specific elements (GSE) have been identified to bind SF-1. Mutation of this putative SF-1 sequence (-134; related to ATG site) in the human GnRHR gene resulted in 80% decrease in promoter activity (Ngan et al. 1999). Furthermore, the reduction of the promoter activity was shown to be pituitary cell specific, since no such inhibition was observed in non-pituitary COS-7 and SKOV-3 cells. The SF-1 binding element was first identified in the human α-subunit gene and shown to be important for gonadotrope-specific expression of α- and LHβ-subunit genes (Horn et al. 1992, Barnhart and Mellon 1994, Halvorson et al. 1996). Taken together, these findings suggest the importance of SF-1 in controlling the basal expression of the GnRHR, as well as glycoprotein α- and LH-β subunit genes at the pituitary level. In rat, transfection studies have demonstrated that the 1.2 kb 5 ' flanking region of the rat GnRHR gene promoter directed the gonadotrope-specific

expression (Pincas et al. 1998). Further analysis identified a multiple regulatory domains that contributed to the cell-specific activity. Deletion of sequences residing from -1140 to -901 (related to ATG start site) resulted in a 43% attenuation of the promoter activity. In addition, further deletion of sequences containing the LH-2 (-869 to -862; related to ATG start site) and SF-1 (-696 to -688; related to ATG start site) motifs led to an additional 24% and 17% decrease in promoter activity, respectively. In contrast, deletion of a sequence located between -629 and -519 resulted in an approximately 2-fold increase in promoter activity (Pincas et al 1998).

1.7.3 Transcription regulation of mouse GnRHR gene by GnRH

Using αT3-1 cells, the regulation of the mouse GnRHR gene by GnRH was studied. A significant increase in luciferase activity was observed in mouse GnRHR promoter-luciferase construct transfected αT3-1 cells after treatment with 100nM of GnRH agonist (GnRHa) (White et al. 1999, Norwitz et al. 1999). Mutational studies using the 600 bp 5' flanking region, which has been shown to mediate basal expression of the mouse GnRHR gene (Duval et al. 1997), located a GnRH-responsiveness fragment, in which a putative AP-1 was identified (White et al. 1999). This GnRHa-induced increase in mouse GnRHR promoter activity was mimicked by phorbol ester treatment, but not by forskolin treatment. Furthermore, pretreatment with a specific protein kinase C inhibitor (GF109203X) blocked the GnRHa- and TPA-induced increase in luciferase activity, suggesting the involvement of the PKC in regulating the expression of the mouse GnRHR gene (White et al 1999). This idea was further supported by depleting the PKC with pretreatment of 10nM TPA for 20h. Other workers also obtained similar results. By the use of progressive 5' deletion mutants of the 1.2 kb mouse GnRHR promoter, a 68 base pair region located between -300 and -232 (related to the major transcriptional start site) was shown to be responsible for the GnRHa-induced stimulation (Norwitz et al. 1999). To examine whether this putative region is sufficient to mediate GnRHa-induced response, a PCR-

generated fragment of the region -387 to -220 was placed in control of the rat growth hormone promoter (GH-P) and transfected into αT3-1 cells. Transfection of wild-type GH-P showed no significant change in luciferase activity in responding to GnRHa treatment. However, a 3' deletion from -387/-220 to -387/-264 resulted in a significant increase in luciferase activity, suggesting the presence of a putative repressor element in this region (-264 to -220). Further deletion and mutational studies have identified two putative GnRH-responsiveness elements, known as SURG-1 (-292/-285) and SURG-2 (-274/-269) (Norwitz et al. 1999). Consensus sequence analysis revealed a putative AP-1 binding element at SURG-2. Furthermore, mutation of the putative AP-1 site resulted in complete elimination of GnRH-induced stimulation, suggesting the importance of this element in GnRH responsiveness in the mouse GnRHR gene. The putative AP-1 binding element identified in this study was the same as that reported elsewhere (White et al. 1999). Taken together, these results confirmed the participation of AP-1 sites in regulating GnRHa-mediated stimulation of GnRHR gene expression. By the use of gel mobility shift assay (GMSA), a specific DNA-protein binding was identified in these regions. Competition and supershift GMSA experiments revealed that a member(s) of the AP-1 (Fos/Jun) heterodimer is responsible for the binding (White et al. 1999, Norwitz et al. 1999). More interestingly, the GnRHa-induced increase in the mouse GnRHR promoter activity was shown not only to be PKC-dependent, but also to depend on mitogen-activated protein kinase (MAPK). The GnRHa- or TPA-induced increase in mouse GnRHR promoter activity was reversed in the presence of 60 μM specific MAPK inhibitor, PD98059 (White et al. 1999).

Similarly, GnRH and TPA treatment resulted in an increase in luciferase activity in mouse GnRHR promoter-luciferase transfected rat somatolactotrope GGH3 cells (Lin and Conn 1999). However, over-expression of MAPK kinase kinase (Raf-1) in GGH3 cells reversed GnRHa-stimulated luciferase activity. Furthermore, inhibition of MAPK activity by PD98059, or by over-expression of kinase-deficient MAPK, enhanced basal and GnRHa-stimulated

luciferase activity (Lin and Conn 1999). These data suggest a different dependence on MAPK pathway in regulating the rat and mouse GnRHR gene.

1.7.4 Transcription regulation of mouse GnRHR gene by cAMP

In the rat pituitary cells, GnRH induced an increase in cAMP levels (Borgeat et al. 1972, Naor et al. 1979, Bourne 1988). Moreover, it was demonstrated that cAMP, or its analogs, mimicked GnRH-induced increase in the GnRHR levels in cultured rat pituitary cells (Young et al. 1984, 1985); this suggests that cAMP may participate in regulating GnRHR gene expression. Recently, the development of a stable rat GnRHR transfected GGH3 cell line revealed the coupling of Gs to GnRHR, and an activation of GnRHR which lead to the production of cAMP and activation of protein kinase A (Kuphal et al. 1994, Kaiser et al. 1997, Lin et al. 1998). By use of these cells as a model, the transcriptional regulation of mouse GnRHR promoter activity was demonstrated to be cAMP dependent (Lin and Conn 1998, Maya-Nunze and Conn 1999). When GGH3 cells transfected with the mouse GnRHR promoter-luciferase construct, and then treated with GnRHa (100nM) for 6h, there was a significant increase in luciferase activity (Lin et al. 1998). This effect was well mimicked by cholera toxin, forskolin or cAMP analog administration (Lin and Conn 1998, Maya-Nunze and Conn 1999). Progressive 5' deletion has identified the CRE located at -107 to -100 (related to the ATG start site). Interestingly, deletion of the DNA fragment from -343 to -335 increased basal activity of the mouse GnRHR promoter. These findings suggest the presence of a CRE and a potential repressor that regulate the expression of the mouse GnRHR gene (Fig. 1.6).

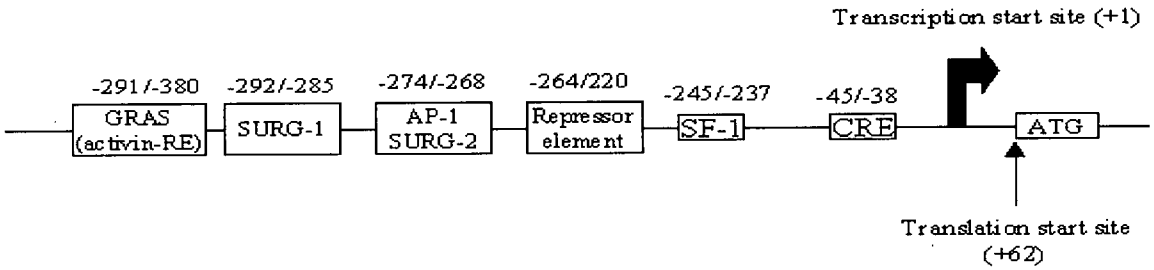


FIG. 1.6 Schematic representation of mouse GnRHR promoter structure. All DNA elements in controlling mouse GnRHR promoter activity were idenitifed experimentally (see text for details). AP-1: activating protein-1; activin-RE: activin-response element; ATG: methionine start codon; CRE: cAMP response element; GRAS: GnRH Receptor Activating Sequence; SF-1: steroidogenic factor-1; SURG-1 and –2: Sequence Underlying Responsiveness to GnRH-1 and –2.

1.8 GnRHR-coupled signal transduction pathway

GnRHR-stimulated gonadotropin release is dependent on intra- and extracellular derived calcium and the stimulation of protein kinase C (Stojilkovic and Catt 1995, Naor 1995). Phospholipase Cβ (PLC) couples to GnRHRs via a Gq/11α interaction (Hsieh and Martin 1992). Activation of PLC results in a hydrolysis of membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-triphosphate and diacylglycerol (DAG) (Fig. 1.7); these agents then mobilize Ca^{2+} from intracellular stores and activate most isoforms of protein kinase C, respectively (Liu 1996). Although it is now generally accepted that Gq/11α is the major G-protein coupled to GnRHR (Hsieh and Martin 1992, Stojilkovic and Catt 1995), evidence from other studies indicated that the actions of the GnRHR were mediated by multiple G-proteins (Andrews et al. 1986, Hawes et al. 1992). Several studies have shown that GnRH stimulates cAMP production in mixed pituitary cell cultures and stimulates the release of gonadotropins; this suggests a potential relationship of GnRH to the cAMP second message system (Borgeat et al. 1972, Noar et al. 1979, van der Merwe et al. 1990). This concept has been strengthened recently by the observation that the highest abundance of the Gsα protein in the pituitary gonadotropes (Wilson et al. 1994), and by the coupling of the cAMP signaling pathway in heterologous expression of rat GnRHR on the rat somatolactotrope GH3 (Kuphal et al. 1994) and baculovirus-insect (sf9) cells (Delahaye et al. 1997), or the mouse GnRHR in monkey kidney COS-7 cells (Arora et al. 1998) and chinese hamster ovary CHO cells (Nelson et al. 1999). In addition, administration of GnRH antagonist, antide, inhibited the GnRHa-induced accumulation of cAMP in CHO cells (Nelson et al. 1999) further suggesting the coupling of GnRHR to Gsα.

Recently, the coupling of GnRHR to Giα protein has also been demonstrated in several cancer cells (Imai et al. 1996, Limonta et al. 1999). In these studies, GnRHa treatment

significantly reduced the forskolin-induced increase of intracellular cAMP levels. In human female reproductive tract tumors, GnRHR is coupled to a 41-kDa Giα protein (Imai et al. 1996). Similarly, Limonta and co-workers (1999) have reported the coupling of the Giα protein-cAMP signal transduction pathway to the GnRHR in the human prostate cancer cells (Fig. 1.7). Desprite of these observations, the exact role of Gs and Gi subunit-mediated cAMP production in GnRH signal transduction remains unclear (Fig. 1.7).

Recently, Stanislaus and co-workers (1998a) have demonstrated a time- and dose-dependent palmitoylation of Gq/11α, Gsα, and Giα in rat gonadotrope and GGH3 cells by GnRH stimulation. This effect was shown to be receptor-mediated, since the GnRH antagonist, antide, did not stimulate palmitoylation of these G-proteins (Stanislaus et al. 1998a). Furthermore, the release of LH and the production of steroids in response to the GnRH agonist, Buserelin, were not completely abolished in Gqα and G11α knockout mice; this further suggests a coupling of multiple G-proteins to GnRHR *in vivo* (Stanislaus et al. 1998b).

1.9. Mitogen-activated protein kinase cascade

It is now cleared that the MAPK cascade can be activated not only by growth factor or cytokine receptors, but also by a variety of G protein-coupled receptors (Wan et al. 1996, Dello Rocca et al. 1997). Recently, several groups have demonstrated that GnRHR activation resulted in activation of the MAPK cascade, including ERK (Sim et al. 1995, Sunaresan et al. 1996, Reiss et al. 1997), c-jun N-terminal protein kinase (JNK) (Levi et al. 1998), and p38 MAP kinase (Roberson et al. 1999). The activation of the ERK pathway is also involved in regulating the expression of gonadotropin gene expression (Sundaresan et al. 1996, Haisenleder et al. 1998). In the case of GnRHR, activation of the MAPK cascade is demonstrated to be PKC-mediated, since this activation is mimicked by phorbol ester treatment. Similarly, depletion of

PKC and inhibition of PKC activity by phorbol ester and PKC inhibitor (GF109203X) pre-treatment, respectively, caused a reduction in MAPK stimulation induced by GnRH (Sim et al. 1995, Reiss et al. 1997, Sundaresan et al. 1996, Roberson et al. 1999) [Fig. 1.7].

In addition to the Gα-proteins, it appears that G-protein βγ-subunits is also able to stimulate MAPK via the activation of Ras (van Biesen et al. 1995, Coso et al. 1996). Several of the intermediate steps in the Gβγ-stimulated MAP kinase pathway are shown to be identical with the receptor tyrosine kinase (RTK)-stimulated signaling cascade, including Shc phosphorylation, Shc/Grb2 association, and Sos activation (van Biesen et al. 1995). Recent study has shown that the Gβγ-mediated MAPK activation is initiated by phosphatidylinositol 3-kinase (PI-3K) activity, followed by a pathway in common with the tyrosine-kinase receptor (Hawes et al. 1996).

It has been shown that cAMP can either attenuate (Wu et al. 1993, Steveson et al. 1993) or activate (Withers 1997, Erhardt et al. 1995) MAPK activity. More recently, analysis of the Gα/MAPK pathway in mutant S49 cells revealed a protein kinase A-dependent activation of MAPK (Wan and Huang 1998). Recent studies using GnRHR transfected GGH3 cells showed that activation of MAPK was achieved by the cAMP/PKA pathway activator in PKC-depleted cells (Han and Conn 1999). Furthermore, the addition of PTX did not affect the cAMP/PKA activator-induced stimulation of MAPK. Taken together, these findings not only support the participating of the PKA signaling pathway in GnRH action, but also demonstrated the involvement of the PKA signal transduction pathway in MAPK activation. Thus, the MAPK activation by GnRHR appears to be mediated by Gq/11, Gs, and Gi/o in different cell types (Fig. 1.7). However, the actual mechanism(s) by which the GnRHR activates the MAPK cascade and the role of intracellular loops in modulating the coupling to multiple MAPK cascades has yet to be determined.

1.10 Purpose of the study

Both gonadotropin-releasing hormone (GnRH) and its receptor (GnRHR) have been detected in pituitary as well as in placenta, outside the hypothalamic-pituitary system. As a first step to understand the molecular mechanisms underlying regulation of GnRH receptor gene expression, functional analysis of the putative promoter by deletion, mutagenesis and transient transfection were carried out to identify the *cis*-acting elements and *trans*-acting transcriptional regulatory factors necessary for regulation and controlling tissue-specific GnRHR gene expression.

The placenta is a transient but vital organ in mammalian reproductive process in maintaining pregnancy. It actively secretes and biosynthesizes various placental hormones and steroids. Although it has been showed in mouse pituitary that the expression of GnRHR gene was constantly regulated by those factors, the transcriptional regulation mechanism for human GnRHR has not yet been elucidated. In the present study, GnRHR gene expression in pituitary and/or placenta under the control of progesterone, second messenger activator and GnRH was investigated in molecular level, and the similarity and difference in the molecular mechanism regulating tissue-specific gene expression can then be elucidated.

1.11 Specific aims

The overall goal of this study is to identify and characterize the transcriptional regulatory factors, and elucidate the molecular mechanisms involved in the regulation of tissue-specific expression of human gonadotropin-releasing hormone receptor gene in pituitary gonadotrope and placental trophoblast cells by functional analysis of the 5'flanking region with methods of deletion, mutagenesis and transient transfection assay using mouse αT3-1 tumor cells and

immortalized extravillous trophoblasts (IEVT) and/or JEG-3 choriocarcinoma cells. The specific aims are listed below.

1. To isolate and characterize the placenta GnRHR full-length cDNA

2. To identify the promoter and transcriptional regulatory sites in the GnRHR gene 5'flanking region which are responsive to the tissue-specific gene expression of the GnRHR in placental trophoblasts.

3. To elucidate the transcriptional regulatory mechanism(s) of the GnRHR gene expression in gonadotrope and trophoblast cells by identifying the cis-acting element and trans-acting factor which mediates the response to progesterone and cAMP/PKA pathway.

4. To evaluate the similarity and difference in tissue-specific GnRHR gene expression regulation.

CHAPTER 2. MATERIALS AND METHODS

2.1 Materials

All chemical reagents were purchased from SIGMA Chemical Co. (Oakville, ON, Canada) or Gibco-BRL Life Technologies (Burlington, ON, Canada). Cell culture media DMEM, supplemented with 4.5 mg/ml glucose, RPMI 1640, trypsin/EDTA solution (0.05% Trypsin, 0.53mM EDTA), fetal bovine serum (FBS), 100 x Penicillin-Streptomycin-Glutamine solution and Gentamicin were purchased from Gibco (Burlington, ON, Canada). Restriction endonucleases and modified enzymes for recombinant DNA manipulation were purchased from Amersham-Pharmacia Biotech, Inc. (Morgan, ON, Canada), New England Biolabs (Mississauga, ON, Canada), or Gibco-BRL Life Technologies (Burlington, ON, Canada). First Strand cDNA Synthesis Kit and T7 DNA Sequencing Kit were purchased from Amersham Pharmacia Biotech, Inc. (Morgan, ON, Canada). Oligodeoxynucleotides corresponding to the putative transcription factor binding elements and their complements were synthesized by the Oligonucletide Synthesis Laboratory in University of British Columbia (Vancouver, BC, Canada). Consensus and mutated oligonucleotides for transcription factor binding sites and antibodies were purchased from Santa Cruz biotechnology Inc. (Santa Cruz, California, USA) or Promega (Nepean, ON, Canada). Monoclonal antibodies against PKC isoforms (Anti-PKC mAB Kit, Cat # S85080) were purchased from Transduction Laboratory Inc. (BD Biosciences, Mississauga, ON, Canada). Bradford Assay Kit was obtained from Bio-Rad (Mississauga, ON, Canada). Human progesterone receptor A (PRA) and progesterone receptor (PRB) expression vectors were kindly provided by Dr. P Chambon (Institut de Genetique et de Biologie Moleculaire et Cellulaire, Universite Louis Pasteur, France).

2.2 Cell culture

2.2.1 Cell lines

The mouse pituitary gonadotrope-derived αT3-1 cells, which expresses high levels of GnRHR (Chapter 1), were generously provided by Dr. PL Mellon, (Department of Reproductive Medicine, The University of California, San Diego, USA). Human embryonic kidney -293 cells (HEK293), African green monkey kidney cells (COS-1), human ovarian carcinoma OVCAR-3 cells, and human choriocarcinoma JEG-3 cells, which resemble characteristics of placenta trophoblasts, were obtained from American Type Culture Collection (Rockville, MD, USA). Human dermal fibroblasts (HDF) and immortalized human extravillous trophoblast cells (IEVT) were provided by Dr. N Auersperg (Department of Obstetrics and Gynaecology, University of British Columbia, Vancouver, Canada) and Dr. PK Lala (Department of Anatomy, University of Western Ontario, Ontario, Canada), respectively. The αT3-1, HEK-293, COS-1, OVCAR-3 and HDF cells were maintained in DMEM, with 4.5 mg/ml glucose with 10% FBS. The JEG-3 and IEVT cells were maintained in RPMI 1640 containing 10% FBS. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were passaged when they reached about 90% confluence using a trypsin/EDTA solution.

2.2.2 Primary Trophoblasts

Primary culture of cytotrophoblasts was isolated from term placental tissues as descried previously (Getsios et al. 1998). The use of these tissues was approved by the Committee for Ethical Review of Research involving Human Subjects at the University of British Columbia. Briefly, fresh placenta was separated from decidual layer and cut into small cube. It was then washed thoroughly in 0.9% w/v saline solution (5 x saline: 45 g NaCl/L water) to remove excess blood and dried briefly in paper towel. Carefully remove villous tissue from fibrous tissue and

blood vessels. The collected villous tissues were weighted and approximately 50 g of tissues were digested with 180 ml Trypsin/DNase solution (0.18 g trypsin and 0.02 g DNase in Ca^{2+} and Mg^{2+} free Hank Balance Salt Solution (HBSS); all reagents were obtained from Sigma) in 500 ml Erlenmeyer flask. The mixture was incubated at 37°C for 20 min. The supernatant was then collected and carefully layered on top of 1.5 ml 10% FBS (Hyclone, Utah, USA) in a 15 ml centrifuge tube. The Trypsin/DNase digestion was repeated for three to four times. Trophoblast cells in the supernatant were collected by centrifugation at 1000 x g for 10 min at 25°C. Cells were then washed in 1 ml DMEM, centrifuged and resuspended in 1 ml pre-warmed (37°C) DMEM. It was then layered on top of a Percoll gradient in a 50 ml centrifugation tube and centrifuged at 500 x g for 30 min at 25°C. The Percoll gradient contained 14 layers (3 ml each) of Percoll began with 70% Percoll (diluted in HBSS) with 5% different between each layer. Carefully removed the gradient from the centrifuge and collected the enriched trophoblast cells from the 45% to 55% layers of Percoll gradient. The mixture was mixed with equal volume of DMEM, centrifuged at 1000 x g for 10 min at 25°C. The isolated trophoblasts (2 x 10^6 cells) were plated in a T-25 culture flask (Falcon) with DMEM containing 10% FBS supplemented with 1 x penicillin-streptomycin-glutamine and 10 μg/ml gentamicin.

2.3 Total RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) amplification

Total cytoplasmic RNA was isolated by the guanidium thiocyanate (GTC)-phenol-chloroform method (Chomcznski and Sacchi 1987). In brief, cells were disrupted in GTC lysis buffer containing 4 M guandium thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5 % w/v N-lauroylsarcosine and 0.1 M β-mercaptoethanol. The lysates was purified by phenol-chloroform extraction. Total RNA was obtained by precipitation with an equal volume of isopropanol overnight at –20°C. The RNA was resuspended in ribonuclease-free diethylpyrocarbonate

(DEPC)-treated water. The concentration and purity of RNA were determined based on absorbance at 260 nm measured by a spectrophotometer (Model DU-64, Beckman). cDNA was synthesized from total RNA using the First Strand cDNA synthesis kit following the manufacturer's instruction. The reaction mixture (15μl), containing 5 μg RNA, 5 μl bulk first-strand reaction mix, 0.2 μg oligo-dT primer and 6 mM dithiothreitol (DTT), was incubated at 37°C for 60 min and terminated by heating at 90°C for 5 min.

To amplify the GnRHR cDNA, 5 μl reverse-transcribed cDNA was subjected to PCR amplification in a 50 μl reaction mix containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.001% w/v gelatin, 10 mM each of dATP, dCTP, dTTP, and dGTP, 2,5 units of Taq polymerase (Gibco); and 20 pmole of each sense (F) and antisence (R) appropriate primers:

GnRHR Full-length :	F1	5' AATATGGCAAACAGTGCCTCTCC3'
	R2	5' CAATCACAGAGAAAAATATCCA 3'
GnRHR fragment:	F3	5' GTATGCTGGAGAGTTACTCTGCA3'
	R4	5' GGATGATGAAGAGGCAGCTGAA3'
GAPDH:	GF	5' ATGTTCGTCATGGGTGTGAACCA 3'
	GR	5' TGGCAGGTTTTTCTAGACGGCAG 3'

Several controls were included to determine the accuracy of the PCR. First, PCR amplification was performed in both the absence of cDNA and reverse transcription reaction to examine the cross-contamination of samples. Second, the integrity of RNA samples was confirmed by gel electrophoresis, and PCR for GAPDH was run in parallel to rule out the possibility of RNA degradation and to justify the quality of the isolated RNA. Finally, since all primer pairs spanned at least one intron, the size of the predicted PCR products ruled out the presence of contaminating genomic DNA in the RNA sample. PCR amplification was carried

out for 35 cycles. For GnRHR, each cycle consisted of denaturation at 94°C for 60 s, primer annealing at 60°C for 65 s, extension at 72°C for 90 s. For GAPDH, PCR amplification was carried out for 25 cycles at 94°C for 60 s, 62°C for 60 s, and 72°C for 90 s. After amplification, PCR products were heated for 15 min at 72°C for completion of strand extension, and 10 μl of the PCR product was used for agarose gel electrophoresis.

For semi-quantitative PCR of GnRHR mRNA levels, amplification of placental GnRHR and GAPDH cDNA were carried out in 30 and 18 cycles, respectively. The PCR products were separated by agarose gel electrophoresis and transferred onto a nylon membrane (Amersham-Pharmacia Biotech, Morgan, ON, Canada) for Southern blotting analysis.

2.4 Southern blot analysis

The membrane was hybridized and detected following the manufacturer's recommended procedures with a digoxigenin (DIG)-dUTP-labeled GnRHR and GAPDH cDNA probe (Boehringer Mannheim, Laval, Canada). Hybridization was performed at 42°C overnight in the presence of 5 x SSC (75 mM sodium citrate, 0.75 M NaCl, pH 7.0), 0.1% w/v N-lauroylsarcosine, 0.02% w/v SDS, 1% w/v blocking reagent and 50% formamide. The membrane was then washed twice with 2 x SSC and 0.1% SDS at room temperature, and followed by twice with 0.1 x SSC and 0.1% SDS at 68°C. After high stringency washing, the membrane was rinsed briefly in washing buffer (maleic acid buffer plus 0.3% Tween-20), and incubated with 1% blocking reagent in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5) for 30 min. An anti-DIG conjugated alkaline phosphatase (diluted in 1:10000 in washing buffer) was added and incubated for additional 30 min. The membrane was then washed twice with washing buffer and equilibrated for 3 min in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5). Chemiluminiscent reaction was stared by the addition of CSPD (1:100), and exposed to Kodak Omat X-ray film. The radioautograms were scanned and

quantified with Scion Image-Released Beta 3b (Scion Corporation, Maryland, USA). The relative GnRHR mRNA level was calculated by normalizing the PCR products for GnRHR against the GAPDH expression.

2.5 Northern blot analysis

Polyadenylated RNA [Poly(A) mRNA] was separated from total RNA by two bindings to Oligo (dT) cellulose according to a procedure modified from Jacobson (1987). Briefly, the Oligo (dT)-celluose (type 3; Sigma Chemical Co.) was first swollen in DEPC-treated water. The fine cellulose was removed by decanting to prevent column from clogging. The saturated Oligo (dT) cellulose was then mixed with elution buffer and packed into a 3 ml syringe. The column was washed with 5 bed volumes of Oligo Binding buffer (0.01 M Tris-HCl pH 7.4, 0.5 M NaCl, 1 mM EDTA, and 0.5% SDS). Total RNA in DEPC-water was heated at 65°C for 10 min and then NaCl was added to give a final concentration of 0.5 M. The RNA sample was then applied onto the column. The flow-through was collected and reheated at 65°C for 3 min before reapply onto the Oligo (dT) column. The column was washed with 3 volumes of Oligo Washing buffer (0.01 M Tris-HCl pH 7.4, 0.1 M NaCl, 1 mM EDTA). The poly(A) mRNA was eluted by applying 4 ml of Oligo elution buffer (0.01 M Tris-HCl pH 7.4, 1 mM EDTA) and precipitated by adding 0.1 volume of 3 M sodium acetate, 2.5 volume of absolute ethanol.

Forty μg of total RNA or 20 to 50 μg of poly (A) mRNA were resolved by formaldehyde denaturing agarose gel electrophoresis and prepared for Northern blotting analysis for βhCG and GnRHR mRNA, respectively. The RNA was incubated at 65°C for 10 min with 12.5 μl formamide, 2.5 μl 10 x MOPS buffer (0.2 M 3-[-N-Morpholino] propane-sulphonic acid, 0.5 M sodium acetate pH 7.0, 0.01 M EDTA) and 4 μl 37% formaldehyde. The denatured RNA was resolved in 1% denatured agarose gel (1 x MOPS, 6.3% formaldehyde) with 1 x MOPS running buffer. The fractionated RNA sample was transferred onto a nylon membrane (Hybond,

Amersham-Pharmacia). The membranes were pre-hybridized and hybridized in standard hybridization solution, containing 50% formamide, 5x SSPE (0.09 M NaCl, 5 mM NaH_2PO_4, 5 mM EDTA pH 7.4), 5 x Denhardt's (0.5 g Ficoll Type 400, 0.5 g polyvinylpyrolidone and 0.5 g bovine serum albumin fraction V), 0.5% SDS, 100 μg/ml denatured herring sperm DNA and P^{32}-labeled probe at 42°C overnight followed by washing in high stringency condition (twice with 0.1x SSPE, 0.1% SDS at 65°C for 10 minutes). Subsequently, the hybridized blots were exposed to Kodak Omat X-ray film. Probe for GnRHR was prepared from the GnRHR PCR product generated from Section 2.3. Probe for βhCG was kindly provided by Dr. C.D. MacCalman (Department of Obstetrics and Gynaecology, The University of British Columbia, Vancouver, Canada) Radiolabeled βhCG and GnRHR probes were prepared with the Random Labeling Kit (Gibco BRL, Burlington, Canada) according to the manufacturer's suggested procedure.

2.6 Molecular Cloning and Sequencing

The PCR products were separated by gel electrophoresis. The DNA was excised and purified from the agarose by hot-phenol method with modification (Falson 1995). Briefly, the excised DNA in agarose gel was re-melted by heating at boiling water for 5 min by adding 1 x TBE containing 0.5 M NaCl. After melting, the mixture was further incubate at 65°C for 10 min. Then, an equal volume of preheated (65°C) phenol was added to the melted gel and mixed. The aqueous phase was separated by centrifugation (14000 rpm for 10 min at 4°C), re-extracted with phenol/chloroform followed by chloroform/isoamyl alcohol, and precipitated with ethanol. The purified PCR products was ligated into PCR II vector (Invitrogen, Carlsbad, CA, USA) at 14°C overnight and transformed into their host bacteria.

Positive clone was checked by restriction enzyme digestion. DNA sequence was determined by dideoxy nucleotide chain termination method using a T7 DNA Polymerase

Sequencing Kit (Pharmacia Biotech, Morgan, Canada). Briefly, 10 pmole of sequencing primer was annealing to 2 μg of denatured plasmid in annealing buffer (0.15 M Tris-HCl pH 7.6, 15 mM $MgCl_2$ and 23 mM DTT) at the final volume of 14 μl. Then 6 μl of 1 x labeling mixture (50 mM NaCl, 32 mM Tris-HCl pH 7.5, 8 mM DTT, 160 μg BSA/ml, 8% glycerol, 0.2 μM each dCTP, dTTP and dGTP, 3.2 units T7 DNA polymerase, 1 μCi α-S^{35}-dATP) was added and incubated for 5 min at room temperature. The reaction was terminated by transferring 4.5 μl of the reaction mixture into 2.5 μl of each dideoxynucleotide terminal reagent (ddGTP, ddATP, ddTTP, and ddATP) at 37°C for 5 min. The sequencing product was resolved by 6% polyacrylamide gel electrophoresis containing 4 M Urea for 8 hours at a constant power of 60 W. The gel was then dried and exposed to X-ray film.

2.7 Preparation of human GnRHR promoter-Luciferase constructs

A 2.7 kb human GnRHR exon 1 fragment subcloned in pBSK II (+) was used as template for PCR amplification using sense (5'GGCCTGCTCTGTTTTAGCACTCTG3') and antisence (5'CTGAAGCTTCCCAGGACAGAGCTTCAAGCCT3'; a *Hind*III recognition site (underlined) was introduced to facilitate cloning procedure) primers to generate a 2297 base pair (bp) human GnRHR 5' flanking region. The PCR product was digested with *Hind*III and ligated into pGL2-Basic vector and named as p2300-LucF. Progressive 5' deletion constructs were prepared using various restriction endonucleases or exonuclease III/SI nuclease digestion (Pharmacia LKB Biotechnology, Piscataway, NJ). The 5' deleted human GnRHR promoter clones p2200Luc, Nde-HLuc, Sac-HLuc, Pst-HLuc, Spe-HLuc, Sty-HLuc and p167-Luc were prepared by digesting the p2300-LucF with *Bgl*I, *Nde*I, *Sac*I, *Pst*I, *Spe*I, *Sty*I and *Hinc* II. Deletion clone p577-Luc, p227-Luc was prepared by exonuclease III/SI nuclease digestion. The 3' deletion human GnRHR promoter clones including p2200/-167Luc, p2200/-421Luc, p2200/-577Luc were obtained by exonuclease III/S1 nucelase digestion. Deletion clones including

p2200/-771Luc, p2200/-1018Luc, p2200/-1346 Luc and p2200/-1671Luc were prepared by digesting p2200Luc with *Spe*I, *Pst*I, *Sac*I and *Nde*I. Deletion mutants p2300/-1346Luc, p2300/-1674Luc, p1671/-1346Luc, p1737/-1346Luc, p2092/-1346Luc were prepared by digesting p2300-LucF with combination of *Hind*III, *Sph*I, *Sin*I, *Nde*I, or *Sac*I. The positive clones were identified by restriction mapping. DNA sequence was determined using a T7 DNA sequencing Kit as described in section 2.6.

2.8 Transient transfections

Transfections were carried out using the calcium phosphate precipitation methodology as previously described (Chen and Okayama 1988). Briefly, 5 x 10^5 of αT3-1 cells, 2.5 x 10^5 of HEK293, COS-1, OVCAR-3 and HDF cells, or 1.5 x 10^5 JEG-3 and IEVT cells were seeded into six-well tissue culture plates before the day of transfection. Two micrograms of the GnRHR promoter-luciferase construct and 0.5 μg RSV-*lac*Z were dissolved in 50 μl 0.1 x TE containing 0.25 M $CaCl_2$ and mixed with 50 μl 2 x BES (50 mM N,N-bis-(2-hydroxyethyl)-2-aminoethanesulforic acid, 280 mM NaCl, and 1.5 mM Na_2HPO_4, pH 6.95). The DNA mixture was incubated for 20 min at room temperature and then applied to the cells. Incubation of the cells with transfection medium was continued for approximately 16h at 37^0C in 3% CO_2. After transfection, the cells were washed twice with culture medium and incubated with normal culture medium containing 10% FBS.

2.9 Luciferase Reporter Assays

Cellular lysates were isolated and assayed for luciferase activity immediately with Enhanced Luciferase Assay Kit (BD PharMingen, Mississauga, Canada) at room temperature. The transfected cells were rinsed twice with phosphate buffered saline (PBS; 137 mM NaCl, 2.7

mM KCl, 8.1 mM Na_2HPO_4, 1.47 M KH_2PO_4) and incubated for 15 min after addition of 200 µl of cell lysis buffer. Cells were then collected by centrifugation at 14000 rpm for 10 s at 4°C. Fifty µl of cell lysates was mixed with 100 µl each of Substrate A and Substrate B, and luminescence was immediately measured by Lumat LB9507 luminometer (EG&G Berthold, Bad Wildbad, Germany). Beta-galactosidase (β-Gal) activity was also measured and used to normalize for varying transfection efficiencies. Chlorophenol red-β-D-galactopyranoside monosodium salt (CPRG) was used as a substrate for the colorimetric determination of β-Gal activity in cell extracts. Briefly, 15 µl of cellular lysates was incubated at 37°C for 10 min or until color change in the presence of 0.75 µl 100 x Mg solution (0.1 M $MgCl_2$, 4.5 M β-mercaptoethonal), 51 µl 0.1 M assay buffer (36 mM NaH_2PO_4, 0.164 M Na_2HPO_4), and 16 µl CPRG (8 mg/ml in 0.1 M assay buffer). After the color development, the reaction was stopped by adding 100 µl 0.1 M $CaCl_2$ and the measured by spectrophotometer at 573 nm. Luciferase units were calculated as luciferase activity/ β-galactosidase.

2.10 Primer Extension

The transcription initiation site was identified by primer extension studies with oligonucleotides PE-A, PE-B and PE-C (Table 2.1) as described previously (Triezenberg 1999). Briefly, each primer was end-radiolabeled with [P^{32}]-ATP by T4 polynucleotide kinase (Gibco) and hybridized with 25 µg of poly(A+) RNA for 90 min at 65°C in final volume of 15 µl containing 1 x hybridization buffer (0.15 M KCl, 10 mM Tris-HCl pH 8.3, 1 mM EDTA) and radiolabeled primer (50,000 cpm). After incubation, the mixture was cooled to 42°C and 30 µl of primer extension reaction mix (30 mM Tris-HCl pH 8.3, 15 mM $MgCl_2$, 8 mM DTT, 6.75 µg actinomycin D, 0.25 mM dNTP, and 20U SuperScript RNase H- Reverse Transcriptase (Gibco)) was added. The mixture was then incubated for 1 h at 42°C, then 105 µl of RNase mix,

containing 20 μg/ml RNase A, 100 μg/ml salmon sperm DNA, 100 mM NaCl, 10 mM Tris-HCl pH 7.5 and 1 mM EDTA, was added and incubated for 15 min at 37°C. The extended products were purified by standard phenol/chloroform extraction and subsequently analyzed on a 6% polyacrylamide/7.0 M urea gel.

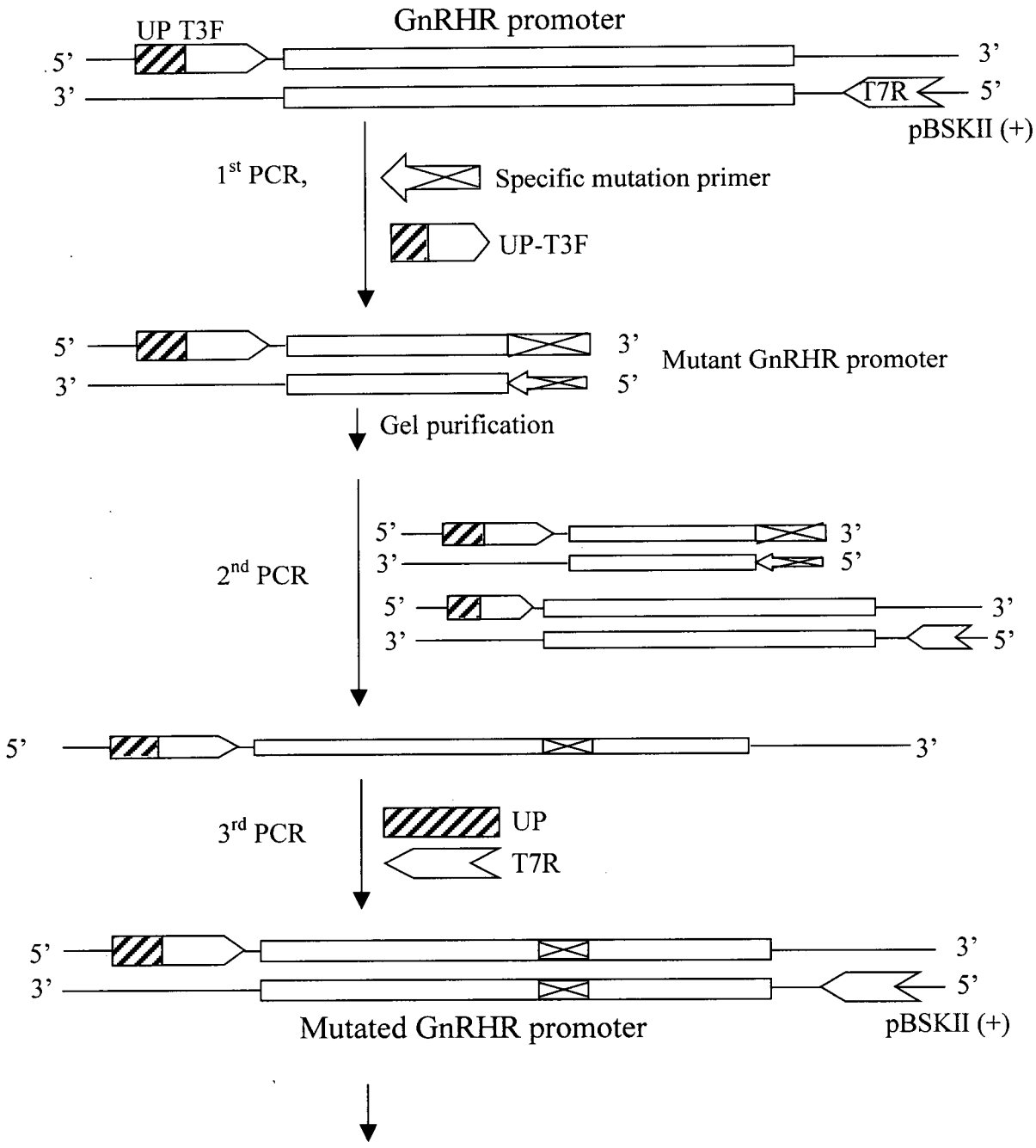
TABLE 2.1 Oligonucleotides used for PCR mutagenesis and primer extension studies. Mutation nucleotides are underlined.

Oligo name	Sequence (5' to 3')	Purpose
PE-A	TCAGCTTCTCTGTGTACTGGCT	Primer extension
PE-B	TTTATTAATCAATCTTACTGAT	Primer extension
PE-C	AAGTTCTGACTCATCTAGGAG	Primer extension
UP-T3F	GTGCCTCTCCTGAACAGCCTCAAGCAATTAACCCTCACTAAAGG	Site-directed mutagenesis
UP	GTGCCTCTCCTGAACAGAATCAA	Site-directed mutagenesis
T7R	CGTAATACGACTCACTATAGG	Site-directed mutagenesis
mP-Oct-1	GGATAACCATTGGAAACGCGGCCGCATATGAAAAGTGTTTGTAAGT	Site-directed mutagenesis
mP-CRE	AGGCTTTGCATATACGCGGCCGCAGTTAATCTGATACGGGC	Site-directed mutagenesis
mP-GATA	GCAATCAGTCTTGAAGCGGCCGCTTTATTTTTCTGAAGAACTA	Site-directed mutagenesis
mP-AP-1	CTACATCAAAACTAAGTTCTGCGGCCGCTAGGAGGAGAATTACAAA AAAAAATTGTGCC	Site-directed mutagenesis
mP-PRE	GTTTTCCTTTTCAAAGCGGCCGCTGAGCACTCGAACACTGGAC	Site-directed mutagenesis
mP-hPRE	AAACTATTAGTGTTAGTCGGCCGTCCAACATACAGATGTA	Site-directed mutagenesis
mP-CRE-1	AACACTGGACAAATATGCGGCCGCAAAATTATTCTACAAC	Site-directed mutagenesis
mP-CRE-2	GAATTTAAAATGCGGCCGCTGATATTATGATTTAAATAAC	Site-directed mutagenesis

2.11 Site-directed mutagenesis

Mutations were introduced by a three-step PCR mutagenesis method as described previously (Chow et al. 1991), using mutagenic primers (a *Not*I restriction enzyme site was introduced to replace the putative transcription factor binding element; underlined at the Table 2.1) and universal primers UP-T3F, UP, and T7R (Table 2.1). The DNA fragment containing sequence for site-directed mutation was first subcloned into pBSK II (+) vector and served as a template for the mutagenesis reaction. Approximately 200ng of purified plasmid was denatured and subjected for the 1st round PCR amplification with universal forward UP-T3F and specific reverse mutational primer (Fig. 2.1). The reaction mixture, containing 50 pmole primers each, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatine, 10 mM dNTP and 2.5 units of Taq polymerase (Gibco) in a volume of 50 μl, was first amplified for 10 cycles of 1 min of denaturation at 95°C, 1 min of annealing at 60°C and 1 min 30 s of extension at 72°C. Then the PCR amplification was carried out in 30 cycles of 1 min denaturation at 94°C and 3 min of annealing and extension at 72°C. A final incubation at 72°C for 15 min was allowed for completed strand extension. The amplified PCR product was visualized by gel electrophoresis and purified as described at above (section 2.6). Second PCR amplification was carried out using the purified 1st round PCR product as primer and the plasmid as template with an initial template denaturation at 94°C for 5 min, following by 10 amplification cycles of 2 min of denaturation at 94°C and 3 min of annealing and extension at 72°C. Then 10 μl of the 2nd PCR reaction mixture was used as the template for the 3rd round of PCR amplification with the universal UP and T7R primers. The PCR amplification was carried out for 40 cycles with 1 min denaturation at 94°C, 1 min annealing at 60°C, and 1 min 30 s extension at 72°C. A final extension at 72°C for 15 min was followed. The expected PCR product was gel purified, subjected to restriction endonucleases digestion and ligated to pGL2-Basic vector (Promega). Mutation was confirmed by restriction enzyme mapping and sequence analysis after the mutagenesis reaction.

FIG. 2.1 Diagrammatic representation of 3 steps PCR mutagenesis. Human GnRHR promoter fragment subcloned into pBSK II (+) vector is used as template for 1st round PCR amplification with specific mutation primer and UP-T3F. The PCR products are purified and subjects to 2nd round PCR amplification. Then, 10 μl of the PCR reaction mix was used for the final PCR amplification with primers UP and T7R. The final PCR products, containing a site-directed mutation, are then digested with restriction enzyme, purified and subcloned into luciferase reporter pGL2-Basic vector.



Restriction enzyme digestion,
Gel Purification,
Cloning into pGL2-Basic

2.12 Preparation of nuclear extracts

Nuclear extracts were prepared from αT3-1, IEVT, and JEG-3 cells according to the methods described previously (Lassar et al. 1991). Briefly, cells at 70% confluency were lysed in 2ml of Lysis Buffer, containing 20mM HEPES pH 7.6, 20% Glycerol, 10mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.1% Triton X-100, 1mM DTT, 1mM PMSF, 10μg/ml leupeptin, 10μg/ml pepstatin, and 100μg/ml aproteinin. Cells were dislodged by scrapping and collected by centrifugation for 5 min at 2000 rpm at 4°C. Nuclei were resuspended at 2.5 x 10^7 nuclear per ml in Nuclear Extraction Buffer (Lysis Buffer plus 500mM NaCl). Nuclei were gently rocked for 1h at 4°C and centrifuged at 10,000 rpm for 10 min, and supernatant was aliquoted, quick frozen in liquid nitrogen, and stored at -80°C.

2.13 Gel Mobility Shift Assay (GMSA)

Oligodeoxynucleotides, corresponding to the putative transcription factor binding site in the human GnRHR 5' flanking region (Table 2.2), for GMSA were end-radiolabeled with T4 polynucleotide kinase (Gibco BRL) in 50 μl reaction mixture containing 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 5 μCi [P^{32}]-ATP, and 0.1 mM spermidine for 1 hour at 37°C. The unincorporated radionucleotides was removed by passage over Sephadex G-50 or G-25 column. GMSA was carried out in 20 μl containing 20 mM HEPES (pH 7.5), 20 mM KCl, 20 mM NaCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 10% glycerol, 2μg poly dI:dC, 5-10μg nuclear proteins, 2 mg/ml of BSA and 20,000 cpm radiolabeled probe.

For the competition assays, the unlabeled DNA (0.1 mM or 200 fold excess) was added simultaneously with the labeled probe. Antibodies used in supershift experiments (Table 2.3) were purchased from Santa Cruz biotechnology Inc. (Santa Cruz, CA) and added to the nuclear extract at room temperature for 1h prior to the addition of labeled probe. The binding mixture

was incubated at room temperature for 20 min and separated in 4 to 8% polyacrylamide gel containing 1 x TBE (Tris-borate-EDTA: 0.09 M Tris-borate and 2 mM EDTA, pH 8.0). Prior to loading of samples, the gel was pre-run for 90 min at 100 V at 4°C. Electrophoresis was carried out at 30 mA at 4°C. The gel was then dried under vacuum and exposed to X-ray film (Kodak X-OMAT AR film; Eastman Kodak) at -70°C for 24h.

TABLE 2.2 Oligonucleotides used for gel mobility shift assay. The sequence of the putative transcription factor binding element was underlined.

Oligo name	Sequence (5' to 3')	Purpose
hGR-AP-1	TCCTAGA*TGAGTCA*GAACTTAG	Probe
mutated hGR-AP-1	TCCTAGA*CGGCCGA*GAACTTAG	Competitor
hGR-CRE	CAGATTAACTGACATGATGTATATGC	Probe
mutated hGR-CRE	CAGATTAAC*CGGCCGGA*TGTATATGC	Competitor
hGR-GATA	AATAAATTATCTTATTCAAG	Probe
mutated hGR-GATA	AATAAA*CGGCCG*TATTCAAG	Competitor
hGR-Oct-1	TTTCATATTTGTATGTGTTTC	Probe
mutated hGR-Oct-1	TTTCAT*ATTCGGCCG*TGTTTC	Competitor
hGR-PRE	GAGTGCTCAACAGTGTGTTTGAAAAGG	Probe
mutated hGR-PRE	GAGTGC*TCAGCGGCCGCTTTG*AAAAGG	Competitor
hGR-AP/CRE-1	AATAATTTTAAGTGAATATATT	Probe
hGR-AP/CRE-2	AATATCATGACTGACATTTTAA	Probe
consensus AP-1	CGCTTGATGACTCAGCCGGAA	Competitor
consensus CRE	AGAGATTGCCTGACGTCAGAGAGCTAG	Competitor
consensus GATA	CACTTGATAACAGAAAGTGATAACTCT	Competitor
consensus Oct-1	TGTCGAATGCAAATCACTAGAA	Competitor
consensus PRE	GATCCTGTACAGGATGTTCTAGCTACA	Competitor
consensus NF-kB	AGTTGAGGGGACTTTCCCAGGC	Competitor
consensus TFIID	GCAGAGCATATAAGGTGAGGTAGGA	Competitor

TABLE 2.3 Antibodies used in the present study

Antibodies	Source	Purpose (concentration)
c-Jun	Santa Cruz Biotech, Inc.	Supershift (2 μg), Western blot (1:2000)
c-Fos	Santa Cruz Biotech, Inc	Supershift (2 μg), Western blot (1:4000)
CREB	Santa Cruz Biotech, Inc	Supershift (2 μg), Western blot (1:3000)
GATA-2	Santa Cruz Biotech, Inc	Supershift (2 μg), Western blot (1:1000)
GATA-3	Santa Cruz Biotech, Inc	Supershift (2 μg), Western blot (1:1000)
Oct-1	Santa Cruz Biotech, Inc	Supershift (2 μg), Western blot (1:2000)
ER	Santa Cruz Biotech, Inc	Supershift (2 μg)
PR	Santa Cruz Biotech, Inc	Supershift (2 μg), Western blot (1:2000)
IgG	Amersham-Pharmacia	Supershift (2 μg)

2.14 Western blot analysis

For Western blot analysis, 1 x 10^6 cells were incubated in 100μl of cell lysis RIPA buffer (containing 1 x PBS (pH 7.4), 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/ml PMSF, 30 μg/ml aprotinin and 10 μg/ml leupeptin) for 15 min on ice. The cellular debris was removed by centrifugation at 14000 rpm for 15min at 4°C. Protein concentration in the cell lysates was determined using a modified Bradford assay Kit. Aliquots (35 μg) were taken from the total cell lysates and subjected to SDS-polyacrylamide gel electrophoresis under reducing conditions. The separated proteins were then electrophoretically transferred onto nitrocellular paper (Hybond-C, Amersham-Pharmacia Biotech, Morgan, Canada). The membranes were blocked with 5% (w/v) non-fat milk in Tris-buffered saline (TBS), containing 20mM Tris-Cl (pH 8.0), 140 mM NaCl and 0.05% (w/v) Tween 20, for at least 1h before the addition of antibodies (Table 2.3) in a final concentration of 0.05 to 0.2 μg/ml. All antibody incubation and washing were performed in Tris buffer saline with 0.05% Tween 20. The Amersham enhanced chemiluminescence system (ECL) was used for detection. Membranes were visualized by exposure to Kodak X-Omat film. The radioautograms were then scanned and quantified with Scion Image-Released Beta 3b software (Scion Corporation, Maryland, USA).

2.15 Progesterone Radioimmunoassay (RIA)

JEG-3 cells were seeded in a density of 1.5 x 10^5 in 35mm dishes one day before treatment. The cells were then treated with corresponding agent for 24h in triplicate for 24 h. The media was removed and stored at -20°C before assay. The cells were lysed with 80 μl of RIPA buffer and the cellular lysates were incubated on ice for 15 min prior to centrifugation. The supernatant was collected and assayed for total protein content using modified Bradford Assay Kit (Bio-Rad) to standardize for cell number. Concentration of progesterone in media was determined by RIA with specific antisera provide by Dr. D.T. Armstrong, University of Western Ontario) as described previously (Li et al. 1993). Briefly, the samples were incubated with 0.5mg/ml rabbit anti-progesterone antisera and 20000 cpm of tracer ([1,2,6,7,17-3H] progesterone; Amersham). A standard was set up with 10 reference concentrations ranging from 0.19-100 ng/ml. All assay contents were diluted in a final assay volume of 300 μl in 0.1 M gelatin phosphate buffered saline (PBSG; 80 g/L NaCl, 2 g/L KCl, 11.5 g/L Na_2HPO_4, 2 g/L NaH_2PO_4 and 0.1% gelatin). After 16h of incubation at 4°C, 500 μl of the cold charcoal:dextan solution (each 0.25% w/v in PBSG) was added to separate bound and unbound antigen. After centrifugation at 4500 rpm for 30 min, 500 μl of the supernatant was removed and diluted with 3 ml of scintillation cocktail (BDH) and counted in a Wallac 1217 Rack beta-counter (LKB Instrument).

2.16 Data analysis

For transfection assay and RIA, data were shown as the means +/- SD of triplicate assays in at least three independent experiments. For Western blot analysis, data are obtained from three independent experiments. All data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test or *t*-test using the computer software PRISM GraphPad Version 2 (GraphPad Software, Inc., San Diego, USA). Data were considered significantly different from each other when $P < 0.05$.

CHAPTER 3. MOLECULAR CLONING OF PLACENTA GnRHR

3.1 Introduction

There is increasing evidence for an extrapituitary function of gonadotropin-releasing hormone (GnRH) in the placenta. Previous studies have reported the presence of GnRH-like material in the placenta that is biochemically and structurally identical to the hypothalamic GnRH (Khodr and Siler-Khodr 1980, Tan and Rousseau 1982). The cDNA for the GnRH precursor in placenta has been isolated (Seeburg and Adelman 1984), and the major site of placental GnRH production was identified to be the cytotrophoblasts (Miyake et al 1982). It is hypothesized that placental GnRH might be involved in the autocrine/paracrine regulation of the biosynthesis of human chorionic gonadotropin (hCG), since GnRH stimulated hCG biosynthesis and secretion from human placental explants (Siler-Khodr et al. 1986, Barnea and Kaplan 1989, Merz et al. 1991). In addition, the secretion of hCG from placental cells was inhibited by treatment with a GnRH antagonist (Siler-Khodr et al. 1983, 1987). This hypothesis is further supported by the observation that the highest GnRH concentrations in the placenta are present in the first trimester of pregnancy, coinciding with the temporal distribution of hCG synthesis (Siler-Khodr et al. 1984).

Using solution hybridization protection assay and *in situ* hybridization assay, the level of GnRH mRNA was found to remain constant throughout gestation (Kelly et al. 1991). In contrast, other studies have demonstrated dynamic changes in human GnRH receptor number and mRNA levels in the placental trophoblast cells at various gestation ages that were functionally correlated to hCG secretion from placental cells (Lin et al. 1995, Bramley et al. 1994). These findings implicate an important role for the GnRHR in regulating hCG secretion during pregnancy.

The human placenta contains specific binding sites for GnRH, which interacts with both GnRH agonist and antagonist analogues (Belisle et al. 1987, Iwashita et al. 1986, Bramley et al. 1992). However, the GnRHR in the placenta has a 10 to 100 fold lower affinity than that of pituitary GnRHR. Although recent studies have suggested that the GnRHR mRNA is expressed in human placental cells (See Chapter 1, Section 1.3.3), the primary structure of the placenta GnRHR remains unknown. To elucidate the primary structure of the placental GnRHR, the full-length cDNA encoding human GnRHR from human placental cells including JEG-3, IEVT and primary trophoblasts culture was characterized.

3.2 Treatments

Pharmacological reagents, including GnRH agonist, D-(Ala^6)-GnRH (GnRHa), and GnRH antagonist (antide), forskolin and the phorbol ester, phorbol 12-myristate 13 acetate (TPA), were purchased from Sigma (Sigma-Aldrich Ltd., Canada). Protein kinase C inhibitor, bisindolymaleimide I (GF109203), adenylate cyclase inhibitor (SQ22536) and protein kinase A inhibitor (PKI 14-22, cell-permeable, Myristoylated) were obtained from Calbiochem (La Jolla, CA). In experiments herein, the effects of the GnRH analogues, forskolin and TPA on GnRHR expression were studied. The cells were treated with the corresponding drugs for 24 h prior to RNA isolation. To inhibit the PKC and PKA activity, cells were pre-treated with corresponding inhibitors for 30 min before stimulations.

3.3.Results

3.3.1 Cloning of human GnRHR full length cDNA from placental cells

Using full-length GnRHR specific primers (F1 and R2), an expected 1003 base pair (bp) PCR product was obtained from JEG-3, IEVT and primary trophoblast cells (Fig 3.1). Human breast carcinoma MCF-7, previously shown to express GnRHR mRNA (Kakar et al 1994), was

used as a positive control. The amplified fragment was identified as GnRHR cDNA by Southern blot hybridization (Fig. 3.1). In addition, the possibility of cross-contamination can be ruled out since no PCR product was observed and detected in all control experiments by both ethidium bromide staining and Southern blotting analysis, respectively. PCR products were cloned and sequence analysis revealed that they were identical to the published pituitary GnRHR cDNA sequence. The expression of GnRHR mRNA was also detected by Northern blotting analysis. As seen in figure 2, a 2.5 kb and 1.2 kb signals were observed in JEG-3, IEVT and breast cancer (MCF-7) cells. Using the same probe, no hybridized signal was observed in human dermal fibroblasts, whereas two bands (3.5 kb and 1.6 kb) were observed in mouse pituitary gonadotrope (αT3-1) cells, as previously reported (Reinhart et al. 1992) [Fig. 3.2].

3.3.2 Regulation of GnRHR mRNA levels in placental cells

To study the regulation of GnRHR in placental cells, a semi-quantitative RT-PCR method was used. PCR optimization for GnRHR (primer pair F3 and R4) and GAPDH (primer pair GF and GR) cDNA showed a linear amplification up to 35 and 25 cycles of amplification, respectively, in both JEG-3 and IEVT cells (Fig. 3.3A and B). Similarly, a linear amplification of mouse GnRHR and GAPDH cDNA were obtained up to 30 and 25 cycles using RNA isolated form αT3-1 cells (Fig. 3.3C). As a result, 30 and 18 cycles of PCR amplification of human placental GnRHR and GAPHD cDNA, and 25 and 18 cycles of PCR amplification of mouse GnRHR and GAPDH cDNA were carried out for subsequent studies.

It has been reported that GnRH (Mason et al. 1994, Kakar et al. 1997) and forskolin (Alarid and Mellon 1995) decrease GnRHR mRNA levels in mouse pituitary gonadotrope (αT3-1) cells. In the present study, a 23% ($P<0.05$ vs control) and 49% ($P<0.001$ vs control) decrease in GnRHR mRNA levels was observed after 24h treatment of αT3-1 cells with 0.1μM GnRHa and 10μM forskolin, respectively (Fig. 3.4A and B). In contrast, no down-regulation of GnRHR mRNA levels was observed in the two placental cell lines after GnRH agonist treatment. Instead, a 43% ($P<0.01$ vs control) and 50% ($P<0.01$ vs control) increase in GnRHR mRNA levels was obtained in JEG-3 cells after 24h of 0.1 μM and 1nM GnRHa treatment, respectively (Fig 3.5A). In IEVT cells, no significant change in GnRHR mRNA levels was observed after 1nM GnRHa treatment, while a stimulation of GnRHR mRNA levels (30%, $P<0.05$) was observed after 0.1μM GnRHa treatment. Treatment with 2IU/ml hCG, 10μM TPA or 10μM forskolin resulted in a significant increase in GnRHR expression from both JEG-3 and IEVT cells (Fig. 3.5A and B).

To confirm that the GnRHa induced effect on GnRHR is a receptor-mediated processes, a GnRH antagonist (antide) was used to treat the placental cells for 24h, alone or in combination with GnRHa. No effect on the human GnRHR expression was observed after antide treatment alone. However, the GnRHa-induced increase in GnRHR expression in JEG-3 and IEVT cells

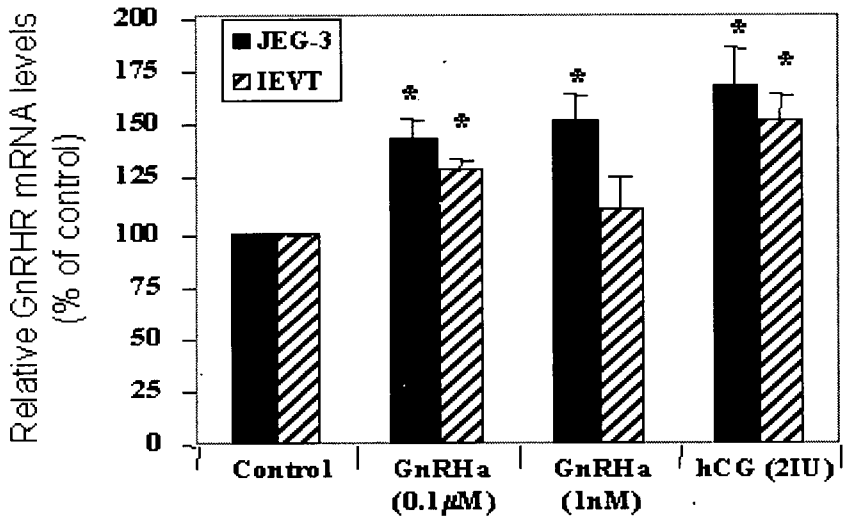
was reversed in the presence of the GnRH antagonist (Fig. 3.6). Similar result was observed using primary trophoblasts culture isolated from term placenta. A 60% increase ($P<0.01$ vs control) in GnRHR mRNA levels was obtained after GnRH agonist treatment and this effect was blocked in the presence of the GnRH antagonist.

FIG. 3.3. Validation of quantitative RT-PCR for GnRHR. Total RNA isolated from human choriocarcinoma JEG-3 [*panel A*], immortalized extravillous trophoblast (IEVT) [*panel B*] and mouse pituitary gonadotrope (αT3-1) [*panel C*] was reversed transcribed. An aliquot of first strand cDNA was amplified for GnRHR (*left panel*) or GAPDH (*right panel*) using different number of PCR cycles. A linear relationship was observed between PCR products and amplification cycle when plotted.

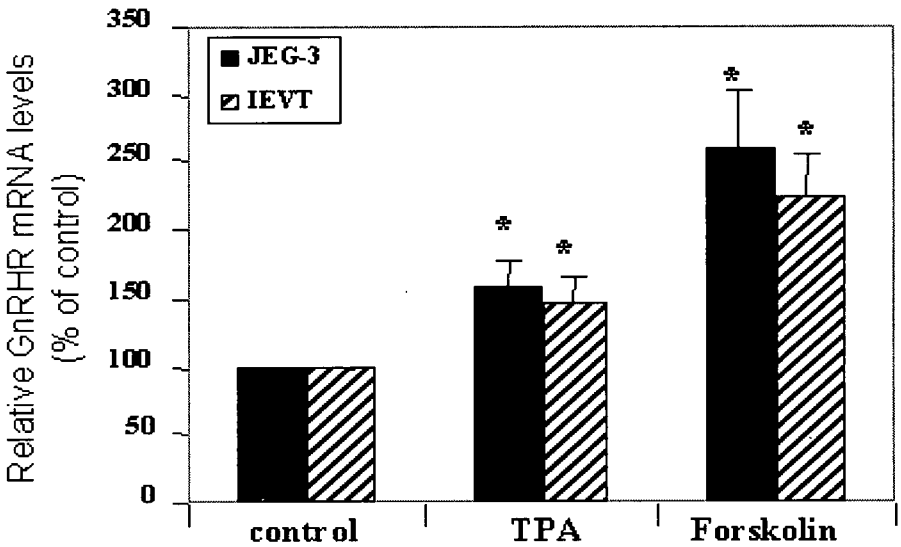
FIG. 3.4 Effects of GnRH agonist (GnRHa) and forskolin on GnRHR expression in αT3-1 cells. Cells were treated with vehicle (control) or corresponding drugs for 24 h before total RNA isolation and analyzed for GnRHR mRNA by semi-quantitative RT-PCR assays. GAPDH mRNA was used as an internal control. Autoradiograms of Southern blot analysis were scanned and quantified. The GnRHR mRNA signal was normalized to the GAPDH internal control for each sample. Mouse pituitary gonadotrope (αT3-1) cells were treated with 0.1 μM GnRHa (*panel A*) or 10 μM forskolin (*panel B*). Results are the mean ± SD from four individual experiments and represented as percentage of control. *, $P<0.05$ from control.

FIG. 3.5 Effects of GnRH agonist (GnRHa), forskolin, phorbol ester (TPA), and human chorionic gonadotropin (hCG) on GnRHR expression in placental cells. Cells were treated with vehicle (control) or corresponding drugs for 24h before total RNA isolation and analyzed for GnRHR mRNA by semi-quantitative RT-PCR assays. GAPDH mRNA was used as an internal control. Autoradiograms of Southern blot analysis were scanned and quantified. The GnRHR mRNA signal was normalized to the GAPDH internal control for each sample. Human choriocarcinoma JEG-3 and immortalized extravillous trophoblasts (IEVT) were treated with GnRHa (0.1 μM and 1 nM) or hCG (2IU/ml) [*panel A*] and TPA (10 μM) or forskolin (10 μM) [*panel B*]. Results are the mean ± SD from four individual experiments and represented as percentage of control. *, $P<0.05$ from control.

A


Relative GnRHR mRNA levels
(% of control)
200
175
150
125
100
75
50
25
0
JEG-3
IEVT
*
Control
GnRHa
(0.1μM)
GnRHa
(1nM)
hCG (2IU)

B


Relative GnRHR mRNA levels
(% of control)
350
300
250
200
150
100
50
0
JEG-3
IEVT
*
control
TPA
Forskolin

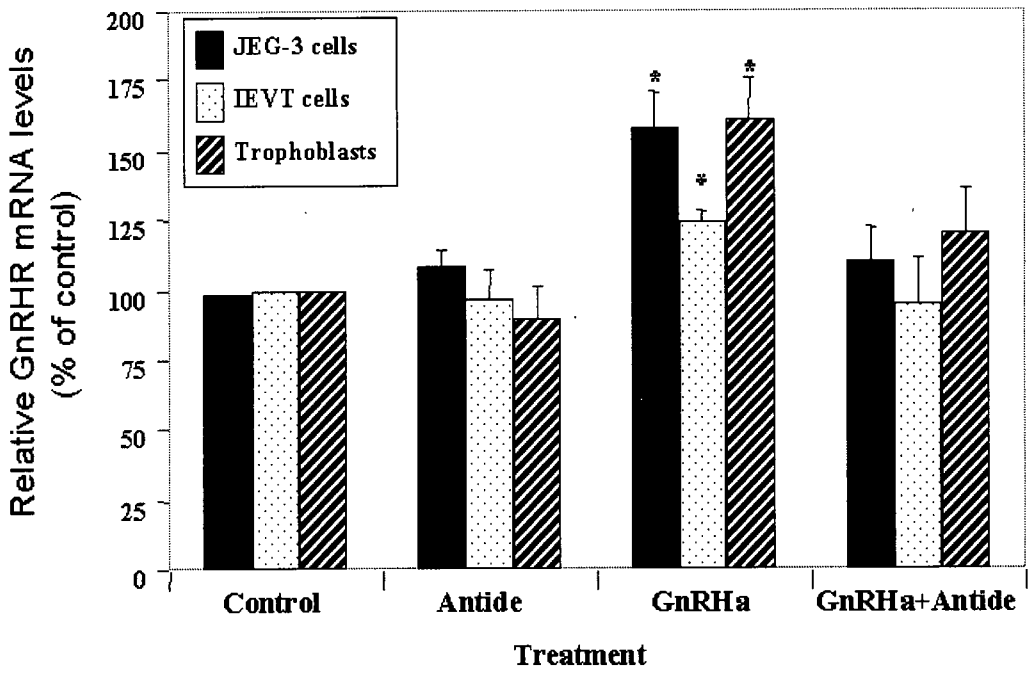


FIG. 3.6 GnRH antagonist, antide, blocks GnRHa-induced GnRHR mRNA expression. Human choriocarcinoma JEG-3, immortalized extravillous trophoblasts (IEVT) and primary trophoblasts culture (Trophoblasts) were treated with vehicle (control), antide (0.1 μM), GnRHa (0.1 μM) in the presence or absence of antide (0.1μM) for 24h. Total RNA was isolated and analyzed for GnRHR mRNA by semi-quantitative RT-PCR. Autoradiograms of Southern blot analysis were scanned and quantified. The GnRHR mRNA signal was normalized to the GAPDH internal control for each sample. Results are the mean±SD from four individual experiments and represented as percentage of control. *, $P<0.05$ from control.

3.3.3 Placental GnRH signaling pathway

As an initial attempt to examine the GnRH-mediated signaling pathway utilized in placental cells to regulate the GnRHR gene expression, JEG-3 cells were pre-treated with a protein kinase C inhibitor (2μM), adenylate cyclase inhibitor (0.5mM), or protein kinase A inhibitor (5μM) for 30min prior GnRH agoinst treatment. Our results indicated that both the PKC and PKA pathways were involved in regulating the GnRHR gene expression, since the GnRH-induced increase in GnRHR expression was attenuated by pretreatment with these inhibitors (Fig. 3.7). In addition, co-treatment of protein kinase C inhibitor and adenylate cyclase inhibitor abolished this stimulatory effect.

3.3.4 GnRH effect on βhCG mRNA levels

Functionally, we observed an increase in βhCG gene expression after GnRHa treatment in JEG-3 cells (Fig.3.8A). A 100% and 65% increase of βhCG mRNA levels was observed after 0.1μM and 1nM GnRH agonist treatment, respectively. Similarly, a 240% and 740% increase in βhCG mRNA levels were measured after 10μM of TPA and forskolin treatment, respectively (Fig. 3.8B). Furthermore, the TPA and forskolin-induced increase in βhCG expression were inhibited in the presence of a protein kinase C inhibitor and an adenylate cyclase inhibitor, respectively (Figs. 3.9A and B). The signal transduction inhibitors used in the present studies was rather specific as no significant non-specific effect was observed. Similarly, the GnRHa-induced βhCG expression was reversed in the presence of either the PKC inhibitor or the adenylate cyclase inhibitor, which was further reduced by co-treatment of PKC inhibitor and adenylate cyclase inhibitor (Fig. 3.9C).

FIG. 3.8 Regulation of βhCG mRNA expression. Total RNA were isolated from human choriocarcinoma JEG-3 after 24h treatment of treatment with vehicle (control) or corresponding drugs. The βhCG and GAPDH mRNA was detected by Northern blot analysis (shown on the upper part of each graph). The radioautograms were scanned and normalized to the corresponding GAPDH signal for each treatment. *Panel A*, JEG-3 cells were treated with 0.1μM or 1nM GnRHa. *Panel B*, cells were treated with 10μM forskolin or 10μM phorbol ester (TPA). Results are mean ± SD from three experiments. *, $P<0.05$ *vs* control.

FIG. 3.9 Effects of a specific protein kinase C inhibitor (GF109203X) and adenylate cyclase inhibitor (SQ22536) on GnRHa-induced βhCG mRNA expression from human choriocarcinoma JEG-3. Total RNA were isolated from JEG-3 cells after 24h treatment of treatment with vehicle (control) or corresponding drugs. The βhCG and GAPDH mRNA was detected by Northern blot analysis (*shown on the upper part of each graph*). The radioautograms were scanned and normalized to the corresponding GAPDH signal for each treatment. JEG-3 cells were treated with 10μM phorbol ester (TPA) [*panel A*], 10μM forskolin [*panel B*], 0.1μM GnRHa [*panel C*] in the presence or absence of 2μM protein kinase C inhibitor (PKCI) and/or 0.5mM adenylate cyclase inhibitor (ACI). Results are mean ± SD from three experiments. a, $P<0.05$ vs control; b, $P<0.05$ *vs* TPA, forskolin or GnRHa treatment.

3.4 Discussion

The identification and characterization of GnRH in the placenta (Khodr and Siler-Khodr 1980, Tan and Rousseau 1982, Miyake et al.1982, Seeburg and Adelman 1984), and its effects in stimulating hCG biosynthesis and secretion (Iwashita et al. 1986, Siler-Khodr et al. 1986, Belisle et al. 1987, Barnea and Kaplan 1989, Merz et al 1991, Bramley et al. 1992), suggest that the placenta possesses an intrinsic GnRH-mediated regulatory system which influences the dynamics of hCG secretion during pregnancy. Regulation of GnRHR gene expression appears to be an important determinant of the concentration of GnRHR in the placenta (Lin et al. 1995). Recently, it has been demonstrated that placental cells express GnRHR (Lin et al. 1995, Wolfahnt et al. 1998, Boyle et al. 1998, Yin et al 1998). In the present study, we have isolated the cDNA encoding the full-length human GnRHR cDNA from two placental cell lines, including JEG-3 and IEVT, and from primary trophoblast cells by RT-PCR amplification. Sequence analysis of the cDNA revealed that the placental GnRHR was same as its counterpart in pituitary, at least at the mRNA level. The difference in GnRH binding observed in placental cells versus pituitary cells may be explained by different post-translational modifications. Moreover, GnRHR expression in the placenta was further substantiated by Northern analysis using poly(A) RNA isolated from IEVT and JEG-3 cells. In the human pituitary, Northern blot analysis detected a predominant mRNA transcript of 4.7-5.0 kb and two minor bands of 2.5 and 1.5 kb (Kakar 1997). The 2.5 kb and 1.2 kb transcripts were observed in both placental cell lines and MCF-7 cells by Northern blot analysis. Interestingly, the major 4.7-5.0 kb transcript was not observed in the extra-pituitary cells used in our study. We suspected that only the shorter GnRHR transcripts are synthesized in extra-pituitary tissues.

We have successfully used semi-quantitative RT-PCR to quantify GnRH and its receptor expression in ovarian cells (Peng et al. 1994, Kang et al. 2000). Although we are able to detect the human GnRHR mRNA by Northern blot analysis, the amounts of total RNA required for

isolating enough poly (A) RNA for regulation studies was enormous. As a result, semi-quantitative RT-PCR was employed to examine GnRHR gene expression in the placental cells. Previous reports have demonstrated that GnRHa (0.1nM to 1μM) and 10μM forskolin treatments resulted in a decrease of the GnRHR mRNA in αT3-1 cells (Mason et al 1994, Alarid and Mellon PL 1995, Kakar et al. 1997). In agreement with these earlier reports, a significant decrease in GnRHR mRNA levels was detected following GnRHa treatment of αT3-1 cells in our study. A similar GnRH-induced decrease in GnRHR mRNA levels was observed in human ovarian cells (Peng et al. 1994, Kang et al. 2000). Interestingly, homologous down-regulation of GnRHR mRNA was not observed in the placental cells. Instead, an increase in GnRHR mRNA level was observed after 0.1μM GnRHa treatment in the two placental cell lines. These results suggest that a different regulatory mechanism may be utilized in the placenta to control the GnRHR mRNA expression.

Functionally, we have demonstrated an increase in βhCG mRNA levels after GnRH agonist treatment in JEG-3 cells, which were mimicked by forskolin and TPA treatments. However, no such effect was observed in IEVT cells. It has been reported that the hCG was only detected in the immortalized IEVT cells but not in the parental cell (Graham et al. 1993). Hence, a different regulatory mechanism may exist in IEVT cells compared to JEG-3 cells. The roles of PKC and PKA pathways in mediating GnRH action in placental cells were further examined pharmacologically. The increase in placental GnRHR and βhCG mRNA levels after GnRHa treatment were reversed by pre-treating the JEG-3 cells with inhibitors of PKC, PKA or adenylate cyclase alone and completely abolished by co-treatment of PKCI and ACI. These findings further suggest that both protein kinase A and protein kinase C pathways are participated in mediating GnRH action in the placental cells. It is well documented that $G_q/_{11}\alpha$ couples to GnRHR (Stojikovic and Catt 1995, Naor et al. 1995). We and others have shown that the stimulation of Ca^{2+} mobilization in human syncytiotrophoblast cells by GnRH through a

receptor mediated event, implicating coupling of the GnRHR to $G_{q/11\alpha}$ in the placenta (Belisle et al. 1989, Mathialagan and Rao 1989). However, others have reported the coupling of $G_s\alpha$ to rat GnRHR (Stanislaus et al. 1998) and $G_i\alpha$ to GnRHR identified from human prostate cancer cells (Limonta et al. 1999). Recent study has demonstrated the direct coupling of $G_s\alpha$ and adenylyl cyclase to mouse GnRHR at the first intracellular loop (Arora et al. 1998). In addition, the lack of complete abolition in GnRHa-induced LH release in the $G_q\alpha$ and $G_{11}\alpha$ knockout mice, further supports the coupling of GnRHR to multiple G-proteins *in vivo* (Stanislaus et al. 1998). Our present finding of a relatively stronger effect of forskolin *vs* GnRHa on βhCG mRNA level, as well as an attenuation of GnRHa-induced increase in GnRHR and βhCG expression by a adenylate cyclase inhibitor, further implicate the coupling of $G_s\alpha$ to the placental GnRHR.

In summary, the present results strongly substantiate the expression of GnRHR in the human placenta and further strengthen the role of GnRH in the autocrine/paracrine regulation of hCG biosynthesis. The demonstration of GnRHa-mediated up-regulation of GnRHR mRNA level in placental cells, suggest that the homologous down-regulation of GnRHR gene seen in the pituitary is absent in the placenta, which may help maintain the GnRH-stimulated hCG secretion throughout pregnancy. In addition, our results further implicate a role for both the PKC and PKA pathways in placental GnRHR signaling and expression.

CHAPTER 4. IDENTFICATION OF PLACENTA-SPECIFIC UPSTREAM GnRHR PROMOTER

4.1 Introduction

The detection of GnRHR mRNA by Northern blot analysis and the isolation of a full-length GnRHR cDNA from the human placental cells, including JEG-3, IEVT and primary culture of trophoblast cells, and the increase in βhCG mRNA levels following 0.1μM GnRHa treatment in JEG-3 cells, supporting the notion that GnRH plays an autocrine/paracrine role in regulating placental function through a receptor-mediated mechanism (Chapter 3). In addition, the change in GnRHR mRNA levels after GnRHa, TPA and forskolin treatment suggest a possible regulation of the gene expression. As the first step towards the understanding of transcription regulation of placental GnRHR gene, we have identified the placental-cells specific promoter.

4.2 Results

4.2.1 Characterization of human GnRHR 5' flanking region in placental cells

The expression of GnRHR mRNA from both JEG-3 and IEVT cells suggest the feasibility of using these placental cells in studying the transcription regulation of this gene in the human placenta. To localize elements within 2.3 kb of the 5' flanking region of the human GnRHR gene that mediate placental cell-specific expression, various 5' and 3'-deletion mutants were constructed and analyzed in JEG-3 and IEVT cells. Transient transfection studies showed similar activity profiles in both cell-lines. Our results revealed that a distal and a proximal region are important for promoter activity in the placental cells (Fig. 4.1 and 4.2). The proximal region was located between nucleotide (nt) -707 to -167 (relative to the translation start site).

Progressive 5'-deletion to the Pst I site (-1018) did not affect the basal promoter activity (Fig. 4.1). Further deleting the sequence from Pst I to Sty I (-707) resulted in an increase in promoter activity (JEG-3 cells; 18 fold vs pGL2-Basic, $P<0.001$, IEVT cells; 13.8 fold vs pGL2-Basic, $P<0.001$), suggesting the presence of a negative regulatory element located within this region. Deletion of the sequences from nt -707 to -407 dramatically reduced the promoter activity. Further removal of DNA sequences from nt -407 to -167 eliminated any residual promoter activity.

The distal promoter region was located between nt -1671 to -1346. Progressive 3' deletion to -421 did not significantly affect the basal promoter activity (Fig. 4.2), while a dramatic increase in promoter activity was obtained after deletion of DNA region between nt -421 to -577 in both JEG-3 (59 fold vs pGL2-Basic, $P<0.001$) and IEVT cells (38.2 fold vs pGL2-Basic, $P<0.001$). This increase in promoter activity was maintained to the deletion of 951bp from the 3' end of the human GnRHR 5' flanking region to -1346. Interestingly, deletion of DNA sequences from nt -577 to -771 resulted in a loss of promoter activity and further deletion of nt -771 to -1018 resumed the maximal promoter activity (Fig. 4.2). These data suggest that a strong positive and negative regulatory regions were located within these two sequences, respectively. The locations of these regulatory regions were the same as those identified by the 5' deletion.

Transient transfection studies with the identified distal and proximal promoter regions introduced into JEG-3 and αT3-1 cells, indicated a possible differential usage of the promoter in the placental and pituitary cells (Fig. 4.3). Although an average of 16 folds increase ($P<0.001$) in luciferase activity was observed in the proximal region-transfected JEG-3 cells, the highest promoter activity (55 fold, $P<0.001$) was observed from the distal region. In contrast, the proximal region was more active in the pituitary gonadotrope αT3-1 cells (47.2 fold vs pGL2-

Basic, P< 0.001), while only an average of 5 folds increase (P<0.01) in luciferase activity was obtained from the distal region-transfected αT3-1 cells.

To better define the distal region important for placental expression, further deletion mutants were generated between nt -2297 and –1346. These constructs were transiently transfected into JEG-3 cells (Fig. 4.4). Deletion of DNA spanning from nt -2297 to -1737 (related to translation start site) did not affect the basal promoter activity. A dramatic decrease (90%) in luciferase activity was observed after deleting the DNA region between -1737 and -1667 (p1667/-1346 Luc), suggesting the importance of this region in mediating the promoter activity. However, removal of DNA sequence from nt -1667 to -1346 (p2300/-1667 Luc) resulted in a minimal promoter activity, even though the retaining of the DNA section from nt -1736 to -1667. Taken together, these data suggest that the upstream 5' flanking region between nt -1737 and -1346 was responsible for maintaining the maximal basal promoter activity.

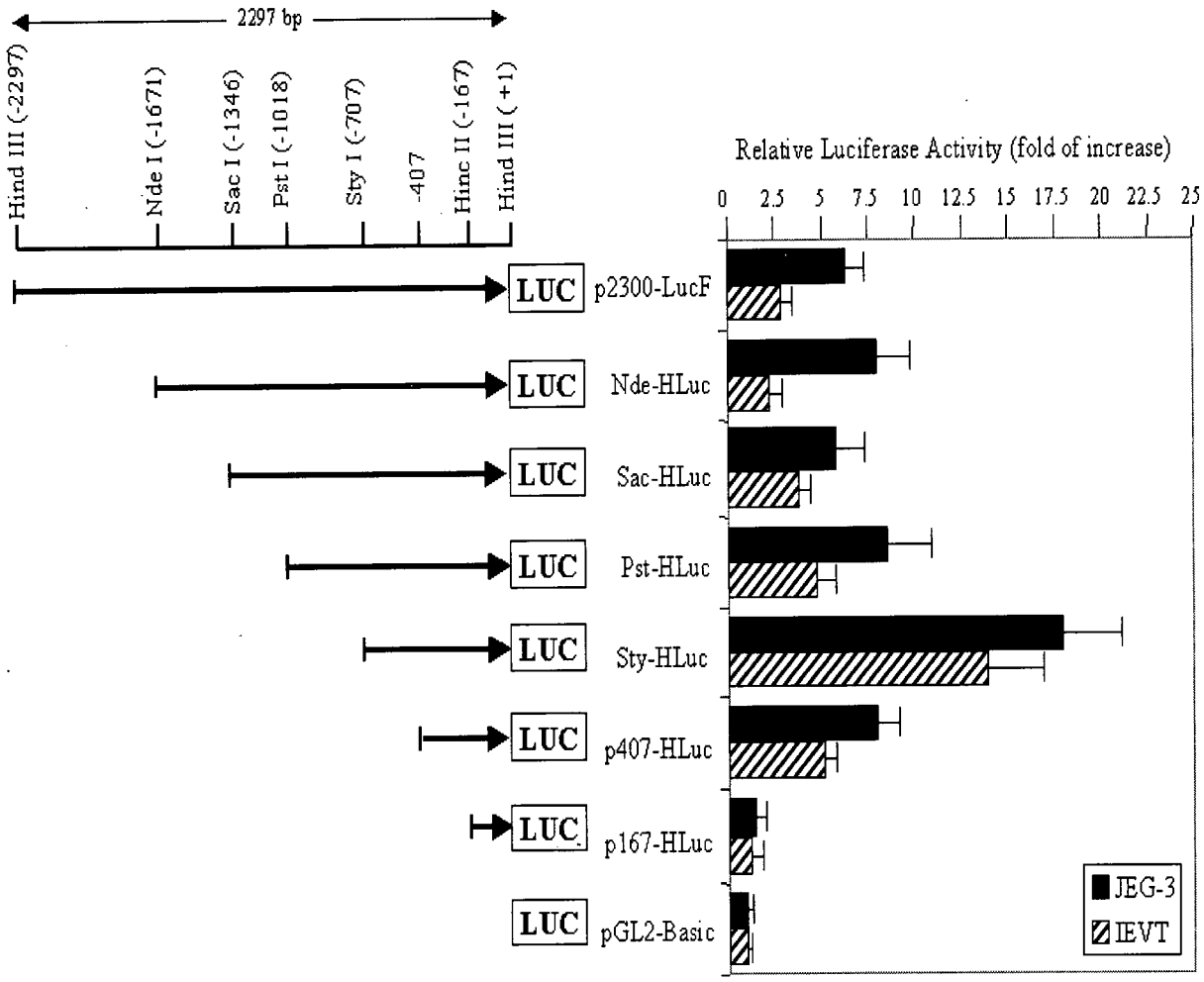


FIG. 4.1 Effects of 5' deletion on the promoter activity of the human GnRHR 5' flanking region. Progressive 5' deletion constructs of p2300-LucF were transiently transfected into JEG-3 and IEVT cells by calcium phosphate precipitation method. The RSV-LacZ vector was co-transfected to normalize for varying transfection efficiencies. Relative promoter activity of each construct is shown as fold increase over a promoterless luciferase control pGL2-Basic, whose activity is set to be 1, after normalized to β-gal activity. Values represent mean +/- S.E. of triplicate experiments.

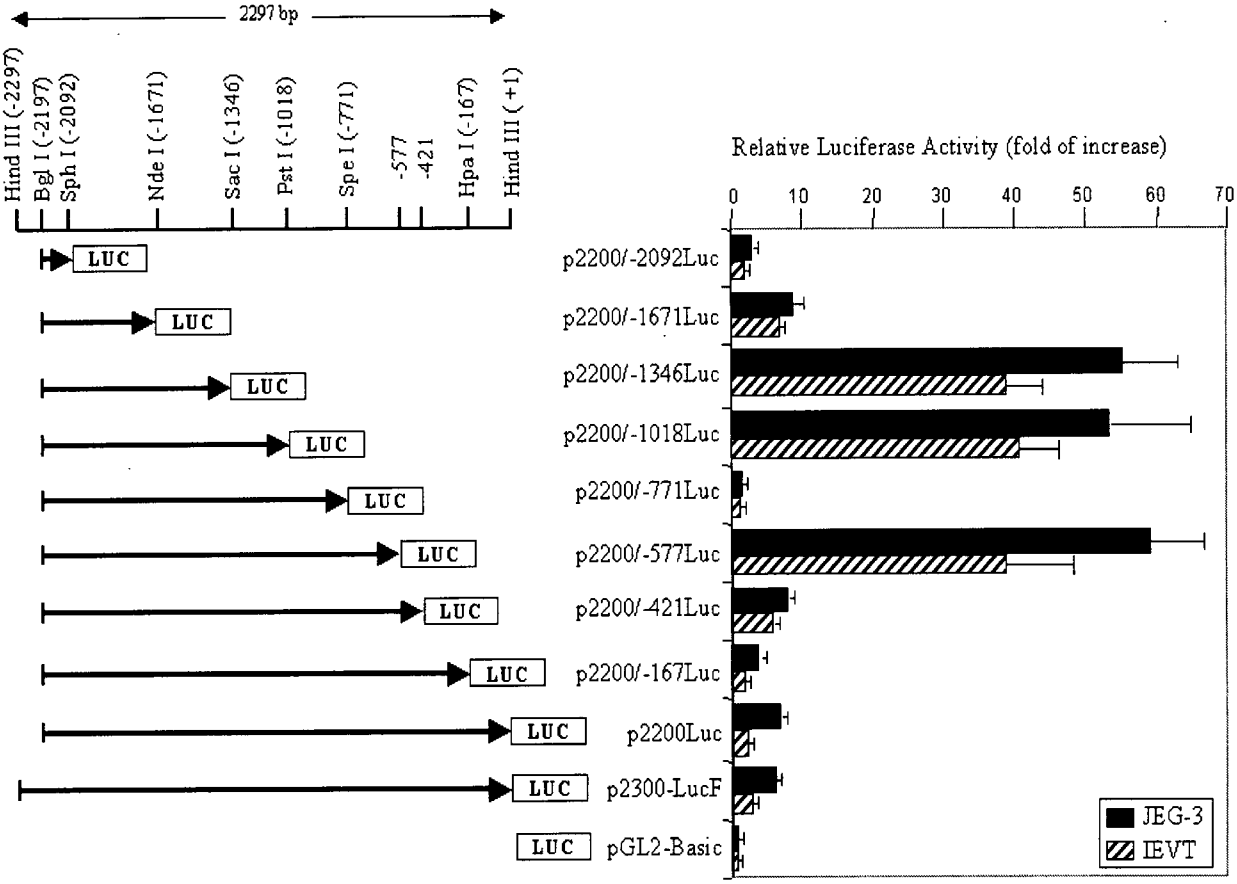


FIG. 4.2 Effects of 3' deletion on the promoter activity of the human GnRHR 5' flanking region. Progressive 3' deletion constructs of p2300-LucF were transiently transfected into JEG-3 and IEVT cells by calcium phosphate precipitation method. The RSV-LacZ vector was co-transfected to normalize for varying transfection efficiencies. Relative promoter activity of each construct is shown as fold increase over a promoterless luciferase control pGL2-Basic, whose activity is set to be 1, after normalized to β-gal activity. Values represent mean +/- S.E. of triplicate experiments.

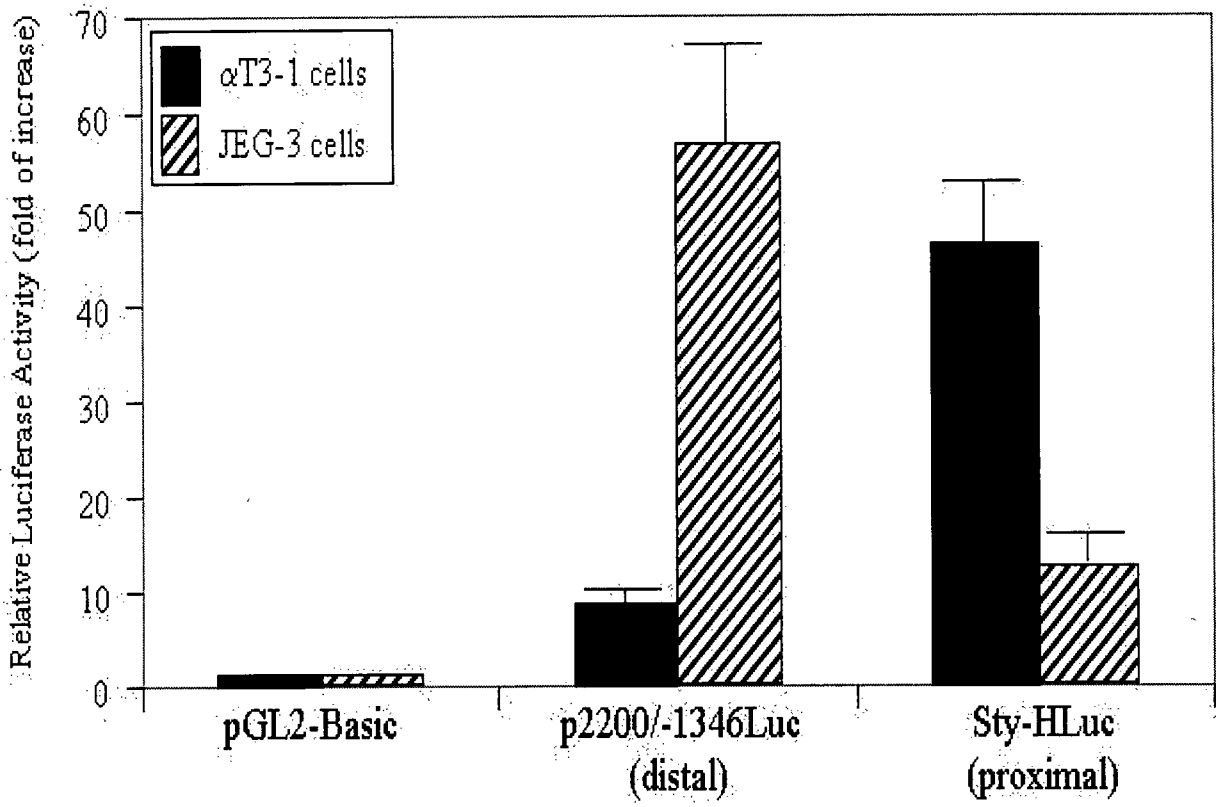


FIG. 4.3 Differential usage of human GnRHR promoters in JEG-3 and αT3-1 cells. The distal and proximal promoters identified in Fig. 1 and 2 were transiently transfection into JEG-3 and αT3-1 cell by calcium phosphate precipitation method. The RSV-LacZ vector was co-transfected to normalize for varying transfection efficiencies. Relative promoter activity of each construct is shown as fold increase over a promoterless luciferase control pGL2-Basic, whose activity is set to be 1, after normalized to β-gal activity. Values represent mean +/- S.E. of triplicate experiments.

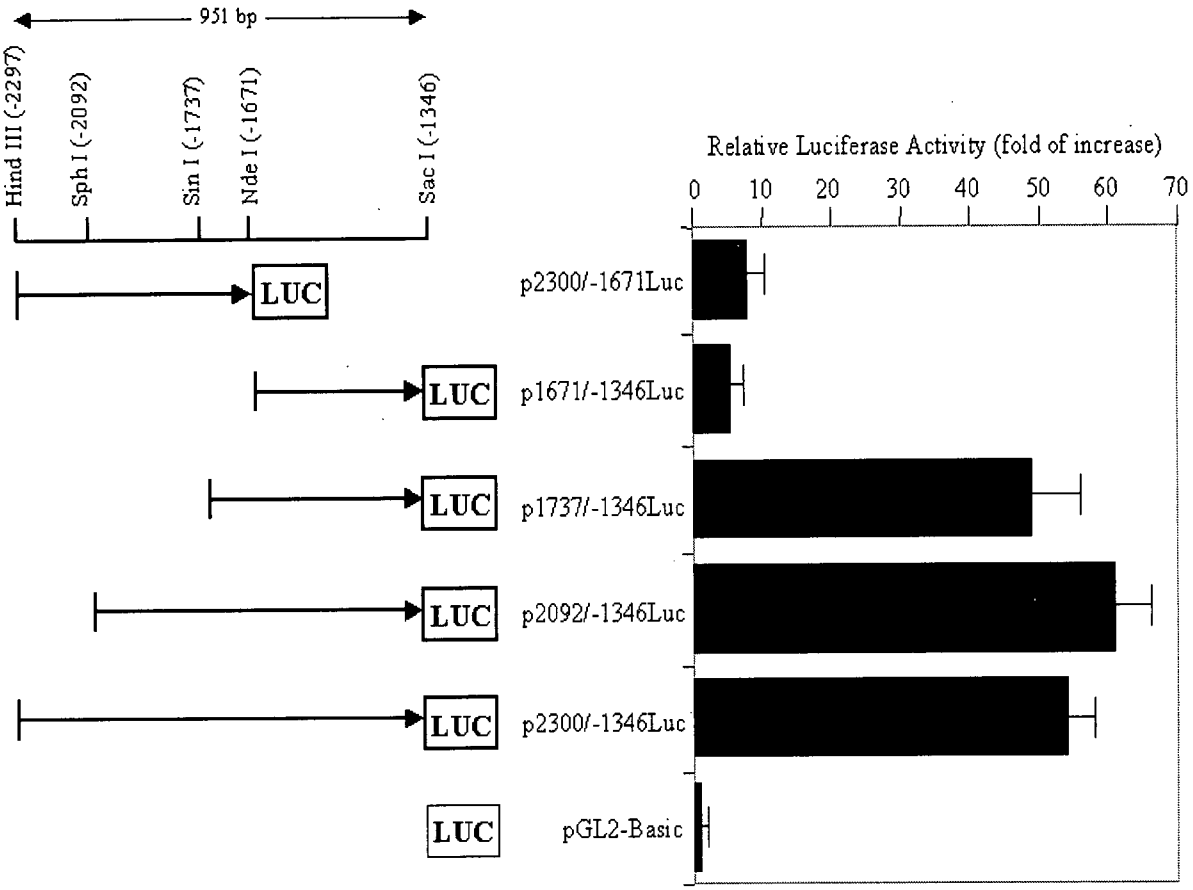


FIG. 4.4 Localization of the minimal promoter region for placenta expression of GnRHR gene. Several deleted constructs of p2300/-1346 Luc was co-transfected with RSV-LacZ vector into JEG-3 cells. Relative promoter activity of each construct is shown as fold increase over a promoterless luciferase control pGL2-Basic, whose activity is set to be 1, after normalized to β-gal activity. Values represent mean +/- S.E. of triplicate experiments.

4.2.2 Characterization of the transcription start site in the human placental GnRHR

To examine the activity of the putative distal and proximal promoter regions *in vivo*, the transcription initiation site for the placental GnRHR gene in JEG-3 cells was examined by primer extension. Using primer PE-A and PE-B (Fig. 4.5A), 4 (indicated by arrows A-D) and 1 (indicated by arrow E) extended products were observed using JEG-3 poly(A+) RNA, respectively, but not in HDF cells (Fig. 4.5B). The location of transcription start sites identified for placental GnRHR were similarly to those reported previously (Fan et al. 1995, Kakar 1997). The most upstream transcription start site (Fig. 4.5B, arrow D) detected by the PE-A primer was further examined (Fig. 4.5C). By the use of upstream primer PE-C, two transcription start sites were identified at nt -1608 and –1629 (relative to translation start site). More importantly, all transcription start sites detected were located within the distal or proximal promoter regions identified in the present study. These results strongly supported the use of these two promoters to drive transcription in the placental JEG-3 cells *in vivo*.

FIG. 4.5 Identification of human GnRHR transcription start site in JEG-3 cells by primer extension. *Panel A*, Diagrammatic representation of the human GnRHR 5' flanking region and the relative position of extension primers (PE-A, PE-B and PE-C). *Panel B,* RNA isolated from HDF and JEG-3 cells were extended with primer PE-A and PE-B. Four (indicated as A-D) and 1 (indicated as E) extended products were obtained from JEG-3 cells using primer PE-A and PE-B, respectively. No signal was obtained from HDF. *GATC*, a sequence reaction used to estimate the size of the extended fragments. *Panel C*, The longest extended product (arrow D in panel B) identified by PE-A was examined by PE-C. Two signals were detected using RNA isolated form JEG-3 cells but not in HDF.

4.2.3 Cell-specific use of upstream promoter

To further examine the specificity of the upstream promoter region (nt -1737 to -1346, related to translation start site) in the placental cells, the p1737/-1346 Luc was transiently transfected into 7 cell lines from different tissues or species including αT3-1, JEG-3, IEVT, HEK293, OVCAR-3, COS-1 and HDF cells (Fig. 4.6). The upstream promoter was not active at all in HDF, but is highly active in placental JEG-3 and IEVT cells. Lower promoter activities were observed in other cell lines tested. These data suggest that this region is specific to placental cell but not in other cell lineage.

4.2.4 Expression of Oct-1, CREB, GATA and AP-1 protein

DNA sequence analysis of the upstream promoter between nt -1737 and -1346 revealed the presence of four putative transcription factor binding sites, including hGR-Oct-1 (5' ATACAAAT 3' located at nt -1718 to -1710, in a reverse orientation, with 87.5% homology to the consensus Oct-1 site), hGR-CRE (5' TGACATGA 3' located at nt -1649 to -1641 with 75% homology to the consensus CRE site), hGR-GATA (5' AGATAA 3' located at nt -1602 to -1597, in reverse orientation, with 100% homology to the GATA site) and hGR-AP-1 (5'TGAGTCA3' located at nt -1518 to -1511 with 100% homology to the consensus AP-1 site). The presence of these motifs suggests the possible regulation of human GnRHR gene expression in the placenta by the transcription factors Oct-1, CREB, GATA-2, GATA-3, c-Jun and c-Fos. The expression of these factors was examined by Western blot analysis using cellular extracts isolated from HDF, COS-1, IEVT, JEG-3, OVCAR-3 and HKE293 cells. As indicated in Fig. 4.7, similar levels of Oct-1, CREB, and c-Fos protein were detected in all the cells. For GATA-2 and GATA-3, the expression levels were higher in placental IEVT and JEG-3 cells. In contrast, the expression level of c-Jun was lower in JEG-3 cells.

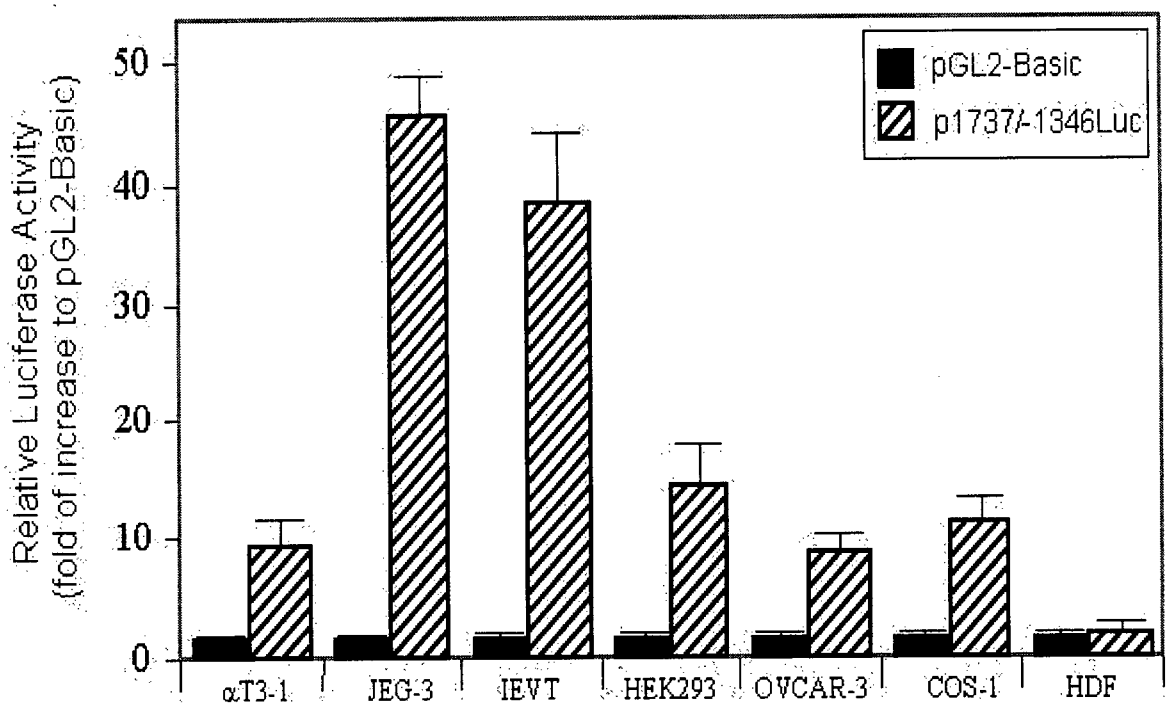


FIG. 4.6 Cell-specific activity of the upstream human GnRHR promoter. Luciferase construct (p1737/-1346 Luc) was co-transfected into αT3-1, JEG-3, IEVT, HEK293, OVCAR-3, COS-1 and HDF with the RSV-LacZ vector to normalize for varying transfection efficiencies. Relative promoter activity of each construct is shown as fold increase over a promoterless luciferase control pGL2-Basic, whose activity is set to be 1, after normalized to β-gal activity. Values represent mean +/- S.E. of triplicate experiments.

4.2.5 Identification of transcription binding sites

The expression of Oct-1, GATA-2, GATA-3, CREB, c-Jun and c-Fos in JEG-3 cells suggest the possible binding of these factors to their corresponding sites in the human GnRHR up-stream promoter. To examine the functional role of these elements in regulating the expression of GnRHR gene in the placental cells, site-specific mutants were constructed and transiently transfected into JEG-3, OVCAR-3, HEK-293 and COS-1 cells (Fig. 4.8). The mutation was introduced by three-steps PCR mutagenesis method (Chapter 2, Section 2.11). The putative transcription factor binding sequence was replaced by a non-related sequence to disturb specific binding. Mutation of the hGR-Oct-1 site resulted in a dramatic decrease in luciferase activity in all the cells tested (77% decrease in JEG-3 cells, $P<0.001$; 48% decrease in OVCAR-3 cells, $P<0.001$; 35% decrease in COS-1 cells, $P<0.001$; and 19% decrease in HEK293 cells, $P<0.05$). Similarly, mutation of the hGR-AP-1 site decreased the luciferase activity in these cells (72% decrease in JEG-3 cells, $P<0.001$; 44% decrease in OVCAR-3 cells, $P<0.001$; 30% decrease in COS-1 cells, $P<0.001$; and 61% decrease in HEK293 cells, $P<0.05$). These results suggest that both Oct-1 and AP-1 functioned constitutively in these cells. On the other hand, mutation of hGR-CRE and hGR-GATA sites caused the loss of luciferase activities only in JEG-3 cells, but not in other cells (Fig. 4.8). These results suggest that these transcription factors are playing specific roles in the placental cells.

To further examine the interaction of these transcription factors in regulating the GnRHR promoter activity, constructs containing multiple mutations were produced and transiently transfected into JEG-3 cells (Fig. 4.9). Promoter-luciferase constructs containing double mutations of hGR-Oct-1 and hGR-AP-1 resulted in a lower promoter activity. Double mutations of hGR-CRE and hGR-GATA, or with AP-1 sites further decreased the promoter activity when compared to the single mutation. Mutations of all 4 putative motifs almost completely eliminated the promoter activity (Fig. 4.9).

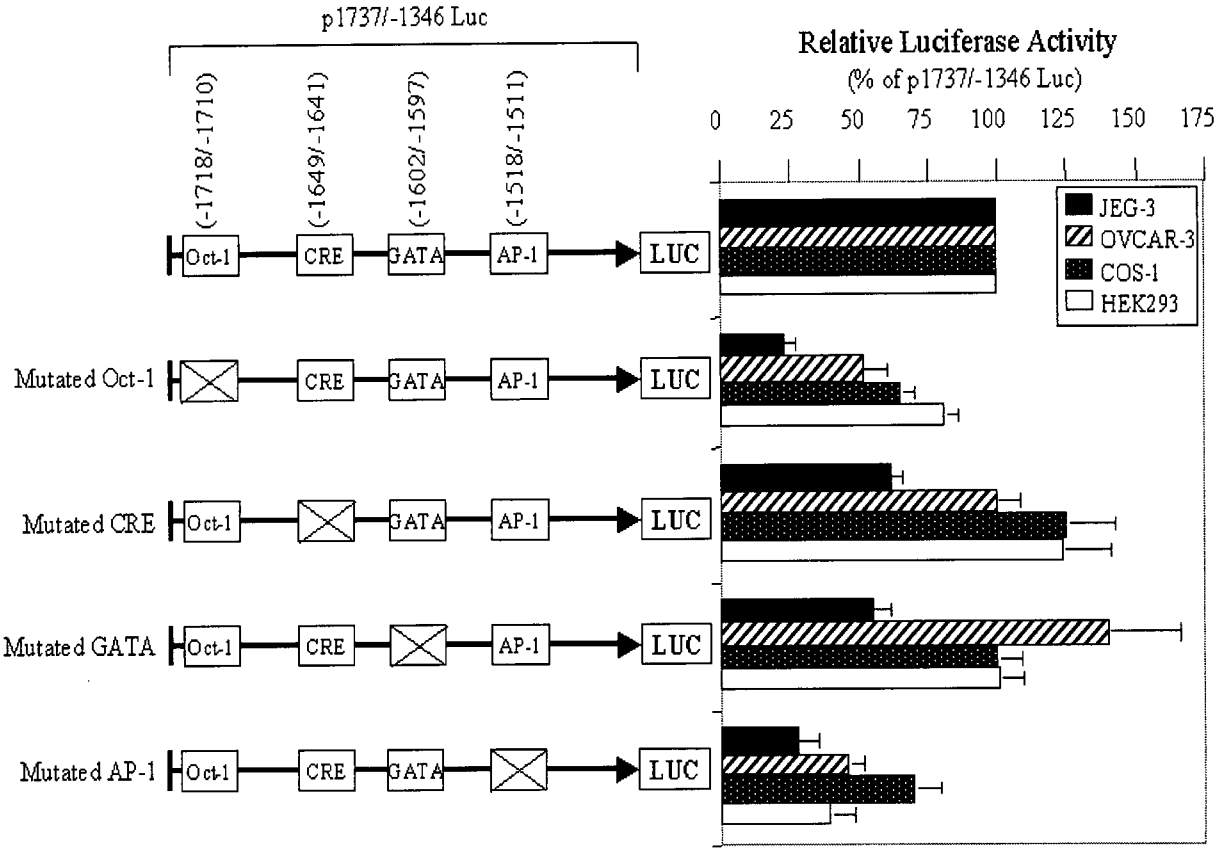


FIG. 4.8. Functional analysis of human GnRHR upstream promoter in different cells. Mutations were introduced by 3-steps PCR mutagenesis as described in *materials and methods*. The mutated promoter constructs were co-transfected with RSV-LacZ vector, to normalize for varying transfection efficiencies, into JEG-3, OVCAR-3, COS-1 and HEK293 cells. The relative activity of each promoter is shown as percentage of p1737/-1346 Luc whose activity is taken as 100%, after normalized to β-gal activity. Values represent mean +/- S.E. of triplicate experiments. The names and the relative position of the putative transcription factors binding sites was given and the mutated element was shown as *crossed box.*

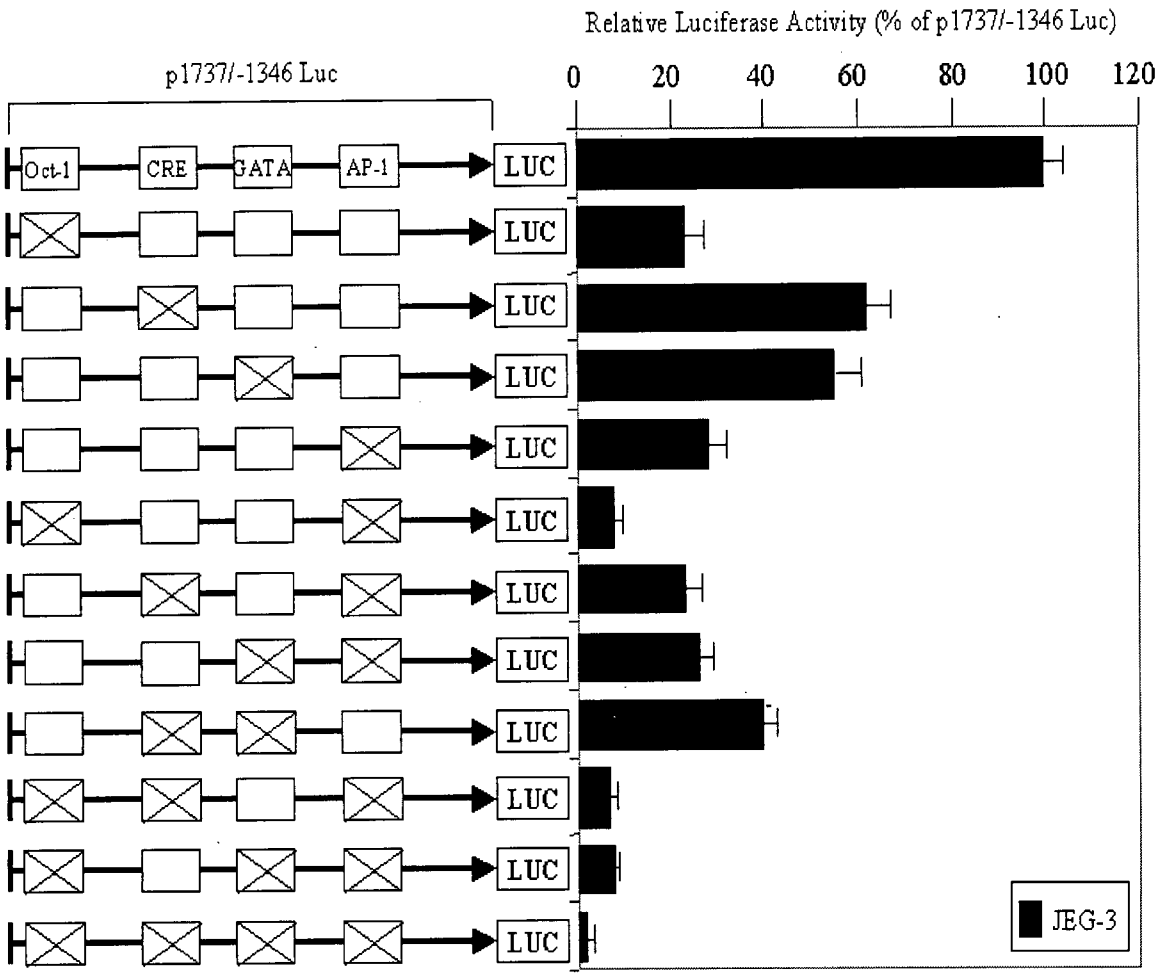


FIG. 4.9 Functional analysis of mutations in multiple elements of the human GnRHR upstream promoter in JEG-3 cells. Single and multiple mutation in p1737/-1346 Luc were introduced by 3-steps PCR mutagenesis as described in materials and methods. Mutation constructs were co-transfected with the RSV-LacZ vector to normalize for varying transfection efficiencies by calcium phosphate precipitation method. The relative promoter activity of each construct is shown as percentage of p1737/-1346 Luc whose activity is taken as 100%, after normalized to β-gal activity. Values represent mean +/- S.E. of triplicate experiments. The names and the relative position of the putative transcription factors binding sites were given. Intact transcription factor binding sites were represented as open box, while mutated elements were shown as crossed box.

4.2.6 The binding of Oct-1, CREB, GATA-2, GATA-3, c-Jun and c-Fos to the putative binding sites.

To confirm the identity of the transcription factors bound to the upstream GnRHR promoter, gel mobility shift assay was preformed with synthetic oligodeoxynucleotides containing the hGR-Oct-1, hGR-CRE, hGR-GATA and hGR-AP-1 sites in the presence of consensus, mutated, non-related oligodeoxynucleotides or antibodies against these transcription factors. Specific DNA-protein complexes were formed with the radiolabelled probes for hGR-Oct-1 (Fig. 4.10A, indicated by arrow A), hGR-CRE (Fig. 4.10B, indicated by arrow B), hGR-GATA (Fig. 4.10C,indicated by arrows C and D) and hGR-AP-1 (Fig. 4.10D, indicated by arrow E) using nuclear extract prepared from JEG-3 cells. These complexes disappeared with competitor DNA fragment (200 fold in excess) containing either the consensus sequences (Oct-1, CRE, GATA, AP-1) or unlabelled probe (hGR-Oct-1, hGR-CRE, hGR-GATA, hGR-AP-1) but not with the mutated competitor mOct-1, mhGR-Oct-1, mCRE, mhGR-CRE, mGATA, mhGR-GATA, mAP-1, mhGR-AP-1, or unrelated sequences (NF-κB or TFIID). Furthermore, the addition of antibodies against these transcription factors either "supershifted" or eliminated the DNA-protein complexes further supporting the binding of the Oct-1 (Fig. 4.11A), CREB (Fig. 4.11B), GATA-2 and GATA-3 (Fig. 4.11C), and AP-1 (Fig. 4.11D) to their corresponding sites in the upstream promoter.

FIG. 4.10 Gel mobility shift assay of JEG-3 nuclear proteins binding to the putative Oct-1, CRE, GATA and AP-1 binding sites in the human GnRHR upstream promoter. Synthetic deoxyribo-oligonucleotide containing the putative upstream GnRHR Oct-1-like (hGR-OCT-1), CRE-like (hGR-CRE), GATA-like (hGR-GATA) and AP-1-like (hGR-AP-1) sequences were P^{32} labeled and incubated with JEG-3 nuclear extracts in the presence of the indicated competitor oligonucleotide (200 fold excess or 0.1 mM). *Panel A*, Specific binding of Oct-1 complex (indicated as arrow A) to the hGR-Oct-1 in the presence of competitor oligonucleotide (lane 1, no competitor; lane 2, mutated consensus Oct-1; lane 3, consensus Oct-1; lane 4, mutated hGR-Oct-1; lane 5, hGR-Oct-1; lane 6; consensus AP-1). *Panel B,* Specific binding of CRE complex (indicated as arrow B) to the hGR-CRE in the presence of competitor oligonucleotide (lane 1, no competitor; lane 2, mutated consensus CRE; lane 3, consensus CRE; lane 4, mutated hGR-CRE; lane 5, hGR-CRE; lane 6; consensus AP-1). *Panel C,* Specific binding of GATA complexes (indicated as arrow C and D) to the hGR-GATA in the presence of competitor oligonucleotide (lane 1, no competitor; lane 2, mutated consensus GATA; lane 3, consensus GATA; lane 4, mutated hGR-GATA; lane 5, hGR-GATA; lane 6; consensus AP-1). *Panel D*, Specific binding of AP-1 complex (indicated as arrow E) to the hGR-AP-1 in the presence of competitor oligonucleotide (lane 1, no competitor; lane 2, mutated consensus AP-1; lane 3, consensus Ap-1; lane 4, mutated hGR-AP-1; lane 5, hGR-AP-1; lane 6; consensus CRE). Unrelated oligonucleotides NF-κB and TFIID were included in each individual experiments as showed in lane 7and lane 8, respectively. .

Fig. 4.11 Identification of transcription factor Oct-1, CREB, GATA-2, GATA-3, c-Fos and c-Jun binding to the upstream GnRHR promoter. Gel mobility shift assay studies were performed as in *FIG. 10* in the presence of antibodies specific to Oct-1, CREB, GATA-2, GATA-3, c-Fos, c-Jun and estrogen receptor (ER). Antibodies were added 1h prior to the addition of JEG-3 nuclear extract. *Panel A*, The P^{32}-labelled hGR-Oct-1 probe was incubated in the presence of antibody against IgG (lane 1), Oct-1 (lane 2), CREB (lane 3) and ER (lane 4). One DNA-complex was "supershifted" by Oct-1 antibody (Indicated as arrow A). *Panel B*, The P^{32}-labelled hGR-CRE probe was incubated in the presence of antibody against IgG (lane 1), CREB (lane 2), c-Jun (lane 3) and ER (lane 4). One DNA-complex was eliminated by CREB antibody (indicated as arrow B). *Panel C,* The P^{32}-labelled hGR-GATA probe was incubated in the presence of antibody against IgG (lane 1), GATA-2 (lane 2), GATA-3 (lane 3), Oct-1 (lane 4) and ER (lane 5). Two DNA-complexes were "supershifted" by the addition of GATA-2 and GATA-3 antibodies (indicated as arrow C and D). *Panel D,* The P^{32}-labelled hGR-AP-1 probe was incubated in the presence of antibody against IgG (lane 1), Oct-1 (lane 2), c-Fos (lane 3), c-Jun (lane 4) and ER (lane 5). One DNA-complex was eliminated by c-Fos and c-Jun antibodies (indicated as arrow E).

4.3 Discussion

4.3.1 Identification of placental cell specific up-stream promoter

GnRH has been shown to regulate the secretion and expression of hCG from the placenta through a receptor mediated process. As the GnRHR number and its mRNA levels change dynamically throughout gestation while the level of GnRH mRNA remains relatively constant, the regulation of GnRHR gene expression most likely plays an important role in mediating the actions of GnRH in the placenta. As the first step to understand the molecular mechanisms regulating the basal GnRHR gene expression in the placenta, 2.3 kb of the human GnRHR gene 5' flanking region was characterized in two placental cell lines, JEG-3 and IEVT. Functional analysis of this region revealed the presence of several TATA and CAAT boxes residing in close proximity to one another in a cluster-like arrangement. We and others have reported the existence of multiple transcriptional start sites of human GnRHR gene using human brain and pituitary RNA (Fan et al. 1995, Kakar 1997). The presence of multiple transcriptional start sites and TATA/CAAT boxes raises the possibility of tissue-specific usage of the GnRHR promoter. Placental-specific expression of GnRHR gene can be controlled at the transcriptional level by two different mechanisms. First, the gene may use the same promoter as in other tissues coupled to a placenta-specific enhancer that binds to placental-specific transcription factors. Examples of the use of placenta-specific enhancer elements include the glycoprotein α subunit gene (Schoderbek et al. 1992, Heckert et al. 1995), human leptin gene (Bi et al. 1997), human chorionic somatomammotropin (hCS) gene (Jiang and Eberhardt 1994) and adenosine deaminase (ADA) gene (Shi et al. 1997). Second, the gene may utilize a tissue-specific promoter such as in the case of the human aromatase cytochrome P-450 gene (Yamada et al. 1995), human leukemia inhibitory factor receptor (LIFR) gene (Wang and Melmed 1998), rat growth hormone-releasing hormone gene (Gonzalez-Crespo and Boronat 1991) and human GnRH gene (Dong et al. 1997). By the use of transient transfection studies, two potential regions, a distal

and a proximal, have been identified to control the expression of the human GnRHR gene in the placental cells. Our comparative analysis of the distal and proximal elements in JEG-3 and αT3-1 cells suggests the differential usage of promoters in the placenta and the pituitary such that the placenta expression is predominantly controlled by the distal promoter and the pituitary expression is regulated by the proximal promoter. We and others have demonstrated that the SF-1 binding element was important in pituitary-specific expression of GnRHR (Duval et al. 1997, Ngan et al. 1999), glycoprotein α-subunit (Barnhart and Mellon 1994.), luteinizing hormone β subunit *in vitro* (Halvorson et al. 1996) and *in vivo* (Keri and Nilson 1996). Similar to these studies, our data further supports the role of SF-1 in expressing human GnRHR gene at the pituitary level as the SF-1 containing proximal promoter was highly active than the non-SF-1 containing distal promoter in αT3-1 cells. In addition, using RNA isolated from the JEG-3 cells, multiple transcription initiation sites for the GnRHR were found to be located in proximity to the distal and proximal promoters. Not only dose these data confirm the expression of GnRHR in the placenta, they also support the use of these promoters *in vivo*. Interestingly, all of the transcriptional start sites identified in the brain, pituitary and placental cells are located correspondingly to the TATA and/or CAAT box clusters, suggesting the importance of these regions for the formation of stable transcription initiation complexes (O'Shea-Greenfield and Smale 1992). Nevertheless, the highest promoter activity observed in JEG-3 and IEVT cells after transfected with the distal promoter, when compared to other cell-lines tested, suggest that this region is predominantly used in the placental cells.

4.3.2 Functional Characterization of Up-stream Promoter

Mapping of this upstream GnRHR promoter identified 4 major transcription factors binding sites namely hGR-Oct-1, hGR-CRE, hGR-GATA and hGR-AP-1 that were functionally involved in regulating the expression of this gene.

4.3.2.1 Octamer binding site (Oct-1)

The octamer sequence motif located between nt -1718 and -1710 (relative to translation start site) was shown to bind transcription factor Oct-1, and deletion or mutation of this motif resulted in a significant decrease in promoter activity. Oct-1 is known to be present in a variety of tissues and cell types that belongs to the POU family of transcription factors (Scheidereit et al. 1988). It has been demonstrated that the transcription regulation of hLIFR (Wang and Melmed 1998) and hCS-B (Jiang and Eberhardt 1994) genes expression in the placental cells was mediated partly through Oct-1. More interestingly, the Oct-1 has demonstrated to position the site of transcription initiation by recruiting TFIIB (Nakshatri et al. 1995). A number of studies have identified that the promoter and cell type-specific function of Oct-1 is partly due to its ability to recruit cofactors into the pre-initiation complex (Gstaiger et al. 1995, Tanaka et al. 1992) as well as other transcription factor (Ullman et al. 1993) in an octamer-site dependent manner. In fact, deletion and mutation in the hGR-Oct-1 motifs resulted in a 90% and 77% decrease in promoter activity, respectively, implicating the importance of this motif in the human GnRHR upstream promoter. Thus, it is possible that the Oct-1 binding site in the upstream promoter is also responsible for recruiting other transcription factors for the formation of initiation complex in the distal promoter. Further experimentation is needed to elucidation of the function of Oct-1 site and Oct-protein in regulating this promoter element.

4.3.2 2 Activating Protein 1 (AP-1)

The identity of the hGR-AP-1 (nt -1518 to -1511) in the human GnRHR upstream promoter was confirmed by gel mobility shift assay. Furthermore, it was shown that c-Jun and c-Fos are responsible for forming the AP-1 site-protein complex. AP-1 protein is a dimeric transcription factor consisting of either Jun/Jun homodimer or Jun/Fos heterodimers (Hai and Curran 1991, Karin et al. 1997), which has been implicated to regulate gene expression, cell

proliferation and transformation (Karin et al. 1997, Angel and Karin 1991). Regulation of gene expression via AP-1 is a complicated process involving a highly ordered cascade so to achieve specific responses to a given stimulus (Hai and Curran 1991, Karin et al. 1997). It has recently been shown that AP-1 involved in controlling the placenta-specific expression of mouse lactogen-I gene (Shida et al. 1993), rat lactogen II (Sun and Duckworth 1999), ovine P-450 side chain cleavage (*CYP11A1*) gene (Pestell et al. 1995), human NAD+-dependent 15-hydroxyprostaglandin dehydrogenase (Greenland et al. 2000), and hCS-A and B genes (Oury et al. 1997). Taken together, we believe that AP-1 plays a role in regulating the expression of human GnRHR gene in the placenta. In fact, this idea was further confirmed by the mutational studies that a significant decrease (72%) of luciferase activity was observed after mutation of the hGR-AP-1 binding site. Recently, we have also reported that the homologous transcriptional down-regulation of human GnRHR gene in the pituitary gonadotrope αT3-1 cells was mediated through an AP-1 site located between nt -1000 and –994 (Appendix I). Although it abolished the GnRH-induced down-regulation, mutation or deletion of this AP-1 site did not significantly affect the basal GnRHR promoter activity in the pituitary. Hence, AP-1 factor may lead to different responses by interacting with different AP-1 sites in different tissues. As both c-Jun and c-Fos belong to multigene families, the differential expression among these multiple gene leads to various composition of AP-1 in different cells and may result in different transcriptional activities and gene specificities (Hai and Curran 1991, De Cesare et al. 1995). In the present study, we had only examined c-Jun and c-Fos, the involvement of other AP-1 proteins in the differential regulation of various promoters is currently underway.

4.3.2.3 cAMP Responsive Element (CRE)

Our data strongly support that the motifs hGR-CRE (nt -1649 to -1641) and hGR-GATA (nt -1602 to -1597) are important for the placenta-specific expression of the human GnRHR

gene. From gel mobility shift assays and antibody "supershift" assays, the binding of CREB to the CRE was confirmed. In human trophoblast cells, cAMP plays a critical role in controlling placenta-specific gene expression. Characterization of the corticotropin-releasing hormone gene promoter indicated that the CRE is essential for gene expression since mutation or deletion of this *cis*-acting element resulted in the loss of expression in the placenta (Scatena and Adler 1998). The glycoprotein hormone α-subunit is expressed in both the pituitary and placenta and the molecular mechanisms controlling its expression have been studied extensively. Analysis of the human α-subunit promoter in placental cells revealed a placenta-specific enhancer region that contains two juxtaposed CRE and an upstream response element (URE). Further studies have identified that the URE contains the αACT, the trophoblast-specific element (TSE) and the URE1 (Heckert et al. 1995, Pittman et al. 1994). Here, the αACT element binds to GATA-2 and GATA-3 (Steger et al. 1994). However, the gonadotrope-specific expression of the α-subunit gene requires different pituitary-specific factors including a pituitary glycoprotein hormone basal element (PGBE), the SF-1, the αBE1, the αBE2 and CRE (Horn et al. 1992, Heckert et al. 1995). It is clear that the CRE of the human α-subunit promoter play an important role in the expression of this gene in both placental and pituitary cells. Similarly, the human GnRHR is also expressed in both pituitary and placenta. We have demonstrated in the present study that the placenta-specific expression of GnRHR is also controlled by the CRE since mutation of the hGR-CRE (nt -1649 to -1641) in the upstream promoter led to a 38% decrease in luciferase activity. Interestingly, the upstream promoter did not confer the gonadotrope-specific expression of the human GnRHR gene, suggesting that this CRE does not participate in pituitary expression of the gene. Instead, the proximal promoter, which contains the SF-1 site, is responsible for the gonadotrope-specific expression (Ngan et al. 1999 and present observation).

4.3.2.4 GATA binding protein

In addition to the CRE, placenta-specific expression of human α-subunit gene is also controlled by GATA-binding protein. GATA-2 and GATA-3 have been reported to be present in JEG-3 cells and to regulate the human gonadotropin α-subunit gene (Steger et al. 1994) and human 17β-hydroxysteroid dehydrogenase type 1 gene (Piao et al. 1997) expression in the placenta. In agreement to these results, our data demonstrated the involvement of the GATA elements in controlling the placenta-expression of the human GnRHR gene. Mutation of the hGR-GATA (nt -1602 to -1597) decreased the upstream promoter activity. By the use of gel mobility shift assay, both GATA-2 and GATA-3 were found to interact to this region. In addition, mutation of this region leads to the decrease in promoter activity only in JEG-3 cells but not in HEK293, OVCAR-3 and COS-1 cells. Similarly, the trophoblast-specific expression of mouse placental lactogen I (PL-I) was found to be controlled by GATA-2 and GATA-3 (Ng et al. 1994) and AP-1 (Shida et al. 1993). The analysis of mouse proliferin (PLF) gene promoter has also revealed a functionally critical AP-1 element (.Mordacq and Linzer 1989) and the presence of GATA-2/3 binding sites (Ng et al. 1994). GATA-2 and GATA-3 knock out mice revealed that placenta lacking GATA-2 and GATA-3 led to a markedly reduction of both PL-I and PLF mRNA expression (Ma et al. 1997). These results further support that the GATA-2 and GATA-3 are important as *in vivo* regulators for placenta-specific gene expression. In both PL-I and PLF promoters, the AP-1 sites were in close proximity to the GATA elements, suggesting that these factors might work together in a complex to regulate the expression of both genes. Likewise, the hGR-GATA and hGR-AP-1 binding sites are also located in proximity to each other, suggesting that similar mechanism may also exist in the human GnRHR gene.

In summary, we have identified an upstream promoter in the human GnRHR gene that is primarily utilized in the placental cells. Mutation studies have identified four transcription elements including Oct-1, CRE, GATA and AP-1, in which the CRE and GATA elements were

showed to be placenta-specific. The identification of CRE and AP-1 motifs in the upstream GnRHR promoter provide a means for regulating the expression of this gene by the PKC and PKA pathways in the transcriptional levels.

CHAPTER 5. REGUALTION OF HUMAN GnRHR GENE TRANSCRIPTION BY PROGESTERONE

5.1 Introduction

Although there is evidence that progesterone (P) inhibits expression of ovine GnRHR gene expression (Chapter 1, Section 1.4.2), no information is available in the role of P in regulating human GnRHR gene expression. Using primary cultures of ovine pituitary cells, P reduced the binding of GnRH (Laws et al. 1990) and decreased amount of GnRHR mRNA (Sakurai et al. 1997, Wu et al. 1994, Kirkpatrick et al. 1998). In light of these studies, it is reasonable to believe that the P can directly regulate the expression of the human GnRHR. As the GnRHR mRNA was detected in both pituitary and placenta, and the placenta is a major site of P secretion, the present study was aimed to understand the molecular mechanism underlying the regulatory role of P on GnRHR gene transcription.

The physiologic effects of P are mediated through a specific nuclear receptor protein, progesterone receptor (PR). Two major isoforms of PR, namely PR-A and PR-B have been described. The large PR-B form contains addition 164-amno acids at the N-terminal that is missing in the truncated PR-A form (Clemm et al. 1995). Hormonal binding to PRs results in the dissociation of heat shock proteins, thereby allowing receptors to form dimers and bind specific nucleotide sequences known as progesterone response element (PRE) (Evans 1988, Tsai and O'Malley 1994). Receptor interaction with PRE can result in either increase or decrease in gene transcription. In cell transfection systems, the two PR isoforms have distinct transcriptional properties. In general, PR-B acts as a stronger transcriptional activator, whereas the transcriptional activity of PR-A was cell- and gene-specific dependence. Interestingly, PR-A also functions as a transcriptional inhibitor of PR-B as well as other steroid receptor family

when PR-A itself is transcriptional inactive (Tora et al. 1988, Vegeto et al. 1993, McDonnell et al. 1994).

5.2 Treatments

Progesterone, mifepristone (RU486), DL-aminoglutethimide (AGT) were purchased form Sigma (Sigma-Aldrich Ltd., Canada). In experiments wherein the effects of P, RU486 on luciferase activity were studied, the cells were treated with corresponding drugs for 24h before luciferase and β-galactosidase activities were measured. Progesterone production in JEG-3 was inhibited by addition of AGT during transfection and the secreted P levels were measured by radioimmunoassay as described (Chapter 2, Section 2.15).

5.3 Results

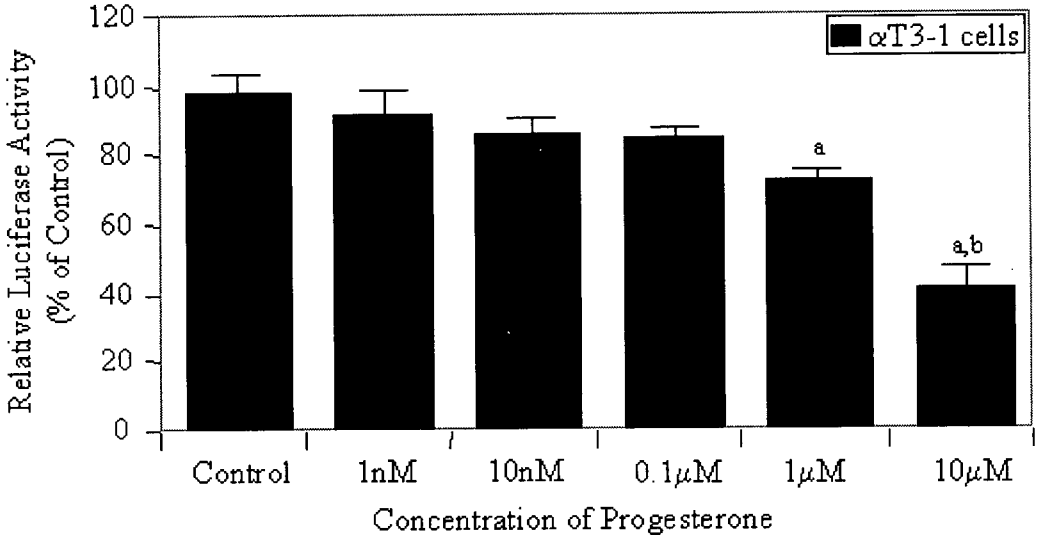
5.3.1 Progesterone regulates human GnRHR promoter activity

To examine the transcriptional regulation of human GnRHR gene expression by progesterone in the pituitary and placenta, full-length human GnRHR promoter-luciferase construct (p2300F-Luc) was transiently transfected into αT3-1 and JEG-3 cells respectively, and treated with increasing concentration of P for 24h. Although a slight decreased (14%, $P>0.05$) in promoter activity in αT3-1 cells was observed with 10nM P treatment, a statistical significant decrease of promoter activity was achieved when treated with 1μM (28%, $P<0.01$) and 10μM (59%, $P<0.001$) of P, respectively (Fig. 5.1A). In addition, this inhibitory effect was showed to be time dependent since the degree of inhibition increased with time of treatment (Fig. 5.1B). These results suggested an inhibitory effect of P on human GnRHR gene transcription at the pituitary level. In contrast, no change in GnRHR promoter activity was observed in JEG-3 cells (Fig. 5.2). As JEG-3 cells produce a high level of P endogenously, we next used a P antagonist, RU486, to indirectly examine the effects of P on human GnRHR promoter activity in JEG-3

cells. Interestingly, a dose- and time-dependent decrease in GnRHR promoter activity were observed after addition of RU486 (Fig. 5.3A and B), suggesting that the P was important in maintaining the expression of human GnRHR gene expression in the placenta. To further confirm the stimulatory role of P in the placenta-expression of human GnRHR, the endogenously production of P from JEG-3 was inhibited by the addition of AGT. A dose-dependent inhibition on P production was observed and the maximum inhibition was achieved at 0.1mM AGT (Fig. 5.4A). Furthermore, inhibition of P production by ATG resulted in a 30% decrease ($P<0.001$) in human GnRHR promoter activity and this decrease in promoter activity was reversed by the replacement of P (Fig. 5.4B). These results strongly implicate a stimulatory role of P in the expression of human GnRHR gene in the placenta.

FIG. 5.1 Dose- and time-dependent regulation of human GnRHR-luciferase vector (p2300-LucF) activity in αT3-1 cells treated with progesterone. *Panel A*, the p2300-LucF transfected cells were treated with varying concentration of progesterone (1 nM to 10 μM) for 24h. *Panel B*, the p2300-LucF transfected cells were treated with 10 μM progesterone for the indicated time points. Relative promoter activity is shown as percentage of control after normalized to β-galactosidase activity. Values represent mean +/- S.E. from triplicate assays in three separate experiments. a, $P<0.01$ from control; b, $P<0.01$ vs the immediately adjacent group on the left.

A


αT3-1 cells
Relative Luciferase Activity (% of Control)
120
100
80
60
40
20
0
a
a,b
Control
1nM
10nM
0.1μM
1μM
10μM
Concentration of Progesterone

B

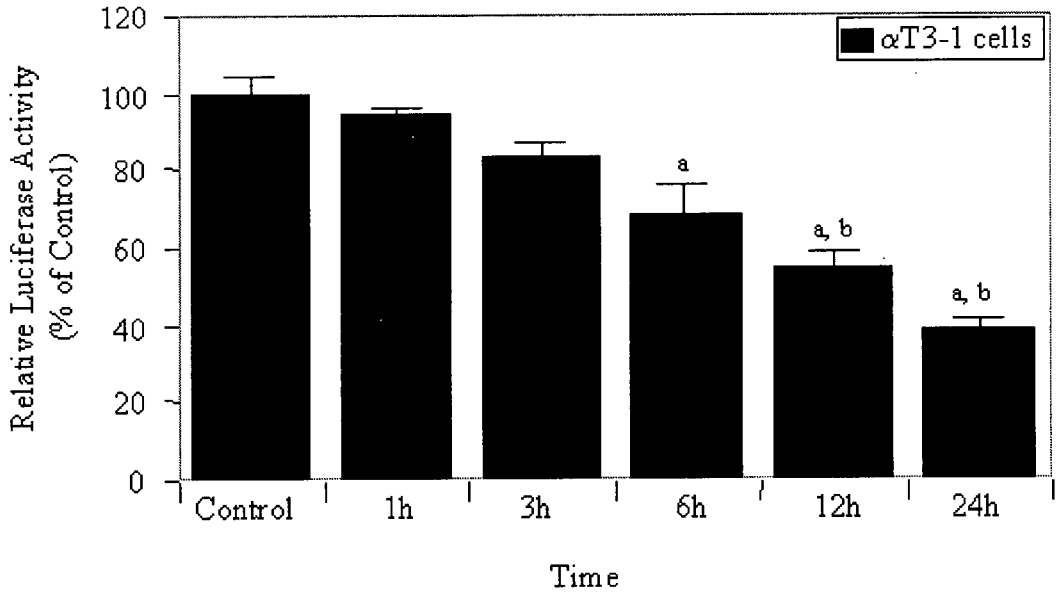
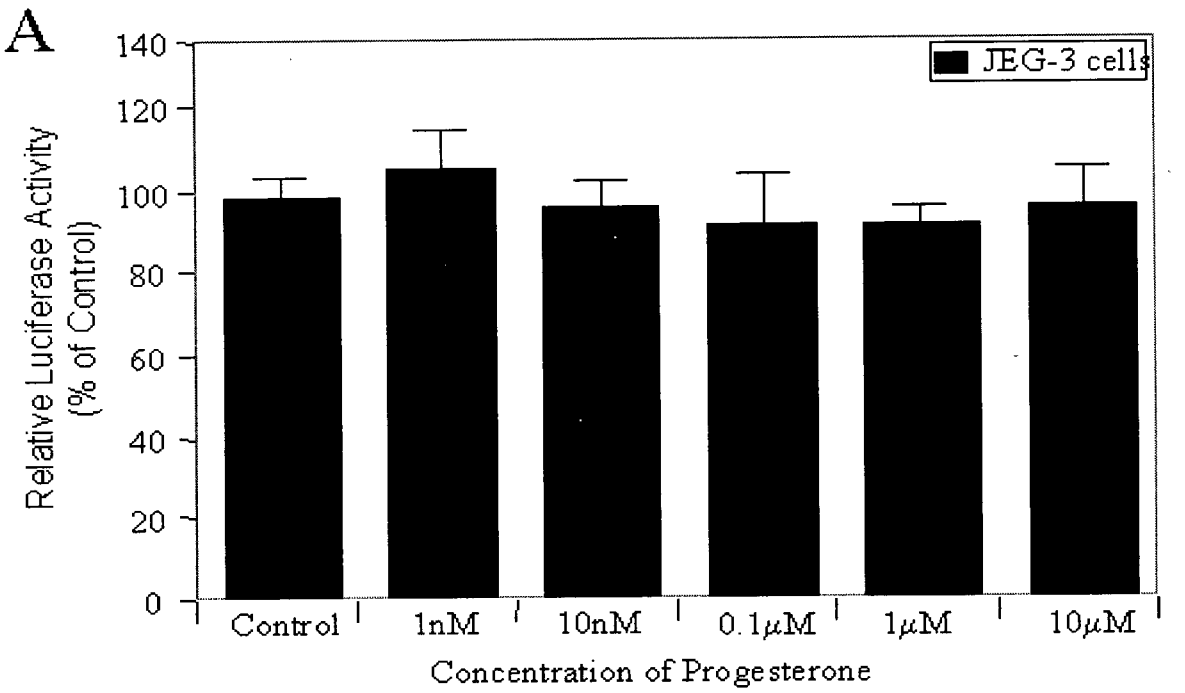

αT3-1 cells
Relative Luciferase Activity (% of Control)
120
100
80
60
40
20
0
a
a, b
a, b
Control
1h
3h
6h
12h
24h
Time

FIG. 5.2 Regulation of human GnRHR-luciferase vector (p2300-LucF) activity in JEG-3 cells treated with progesterone. *Panel A*, the p2300-LucF transfected cells were treated with varying concentration of progesterone (1 nM to 10 μM) for 24h. *Panel B*, the p2300-LucF transfected cells were treated with 10 μM progesterone for the indicated time points. Relative promoter activity is shown as percentage of control after normalized to β-galactosidase activity. Values represent mean +/- S.E. from triplicate assays in three separate experiments.


A
JEG-3 cells
Relative Luciferase Activity
(% of Control)
140
120
100
80
60
40
20
0
Control
1nM
10nM
0.1μM
1μM
10μM
Concentration of Progesterone

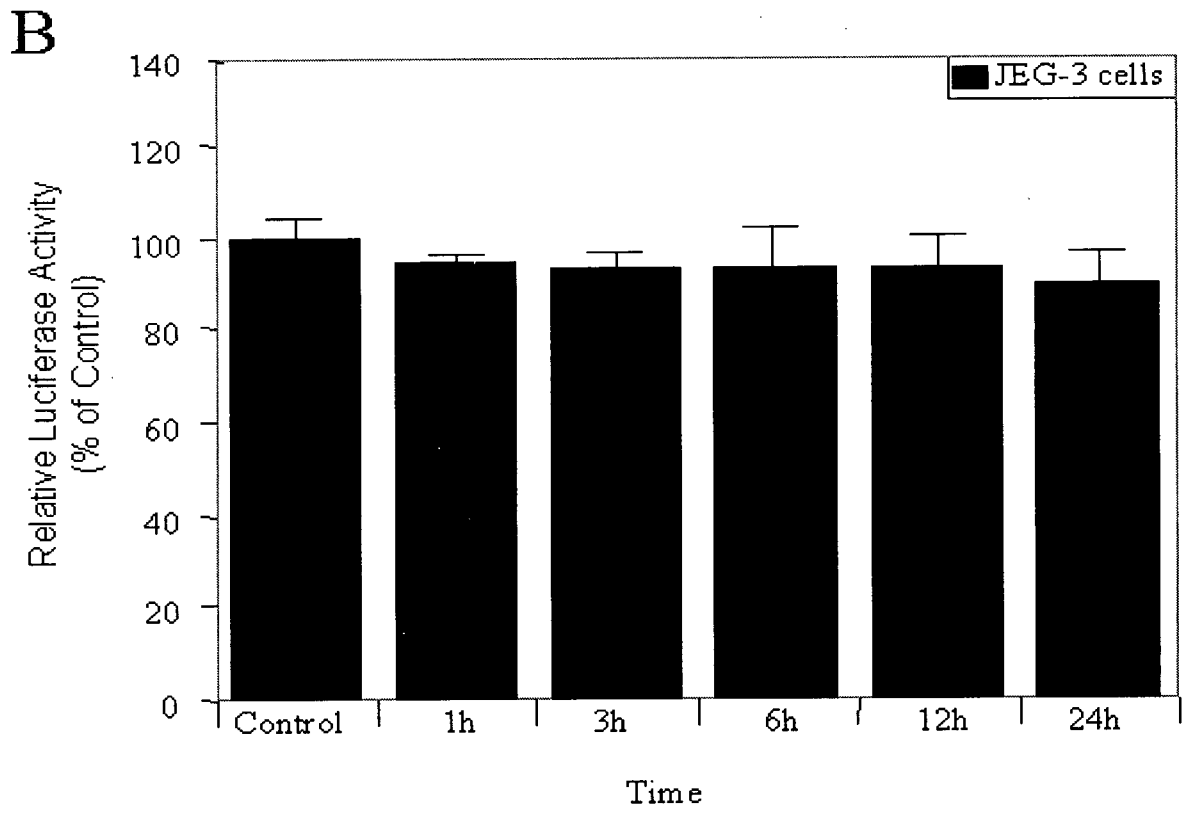
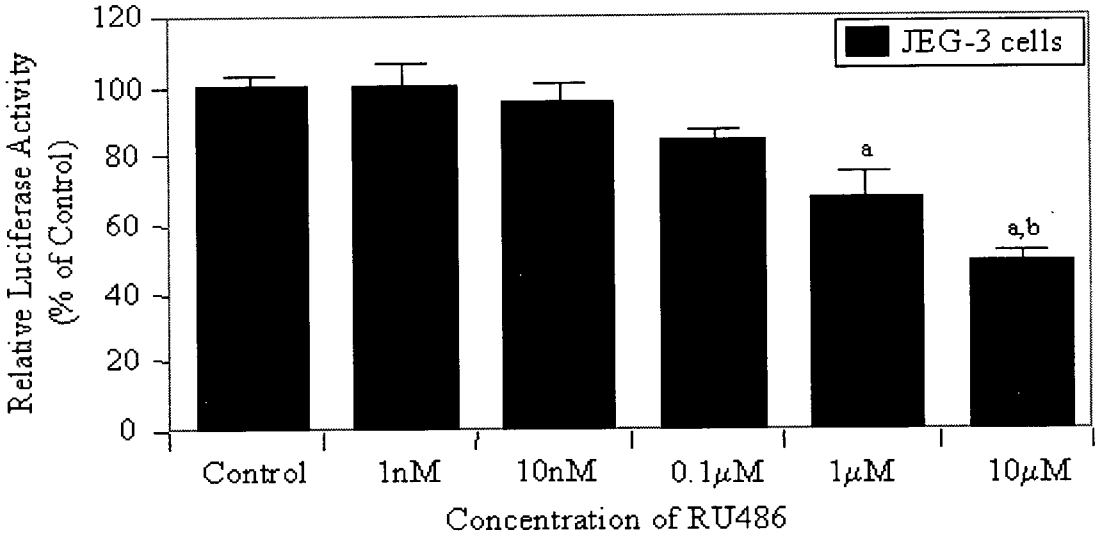

B
JEG-3 cells
Relative Luciferase Activity
(% of Control)
140
120
100
80
60
40
20
0
Control
1h
3h
6h
12h
24h
Time

FIG. 5.3 Dose- and time-dependent regulation of human GnRHR-luciferase vector (p2300-LucF) activity in JEG-3 cells treated with RU486. *Panel A*, the p2300-LucF transfected cells were treated with varying concentration of RU486 (1 nM to 10 μM) for 24h. *Panel B*, the p2300-LucF transfected cells were treated with 10 μM RU486 for the indicated time points. Relative promoter activity is shown as percentage of control after normalized to β-galactosidase activity. Values represent mean +/- S.E. from triplicate assays in three separate experiments. a, $P<0.01$ from control; b, $P<0.01$ vs the immediately adjacent group on the left.

A


JEG-3 cells
Relative Luciferase Activity (% of Control)
120
100
80
60
40
20
0
a
a,b
Control
1nM
10nM
0.1μM
1μM
10μM
Concentration of RU486

B

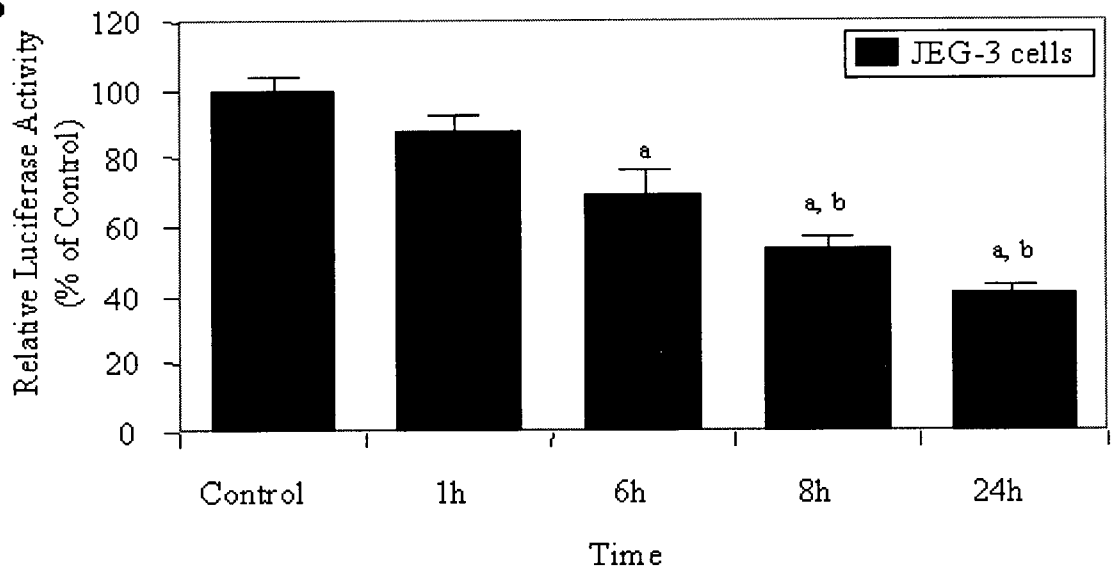
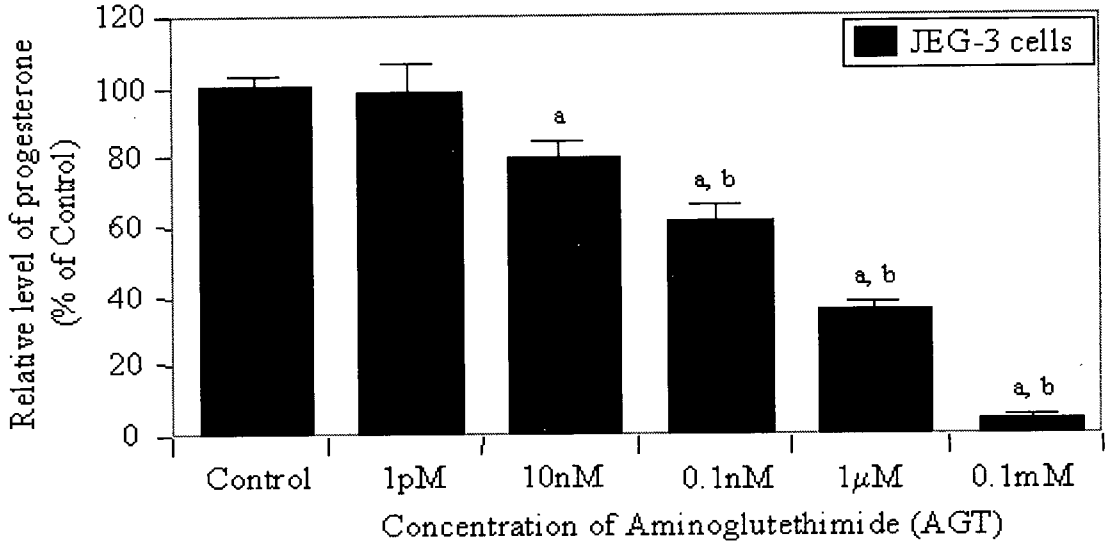
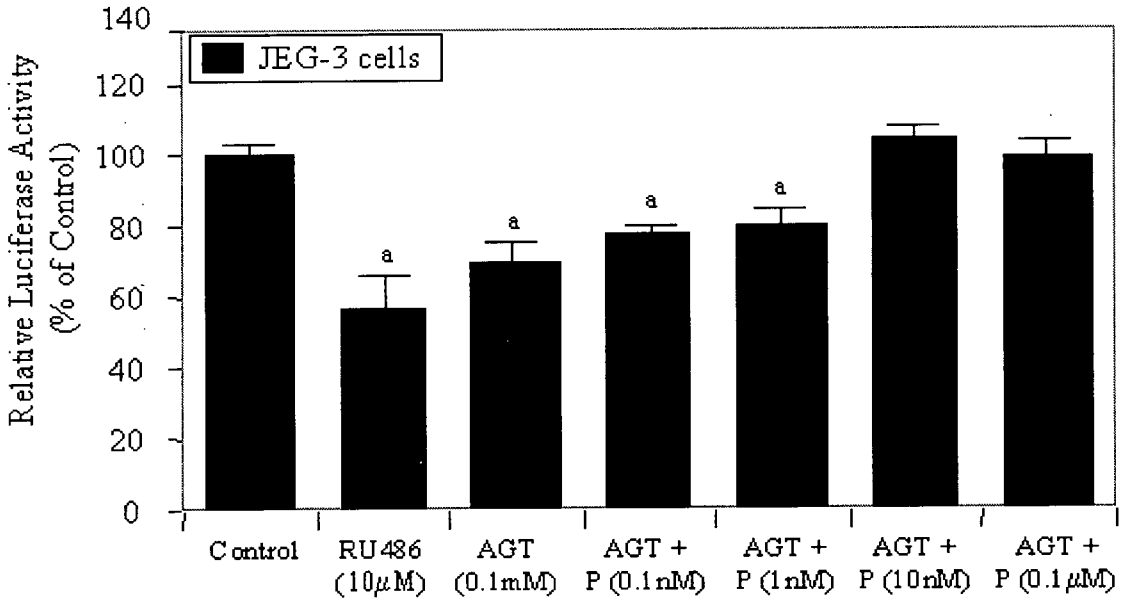

JEG-3 cells
Relative Luciferase Activity (% of Control)
120
100
80
60
40
20
0
a
a, b
a, b
Control
1h
6h
8h
24h
Time

FIG. 5.4 Effects of progesterone (P) in human GnRHR-luciferase vector (p2300-LucF) activity in JEG-3 cells. *Panel A*, human choriocarcinoma JEG-3 cells were treated with varying concentration (1 pM to 0.1 mM) of DL-aminoglutethimide (AGT) for 24h. The production of progesterone was measured by radioimmunoassay. Values represent mean +/- S.E. from triplicate assays in two separate experiments. a, $P<0.01$ from control; b, $P<0.001$ vs the immediately adjacent group on the left. *Panel B*, the production of progesterone from JEG-3 cells was inhibited with 0.1 mM AGT treatment during transfection. After transfection, the cells were incubated for additional 24h in the presence of 0.1 mM AGT and increasing concentration of P (0.1 nM to 0.1 μM). Relative promoter activity is shown as percentage of control after normalized to β-galactosidase activity. Values represent mean +/- S.E. from triplicate assays in three separate experiments. a, $P<0.01$ from control.


JEG-3 cells
Relative level of progesterone (% of Control)
120
100
80
60
40
20
0
a
a, b
a, b
a, b
Control
1pM
10nM
0.1nM
1μM
0.1mM
Concentration of Aminoglutethimide (AGT)


JEG-3 cells
Relative Luciferase Activity (% of Control)
140
120
100
80
60
40
20
0
a
a
a
a
Control
RU486 (10μM)
AGT (0.1mM)
AGT + P (0.1nM)
AGT + P (1nM)
AGT + P (10nM)
AGT + P (0.1μM)

5.3.2 Progesterone regulates GnRHR mRNA levels in human placental cells

The stimulatory role of P in human GnRHR gene expression was further examined by the quantitative RT-PCR methodology. Total RNA was isolated from JEG-3 cells after RU486, AGT, or 0.1 mM AGT plus 0.1 μM P treatment. The relative GnRHR mRNA level was decreased by 14% ($P<0.05$) and 33% ($P<0.01$) after 0.1 μM and 10 μM RU 486 treatment, respectively (Fig. 5.5). Similarly, inhibition of P production in JEG-3 cells by 0.1 mM AGT treatment resulted in a 20% ($P<0.05$) decrease in GnRHR mRNA levels (Fig. 5.5). In addition, replacement of P in AGT-treated JEG-3 cells reversed the reduction in GnRHR mRNA levels induced by the blockage of endogenously P production, indicating the stimulatory role of P in placental GnRHR gene expression (Fig. 5.5).

5.3.3 Localization of progesterone response region in human GnRHR 5' flanking region

To localize a specific region that mediates the progesterone effects on the 2.3kb 5' flanking region of the human GnRHR gene, a series of 5' deletion mutants were analyzed in αT3-1 and JEG-3 cells and treated with 10 μM P and 10 μM RU486, respectively. Transient transfection studies showed similar results for both cell-lines (Fig. 5.6). Progressive 5'-deletion up to nt -577 did not affect the P- and RU486-induced inhibition in the human GnRHR promoter activity in αT3-1 and JEG-3 cells, respectively (Fig. 5.6B and C). Further deleting the sequence from nt -577 to -227 (relative to translation start site) resulted in a loss of the response in P- and RU486-induced inhibition in both cells. Taken together, these data suggest that the 350bp region (located between nt -577 and -227) containing the putative transcription factor(s) binding site that was important in mediating the P-mediated effects on human GnRHR promoter activity in both pituitary and placenta.

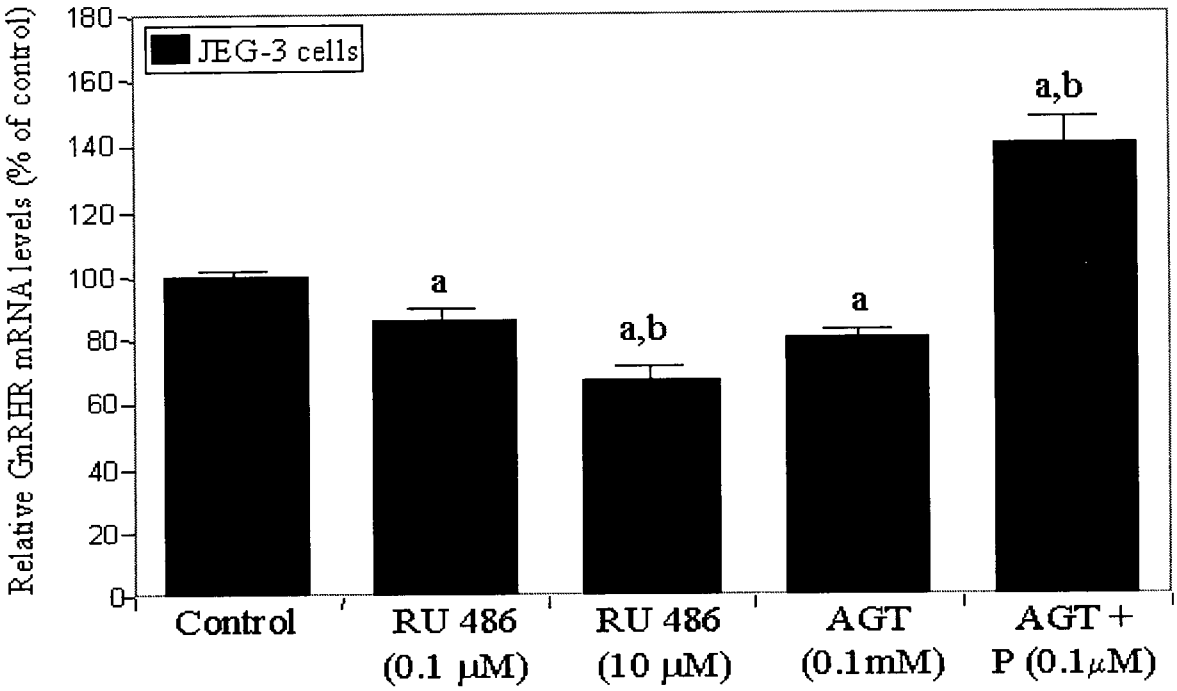
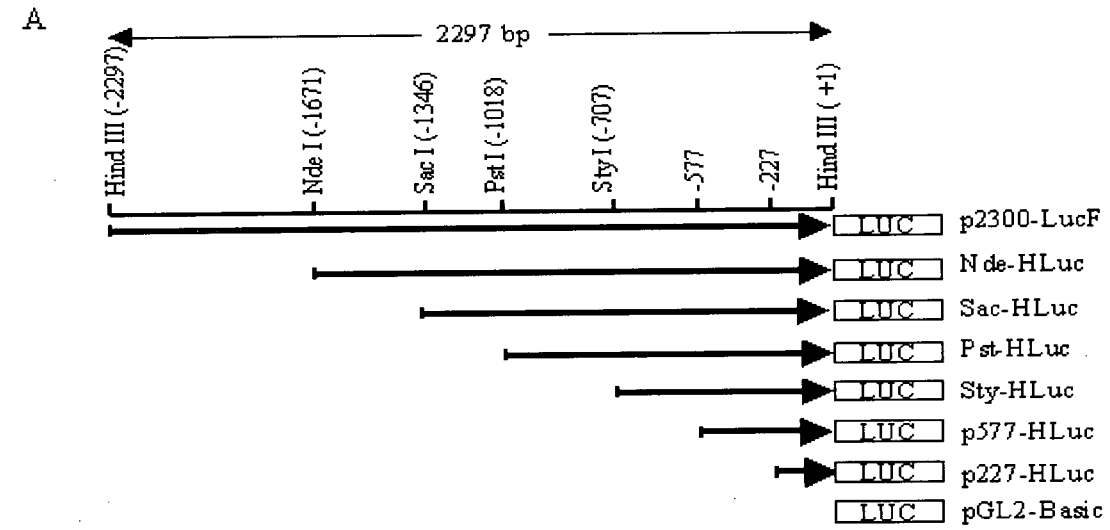
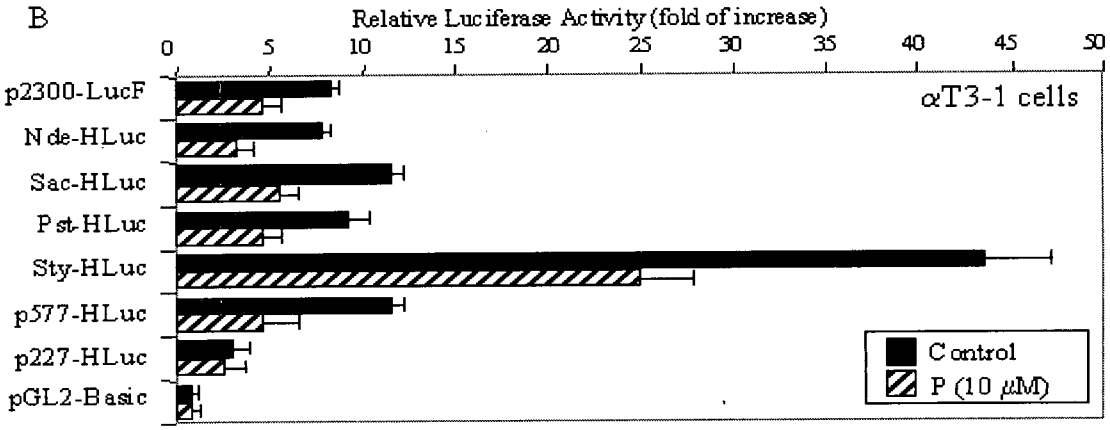
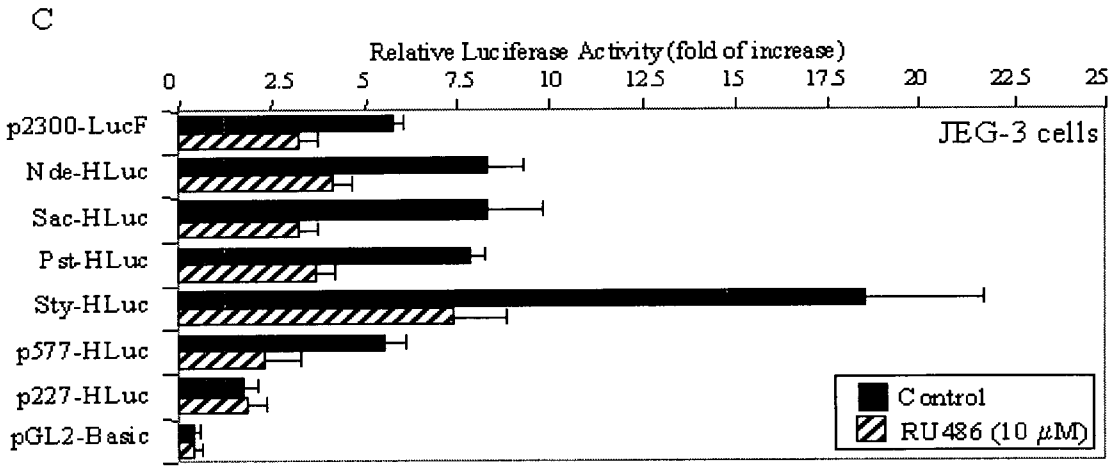


FIG. 5.5 Effects of RU 486, DL-aminoglutethimide (AGT) and progesterone (P) on GnRHR expression in JEG-3 cells. Cells were treated with vehicle (control), 0.1 μM RU 486, 10 μM RU486, 0.1 mM AGT, or 0.1 mM AGT plus 0.1 μM P for 24h before total RNA isolation and analyzed for GnRHR mRNA by semi-quantitative RT-PCR assays. GAPDH mRNA was used as an internal control. The GnRHR mRNA signal was normalized to the GAPDH internal control for each sample. Results are the mean ± SD from three individual experiments and represented as percentage of control. a, $P<0.05$ from control; b, $P<0.05$ vs the immediately adjacent group on the left.

FIG. 5.6 Localization of the progesterone responsive region in the human GnRHR gene. *Panel A*, diagrammatic representation of progressive 5' deletion constructs of p2300-LucF. Deletion mutants were transiently transfected into αT3-1 (*Panel B*) and JEG-3 (*Panel C*) cells by calcium phosphate precipitation method and treated with 10 μM progesterone (P) and 10 μM RU486, respectively, for 24h. The RSV-LacZ vector was co-transfected to normalize for varying transfection efficiencies. Relative promoter activity of each construct is shown as fold increase over a promoterless luciferase control pGL2-Basic, whose activity is set to be 1, after normalized to β-galactosidase activity. Values represent mean +/- S.E. of triplicate assays in three separate experiments.


A
2297 bp
Hind III (-2297)
Nde I (-1671)
Sac I (-1346)
Pst I (-1018)
Sty I (-707)
-577
-227
Hind III (+1)
LUC
p2300-LucF
Nde-HLuc
Sac-HLuc
Pst-HLuc
Sty-HLuc
p577-HLuc
p227-HLuc
pGL2-Basic


B
Relative Luciferase Activity (fold of increase)
0
5
10
15
20
25
30
35
40
45
50
p2300-LucF
Nde-HLuc
Sac-HLuc
Pst-HLuc
Sty-HLuc
p577-HLuc
p227-HLuc
pGL2-Basic
αT3-1 cells
Control
P (10 μM)


C
Relative Luciferase Activity (fold of increase)
0
2.5
5
7.5
10
12.5
15
17.5
20
22.5
25
p2300-LucF
Nde-HLuc
Sac-HLuc
Pst-HLuc
Sty-HLuc
p577-HLuc
p227-HLuc
pGL2-Basic
JEG-3 cells
Control
RU486 (10 μM)

5.3.4 Identification of transcription binding sites

DNA analysis of the DNA region between -577 and -227 identified one putative PRE binding site, namely hGR-PRE (5' TCAACAGTGTGTTTG3' located at nt -535 to -521; with 75% homology to the consensus PRE site) and a half PRE binding site, namely hGR-hPRE (5'AGAACA3' located at nt -402 to -397; with 100% homology to the half consensus PRE site). To examine the role of these putative PRE motifs in controlling the expression of GnRHR gene, the two putative PRE motifs were mutated in Sty-HLuc, as the highest promoter activity was obtained from this construct (Fig. 5.6). The mutation was introduced by three-steps PCR mutagenesis method (Chapter 2, Section 2.11). The putative transcription factor binding sequence was replaced by a non-related sequence to disturb specific transcription factor binding. Mutation of the putative hGR-PRE did not only result in reducing the P-induced inhibition but also increased the basal luciferase activity in αT3-1 cells (Fig. 5.7A). A 60% increase ($P<0.001$) in basal promoter activity was observed after site-directed mutation of the hGR-PRE but not in mutated hGR-hPRE. Although the mutation of hGR-PRE significantly reduced the P-induced decrease in promoter activity from 54% to 19% ($P<0.001$), it did not completely eliminate the P-induced inhibition on promoter activity. Mutation of hGR-hPRE did not affect the basal promoter activity, or eliminate the P-mediated action in transfected αT3-1 cells.

In JEG-3 cells, mutation of hGR-PRE lead to a 35% decrease ($P<0.001$) in basal promoter activity (Fig. 5.7B). Again, no such reduction in promoter activity was observed in mutated hGR-hPRE. In addition, mutation of hGR-PRE completely abolished the RU486-induced decrease in promoter activity in the placental cells (Fig. 5.7B). Taken together, these results suggested that the hGR-PRE located at nt -535 to -521 plays an important role in mediating P effect on GnRHR transcription.

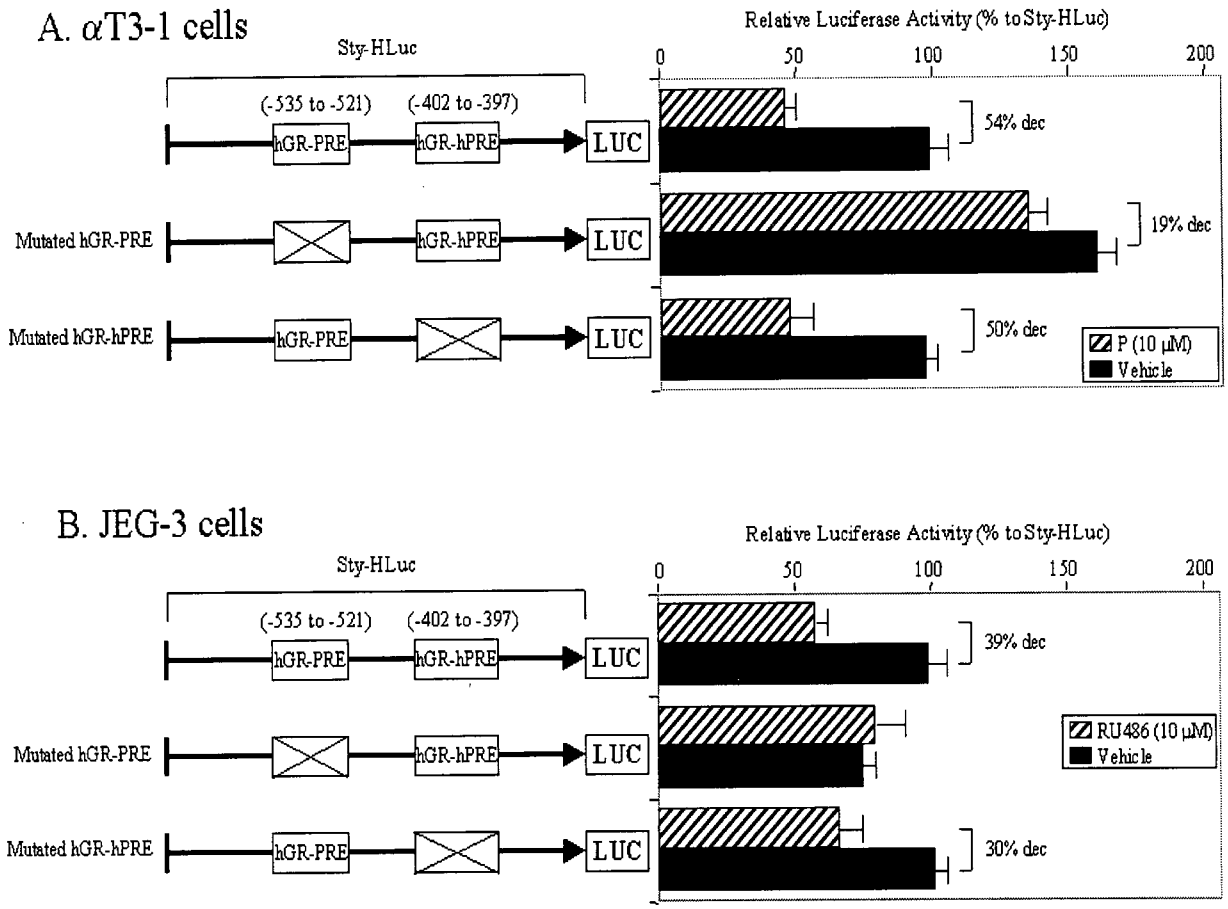


FIG. 5.7 Functional analysis of putative progesterone response element (PRE) in human GnRHR promoter. Mutations were introduced by 3-steps PCR mutagenesis as described in *Materials and Methods*. The wild-type and mutated promoter constructs were co-transfected with RSV-LacZ vector, to normalize for varying transfection efficiencies, into αT3-1 and JEG-3 cells. *Panel A*, the transfected αT3-1 cells were treated with 10 μM of progesterone (P) for 24h. *Panel B*, the PRE mutants transfected JEG-3 cells were treated with 10 μM RU486 for 24h. The relative basal activity of each promoter mutants is shown as percentage of vehicle treated (control) Sty-HLuc, whose activity is taken as 100%, after normalized to β-gal activity. The percentage of inhibition is calculated by comparing to individual control. Values represent mean +/- S.E. of triplicate assays in three separate experiments. The names and the relative position of the putative transcription factors binding sites are given and the mutated element was shown as *cross box*. The relative change in promoter activity after treatment was indicated.

5.3.5 Effects of co-transfection of progesterone receptor on human GnRHR promoter activity in αT3-1 and JEG-3 cells

The expression of PRs in αT3-1 and JEG-3 cells were examined by Western blot analysis. Aliquots of protein, 50 μg for αT3-1 cells and 30 μg for JEG-3 cells, was separated in SDS-PAGE and detected with corresponding antibodies. As seen in Fig. 5.8A, both PR-A and PR-B were expressed in αT3-1 and JEG-3 cells using protein isolated from two different cultures. Interestingly, the levels of PR-A in JEG-3 cells was very low when compared to PR-B levels. Although both PR-A and PR-B isoforms was detected in αT3-1 cells, the PRs levels in αT3-1 cells was relatively lower than that in JEG-3 cells. To further evaluate the role of human PR-A and PR-B in mediating the P action at the pituitary and placenta levels, the human PR-A and PR-B expression constructs were co-transfected into αT3-1 and JEG-3 cells. Co-transfection of human PR-A and PR-B expression vectors resulted in an increase in PR-A and PR-B levels (Fig. 5.8B).

No significant change in promoter activity was observed after treated with 0.1 μM P for 24h at the Sty-HLuc transfected αT3-1 cells (Fig. 5.9). However, over-expression of human PRs in αT3-1 cells increased the sensitivity towards P treatment. A significant decrease (52%, $P< 0.001$) in promoter activity was achieved in 0.1 μM of P treatment after co-transfected with 1 μg of PR-B expression vector (Fig. 5.9A). Similarly, co-transfection with 1 μg of PR-A expression vector resulted in a slightly decrease (20%, $P<0.05$) in the promoter activity after treated with 0.1 μM of P. Nevertheless, these data suggest that PR-B play a more active role than PR-A in mediating P-induced inhibitory effects on the pituitary, since a higher inhibition in promoter activity was observed in PR-B co-transfected cells after P treatment. The functional role of the PRE in responding to the human PRs in mediating the P-induced decrease in promoter activity at the pituitary was also examined in the hGR-PRE and hGR-hPRE mutated constructs. Again, an increase in basal promoter activity was observed after the mutation of hGR-PRE, and this

mutation eliminated and reduced the degree of inhibition induced by 0.1 μM P after over-expression of human PR-A and PR-B (from 52% to 17%), respectively (Fig. 5.9A).

In the placenta JEG-3, over-expression of hPR-A resulted in a 51% decrease ($P<0.001$) in basal promoter activity (Fig. 5.9B). In contrast, over-expression of hPR-B lead to an increase (39%, $P<0.001$) in promoter activity (Fig. 5.9B). In addition, co-expression of hPR-A and hPR-B counteract each other function in regulating the promoter activity. These results suggested that the balance between PR-A and PR-B expression in the placenta plays an important role in controlling the expression of this gene, whereas PR-B stimulates and PR-A inhibits the promoter activity. Further studies have shown that the hGR-PRE solely mediated the hPR-B stimulated increase in human GnRHR promoter activity as the mutation of this binding site eliminated the increase in luciferase activity (Fig. 5.9B). Although the mutation of hGR-PRE decreased the inhibitory effect of over-expression of hPR-A (from 51% to 23%), it did not completely eliminate this effect implicating that an addition regulatory mechanism is involved in mediating the PR-A action.

5.3.6 Binding of PR to the putative hGR-PRE.

To confirm the identity of the transcription factor bound to the hGR-PRE, gel mobility shift assay was preformed with a synthetic oligodexoynucleotide containing the putative hGR-PRE binding site in the presence of consensus and mutated PRE, hGR-PRE, non-related oilgodeoxynucleotide or antibody against the PR. Specific DNA-protein complexes were observed using the nuclear extract isolated from αT3-1 cells, after cotransfected with both human PR-A and PR-B expression vectors and treated with P, (Fig. 5.10A, indicated as arrows 1, 2 and 3). Similarly, specific DNA-protein complexes were obtained using nuclear extract isolated from JEG-3 cells (Fig. 5.10B, indicated as arrows 4 and 5). These complexes were eliminated (Fig. 5.10A and B) with the addition of increasing competitor DNA fragment (200

fold in excess) containing a consensus PRE (lane 3) and hGR-PRE (lane 5) but not with a competitor containing mutated PRE (lane 2), mutated hGR-PRE (lane 4) or non-related sequence NF-κB (lane 6) or TFIID (lane 7). Furthermore, the addition of an antibody against the PR "supershifted" the DNA-protein complexes, further supporting the binding of the PR to this binding site (Fig. 5.11A and B).

FIG. 5.10 Gel mobility assay of the putative hGR-PRE binding sites in the human GnRHR gene. Synthetic deoxyribonucleotide containing the putative PRE sequences (hGR-PRE) in human GnRHR gene was P^{32} labeled and incubated with nuclear extract isolated from human PR-A and PR-B co-transfected, progesterone treated αT3-1 cells (*Panel A*) or JEG-3 cells (*Panel B*) in the presence of 200 fold excess indicated competitor oligonucleotide. Specific DNA-protein complexes formed to the hGR-PRE in nuclear extract isolated from αT3-1 (indicated as arrow 1, 2 and 3) and JEG-3 (indicated as arrow 4 and 5) cells were eliminated in the presence of competitor oligonucleotide (lane 1, no competitor; lane 2, mutated consensus PRE; lane 3, consensus PRE; lane 4, mutated hGR-PRE; lane 5, hGR-PRE; lane 6, consensus NF-κB and lane 7, consensus TFIID).

FIG. 5.11 Identification of progesterone receptor (PR) binding to the hGR-PRE. Gel mobility assay studies were performed as in FIG. 10 in the presence of antibodies specific to PR, Oct-1 and GATA-2. Antibodies were added 1h prior to the addition of nuclear extract isolated from human PR-A and PR-B cotransfected, progesterone treated αT3-1 cells (*Panel A*) and JEG-3 cells (*Panel B*). The P^{32}-labelled hGR-PRE probe was incubated in the presence of antibody against IgG (lane 1), PR (lane 2), Oct-1 (lane 3) and GATA-2 (lane 4). DNA-protein complexes were "supershifted" by the addition of antibody against PR (indicated as arrow Shifted) but not by antibodies against IgG, Oct-1 and GATA-2.

5.4 Discussion

While P is the dominant steroid present in the circulation during human gestation, pituitary responsiveness to GnRH is reduced during this period (Reyes et al. 1976, Rubinstein et al. 1978,Sowers et al. 1978). It has been showed that P negatively regulated the hypothalamic-pituitary functions through a negative feedback mechanism in animals (Sagrillo et al. 1996, Schumacher et al. 1999) and in human (Poindexter et al. 1993, Alexandris et al. 1997). Recent studies in the ovine have demonstrated that the numbers of GnRHR were relatively lower during the luteal phase of the estrous cycle (Crowder and Nett 1984) and induction of luteolysis by prostaglandin F2α resulted in an increase in both GnRHR numbers and mRNA levels *in vivo* (Turizillo et al. 1994), suggesting a negative regulatory effect of P in GnRHR expression. In subsequent studies using primary culture ovine pituitaries, a decrease in GnRHR mRNA levels was observed after P-treatment (Sakurai et al. 1997,Wu et al. 1994, Kirkpatrick et al. 1998) further supporting a role of P in regulating the ovine GnRHR gene expression.

5.4.1 P regulates human GnRHR promoter activity

We have observed a P-induced decrease in human GnRHR promoter activity at the pituitary cells. This observation was in agreement with the results obtained from the studies in the ovine that P negatively regulates the expression of GnRHR gene. Interestingly, this P-induced inhibition on human GnRHR promoter activity could not be observed in the placental JEG-3 cells, which also possess the GnRH-GnRHR system (Chapter 3). Instead, using RU486 to block endogenous P action and AGT to inhibit P production resulted in a decrease in human GnRHR promoter activity, suggesting the importance of P in maintaining the expression of GnRHR at the placenta. GnRH has been shown to stimulate the secretion of hCG from the placenta (Merz et al. 1991) which is important in maintaining the early pregnancy by stimulating the P production from corpus luteum (Fritz and Speroff 1982). The lack of the negative

regulatory system in the expression of placental GnRHR by the P may help sustain the GnRH-stimulated hCG production through pregnancy.

5.4.2 Progesterone response element in human GnRHR promoter

Progressive 5' deletion of the human GnRHR 5' flanking region and mutational studies have identified a putative PRE, namely hGR-PRE located at -535 to -521, which participates in P-mediated action. Our data showed that the very same region mediated the P-induced inhibition and stimulation actions on pituitary and placental cells, respectively. Identity of the transcription factor that bound to this region has been demonstrated containing PR by supershift assay. P mediates its biological activity following its interaction with a specific receptor within the target cells nuclei. Progesterone receptors are a ligand-inducible transcriptional regulator that controls gene expression upon binding at the PRE in the vicinity of target promoters or influence gene expression by interaction with other transcription factors independently of PRE (Truss and Beato 1993). The expression of PR-A and PR-B isoforms in the JEG-3 and αT3-1 cells were examined by the use of Western blot analysis. The expression levels of PR-A and PR-B were similar in the αT3-1 cells, whereas PR-B in the JEG-3 cells is much higher than the PR-A. The requirement of relatively higher concentration (1 μM) of P to achieved the statistically significant decrease in human GnRHR promoter activity in αT3-1 cells may due to the present of low levels of PRs or the use of mouse PRs in studying the regulation of human GnRHR gene.

5.4.3 Role of progesterone receptor in mediating P effects on human GnRHR promoter

In order to examine the role of human PR in controlling the expression of human GnRHR gene, αT3-1 cells were co-transfected with human PR-A or PR-B expression vector. Over-expression of human PRs in the αT3-1 cells resulted in a decrease in GnRHR promoter activity after 0.1 μM P treatment. The same results obtained from wide-type αT3-1 and human

PRs co-transfected αT3-1 cells after P treatment further implicate the potential value of these cells in studying the P-induced regulation of human GnRHR gene. Although over-expression of both human PR-A and PR-B isoforms resulted in increase sensitivity towards P treatment, it seems that PR-B plays a more important role in mediating the P-induced inhibitory action. This postulation was based on the observations that 1) a higher degree in P-induced decrease in human GnRHR promoter was obtained from PR-B co-transfected cells, and 2) mutation of hGR-PRE completely eliminated the PR-A mediated inhibitory effects but not PR-B mediated action. Although the presence and distribution of steroid receptors in the normal human pituitary has not been reported, recent studies have demonstrated the expression of PR in human pituitary adenomas and supported the direct action of P in the human pituitary (Jaffrain-Rea et al. 1996).

In agreement to our Western blot results in PRs expression in the JEG-3 cells, expression of PRs in the human placenta has also been demonstrated (Rossmanith et al. 1997, Shanker et al. 1997, Cudeville et al. 2000). Thus, human placenta is very likely a target tissue for the action of P. In fact, recent reports describing the effects of P on expression of chorionic gonadotropin α– and β– subunits genes in the human placenta (Rao et al. 1995) and corticotropin releasing hormone gene in human trophoblast cell cultures (Karalis and Majzoub 1996) provide evidence that placenta itself can be a target tissue for the action of P. To further study the role of these receptors in mediating the P-induced effect in placenta, human PR-A and PR-B expression vectors were cotransfected into JEG-3 cells. Interestingly, our results showed that PR-A and PR-B have a differential function in regulating the expression of human GnRHR gene in the placenta cells. Similar to the data obtained from the pituitary cells, over-expression of PR-A resulted in a decrease in human GnRHR promoter activity. However, over-expression of PR-B increased the human GnRHR promoter activity in the placental cells, while this over-expression lead to a decrease in promoter activity at the pituitary cells. These results support the P-stimulatory role of GnRHR gene expression in the placenta, since a much higher level of PR-B

was detected in the JEG-3 cells. Taken together, these results indicating that PR-A is mainly involved in down-regulating, while PR-B up-regulating, the transcription of human GnRHR in pituitary and placenta.

The PR-A and PR-B isoforms arise by the transcription of same gene with the use of different promoter region (Kastner et al. 1990) suggests that independent regulation of these promoter may occur (Meyer et al. 1992). It is now clear that their relative levels are under hormonal control (Numan et al. 1999). Thus, different hormonal input in the pituitary and placenta may affect the expression levels of PRs. As both PR-A and PR-B are capable of binding P, dimerizing and interacting with PRE, the levels of PRs as well as the stoichiometric ratio of PR-A and PR-B within a target cell under specific physiologic condition would be expected to alter the relative complement of dimeric complex and exert significant impact on the overall cellular response to P. To add to the complexity of the PRs regulatory system, a third PR isoform has recently been identified in T47D human breast cancer cells and named as PR-C (Wei and Miner 1994). This N-terminally truncated PR-C isoform arises from the use of translation start site downstream of the translation site for PR-A and PR-B at the same gene. The PR-C contains only the second zinc-finger of the DNA-binding domain but lacking of the first one. Alone, PR-C is transcriptionally inactive but able to regulate the PR-A and PR-B transcriptional activity when co-expressed (Wei et al. 1996). Recent studies have also demonstrated that transactivation by PRs might involve additional cofactors including co-activator or co-repressor (Glass et al. 1997, Robyr et al. 2000). It is possible that different co-activators or repressors were existed in the pituitary and placenta cells which resulted in a different response in PR-B mediated action. In addition, there is evidence for the interaction between steroid hormone receptors, including PR, and other transcription factors, such as octamer transcription factor 1 (Oct-1), activating protein-1 (AP-1) and Stat in controlling gene expression at the transcriptional levels (Bruggemeier et al. 1991, Bamberger et al. 1996,

Stoecklin et al. 1999). Hence, the incomplete elimination of PR-B and PR-A mediated decrease in promoter activity in mutated hGR-PRE construct in αT3-1 and JEG-3 cells, respectively, may due to fact that an additional transcription regulatory mechanism(s) is used.

In summary, we have demonstrated a direct action of P in regulating the human GnRHR gene expression at the transcriptional levels through binding to a putative hGR-PRE motif. Over-expression of human PR-A shows a transcriptional inhibition action in GnRHR gene expression. In contrast, PR-B exhibits a cell-dependent differential transcriptional activity in which it acts as transcriptional activator in placenta cells but a transcriptional repressor in pituitary cells.

CHAPTER 6. UP-REGULATION OF GnRHR TRANSCRIPTION BY 3',5'-CYCLIC ADENOSINE MONOPHOSPHATE/ PROTEIN KINASE A PATHWAY

6.1 Introduction

Cyclic adenosine monophosphate (cAMP) generation is one of the common mechanisms by which extracellular signals are transduced into biological responses in mammalian cells (McKnight 1991,Daniel et al. 1998). Early studies using transient transfection system have demonstrated an increase in rat (Reinhart et al. 1997) and mouse (Lin and Conn 1998) GnRHR promoter activity in pituitary cells after forskolin, cholera toxin (CTX) or cAMP stimulation. Further studies have identified a putative CRE in the mouse GnRHR promoter that is responsible for cAMP-mediated transcriptional activation (Maya-Nunez and Conn 1999).

In human trophoblast cells, cyclic adenosine monophosphate (cAMP) plays a critical role in controlling placenta-specific gene expression. Characterization of the human corticotropin-releasing hormone gene promoter (Spengler et al. 1992, Scatena and Adler 1998), the human cytochrome P450 side-chain cleavage (CYP11A) gene promoter (Moore et al. 1992) and the human glycoprotein hormone α-subunit gene promoter (Bokar et al. 1989, Heckert et al. 1995), revealed that the CRE is essential for the gene expression in the placenta. In particular interest, the CRE was also found to be essential for the pituitary expression of glycoprotein α-subunit gene (Schoderbek , 1992, Heckert et al 1995). The increased in GnRHR mRNA levels in placental JEG-3 and IEVT cells after forskolin treatment suggesting a possible regulation of GnRHR gene transcription by cAMP/PKA pathway (Chapter 3). In an attempt to elucidate the role of cAMP/PKA pathway in controlling human GnRHR gene expression in transcriptional

level, the human GnRHR 5' flanking region was examined by transient transfection into αT3-1 and JEG-3 cells.

6.2 Pharmacological treatments

Pharmacological reagents, including forskolin, 8-Bromoadenosine-3',5'-cyclic monophosphate (8-Br-cAMP), cholera toxin (CTX) and hCG were purchased from Sigma (Sigma-Aldrich Ltd., Canada). PACAP 38, adenylate cyclase inhibitor (SQ22536) and cell permeable protein kinase A inhibitor 14-22 amide, myristoylated (PKAI) were purchased from Cal-biochem (La Jolla, CA). In experiments wherein the effects of PACAP, forskolin, 8-Br-cAMP and CTX on GnRHR-Luc activity were studied, the cells were treated with various amounts of chemical for indicated time before luciferase activity assay. To study the role of PKA and AC, transfected cells were pre-incubated with the corresponding inhibitors for 30 min before treatments.

6.3 Results

6.3.1 Effects of Forskolin, CTX and 8-Br-cAMP on GnRHR -Luc gene expression

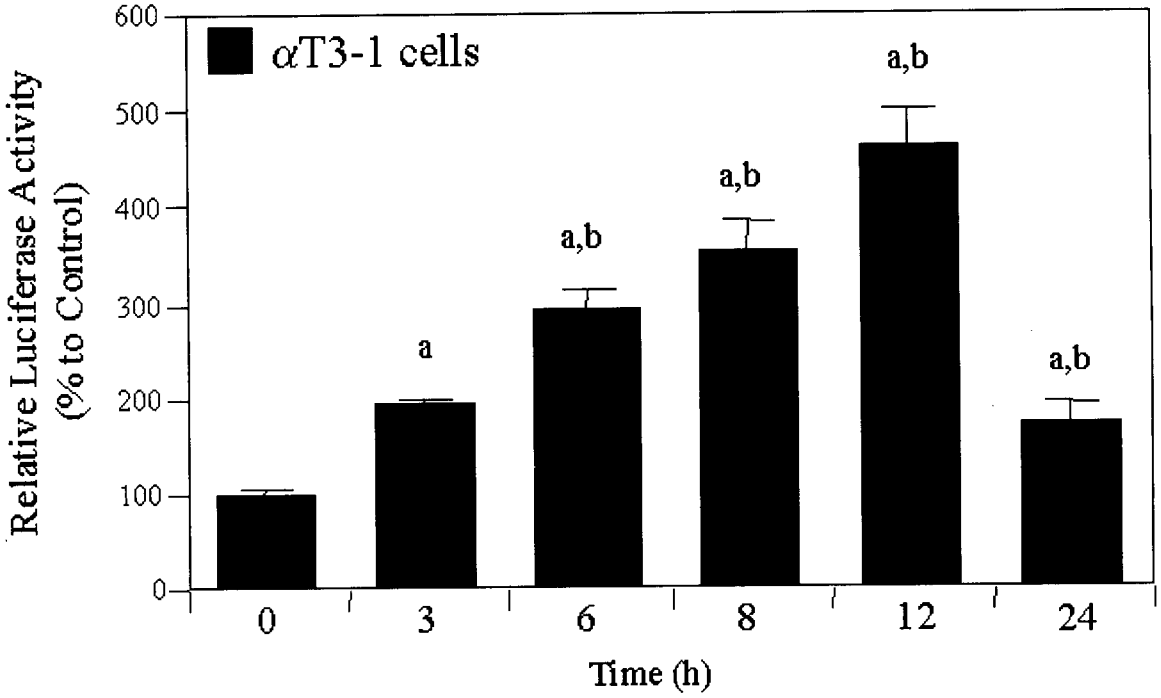
To examine the possible transcriptional regulation of human GnRHR gene by cAMP/ PKA pathway, forskolin was administrated to the αT3-1 cells transfected with the full length 5' flanking region. A time and dose-depended increase in the GnRHR-promoter activity were observed (Fig. 6.1). A substantially increase in luciferase activity was observed after 3 h of forskolin treatment (10 μM) and reached maximal induction (4.3 folds, $P<0.001$) after 12 h treatment. However, prolonged (24 h) forskolin treatment did not further increase the promoter activity (Fig. 6.1A). Similarly, a substantial increase (5.8-fold, $P<0.001$) in luciferase activity was observed after 3 h of 10 μM forskolin treatment in p2300-LucF transfected JEG-3 cells (Fig. 6.2). However, extended forskolin stimulation (6–24 h) did not further increase the promoter activity (Fig. 6.2A). Further study has shown that the induction of promoter activity in both αT3-

1 and JEG-3 cells was dependent on the dosage with the maximum increase achieved at 10 μM of forskolin (Fig. 6.1B and 6.2B).

As forskolin is known to increase intracellular cAMP concentrations by activating adenylate cyclase, to further examine the role of adenylate cyclase in stimulating the GnRHR promoter activity, CTX was used in addition to forskolin. Cholera toxin increases cAMP production by activating adenylate cyclase constitutively. Treatments of transfected αT3-1 and JEG-3 cells with 1 μM CTX was able to mimic the stimulatory effect of forskolin (Fig. 6.3). Increasing the concentration of CTX to 5 μM further enhance the stimulation. Similarly, administrating a cAMP analog (1 mM or 5 mM 8-Br-cAMP) was also able to increase the GnRHR-promoter activity. These results demonstrate that increased intracellular cAMP concentrations would result in enhanced GnRHR gene transcription.

FIG. 6.1 Time- and dose-dependent regulation of human GnRHR-luciferase vector (p2300-LucF) activity in αT3-1 cells treated with forskolin. (*Panel A*) The p2300-LucF transfected αT3-1 cells were treated with 10 μM of forskolin for the indicated times. The RSV-LacZ vector was co-transfected to normalize for varying transfection efficiencies (*Panel B*) The mouse αT3-1 cells were transfected with p2300-LucF and varying concentrations of forskolin (10 nM to 0.1 mM) were added to the medium. The cells were collected for luciferase activity measurement after 12 h treatment. Luciferase units were calculated as luciferase activity/ β-galactosidase activity and presented as percentages of control and means +/- SD from triplicate assays in three separate experiments. a, $P < 0.01$ from control; b, $P < 0.05$ from the immediately adjacent group on the left.

A


αT3-1 cells
Relative Luciferase Activity (% to Control)
600
500
400
300
200
100
0
a
a,b
a,b
a,b
a,b
0
3
6
8
12
24
Time (h)

B

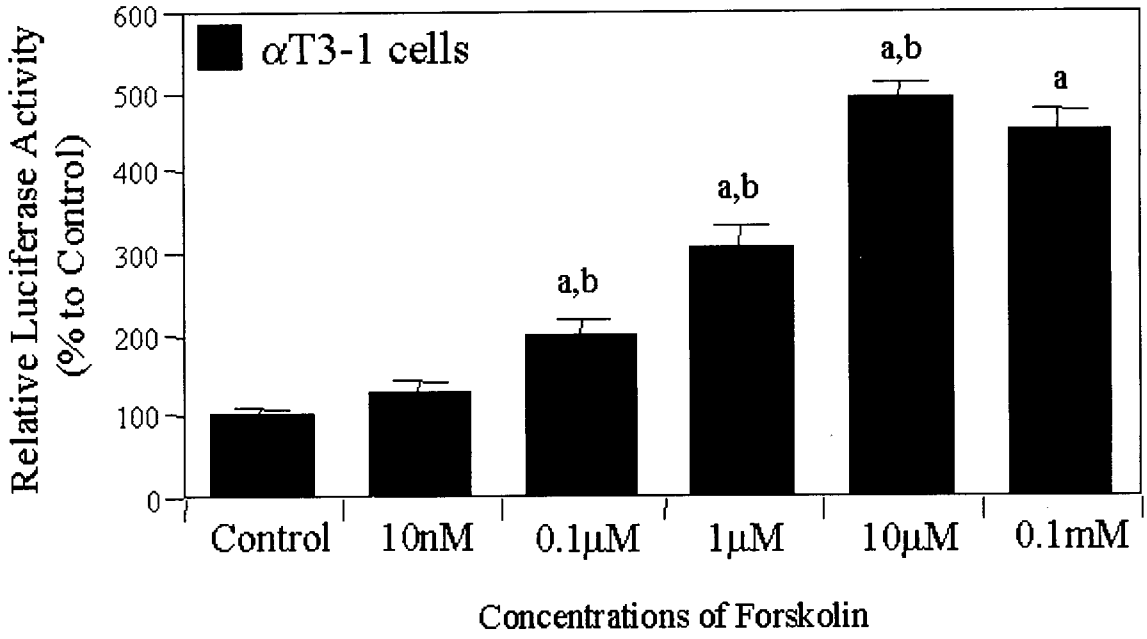
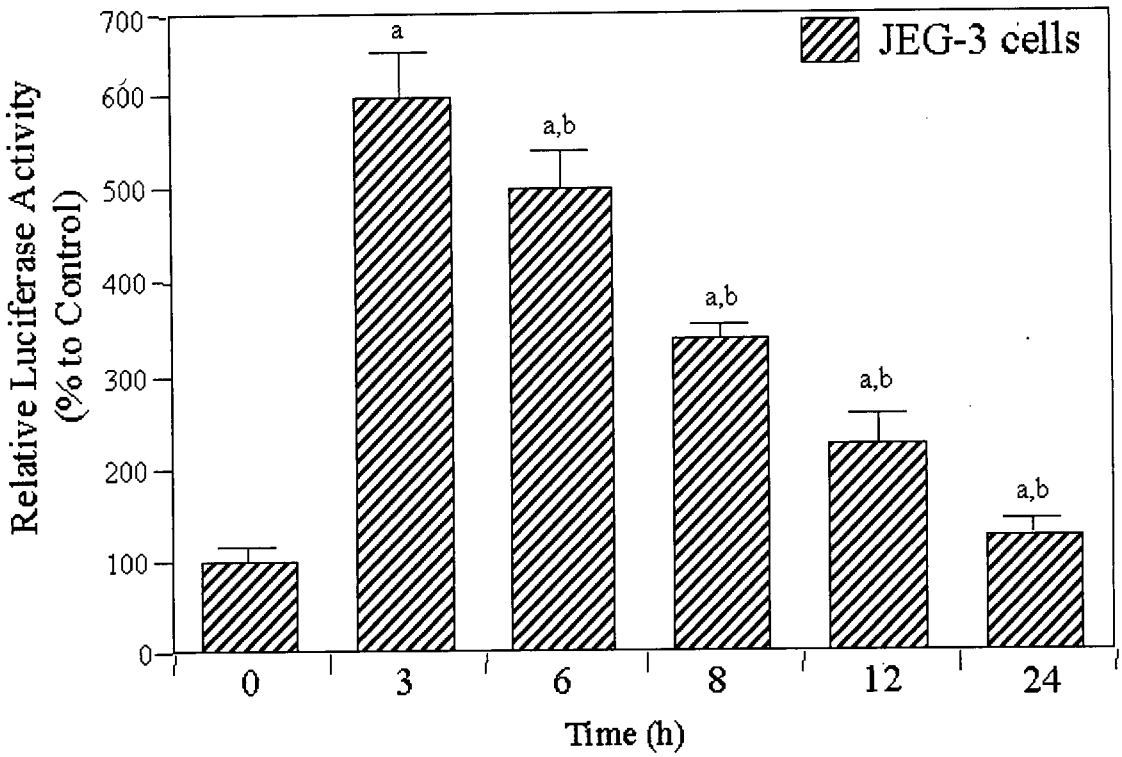

αT3-1 cells
Relative Luciferase Activity (% to Control)
600
500
400
300
200
100
0
a,b
a,b
a,b
a
Control
10nM
0.1μM
1μM
10μM
0.1mM
Concentrations of Forskolin

FIG. 6.2 Time- and dose-dependent regulation of human GnRHR-luciferase vector (p2300-LucF) activity in JEG-3 cells treated with forskolin. (*Panel A*) The p2300-LucF transfected JEG-3 cells were treated with 10 μM of forskolin for the indicated times. The RSV-LacZ vector was co-transfected to normalize for varying transfection efficiencies (*Panel B*) The placental JEG-3 cells were transfected with p2300-LucF and varying concentrations of forskolin (10 nM to 0.1 mM) were added to the medium. The cells were collected for luciferase activity measurement after 3 h treatment. Luciferase units were calculated as luciferase activity/ β-galactosidase activity and presented as percentages of control and means +/- SD from triplicate assays in three separate experiments. a, $P < 0.01$ from control; b, $P < 0.05$ from the immediately adjacent group on the left.

A


JEG-3 cells
Relative Luciferase Activity
(% to Control)
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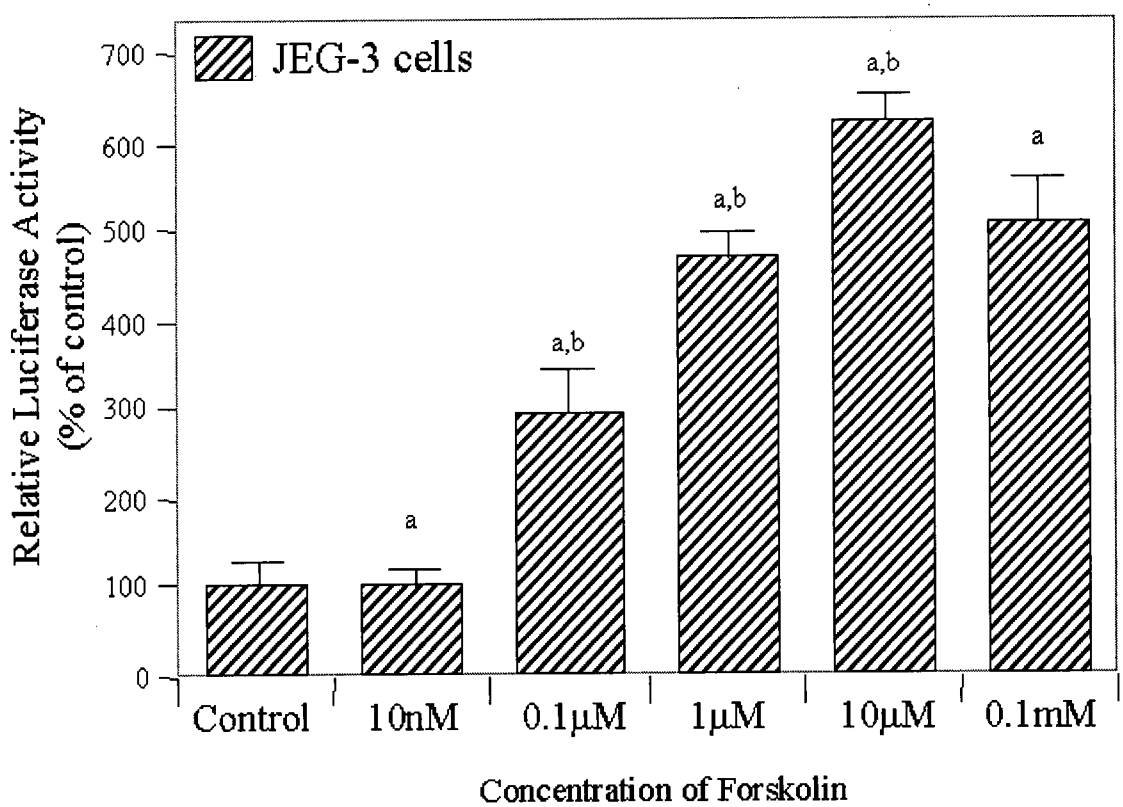
600
500
400
300
200
100
0
a
a,b
a,b
a,b
a,b
0
3
6
8
12
24
Time (h)

B


JEG-3 cells
Relative Luciferase Activity
(% of control)
700
600
500
400
300
200
100
0
a
a,b
a,b
a,b
a
Control
10nM
0.1μM
1μM
10μM
0.1mM
Concentration of Forskolin

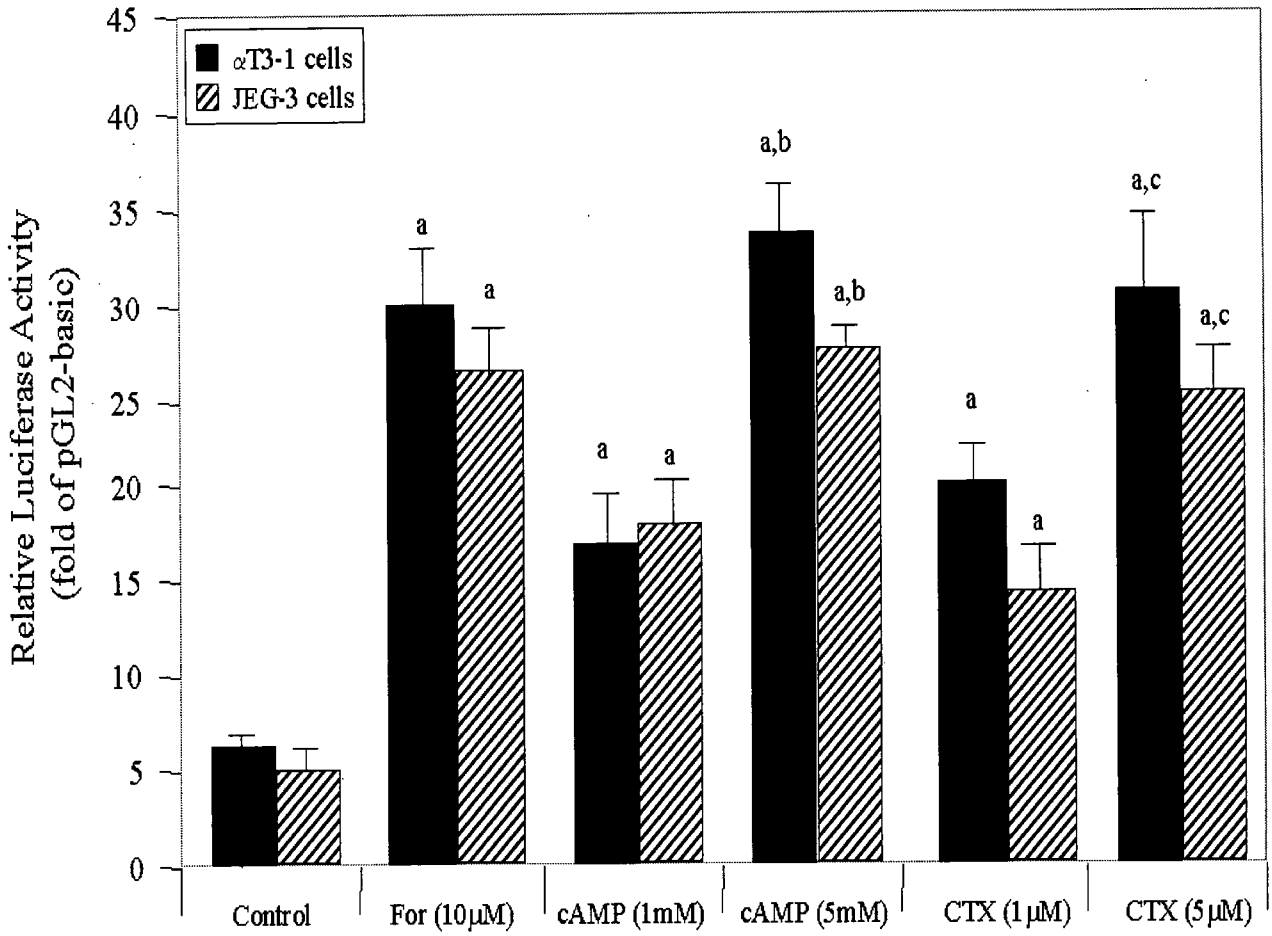


FIG. 6.3 Effects of forskolin (For), 8-Br-cAMP (cAMP) or cholera toxin (CTX) on human GnRHR promoter activity. Cellular lysates were harvested 12 h and 3 h post-transfection for αT3-1 and JEG-3 cells, respectively. The RSV-LacZ vector was co-transfected to normalize for varying transfection efficiencies. The p2300-LucF transfected αT3-1 or JEG-3 cells were treated with vehicle (control), 10 μM For, 1 mM or 5 mM cAMP, or 1 μM or 5 μM CTX. Luciferase units were calculated as luciferase activity/ β-galactosidase activity. Data were presented as means +/- SD of three individual experiments with triplication. a, $P < 0.001$ from the control; b, $P < 0.001$ from the cAMP (5 mM) vs cAMP (1 mM); c, $P<0.001$ from CTX (5 μM) vs CTX (1 μM).

6.3.2 Involvement of PKA and adenylate cyclase in up-regulation of human GnRHR gene

As cAMP is known to activate protein kinase A activity, to dissect the mechanism by which cAMP/forskolin/CTX induced GnRHR promoter activity, the p2300-LucF transfected cells were treated with either adenylate cyclase inhibitor (ACI) or PKAI before forskolin stimulation. In the presence of 8 μM PKAI, the forskolin-induced stimulation of GnRHR promoter activity in aT3-1 and JEG-3 cells was reduced by 50% ($P<0.01$, vs Forskolin only) and 32.4% ($P<0.01$ vs Forskolin only), respectively. These results suggest that the activation of PKA was important in regulating the GnRHR promoter activity (Fig. 6.4). In another experiment, pretreating the transfected cells with adenylate cyclase inhibitor (0.5 mM) dramatically decreased the responsiveness to forskolin-induced stimulation on GnRHR promoter activity to 41.6% ($P<0.001$ vs Forskolin only) and 42% ($P<0.01$ vs Forskolin only) in αT-1 and JEG-3 cells, respectively (Fig. 6.4). These results show that the production of cAMP and activation of protein kinase A was vital for increasing GnRHR gene expression.

6.3.3 Effects of PACAP or hCG in human GnRHR gene transcription

PACAP was showed to bind type 1 PVR and activates adenylate cyclase leading to cAMP production in pituitary cells (Rawlings and Hezareh 1996). Treatment of p2300-LucF transfected αT3-1 cells with increasing concentrations of PACAP for 12h showed an increase in promoter activity (Fig. 6.5A). A maximum increase (5.52 fold vs control, $P<0.001$) in promoter activity was achieved at 10 nM PACAP. In addition, this PACAP-induced increase in promoter activity was reversed in the presence of 0.5mM ACI (Fig. 6.5B). Similarly, administrating of 2IU/ml hCG to p2300-LucF transfected JEG-3 cells led to 2-fold increase in promoter activity (Fig. 6.6). Taken together, these results indicate that hormones, which activate adenylate cyclase, may increase the expression of human GnRHR gene in transcriptional level.

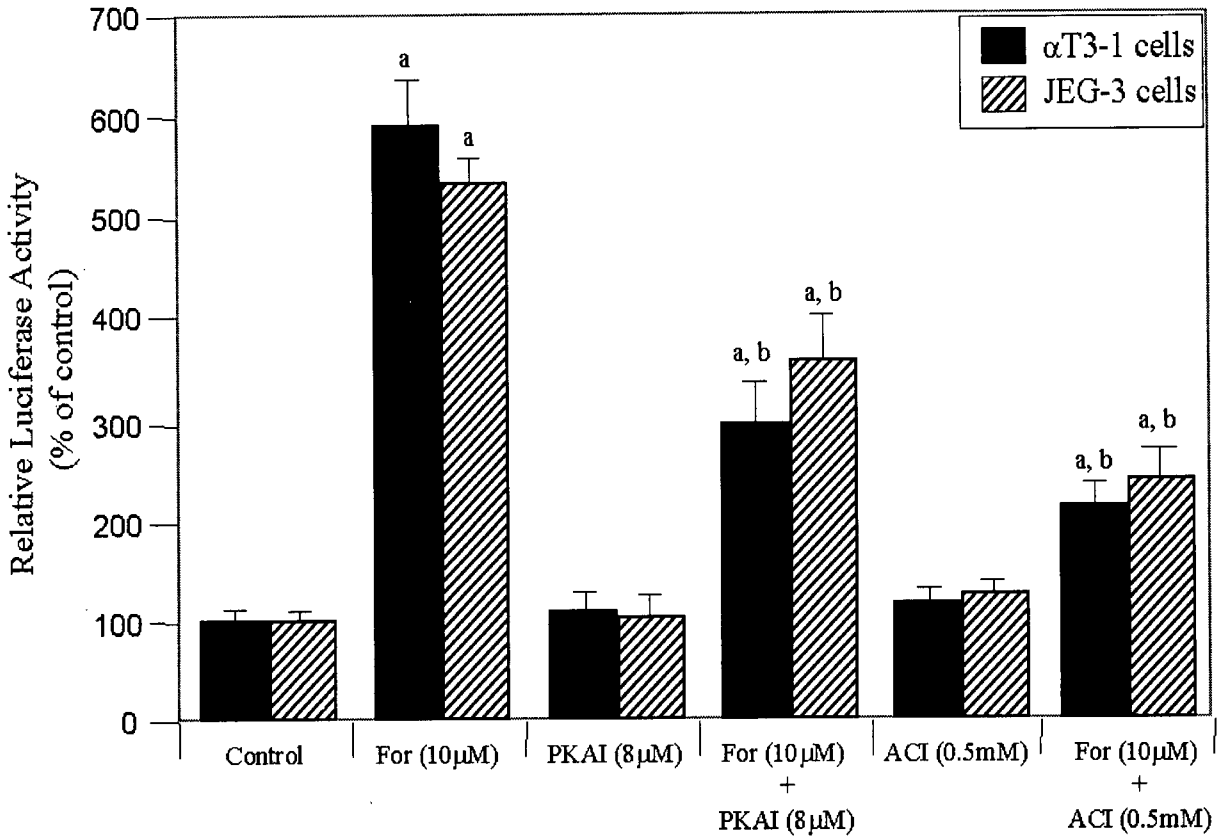
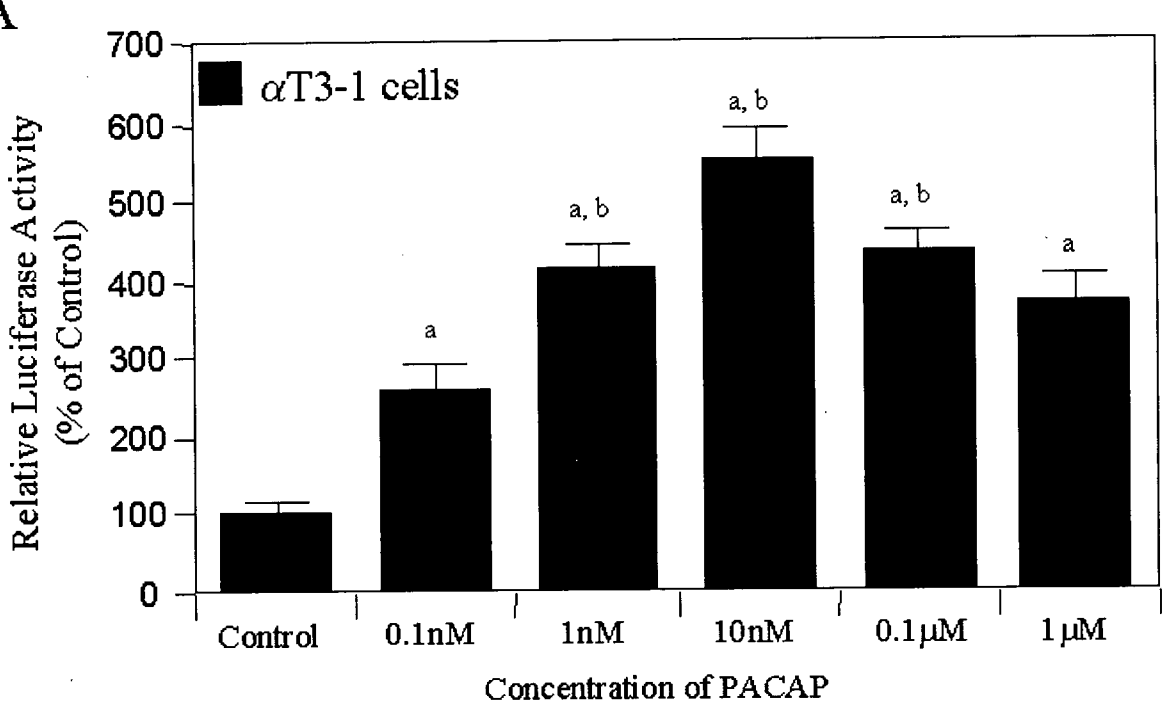


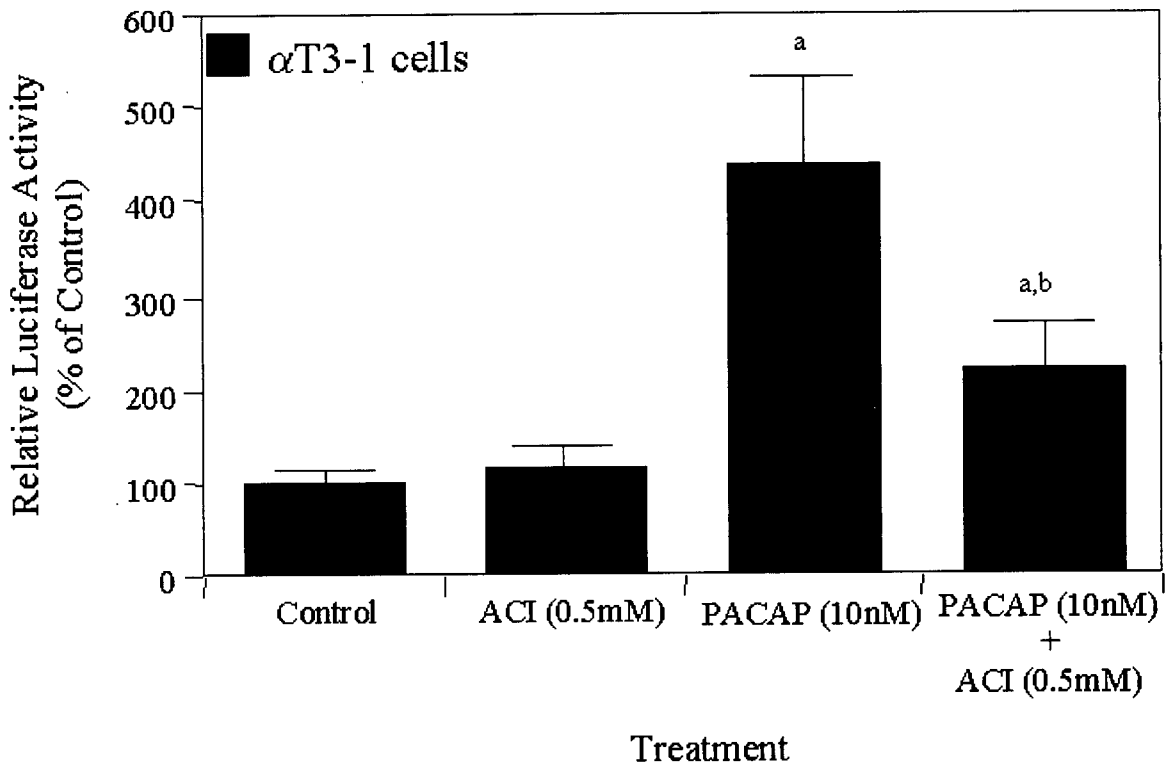
FIG. 6.4 Effects of the protein kinase A inhibitor (PKAI) and adenylate cyclase inhibitor (ACI) on forskolin-induced stimulation of human GnRHR promoter activity. Cells were harvested 12h (αT3-1 cells) or 3h (JEG-3 cells) post-transfection. PKAI or ACI was applied 30 min prior to forskolin (For) treatment. The RSV-LacZ vector was co-transfected to normalize for varying transfection efficiencies. The p2300-LucF transfected cells were treated with vehicle (control), 10 μM For, 8 μM PKAI, both For and PKAI, 0.5 mM ACI or For plus ACI. Luciferase units were calculated as luciferase activity/ β-galactosidase activity. Data were presented as percentages of control and means +/- SD of three individual experiments with triplication. a, $P <$ 0.001 from control; b, $P < 0.001$ from forskolin alone treatment.

FIG. 6.5 Effects of pituitary adenylate cyclase activating polypeptide 38 (PACAP) and adenylate cyclase inhibitor (ACI) on PACAP-induced stimulation on human GnRHR promoter activity. Cells were harvested 12 h post-transfection. The RSV-LacZ vector was co-transfected to normalize for varying transfection efficiencies. (*Panel A*) The p2300-LucF transfected αT3-1 cells were treated with varying concentration of PACAP (0.1 nM to 1 μM). (*Panel B*) The p2300-LucF transfected αT3-1 cells were pre-treated with 0.5 mM ACI was for 30 min prior to 10 nM PACAP treatment. The RSV-LacZ vector was co-transfected to normalize for varying transfection. Luciferase units were calculated as luciferase activity/ β-galactosidase activity. Data were presented as percentage of control and means +/- SD of three individual experiments with triplication. a, $P < 0.001$ from the control; b, $P < 0.01$ from the immediately adjacent group on the left.

A


αT3-1 cells
Relative Luciferase Activity (% of Control)
700
600
500
400
300
200
100
0
a
a, b
a, b
a, b
a
Control
0.1nM
1nM
10nM
0.1μM
1μM
Concentration of PACAP

B


αT3-1 cells
Relative Luciferase Activity (% of Control)
600
500
400
300
200
100
0
a
a,b
Control
ACI (0.5mM)
PACAP (10nM)
PACAP (10nM) + ACI (0.5mM)
Treatment

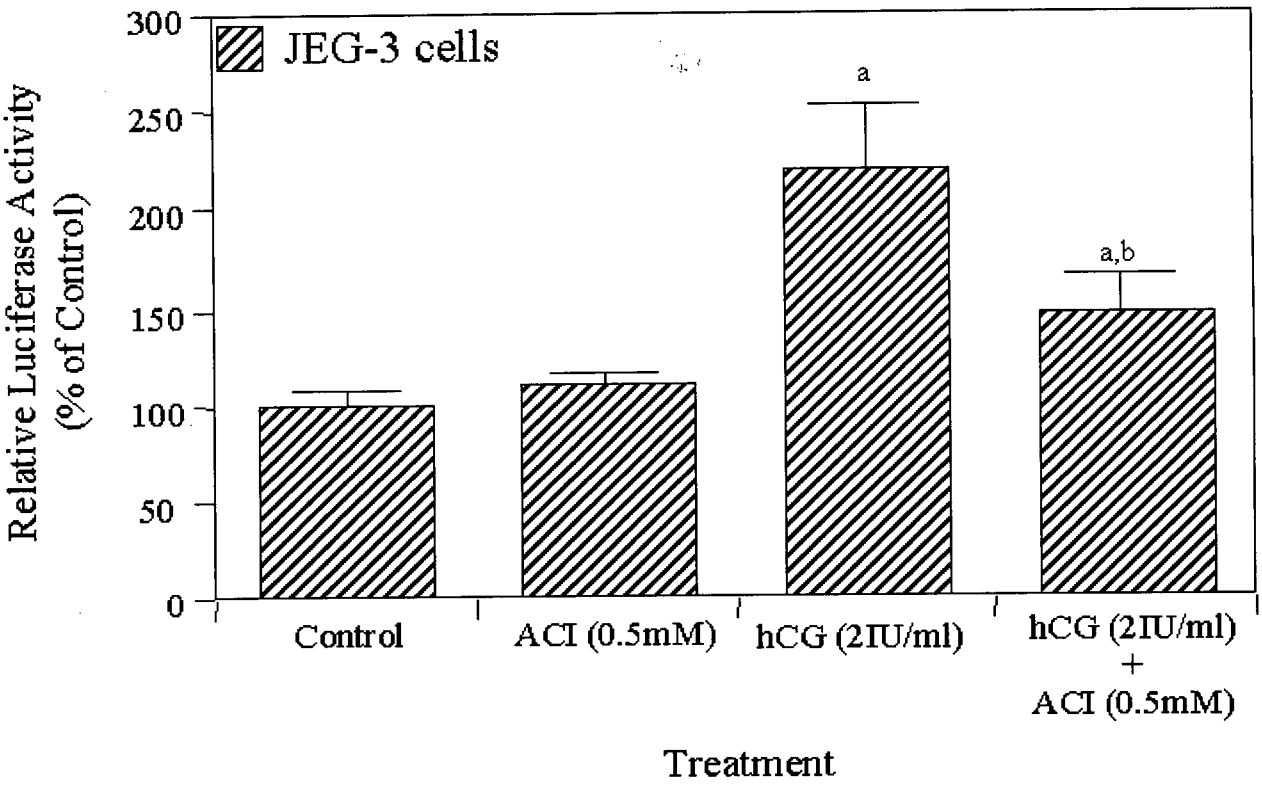


FIG. 6.6 Effects of human chorionic gonadotropin (hCG) and adenylate cyclase inhibitor (ACI) on hCG-induced stimulation on human GnRHR promoter activity. Cells were harvested 3h post-transfection. The RSV-LacZ vector was co-transfected to normalize for varying transfection efficiencies. The p2300-LucF transfected JEG-3 cells were pre-treated with 0.5 mM ACI for 30 min prior to 2 IU/ml hCG treatment. The RSV-LacZ vector was co-transfected to normalize for varying transfection. Luciferase units were calculated as luciferase activity/ β-galactosidase activity. Data were presented as percentage of control and means +/- SD of three individual experiments with triplication. a, $P < 0.001$ from the control; b, $P < 0.01$ from LCG alone.

6.3.4 Localization of cAMP responding region on human GnRHR promoter

To localize the specific promoter region that mediates the responsiveness to cAMP/PKA up-regulation of GnRHR promoter activity, progressive 5' deletion constructs containing fragments of the human GnRHR promoter were fused to a luciferase reporter gene and transfected into αT3-1 and JEG-3 cells (Fig. 6.7A). Removal of DNA sequence between nt –2297 to –1346 (relative to translation start site) resulted in a 19% ($P<0.05$) and 15% ($P<0.05$) decrease in forskolin-induced increase in promoter activity in αT3-1 and JEG-3 cells, respectively (Fig. 6.7B and C). Nevertheless, the responsiveness to forskolin-induced stimulation in promoter activity was maintained. Interestingly, the maximum response to forskolin was restored after deletion of DNA fragment from nt –1346 to –707 (Fig. 6.7). Further deletion to 577 bases away from translation start site slightly decreased 15% ($P<0.05$) and 10% ($P>0.05$) the forskolin-induced effect in αT3-1 and JEG-3 cells, respectively. However, deletion of DNA sequence between nt –577 to –227 eliminated the forskolin action in both cells (Fig. 6.7B and C). These data suggest that the complete responsiveness towards cAMP stimulation was controlled by interaction of varies region in the human GnRHR, whereas the DNA fragment between nt –577 and –227 play an essential role to maintain basal forskolin-induced stimulatory effect.

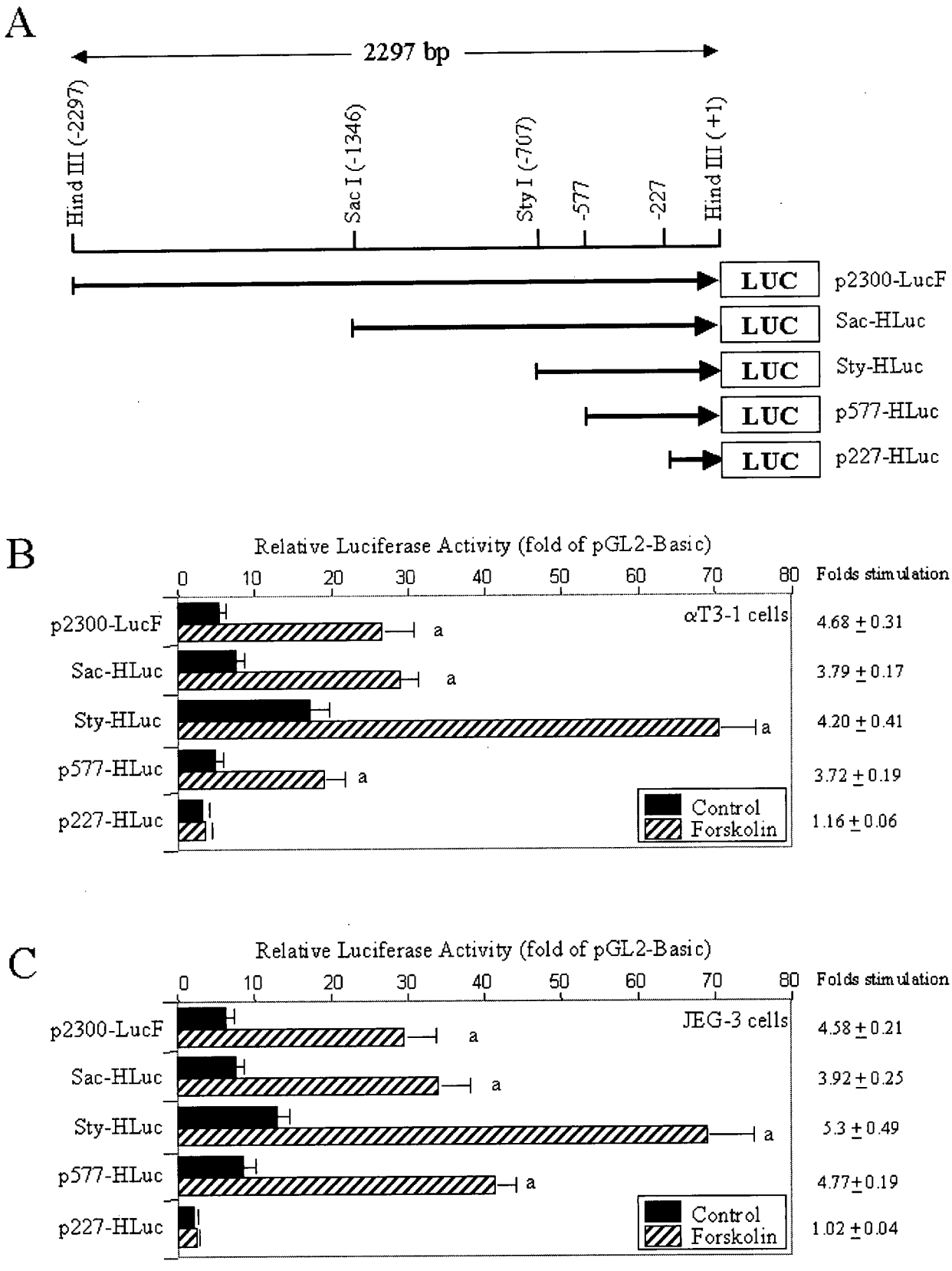
6.3.5 Identification of cAMP response element in the human GnRHR 5'-flanking region

Sequence homology search by MatInspector Professional analysis (Quandt et al. 1995) of this 351 bp fragment (nt -577 to -227) did not identify any consensus CRE. However, it revealed the existence of two potential AP-1/CREB binding sites, namely hGR-AP/CRE-1 (located at nt –568 to –561, 5'TTAAGTGA3', in complementary orientation, with 75% and 71.5% homology to consensus CRE and AP-1, respectively) and hGR-AP/CRE-2 (located at nt –340 to –333, 5'TGACTGAC3', with 50% and 86% homology to consensus CRE and AP-1, respectively). To

examine the role of these binding sites in mediating the forskolin stimulatory effects, the two putative AP-1/CREB binding sites were mutated in Sty-HLuc (Fig. 6.8). The mutation was introduced by three-steps PCR mutagenesis method (Chapter 2, Section 2.11). The putative transcription factor binding sequence was replaced by a non-related sequence to disturb specific transcription factor binding. In αT3-1 cells, mutation of the putative hGR-AP/CRE-1 did not only result in a 38% ($P< 0.001$) decrease in the forskolin-induced stimulation, but also decreased (22%, $P<0.01$) in basal promoter activity in αT3-1 cells (Fig. 6.8A). Similarly, mutation of the hGR-AP/CRE-2 caused a 32% ($P<0.001$) decrease in the forskolin stimulation in the promoter activity (Fig. 6.8A). Interestingly, this site-directed mutation resulted in a 29% ($P<0.01$) increase in basal promoter activity.

In JEG-3 cells, mutation of hGR-AP/CRE-1 and hGR-AP/CRE-2 resulted in a 23% ($P<0.01$) and 35% decrease in responding to forskolin stimulation, respectively (Fig. 6.8B). Interestingly, mutation of hGR-AP/CRE-1 led to a 40% ($P<0.01$) increase (40%, $P<0.01$), but mutation of hGR-AP/CRE-2 caused a 20% ($P<0.05$) decrease in basal promoter activity in JEG-3 cells (Fig. 6.8B) that is opposite to the results observed in αT3-1 cells. Nevertheless, these results suggest that both elements were involved in controlling the basal promoter activity. Double mutation of both AP-1/CREB binding sites did not only further reduce the responsiveness to forskolin stimulation (53.5% decrease, $P<0.001$), but also compensate the decreasing and increasing effects in the basal promoter activity caused by respective single mutations (Fig. 6.8). Mutation of a putative progesterone response element (PRE) did not affect the forskolin-induced increase in promoter activity further demonstrating the specificity of the two AP-1/CREB binding sites in responding to the forskolin stimulation (Fig. 6.8).

FIG. 6.7 Localization of the forskolin-responsive region in the human GnRHR 5' flanking region. Progressive 5' deletion constructs were transiently transfected into αT3-1 and JEG-3 cells and treated with 10 μM of forskolin for 12h (*panel B*) or 3h (*panel C*), respectively, before being harvested for luciferase activity measurement. The RSV-LacZ vector was co-transfected to normalize for varying transfection efficiencies. Relative promoter activity of each construct is shown as fold increase over a promoterless luciferase control pGL2-Basic, whose activity is set to be 1, after normalized to β-galactosidase activity and presented as means +/- SD of three individual experiments with triplication. The fold of increases in promoter activity after forskolin-stimulation was indicated. a, $P < 0.001$ from individual control.


A
2297 bp
Hind III (-2297)
Sac I (-1346)
Sty I (-707)
-577
-227
Hind III (+1)
LUC
p2300-LucF
Sac-HLuc
Sty-HLuc
p577-HLuc
p227-HLuc
B
Relative Luciferase Activity (fold of pGL2-Basic)
0 10 20 30 40 50 60 70 80
Folds stimulation
αT3-1 cells
p2300-LucF
Sac-HLuc
Sty-HLuc
p577-HLuc
p227-HLuc
a
4.68 ± 0.31
3.79 ± 0.17
4.20 ± 0.41
3.72 ± 0.19
1.16 ± 0.06
Control
Forskolin
C
Relative Luciferase Activity (fold of pGL2-Basic)
0 10 20 30 40 50 60 70 80
Folds stimulation
JEG-3 cells
p2300-LucF
Sac-HLuc
Sty-HLuc
p577-HLuc
p227-HLuc
a
4.58 ± 0.21
3.92 ± 0.25
5.3 ± 0.49
4.77 ± 0.19
1.02 ± 0.04
Control
Forskolin

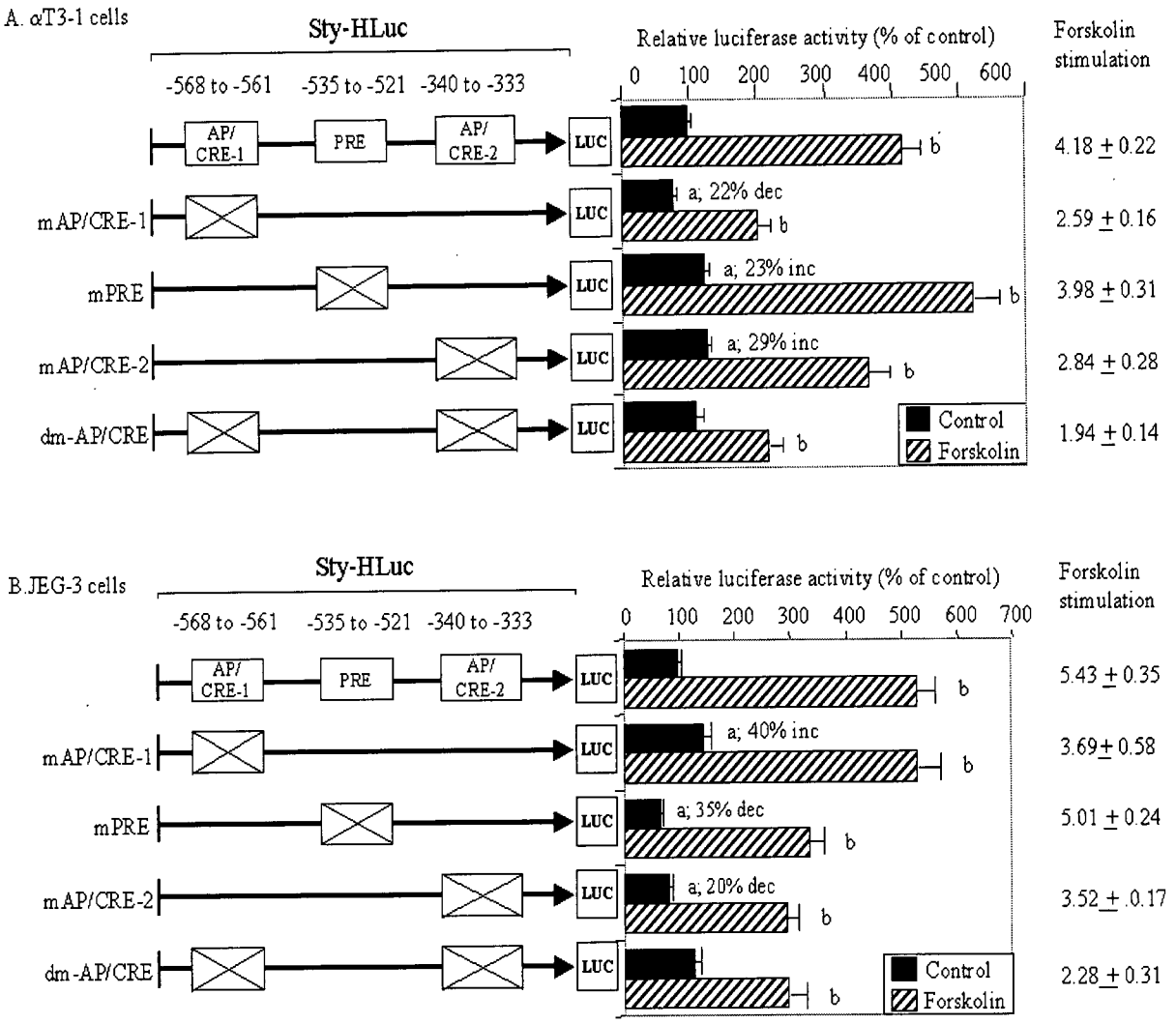


FIG. 6.8 Effect of mutations in the putative AP-1/CREB binding sites on forskolin responsiveness of the human GnRHR gene. Single mutation; mAP/CRE-1, mPRE and mAP/CRE-2, and double mutation (dm-AP/CRE) in Sty-HLuc constructs were transiently transfected into αT3-1 and JEG-3 cells, and treated with 10 μM of forskolin for 12h and 3h, respectively, before being harvested for luciferase activity measurement. The RSV-LacZ vector was co-transfected to normalize for varying transfection efficiencies. The relative basal activity of each promoter mutants is shown as percentage of vehicle treated (control) Sty-HLuc, whose activity is taken as 100%, after normalized to β-gal activity. The fold of stimulation is calculated by comparing to individual control. Values represent mean +/- SD of triplicate assays in three separate experiments. The names and the relative position of the putative transcription factor binding sites are given and the mutated element was shown as *cross box.* The fold of increases in promoter activity after forskolin-stimulation was indicated. a, $P < 0.05$ from the control of Sty-HLuc; b, $P < 0.001$ from individual control.

6.3.6 Binding of nuclear protein to the putative hGR-AP/CRE

To confirm the identity of the transcription factor bound to the two putative AP-1/CREB binding sites, GMSA was preformed with a synthetic oligodeoxynucleotide containing the putative hGR-AP/CRE-1 and hGR-AP/CRE-2 binding site in the presence of consensus and mutated AP-1, CRE, non-related oligodeoxynucleotide or antibody against the CREB. Specific DNA-protein complexes were observed using the nuclear extract isolated from αT3-1 cells, before and after treatment of 10μM forskolin for 30 min. An increase in DNA-protein complex intensity was observed after forskolin stimulation (Fig. 6.9 and Fig 6.10). Using hGR-CRE-1 as a probe, three DNA-protein complexes were formed (Fig. 6.9, indicated by the complexes A, B and C). The specific complex A was eliminated with the addition of increasing competitor DNA fragment (200 fold in excess or 0.1 mM) containing a consensus CRE (lanes 5 and 12) but not with a competitor containing mutated AP-1 (mAP-1; lanes 2 and 9), consensus AP- 1 (lanes 3 and 10) mutated CRE (mCRE; lane 4 and lane 11), non-related sequence NF-κB (lanes 6 and 13) or TFIID (lanes 7 and 14). GMSA of hGR-AP/CRE-2 revealed a change in DNA-complex composition after forskolin treatment (Fig. 6.10). Using nuclear extract isolated before and after forskolin treatment, two DNA-protein were observed, as indicated by complexes A and B. Interestingly, the specific complex A before forskolin was only eliminated by the addition of consensus AP-1 sequence (lane 3) but not by others sequences (Fig. 6.10). In contrast, after forskolin treatment, this complex was mainly eliminated by the addition of consensus CRE sequence (lane 12), but only slightly eliminated with the consensus AP-1 sequence (lane 10).

GMSA was also preformed using nuclear extract isolated from JEG-3 cells. Generally, there was an increase in DNA-protein complex intensity with nuclear extract isolated after forskolin treatment (Fig. 6.11 and 6.12). Using hGR-AP/CRE-1 as a probe, 4 DNA-protein complexes were obtained (Fig. 6.11, indicated by the arrow A-D), while complexes A and B was eliminated with the addition of increasing (200 fold in excess) competitor DNA containing

consensus CRE (lane 5 and 12), but not with a competitor containing mutated AP-1 (lane 2 and 9), consensus AP-1 (lane 3 and 10), mutated CRE (lane 4 and 11), non-related sequence NF-kB (lane 6 and 13) or TFIID (lane 7 and 14). Similarly results were observed in hGR-AP/CRE-2 probe (Fig. 6.12).

To further examine the identity of these proteins, antibody supershift assay was performed using nuclear extract isolated from αT3-1 (Fig. 6.13) and JEG-3 (Fig. 6.14) cells after forskolin stimulation. The addition of an antibody against the CREB eliminated the DNA-protein complexes, further supporting the binding of the CREB to these binding site.

6.4 Discussion

6.4.1 Transcription regulation of human GnRHR gene by cAMP/PKA pathway

It has been shown that the regulation of GnRHR gene expression is controlled, at least in part, at the transcriptional levels (Chapter 1, Section 1.7). Early studies using transient transfection system have demonstrated an increase in rat GnRHR promoter activity in aT3-1 and GT1-7 cell-lines after 10 uM forskolin stimulation (Reinhart et al. 1997). Similarly, an increase in mouse GnRHR promoter was also been observed in GGH3 cells after CTX or cAMP stimulation (Lin and Conn 1998). Further studies in mouse GnRHR promoter have identified a putative CRE located between –107 and –100 (5' TGACGTTT3'; relative to transcription start site) that is responsible for the transcriptional activation of the mouse GnRHR gene by cAMP (Maya-Nunez and Conn 1999). These data suggest the possible role of cAMP in up-regulating the GnRHR gene transcription. In the present study, a luciferase reporter gene vector containing 2297 bp human GnRHR 5'-flanking region was used to examine the transcriptional up-regulation of human GnRHR gene expression through the cAMP/PKA pathway. An increase in human GnRHR-Luc gene expression is stimulated by treatment of adenylate cyclase activators, including forskolin and CTX, as well as by a cAMP analog (8-Br-cAMP). These results demonstrate that cAMP regulates GnRHR gene transcription in human as in rodent. Interestingly, prolong activation by forskolin (24h) did not further increase the GnRHR promoter activity. Instead, a decrease in human GnRHR promoter activity was observed. It has been demonstrated that activation of protein kinase A was capable to alter the expression of PKA subunits (Garrel et al. 1995). A decrease in catalytic subunits and increase in regulatory subunit levels were observed in response to sustained activation of PKA system by forskolin and cAMP analogs (Houge et al. 1990, Richardson et al. 1990, Garrel et al. 1995). This negative feedback regulatory mechanism helps to maintain cellular homeostasis under prolonged hormonal or neurohormonal stimulation and prevents it from over stimulation.

A pharmacological approach was used to further define the signaling pathway in controlling the up-regulation of GnRHR gene expression by the forskolin stimulation. By the use of specific inhibitors for PKA and AC, the forskolin-induced increase in human GnRHR promoter activity was demonstrated to be PKA and AC dependent. The results in the present study suggested the possibility of regulating human GnRHR gene expression by hormones that activate cAMP/PKA pathway. The demonstration of an increase in human GnRHR promoter activity in pituitary cells after PACAP treatment and in placental cells after hCG administration further support the notion that cAMP/PKA regulates the GnRHR gene transcription.

6.4.2 Role of cAMP response element in human GnRHR gene

Progressive 5' deletion assay revealed several potential cAMP responding regions. Among them the sequence between nt –577 to –227 appears to play a major role in maintaining the basal cAMP stimulation. Although no CRE consensus motif was located within this region, two putative AP-1/CREB binding sites were functionally identified to be involved in mediating cAMP-induced stimulatory effects by mutation studies and GMSA. Using consensus AP-1 and CRE motifs, and antibodies against CREB, c-Jun and c-Fos, hGR-AP/CRE-1 was found mainly bind CREB in both αT3-1 and JEG-3 cells. Similarly, hGR-AP/CRE-2 bound mainly CREB in JEG-3 cells. In contrast, hGR-AP/CRE-2 was found to bind both AP-1 and CREB in αT3-1 cells, mainly to AP-1 before and to CREB after forskolin stimulation. There is evidence that despite their structural and functional uniqueness, the CREB and AP-1 binding protein are not only capable of binding to their cognate binding sites, but are also able to exchange partners and form heterodimers with altered binding affinities (Hai and Curran 1991, Chatton et al. 1994, De Cesare et al. 1995, Millhouse et al. 1998). Thus, AP-1 has been shown to bind to CRE motif (Sassone-Corsi et al. 1990). Likewise, CREB has been shown to bind AP-1 site (Masquilier and Sassone-Corsi 1992). Recent studies have also demonstrated a single AP-1/CREB binding site

in human histone gene H3.3B that binds protein complex containing AP-1 and CREB (Witt et al. 1998). Hence, it is not unexpected that the AP-1/CREB binding sites located in the present study are responsible for the forskolin-stimulatory effects. Site-directed mutagenesis coupled with functional studies confirmed that these two AP-1/CREB binding sites in human GnRHR promoter played a role in mediating the forskolin-stimulatory effect.

6.4.3 Role of CREB and AP-1 in human GnRHR gene transcription

In addition, mutation of hGR-AP/CRE-1 and hGR-AP/CRE-2 in αT3-1 cells resulted in a 22% decrease and 29% increase in basal promoter activity, respectively. Interestingly, an opposite effect was obtained in JEG-3, where as 40% increase and 20% decrease in basal activity was observed in hGR-AP/CRE-1 and hGR-AP/CRE-2 transfected JEG-3 cells, respectively. These results indicate that this element is not only functionally involved in mediating the cAMP-induced promoter activity but also involved in basal promoter activity. However, their effects were cell-dependent. Although CREB is one of the best-studied links between activation of cAMP/PKA pathway and gene expression, it is now clear that the versatility of the nuclear response to cAMP is provided by interplay of addition transcription factors including cAMP response element modulator (CREM) (Daniel 1998). Both CREB and CREM are originally identified as responsive to cAMP-dependent signaling pathway (Montminy 1997)) and showed a high degree of sequence homology (Daniel 1998). As these factors belong to the basic leucine zipper (bZIP) protein class, it may allow potential dimerization between different transcription factors (Daniel 1998). Interestingly, CREM family not only contains trans-activator but also negative repressor (Foulkes et al. 1991). It has been demonstrated that the CREM α, β, and γ functioned as antagonists of cAMP-induced transcription either by binding to CRE as nonactivating homodimers or heterodimer, thereby blocking CRE-binding activator (Foulkes et al. 1991). Furthermore, by the use of an alternative promoter, a truncated product,

ICER is produced from the CREM gene (Molina et al. 1993). This protein contains only the DNA-binding domain and does not depend on phosphorylation by PKA for their activation (Molina et al. 1993). The intact DNA-binding domain directs specific ICER binding to the consensus CRE. Importantly, ICER is able to heterodimerize with other members in CREM and CREB family and functions as a powerful repressor of cAMP-induced transcription (Molina et al. 1993). Therefore, the differential expression of varies CREB, CREM, and ICER levels in αT3-1 and JEG-3 cells will certainly affect the composition of transcription factors that bound to these elements.

The demonstration of AP-1 binding to hGR-AP/CRE-2 and a 29% increase in basal promoter activity after mutation of these site in αT3-1 imply that the AP-1 binding may suppress the basal activity of this promoter. Interestingly, the increase in CREB binding to this site reverse this regulatory effect from inhibitory to stimulatory, suggesting that an increase in CREB, presumably after PKA activation by forskolin-treatment, compete for AP-1 to the hGR-AP/CRE-2 binding site. These data not only confirm the role of this motif in mediating cAMP action, but also underscore the complex regulatory machinery in gene expression, such that the expression of GnRHR gene was constantly under fine-tuning by the availability of different transcription factors.

6.4.4 Involvement of other transcription factors in mediating cAMP responsiveness

Although double mutation of both AP-1/CRE binding sites further reduced the responsiveness to forskolin stimulation, it did not completely eliminate this effect. These data suggest that other regulatory elements were involved to attain the maximal response. It has been shown that other transcription factors, such as AP-2, NF-kB and Sp1 (Momoi et al. 1992, Daniel et al. 1998) can also mediate cAMP stimulation in gene expression. Of particular interest to the present study, an orphan nuclear receptor steroidogenic factor-1 (SF-1) was demonstrated to

mediate the cAMP-induced transcriptional activation (Michael et al. 1995, Zhang and Mellon 1996). Analysis of the 351 bp fragment between –577 to –227 revealed a putative SF-1 binding site, located in –404 to –396, suggesting the possibility of this GSE in mediating the forskolin-stimulatory effect. The co-operative action of this GSE and/or other transcription factor binding sites with the two putative AP-1/CRE binding sites need to be explored.

6.4.5 GnRH activates cAMP/PKA pathway

It is well characterized that agonist occupancy of GnRHR results in a Gq/11-mediated activation of PLCβ, with consequent generation of diacylglycerol and inositol phosphates, which in turn activates protein kinase C (PKC) and elevates cytosolic Ca^{2+}, respectively (Stojilkovic and Catt 1995). However, there is also evidence that GnRHR is coupled to cholera toxin sensitive G-protein and stimulates cAMP production. This potential role of cAMP in GnRH signaling was firstly reported in the rat pituitary (Borgeat et al. 1972, Beaulieu et al. 1975). Furthermore, expression of rat GnRHR on the rat somatolactotrope GH3 (Kuphal et al. 1994) and baculovirus-insect (sf9) cells (Delahaye et al. 1997), or the mouse GnRHR in monkey kidney COS-7 cells (Arora et al. 1998) and in Chinese hamster ovary CHO cells (Nelson et al. 1999) demonstrated the GnRHa-induced release of cAMP. This concept has been strengthened recently by the observation that the highest abundance of the Gsα protein in the pituitary gonadotropes (Wilson et al. 1995). Together, these observations have led to the view that, besides PLC/PKC pathway, cAMP might also act as a second messenger for GnRHR. In fact, our results in GnRHa-induced increase in GnRHR and βhCG mRNA levels in JEG-3 cells was mediated by PKA pathway indicate the potential coupling to cAMP/PKA pathway, at least in the human placenta (Chapter 3). Based on our current data, we hypothesize that one of the possible mechanism by which the up-regulation of human GnRHR gene expression is controlled by cAMP mediated pathway after the binding of GnRH to its receptor. However, this mechanism

cannot be tested in the present study using αT3-1 cells since the administration of GnRH to αT3-1 cells did not stimulate the accumulation of cAMP (Horn et al. 1991). However, the increase in human GnRHR promoter after forskolin, cholera toxin, or cAMP stimulation in both pituitary and placenta cells support this idea. In addition, cAMP and forskolin has been demonstrated to increase the number of GnRH receptor in cultured rat pituitary gonadotrope cells (Clayton et al. 1985, Clayton 1989). Lin and coworkers (1998) have also demonstrated an increase in mouse GnRHR promoter activity in GGH3 cells after a GnRH agonist (Buserelin) treatment. This stimulation was reversed by the co-administration of ACI (SQ22536) or mimicked by both CTX and cAMP further supporting this notion.

In conclusion, we have demonstrated an increase in human GnRHR promoter activity after activation of cAMP/PKA pathway. Two putative AP-1/CREB binding sites were identified and functionally participated, at least in part, in mediating the basal and cAMP-induced increase in promoter activity.

CHAPTER 7. SUMMARY

The recent molecular cloning of the GnRHR cDNA from mammals leads to a rapid progress in our understanding of the structure of this receptor, its interaction with ligand, and its signaling pathway after activation. It is now clear that regulation of the level and responsiveness of the GnRHR is critical to the maintenance of normal reproductive function and to the effectiveness of GnRH analogs in a variety of clinical application, and the change in GnRHR mRNA level is one of the mechanisms for regulating the expression of the GnRHR (see Chapter 1 for review). The mechanism by which the expression of one gene can be specifically directed in a range of functionally and developmental diverse tissues might be based on the usage and/or presence of tissue-specific promoter or transcription regulator. These regulators are able to govern the expression of the gene by virtue of specific recognition sites that binds DNA and enhance or repress transcription. Although the isolation and the initial characterization studies of the mouse GnRHR 5' flanking DNA have identified several DNA binding domains that are responsible for basal expression and/or GnRH-mediated homologous regulation of this gene in the pituitary (Albarracin et al. 1994, Clay et al. 1995, Duval et al. 1997, Norwitz et al. 1999, White et al. 1999), the transcription regulation of human GnRHR gene in the pituitary is still unknown. In addition, the recent identification of GnRH-GnRHR system in the placenta prompts us to ask the mechanism(s) in controlling the expression of human GnRHR gene in these two tissues.

Using PCR amplification and *in situ* hybridization methodology, the expression of GnRHR mRNA was demonstrated (Bramley et al. 1994, Lin et al. 1995, Yin et al. 1998). However, this technique only detected a fragment of the GnRHR mRNA. Recent studies using human pituitary poly (A) RNA as a template for PCR amplification, a truncated GnRHR cDNA

subtype was identified, in addition to the wild type GnRHR (Grosse et al. 1997). The truncated cDNA arises from alternative splicing by accepting a cryptic splicing acceptor site in exon 2. In addition, a third GnRHR transcript variant was also identified, and the expression pattern of these variants was demonstrated to be cell specific (Kottler et al. 1999). Interestingly, a recent study has reported the identification of second human GnRHR gene (Millar et al. 1999). However, sequence analysis revealed the lack of exon 1 in this gene, and the presence of a putative carboxyl-terminal tail from the deduced cDNA sequence; this tail is absent in all of the isolated mammalian GnRHR cDNA. By the use of PCR amplification and Northern blot analysis, this gene has been shown to express in a wide range of tissues, but the principal product is a transcript of the antisense DNA strand (Millar et al. 1999). Sequence analysis of this antisense transcript reveals no significant open reading frame. It suggested that a functional protein is not translated from this gene. As the GnRHR in the placenta has a 10 to 100 fold lower affinity than that of the pituitary counterpart, it is possible that the placenta GnRHR mRNA detected previously may 1) have a different primary structure, 2) not expressing full-length mRNA and not code for a functional protein, or 3) have a different post-translation modification. To confirm the expression and elucidate the structure of the placenta GnRHR, the full-length cDNA encoding human GnRHR from human placental cells was characterized. Our results revealed that the human placental GnRHR has the same primary structure as in the pituitary; therefore, the different in GnRH affinity may due to the post-translation modification (Chapter 3). Nevertheless, two GnRHR subtypes have recently been isolated from goldfish (Illing et al. 1999). By the use of PCR amplification with PCR primers encoding the 3rd extracellular loop of GnRHR (which is known to be necessary for the determination of ligand specificity), Troskie and coworkers (1998) reported three putative GnRHR subtypes in vertebrates; this suggested the possible presence of multiple GnRHRs, even in the human, although, the presence of a second functional GnRHR remains to be demonstrated.

The isolation of full-length placental GnRHR from JEG-3 cells suggests the feasibility of studying the transcription regulation of this gene in these cells. By the use of transient transfection assay, the expression of placental GnRHR gene transcription was controlled mainly by the upstream promoter. In addition, this promoter was found to be placental-cells specific. Further studies have identified 4 transcription factor binding sites, namely hGR-Oct-1, hGR-CRE, hGR-GATA and hGR-AP, which involved in regulating the basal promoter activity (Chapter 4).

It has been demonstrated in ovine that P negatively regulates the pituitary GnRHR gene expression (Sakurai et al. 1997, Kirkatrick et al. 1998). The regulatory role of P in controlling the GnRHR gene transcription in both pituitary and placenta levels is examined in the present study. As expected, P negatively regulates the human GnRHR gene transcription in pituitary cells. In contrast, P acts as a positive regulator in mediating the placental GnRHR gene expression (Chapter 5). By the use of progressive 5'deletion luciferase constructs, mutation coupled with functional studies and GMSA, a putative PRE, namely hGR-PRE, was identified to be mediated, at least in part, to the P-induced inhibitory and stimulatory effects in human GnRHR gene transcription in the pituitary and placenta, respectively. However, mutation of this PRE dose not completely eliminates the P-induced effects. Over-expression of human PRA and PRB in αT3-1 cells shows that both PR-A and PR-B play a role in mediating the inhibitory effect of P. Similarly, over-expression of PR-A resulted in a decrease in human GnRHR promoter activity in JEG-3 cells. Interestingly, over-expression of PRB increase the human GnRHR promoter activity in the JEG-3 cells, suggesting a differential role of PRA and PR-B in regulating the expression of this gene in the placental (Chapter 5).

It has been shown in the rat (Reinhart et al. 1997) and mouse (Lin and Conn 1998) GnRHR promoter that forskolin, cholera toxin or cAMP analog treatment resulted in an increase in promoter activity. The demonstration of increase in GnRHR mRNA levels in placenta by

GnRHa, the blockage of this effect by the administration of adenylate cyclase inhibitor, and mimicking this stimulatory effect by forskolin treatment suggest the potential up-regulation of GnRHR gene expression by cAMP/PKA pathway (Chapter 3). Our results indicate that activation of cAMP/PKA pathway by pharmacological agents in the pituitary and placenta increases the human GnRHR gene transcription (Chapter 6). In addition, this stimulatory action can be mimicked by the administration of PACAP and hCG in the pituitary and placental cells, respectively. These results imply that any hormones or regulator, which activates cAMP/PKA pathway in the pituitary and placenta, may increase the human GnRHR gene expression. Two CREs, namely hGR-AP/CRE-1 and hGR-AP/CRE-2, are identified and showed to mediate, at least in part, cAMP-induced increase in human GnRHR promoter activity. However, mutation of both CREs does not abolish the cAMP-induced stimulatory effect, suggesting an involvement of additional mechanism or transcription factor to attain the full response. More interestingly, hGR-AP/CRE-2 showed to bind both AP-1 and CRE.

Progesterone and hCG production during human gestation play an important role in maintenance of pregnancy (Falcone and Little 1994). The stimulatory role of P and hCG in placental GnRHR expression may help to sustain the expression of GnRHR in responding to placenta GnRH-induced hCG production (Siler-Khodr et al. 1986, Barnea and Kaplan 1989, Merz et al. 1991) throughout the pregnancy.

In conclusion, the molecular cloning of human GnRHR full-length cDNA from human placental cells strengthens the autocrine/paracrine role of GnRH in the placenta. The identification of various transcription factor binding sites in the human GnRHR 5'-flanking region in controlling the basal expression as well as hormonal-mediated regulation of this gene underscore a complex regulatory mechanism, which is constantly under fine-tuning by the availability of different transcription factors and different signaling input in controlling the human GnRHR gene expression at the transcriptional level.

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APPENDIX 1. TRANSCRIPTION DOWN-REGULATION OF GnRHR GENE BY GnRH IN PITUITARY

A1.1 Introduction

Treatment with GnRH results in a biphasic response in the gonadotropes with respect to GnRHR number. Short-term pulsatile treatment results in GnRHR up-regulation whereas prolonged exposure to high concentrations of GnRH induces GnRHR down-regulation (see Chapter 1 for review). Clinically, GnRH analogues have proven to be efficacious in treating a wide varity of gonadal hormone-dependent disorders, such as endometriosis, precocious puberty and polycystic ovarian syndrome (Faure et al. 1993). In addition, GnRH agonists have been used extensively in assisted reproductive technologies (Gordon and Hodgen 1992, Faure et al. 1993). The clinical applications of GnRH agonists are based primarily on the decrease in gonadotropin release as a result of GnRH receptor down-regulation by continuous GnRH agonist administration (Barbieri 1992, Emons and Schally 1994). To date, the molecular mechanisms underlying the transcriptional regulation of the human GnRHR gene in pituitary after continuous GnRH treatment remains poorly understood. As the first step to understand the possible transcriptional regulation of the human GnRHR gene by GnRH, a 2297 base pair (bp) 5' flanking region of the human GnRHR gene was functionally characterized by luciferase reporter gene assays in pituitary gonadotrope-derived cell line (αT3-1).

A1. 2 Materials and Methods

A.1.2.1 Site-directed mutagenesis

Mutations were introduced by a three-step PCR mutagenesis method as described in Chapter 2 (section 2.11) using mutagenic primers mAP-1(-1000) and mAP-1(-943), and universal primers UP-T3F, UP, and T7R (Table A1.1).

A2.2.2 Transient transfections and reporter assay.

Transfections were carried out using the Lipofectin procedure as recommended by the manufacturer (Gibco-BRL Life Technologies, Burlington, Canada). In order to correct for different transfection efficiencies of various luciferase constructs, the RSV-LacZ plasmid was cotransfected into cells with the GnRHR promoter-luciferase construct. Briefly, $5x10^5$ cells were seeded into 6-well tissue culture plate 24 h prior to the day of transfection. Five μg of the promoter-luciferase construct and 2.5 μg of RSV-LacZ were combined with 8 μl of lipofectin reagent in 200 μl of serum-free medium. Lipofectin and DNA were incubated together for 45 min at room temperature. It was then diluted to 1 ml with serum-free medium and applied to the cells. Incubation of the cells with transfection cocktail was continued for 24 h at 37°C. Then, 1 ml of medium containing 20% of FBS was added. Cell lysate was prepared 24h later with 200 μl of the luciferase reporter buffer (Promega). Cellular lysates were assayed for luciferase activity immediately. Luminescence was measured using the TROPIX OPIOCOMP I Luminometer (Bio/Can Scientific, Mississauga, Canada). Beta-galactosidase activity was also measured and used to normalize the transfection efficiency. Promoter activity was calculated as luciferase activity/ β-galactosidase activity. A promoterless pGL2-Basic vector was included as a control in the transfection experiments.

A.1.2.3 Pharmacological treatments

In time-course experiments, treatments of the transfected cells were initiated at the same time with the indicated agents, and the treated cells were harvested at each time-point of interest. In experiments wherein the effects of GnRHa, antide and TPA on GnRHR-Luc activity were studied, the cells were treated with the corresponding drug for 24h before luciferase and β-galactosidase activities were measured.

A.1.2.4 Gel Mobility Shift Assay (GMSA)

Oligodeoxynucleotides corresponding to the putative AP-1 element (hG-AP-1) at the human GnRHR 5' flanking region (-1004 to -988), mutated hG-AP-1a, mutated hG-AP-1b and their complements (Table A1.1) were used in GMSA as described in Chapter 2 (section 2.13).

TABLE A1.1. Oligonucleotides used in this study. Mutation nucleotides are underlined.

Oligo name	Sequence (5' to 3')	Purpose
UP-T3F	GTGCCTCTCCTGAACAGCCTCAAGCAATTAACCCTCACTAAAGG	Site-directed mutagenesis
UP	GTGCCTCTCCTGAACAGAATCAA	Site-directed mutagenesis
T7R	CGTAATACGACTCACTATAGG	Site-directed mutagenesis
mAP-1 (-1000)	TTAAATCACCAGCTTGATTTGGCGGCCGCCAACAAGTTTGCTTCTGCA	Site-directed mutagenesis
mAP-1 (-943)	CATGGTGAGTGCTGGATAGCGGCCGCTCAATACTGGTAAGACTTACA	Site-directed mutagenesis
hG-AP-1	TTGGCATTAGACAAATCAA	Probe
mutated hG-AP-1a	TTGGCATTACGGAAATCAA	Competitor
mutated hG-AP-1b	TTGGCATCCGACAAATCAA	Competitor
consensus AP-1	CGCTTGATGACTCAGCCGGAA	Competitor
mutated AP-1	CGCTTGATGACTTGGCCGGAA	Competitor
PRE	GATCCTGTACAGGATGTTCTAGCTACA	Competitor

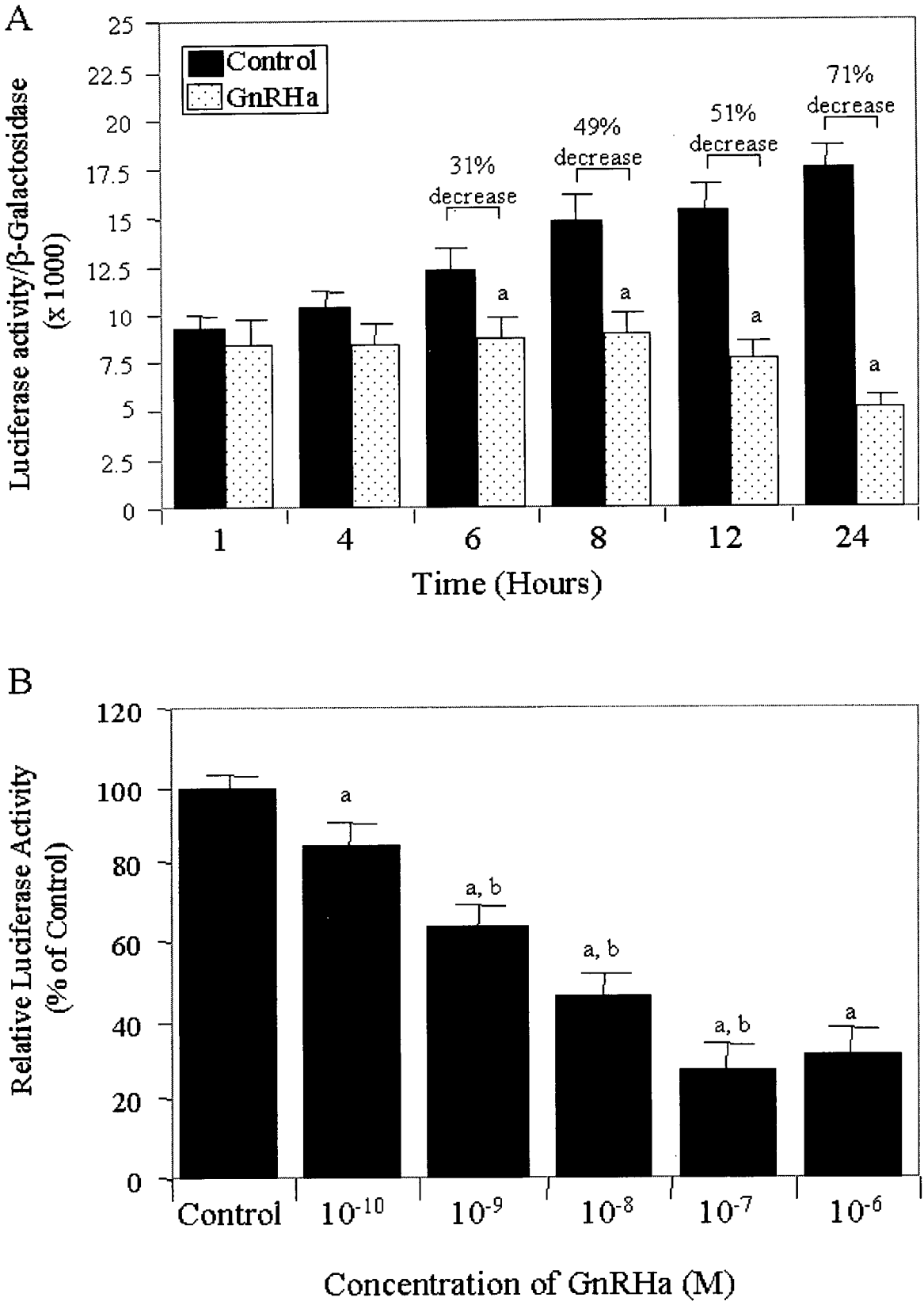
A.1.3 Results

A.1.3.1 Effects of GnRHa, antide and TPA on human GnRHR promoter-luciferase activity in pituitary

As human pituitary gonadotrope cells were unavailable to us, we have used the mouse αT3-1 pituitary tumor cell line, which expresses high levels of GnRHR, as an experimental model for our transient transfection assays. To determine the effects of GnRHa, antide and TPA on the promoter activity of the human GnRHR 5' flanking region, p2300-LucF was transiently transfected into αT3-1 cells and treated with the corresponding pharmacological agent prior to the measurement of the luciferase activity. A dose- and time-dependent decrease in the human GnRHR promoter activity was observed after GnRHa treatment (Fig. A1.1). A longer post-transfection incubation resulted in increased basal luciferase activity. A significant decrease in luciferase activity (31%, $P < 0.001$ vs control) was observed after 6 h of 0.1 μM GnRHa treatment and a maximum inhibition (71%, $P<0.001$) was reached after 24h of 0.1 μM GnRHa treatment (Fig. A1.1A). In addition, this inhibitory effect was observed when treating the p2300-LucF transfected cells with as low as 0.1nM GnRHa for 24h (Fig. A1.1B). In order to investigate whether the activation of GnRHR is essential for inhibiting the human GnRHR promoter activity, a GnRH antagonist, antide, was used to treat the p2300-LucF transfected αT3-1 cells for 24 h, alone or in combination with GnRHa. No significant change of the human GnRHR promoter activity was observed after antide treatment alone, and the magnitude of inhibition by GnRHa was reduced from 55% to 20% ($P < 0.001$) and completely blocked in the presence of 0.1μM and 10μM of antide, respectively (Fig. A1.2). These results suggest that the activation of GnRHR by GnRHa is important and results in activation of an intracellular mechanism that subsequently inhibits human GnRHR gene expression in the pituitary cells. Similar to the results obtained from the GnRHa treatment, a dose- and time-dependent inhibition of human GnRHR promoter activioty was observed after TPA treatment (Fig. A1.3). A significant decrease (30%,

$P<0.01$) in luciferase activity was observed after 6h of 10μM TPA treatment, and the degrees of inhibition increased with time of culture (Fig. A1.3A). Mimicking the inhibitory effect of GnRHa on the GnRHR promoter activity by the administration of TPA suggesting that the protein kinase C pathway is involved in regulating the human GnRHR gene expression in the pituitary gonadotropes at the transcriptional level.

FIG. A1.1 Time- and dose-dependent regulation of human GnRHR-luciferase vector (p2300-LucF) activity in αT3-1 cells treated with GnRH agonist (GnRHa). (*Panel A*) The p2300-LucF transfected αT3-1 cells were treated with 0.1 μM of GnRHa for the indicated times. Treatments were initiated at the same time, and the control and treated cells were harvested at each time point of interest. The RSV-LacZ vector was co-transfected to normalize for varying transfection efficiencies. Luciferase units were calculated as luciferase activity/ β-galactosidase activity and presented as means +/- SD from triplicate assays in four separate experiments. (*Panel B*) The mouse αT3-1 cells were transfected with p2300-LucF and varying concentrations of GnRHa (10^{-6}M to 10^{-10}M) were added to the medium. The cells were collected for luciferase activity measurement after 24 h treatment. Data were presented as percentages of control and means +/- SD from triplicate assays in four separate experiments. a, $P < 0.001$ from control; b, $P < 0.05$ vs the immediately adjacent group on the left.


A
Luciferase activity/β-Galactosidase (x 1000)
25
22.5
20
17.5
15
12.5
10
7.5
5
2.5
0
Control
GnRHa
31% decrease
49% decrease
51% decrease
71% decrease
a
1
4
6
8
12
24
Time (Hours)
B
Relative Luciferase Activity (% of Control)
120
100
80
60
40
20
0
a
a, b
a, b
a, b
a
Control
10-10
10-9
10-8
10-7
10-6
Concentration of GnRHa (M)

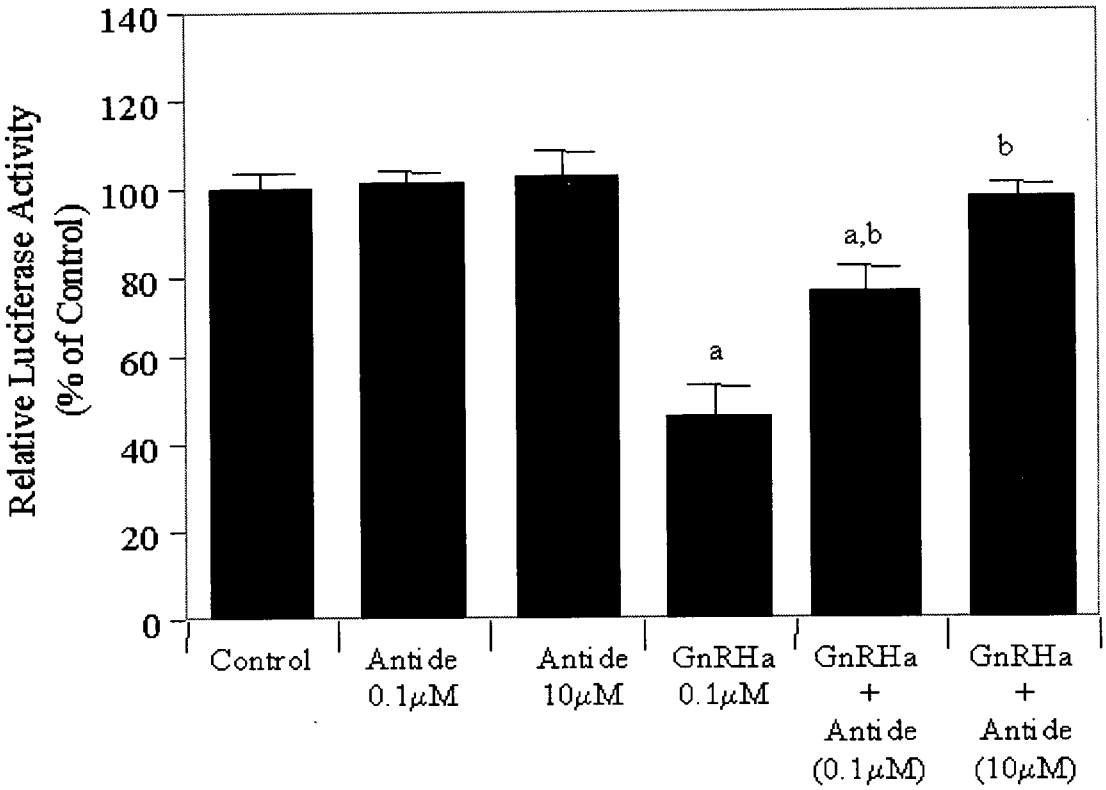
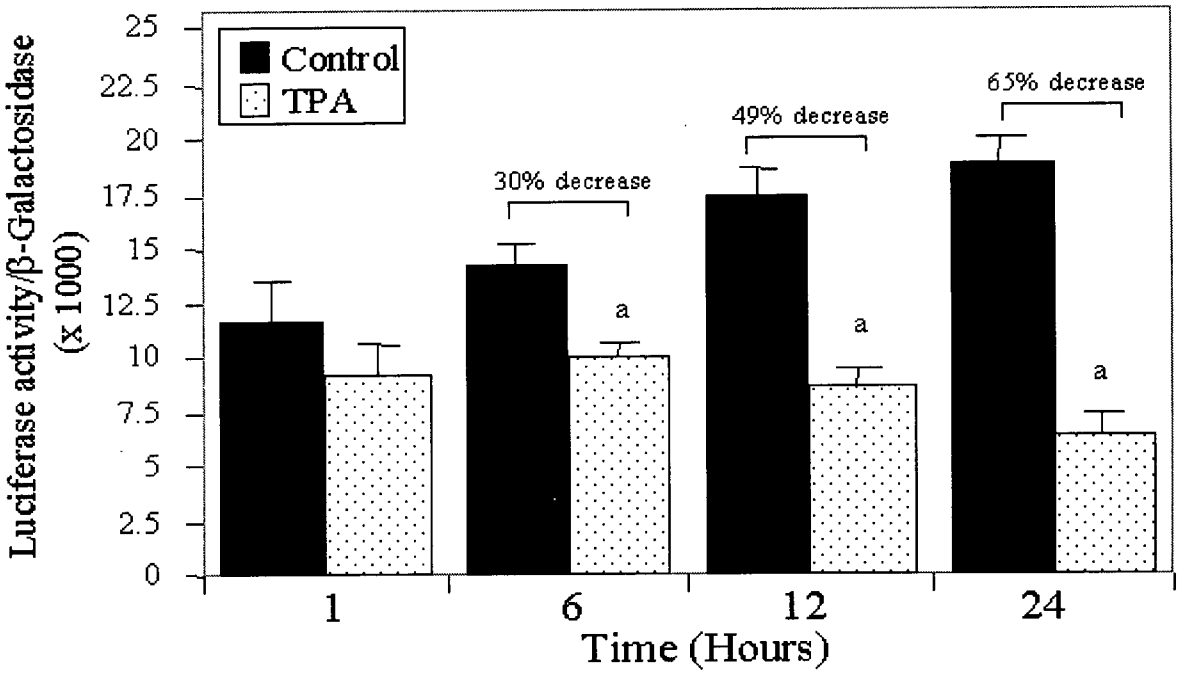


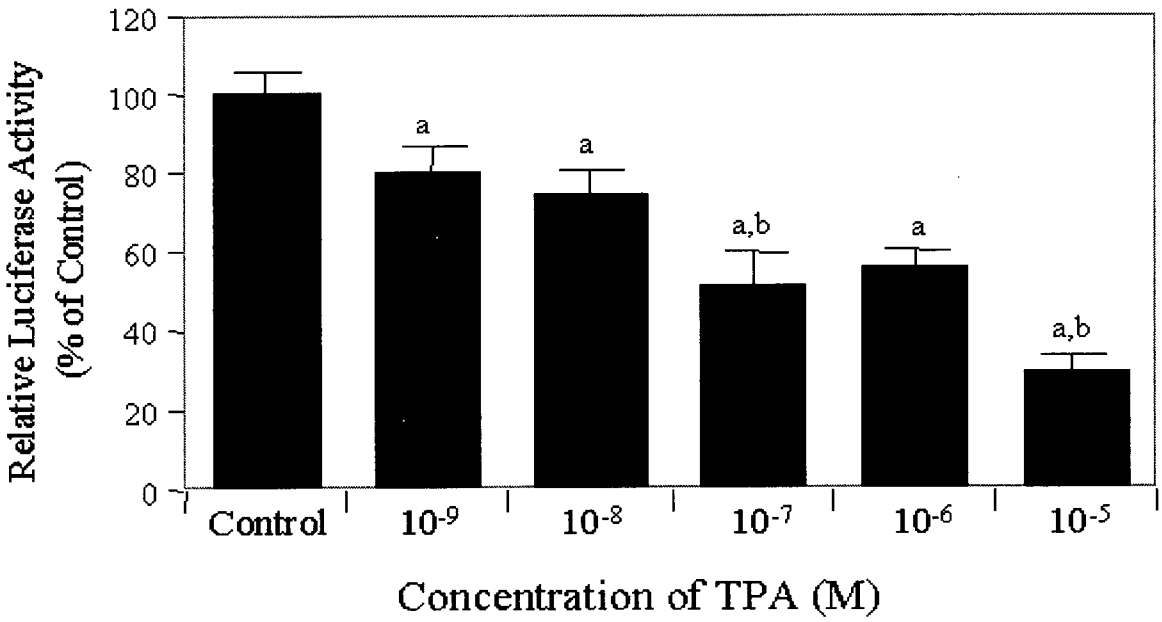
FIG. A1.2 Effects of GnRH antagonist (antide) on GnRHa-mediated inhibition of human GnRHR promoter activity. αT3-1 cells were transfected with p2300-LucF. Cells were harvested 24h post-transfection and were treated with vehicle (control) , 0.1 μM GnRHa, 0.1 μM or 10 μM antide, or both GnRHa and antide for 24 h. The RSV-LacZ vector was co-transfected to normalize for varying transfection efficiencies. Luciferase units were calculated as luciferase activity/ β-galactosidase activity. Data were presented as percentages of control and means +/- SD of three individual experiments with triplication. a, $P < 0.001$ from the corresponding control; b, $P < 0.01$ from the immediately adjacent group on the left.

FIG. A1.3 Time- and dose-dependent regulation of human GnRHR-luciferase vector (p2300-LucF) activity in αT3-1 cells treated with the phorbol ester (TPA). (*Panel A*) The p2300-LucF transfected αT3-1 cells were treated with 10 μM of TPA for the indicated times. Treatments were initiated at the same time, and the control and treated cells were harvested at each time point of interest. The RSV-LacZ vector was co-transfected to normalize for varying transfection efficiencies. Luciferase units were calculated as luciferase activity/ β-galactosidase activity and presented as means +/- SD from triplicate assays in four separate experiments (*Panel B*) The mouse αT3-1 cells were transfected with p2300-LucF and varying concentrations of TPA (10^{-5}M to 10^{-9}M) were added to the medium. The cells were collected for luciferase activity measurement after 24 h treatment. Data were presented as percentages of control and means +/- SD from triplicate assays in four separate experiments. a, $P < 0.001$ from control; b, $P < 0.05$ from the immediately adjacent group on the left.

A


Control
TPA
30% decrease
49% decrease
65% decrease
a
a
a
Luciferase activity/β-Galactosidase (x 1000)
25
22.5
20
17.5
15
12.5
10
7.5
5
2.5
0
1
6
12
24
Time (Hours)

B

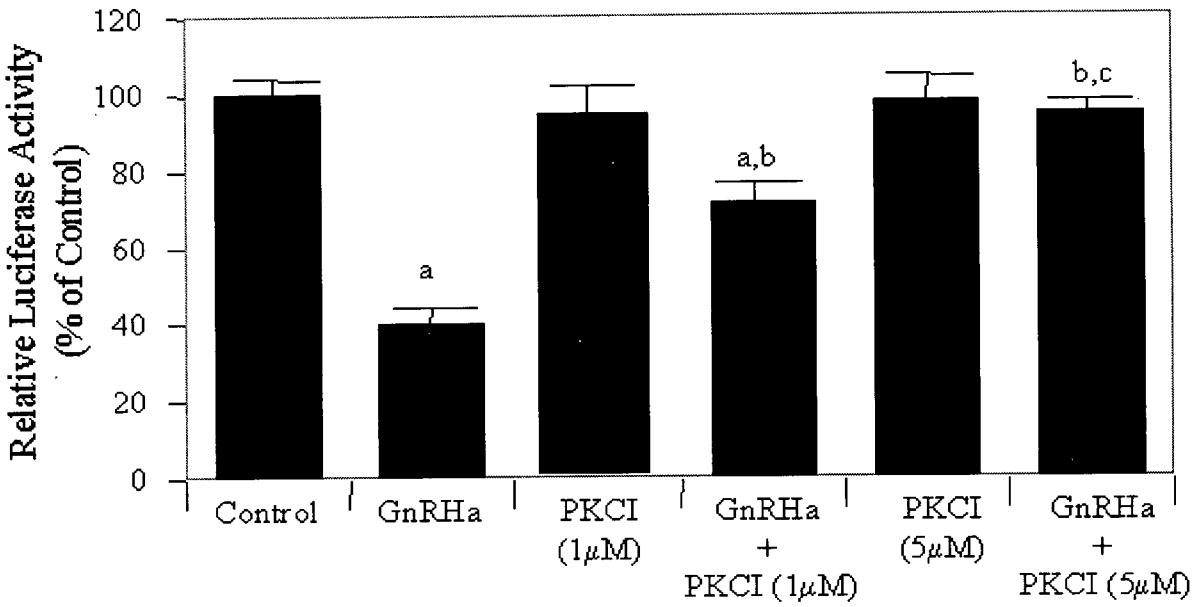

Relative Luciferase Activity (% of Control)
120
100
80
60
40
20
0
a
a
a,b
a
a,b
Control
10-9
10-8
10-7
10-6
10-5
Concentration of TPA (M)

A.1.3.2 Involvement of protein kinase C in the inhibitory effect of GnRH on GnRHR promoter activity in pituitary

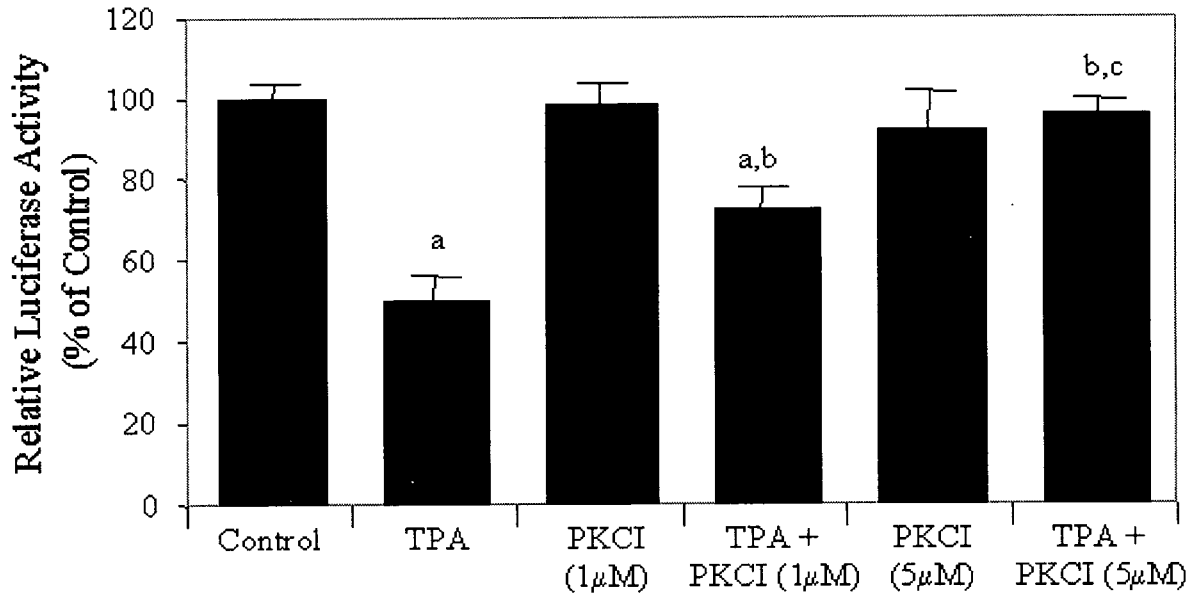
To investigate the possible role of the PKC pathway in regulating the human GnRHR gene expression, a highly specific PKC inhibitor (GF109203X) was used to block the PKC-dependent pathway. The αT3-1 cells were transiently transfected with p2300-LucF and treated with the vehicle, 1 μM or 5 μM GF109203X, 0.1 μM GnRHa or 10 μM TPA alone, or 0.1 μM GnRHa or 10 μM TPA plus GF109203X (Fig. A1.4). Administration of GF109203X alone did not affect the GnRHR promoter activity. However, in the presence of 1μM of GF109203X, the inhibitory effects of GnRHa and TPA on the human GnRHR promoter activity was reduced from 62.5% to 30% ($P < 0.01$) and 57% to 30% ($P < 0.05$), respectively (Fig. A1.4A and B). These inhibitory effects were completely reversed by 5 μM of GF109203X. These results indicate the participation of the PKC pathway in controlling the human GnRHR gene expression at the transcriptional level after GnRHa treatment. The role of PKC in regulating the transcriptional activation of GnRHR gene was further examined by depleting the PKC with pretreatment of 0.1 μM TPA during transfection (Fig. A1.5). The PKC-depleted transfected αT3-1 cells were then treated with vehicle, 0.1 μM GnRHa, or 10 μM TPA for additional 24 h. By the use of Western blot analysis (Fig. A1.5A), pretreatment of αT3-1 cells with TPA resulted in a significant decrease in PKCα (75%, $P<0.001$), PKCβ (60%, $P<0.001$), PKCδ (80%, $P<0.001$) and PKCε (40%, $P<0.001$) levels with no significant decrease in basal luciferase activity (Fig. A1.5B). However, TPA-induced inhibition of GnRHR promoter activity was completely blocked in the PKC-depleted cells. Similarly, TPA-pretreatment resulted in significant reduction on GnRH-induced inhibition of GnRHR promoter activity (Fig.A1.5B). These results indicate that the PKC pathway is important in regulating, at least in part, the human GnRHR gene expression at the transcriptional level.

FIG. A1.4 Effects of the protein kinase C inhibitor (PKCI), GF109203X, on (A) GnRHa- and (B) TPA-mediated inhibition of human GnRHR promoter activity. Cells were harvested 24 h post-transfection. GF109203X was applied 30 min prior to GnRHa and TPA treatment. The RSV-LacZ vector was co-transfected to normalize for varying transfection efficiencies. The p2300-LucF transfected αT3-1 cells were treated with vehicle (control) , 1 μM or 5 μM GF109203X, 0.1 μM GnRHa, both GnRHa and GF109203X, 10 μM TPA, and both TPA and GF109203X for 24 h. Luciferase units were calculated as luciferase activity/ β-galactosidase activity. Data were presented as percentages of control and means +/- SD of three individual experiments with triplication. a, $P < 0.001$ from the corresponding control; b, $P < 0.001$ from GnRHa or TPA alone treatment; c, $P < 0.05$ from GnRHa/TPA + PKCI 1μM treatment.

A


Relative Luciferase Activity
(% of Control)
120
100
80
60
40
20
0
a
a,b
b,c
Control
GnRHa
PKCI
(1μM)
GnRHa
+
PKCI (1μM)
PKCI
(5μM)
GnRHa
+
PKCI (5μM)

B


Relative Luciferase Activity
(% of Control)
120
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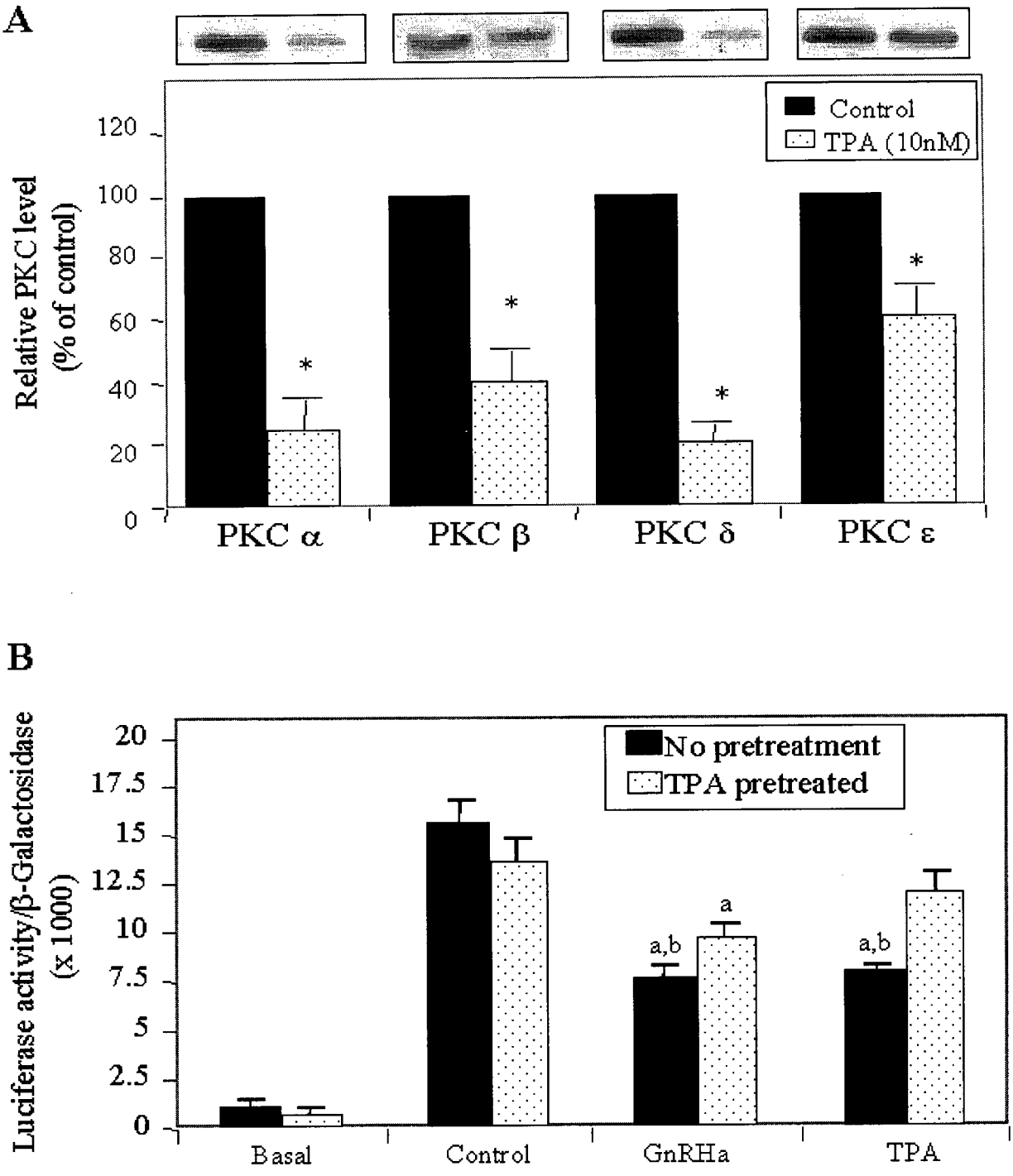
Control
TPA
PKCI
(1μM)
TPA +
PKCI (1μM)
PKCI
(5μM)
TPA +
PKCI (5μM)

FIG. A1.5 Effects of PKC down-regulation on GnRHa and TPA-mediated inhibition of human GnRHR promoter activity. (*Panel A*) Western blot analysis of PKC levels in αT3-1 cells after exposure of the cells to 0.1 μM TPA for 24h. Cell lysates were separated in SDS-polyacrylamide gel. The radioautogram were scanned (shown on the upper part of the graph) and quantified. Data were presented as percentage of control and means +/- SD of three individual experiments. *, $P < 0.001$ from contorl. (*Panel B*) To deplete PKC, one-half of the cells were treated with 10 nM of TPA during transfection, while the other half treated with vehicle. One set of the cells were collected after transfection (basal), remaining cells were washed once and treated with vehicle (control), 0.1 μM GnRHa or 10 μM TPA for addition 24h. The RSV-LacZ vector was co-transfected to normalize for varying transfection efficiencies. Luciferase units were calculated as luciferase activity/ β-galactosidase activity and presented as means +/- SD of three individual experiments with triplication. a, $P < 0.001$ from the corresponding control; b, $P < 0.001$ from the TPA pretreated group.


A
Relative PKC level
(% of control)
120
100
80
60
40
20
0
Control
TPA (10nM)
*
*
*
*
PKC α
PKC β
PKC δ
PKC ε
B
Luciferase activity/β-Galactosidase
(x 1000)
20
17.5
15
12.5
10
7.5
5
2.5
0
No pretreatment
TPA pretreated
a,b
a
a,b
Basal
Control
GnRHa
TPA

A.1.3.3 Identification of the human GnRHR promoter sequence required for the GnRH-mediated inhibition in pituitary

To localize a specific region that mediates the responsiveness to GnRHa down-regulation of GnRHR promoter activity, progressive deletion constructs containing fragments of the human GnRHR 5' flanking region were fused to a luciferase reporter gene and transfected into αT3-1 cells. All deletion clones were functionally active in αT3-1 cell with a minimal 7 folds increase in luciferase activity when compared to pGL2-Basic, except p167-HLuc (Fig. A1.6). Deletion in the human GnRHR 5' flanking sequences up to the Pst I site (1018 bases away from the translation start site) did not affect its responsiveness to GnRHa inhibition. However, further deletion to the Spe I site (771 bases away from the translation start site) abolished the GnRHa-induced inhibitory effects on luciferase activity (Fig. A1.6). These results suggest that this 248 bp DNA fragment located between Pst I and Spe I sites in the human GnRHR 5' flanking region is responsible for the GnRH-mediated down-regulation of human GnRHR gene expression in αT3-1 cells.

A.1.3.4 Identification of putative AP-1 binding in the 248 bp GnRHR 5' flanking region

Sequence analysis of this 248 bp fragment reveals the existence of two potential AP-1 binding sites, located at nt -1000 to -994 (5'TTAGACA3'; in complementary orientation) and nt -943 to -937 (5'TGAATAA3'), with 87% of sequence identity to the AP-1 concensus sequence (the different nucleotide from the consensus AP-1 sequence was underlined). Gel mobility shift assays were conducted using the 248 bp DNA (nt -1018 to –771; related to translation start site) as a probe and nuclear extracts isolated from αT3-1 cells after GnRHa treatment. As shown in figure A1.7, there were 3 DNA-protein complexes formed with the 248 bp DNA fragments. The specificity of these complexes was confirmed by the addition of both specific (unlabelled 248 bp probe; -1018 to -771) and non-specific (unlabelled DNA fragment; -2300 to –2000 related to

translation start site) DNA competitors (Fig. A1.7; lane 2 and lane 3). Furthermore, the competition with increasing concentrations of unlabelled AP-1 consensus element displaced the binding of two sequence-specific protein complexes (Fig. A1.7; band 1 and band 2), suggesting the presence of AP-1 binding sites within this region.

A.1.3.5 AP-1 mediates GnRHa-induced transcriptional down-regulation of GnRHR gene in pituitary

To test the role of these AP-1 binding sites in mediating GnRHa-induced inhibitory effect, the two putative AP-1 binding sites were mutated in SHLuc (Fig. A1.8). The mutated constructs were transiently transfected into αT3-1 cells and treated with 0.1μM of GnRHa for 24h. Site directed mutation of the putative AP-1 binding site located at nt -943 to -937 did not alter GnRHa-induced inhibition of GnRHR promoter activity. However, mutation of the AP-1 binding site at nt -1000 to -994 abolished the GnRHa induced inhibition (Fig. A1.8).

A.1.3.6 The putative AP-1 response element binds c-Fos and c-Jun protein from αT3-1 cells after GnRHa treatment

To confirm the identity of the AP-1 binding site in the human GnRHR promoter, gel mobility shift assay was repeated with a synthetic oligodeoxynucleotide containing the putative AP-1 binding element (hG-AP-1; -1000 to -994) in the presence of AP-1 consensus element, and antibodies against c-Fos and c-Jun. As seen in figure A1.9, two DNA-protein complexes were formed (band 1 and band 2) using nuclear extract isolated from GnRHa-treated αT3-1 cells. These bands were eliminated by incubation with increasing competitor DNA fragment containing a consensus AP-1 site (AP-1) or unlabelled probe but not with a competitor containing mutated AP-1 site, mutated hG-AP-1 site or non-related sequence (PRE) [Fig. A1.9].

Furthermore, the addition of anti-c-Jun or anti-c-Fos antibodies "supershifted" these complexes, supporting the binding of c-Jun and c-Fos to the hG-AP-1 (Fig. A1.10).

A.1.3.7 Regulation of AP-1 protein by GnRHa

The confirmation of AP-1 binding in the GnRHR promoter suggests that the regulation of AP-1 production, including c-Jun and/or c-Fos, by GnRH might be the molecular mechanism in controlling the expression of this gene. To test this possibility, Western blot analysis was performed using protein isolated from GnRHa-treated αT3-1 cells. A 100% increase ($P< 0.05$) in c-Jun protein was observed after 3h of 0.1 μM GnRHa treatment and maintained up to 24h (Fig. A1.11A). Similarly, a significant increase in c-Fos levels (50%, $P< 0.05$) was observed after GnRHa treatment (Fig. A1.11B). The GnRHa-mediated regulation of AP-1 protein production was further examined by gel mobility assay using hG-AP-1. Interestingly, a differential AP-1 binding was observed. Only one DNA-protein complex was formed with the nuclear extract isolated from non-GnRHa-treated αT3-1 cells, while two DNA-protein complexes were formed with the GnRHa-treated nuclear extract (Fig. A1.12A). The increase in DNA-protein complex intensity implied that more AP-1 like protein was available after GnRHa treatment. In addition, the DNA-protein complex formed with nuclear extract isolated from non-GnRHa-treated cells was "supershifted" only by anti-c-Jun but not by anti-c-Fos antibodies (Fig. A1.12B). Similar to Fig. A1.10, the DNA-protein complexes formed by nuclear extract isolated from GnRHa-treated αT3-1 cells were supershifted by both c-Jun and c-Fos antibodies (Fig. A1.12C). These results suggest that a different composition of AP-1 complex, particularly with c-Fos, may bind to the hG-AP-1 site after GnRHa-treatment.

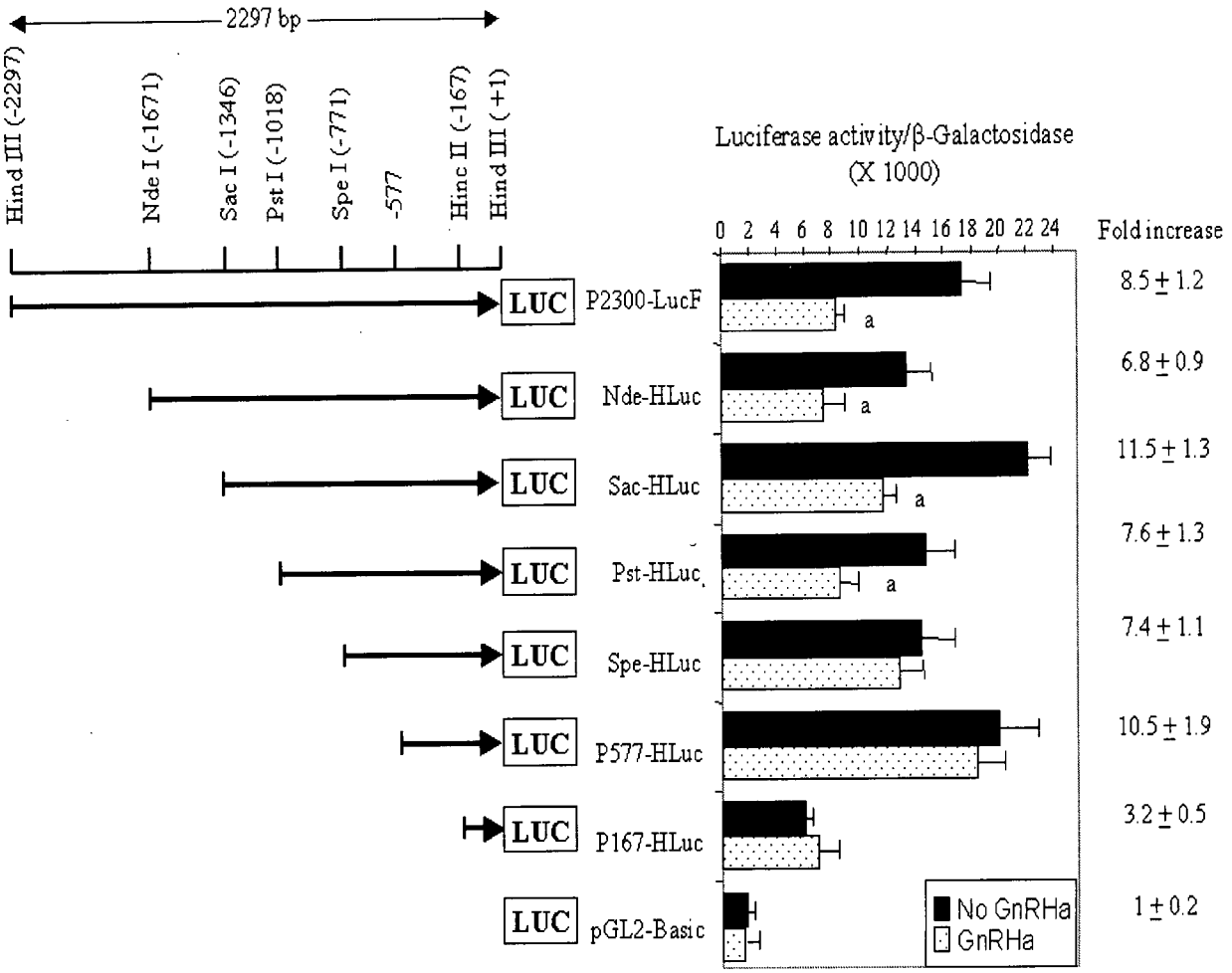


FIG. A1.6 Localization of the GnRH-responsive region in the human GnRHR 5' flanking region. Progressive 5' deletion constructs were transiently transfected into αT3-1 cells and treated with 0.1 μM of GnRHa for 24 h before being harvested for luciferase activity measurement. The RSV-LacZ vector was co-transfected to normalize for varying transfection efficiencies. Luciferase units were calculated as luciferase activity/ β-galactosidase activity and presented means +/- SD of four individual experiments with triplication : a, $P < 0.001$ from no GnRHa treatment. The fold increase was calculated by comparison with the promoterless luciferase vector (pGL2-Basic).

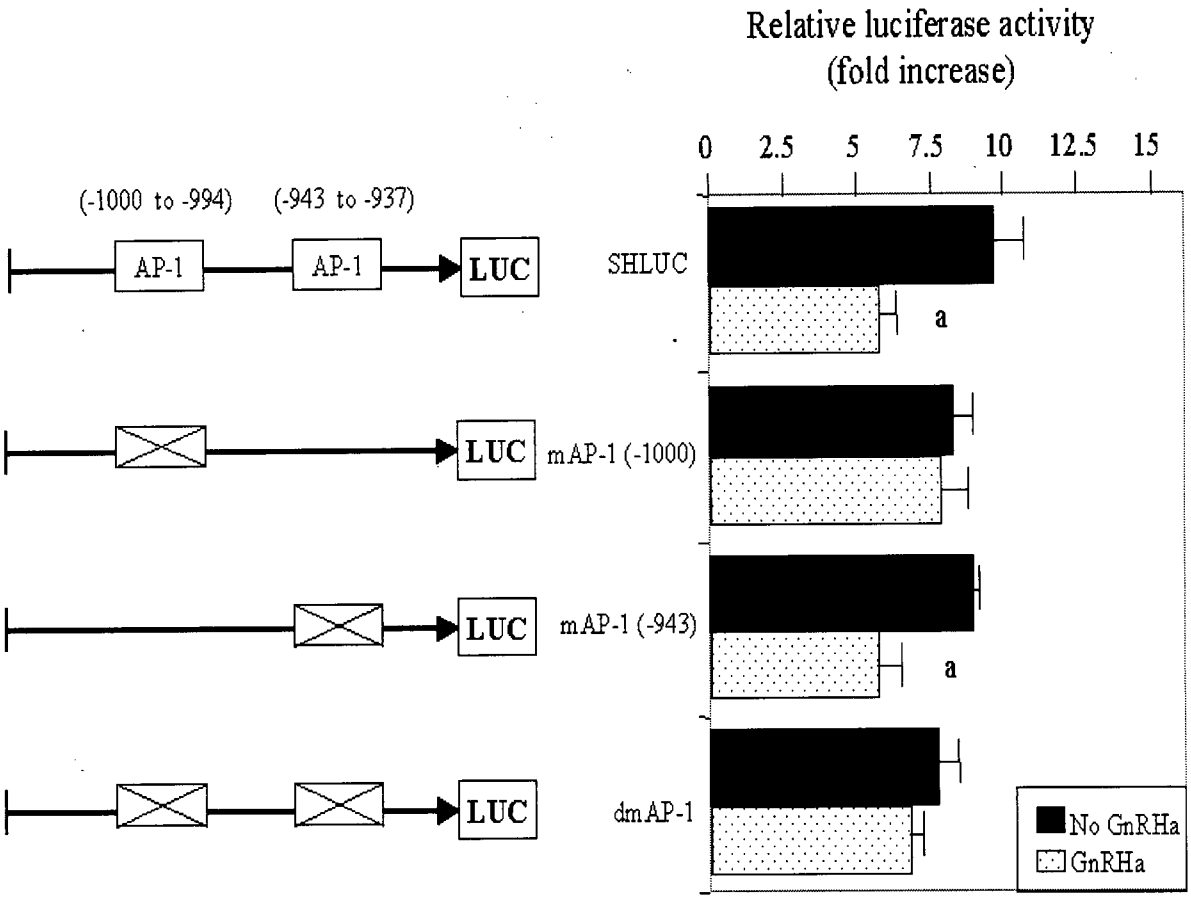


FIG. A1.8 Effect of mutations in the putative AP-1 binding sites on GnRH responsiveness of the human GnRHR gene. Single mutation, mAP-1(-1000) and mAP-1(-943), and double mutation (dmAP-1) SHLuc constructs were transiently transfected into αT3-1 cells and treated with 0.1 μM of GnRHa for 24 h before being harvested for luciferase activity measurement. The RSV-LacZ vector was co-transfected to normalize for varying transfection efficiencies. Luciferase units were calculated as luciferase activity/ β-galactosidase activity. The fold increase was calculated by comparison with the promoterless luciferase vector (pGL2-Basic). Data were presented as means +/- SD of four individual experiments with triplication : a, $P < 0.001$ from no GnRHa treatment.

A.1.4 Discussion

A.1.4.1 Transcription down-regulation of human GnRHR by GnRHa

It has been shown that GnRH can down-regulate GnRHR number (Uemura et al. 1992, Anderson et al. 1995) and mRNA levels in the pituitary cells of rat (Bauer-Dantoin et al. 1995), sheep (Turzillo et al. 1995, Wu et al. 1994) and cow (Vizcarra et al. 1997). Studies of transcriptional regulation of GnRHR gene expression in gonadotrope cells have been reported since the isolation of GnRHR gene 5' flanking from the mouse (Albarracin et al. 1994, Clay et al. 1995) and human (Fan et al. 1995, Kakar 1997). It has been shown that the preferential expression of mouse and human GnRHR in gonadotropes was controlled by the existence of several transcriptional factors including steroidogenic factor-1 (SF-1) and its binding sites in the mouse GnRHR 5' flanking region (Duval et al 1997, Ngan et al. 1999). Although the 5' flanking regions of the GnRHR gene from human (Fan et al. 1995, Kakar 1997) have been isolated, the molecular mechanism(s) controlling the transcriptional regulation of the GnRHR gene expression in the human remains unclear. In the present study, a 2297 bp of the human GnRHR gene 5' flanking region was used to examine the transcriptional regulation of human GnRHR gene by GnRHa and TPA in αT3-1 cells. As no human pituitary gonadotrope cells were available, we have employed mouse gonadotrope-derived αT3-1 cells as an experimental model in the present study. Our results suggest that one potential mechanism for down-regulation of the human GnRHR is by decreasing its gene expression, since continuous administration of GnRH resulted in the reduction of the human GnRHR promoter activity. Prolonged incubation (24 h) of the transfected αT3-1 cells with a GnRH agonist inhibits the human GnRHR promoter activity in a dose-dependent manner. In addition, our results also indicate that activation of GnRHR by GnRH is essential for the regulation of GnRHR gene activity since no reduction in luciferase activity has been observed after antide treatment alone, and the inhibitory effect of

GnRHa on human GnRHR promoter activity was reversed in the presence of the GnRH antagonist.

Interestingly, no stimulatory effect on human GnRHR promoter activity by GnRHa treatment was observed as in the mouse gene. In these study, an increase in mGnRHR promoter activity was resulted in the transfected αT3-1 cells after 4h of 0.1μM GnRH treatment (White et al. 1999, Norwitz et al. 1999). Further analysis of the time-course of the GnRH response revealed a higher effect at 6h treatment (White et al. 1999). Although the basal luciferase activity was increased by prolonged transfection, it reduced the responsiveness of the transfected cells to GnRH treatment (Norwitz et al. 1999). We did not observe any increase in the human GnRHR promoter activity in 4h and 6h of 0.1μM GnRHa treatment. Instead, a significant inhibition on the human GnRHR promoter activity was obtained after 6h of 0.1μM GnRHa treatment. This difference is possibly due to the different experimental conditions in the present study in comparison to the mouse studies: 1) transient transfection was carried out by lipofectin in contrast to the calcium phosphate precipitation; 2) the incubation time for the transfection reaction was 24h in our study but 30min (White et al. 1999) or 4h (Norwitz et al. 1999) in the mouse studies; 3) a mouse gonadotrope-derived cell-line was used in this study to examine the regulation of human gene, it is possible that a species-specific regulator(s) required to control the increase in expression of human GnRHR gene are not present in the αT3-1 cells; or 4) a different regulatory mechanism was used in controlling human gene vs rodent gene.

A1.4.2 Role of PKC in mediating GnRHa action

It is well known that agonist occupancy of GnRHR results in a Gq/11-mediated activation of PLCβ, with consequent generation of diacylglycerol and inositol phosphates, which in turn activates protein kinase C and elevates cytosolic Ca^{2+}, respectively (Stojilkovic and Catt 1995). We have shown in the present study that the activation of the PKC pathway is involved in

controlling the human GnRHR gene expression at the transcriptional level. The inhibition of human GnRHR promoter activity by GnRHa can be mimicked by TPA administration. Our data from the experiments using the specific PKC inhibitor, GF109203X, further confirmed that GnRH acts through a PKC-dependent pathway in regulating the transcription of human GnRHR gene. The role of PKC in regulating the transcriptional activation of GnRHR gene was further demonstrated by depleting the PKC with pretreatment of 0.1 μM of TPA. In agreement with previous studies (Poulin et al. 1998), we observed a significant decrease in PKC-α, -β, -δ, and –ε isoforms after PKC depletion. In addition, depletion of endogenous PKC eliminated the TPA-induced inhibition of GnRHR promoter activity further supported the role of PKC. However, the GnRHa-induced inhibition on GnRHR promoter activity cannot be abolished completely after depletion of PKC by TPA pretreatment, suggesting an additional mechanism might be involved. It has been recently shown that the desensitizing effect of GnRH remained unchanged despite of the presence of PKC inhibitor or down-regulation of PKC by TPA pretreatment in αT3-1 cells (Poulin et al. 1998). Apart from the PLCβ, phospoholipase A2 (PLA) and phospholipase D (PLD) are also activated by GnRH which resulted in a production of arachidonic acid (AA) and phosphatidic acid (PA), respectively (Netiv et al. 1991, Ben-Menahem et al. 1994, Zheng et al. 1994, Poulin et al. 1998). Phosphatidic acid can be converted to DAG, by a specific PA-phosphophydrolase, which activates Ca^{2+} independent PKC isoforms such as novel PKC (Nishizuka 1992). In addition, arachidonic, oleic, linoleic and linolenic acids (derived from PA via PLA) were found capable of supporting the activation of specific PKC isoforms (Bell and Burns 1991). Recent studies have demonstrated that GnRH activates and regulates various PKC isoforms in αT3-1 cells (Shraga-Levine et al. 1994, Kratzmeier et al. 1996, Harris et al. 1997, Poulin et al. 1998). In particular interest, GnRH activated PKCζ (Kratzmeier et al. 1996), which is insensitive to either GnRH or TPA down-regulation (Poulin et al. 1998). Perhaps the

stimulation of this TPA-insensitive PKC isoform after GnRHR activation is also involved in regulating the expression of human GnRHR gene.

The mechanism(s) underlying GnRHR homologous desensitization remains unclear and might be different from other G protein coupled receptors (Ferguson et al. 1996) due to the lack of C-terminal tail and parts of the third intracellular loop (McArdle et al. 1999). Since GnRH exposure to gonadotropes resulted in a decrease of GnRHR number and mRNA levels, modification of GnRHR synthesis has been proposed as a mechanism of GnRHR homologous desensitization. Thus decrease of the GnRHR promoter activity may provide one possible mechanism of GnRHR desensitization.

A.1.4.3 Identification of GnRHa responding region and role of AP-1

This is the first demonstration of GnRH down-regulation of the human GnRHR promoter activity. Using deletion, mutation and gel mobility shift studies, a putative AP-1 binding site located between -1000 and -994 was identified to be responsible for this inhibitory effect. Interestingly, AP-1 has been shown to be important in regulating gonadotrope-specific expression of the mouse GnRHR and to mediate GnRH-induced increase in mGnRHR promoter activity, since the mutation of the AP-1 site resulted in a significant loss of promoter activity as well as GnRH responsiveness (Duval et al. 1997, White et al. 1999, Norwitz et al. 1999). However, we did not observe any significant loss of promoter activity after mutating the AP-1 site located at –1000 to –994, suggesting that this AP-1 site may not regulate basal human GnRHR gene expression. It has been further supported by the observation that the deletion of this AP-1 site form Pst-Hluc to Spe-Hluc did not affect the basal promoter activity. It has been demonstrated in the rodent GnRH gene that a 1-bp difference from the consensus AP-1 binding element in the human GnRH gene resulted in a loss of DNA-protein binding (Zakaria et al. 1996). Indeed, there are two nucleotides different between the identified AP-1 binding site in the

mouse (TGACTCA) and human (TGTCTAA) GnRHR genes, suggesting that a different regulatory mechanism may be used in controlling the GnRHR gene expression in rodent and human. Nevertheless, a recent study has demonstrated that the inhibition of rat GnRH gene expression at the transcriptional level by phorbol ester may be mediated by the AP-1, since deletion of a putative AP-1 binding site abolished this TPA inhibition (Bruder and Wierman 1994). Similarly, over-expression of c-Jun and c-Fos in rat pituitary cells significantly reduced gonadotropin α-subunit promoter activity (Colin and Jameson 1994). These studies support the potential role of AP-1 in down-regulation of gene expression.

Activation of GnRHR in primary cultures of rat pituitary gonadotropes and αT3-1 cells by GnRH caused an increase in mRNA levels of c-Jun, c-Fos and JunB (Cesnjaj et al. 1994). The GnRHa-induced expression of these genes was mimicked by activation of protein kinase C by phorbol ester. In addition, depletion of cellular protein kinase C by prior treatment of TPA reduced GnRH- and TPA-induced expression of these genes, further supporting the role of protein kinase C in mediating the GnRH stimulatory effect (Cesnjaj et al. 1994). In agreement with these studies, a significant increase in c-Fos and c-Jun protein levels was observed after GnRHa treatment in the present study by Western blot analysis. Using nuclear extracts from αT3-1 cells, with or without GnRHa treatment, a differential binding of AP-1 was observed in hG-AP-1. Our data suggested that the possible mechanism in GnRHa-mediated inhibition in human GnRHR promoter activity may be the result of changing in AP-1 composition, since no retarded migration of the AP-1 complex by c-Fos antibody in non GnRHa-treated nuclear extract. Furthermore, a second DNA-protein complex, which can be retarded by both c-Jun and c-Fos antibodies, was only observed in GnRHa-treated nuclear extract. These results indicated that c-Fos plays an important role in mediating the GnRHa-induced inhibition of the human GnRHR gene expression. Several studies have demonstrated the negative transcriptional regulatory action of c-Fos (Burder et al. 1996, Jin and Howe 1999). A dose-dependent decrease

in rat GnRH promoter activity (Burder et al. 1996) and clusterin promoter activity (Jin and Howe 1999) was observed with co-transfection of increasing c-Fos expression vector. Furthermore, this inhibitory effect was reversed in the presence of mutant c-Fos expression vector (Burder et al. 1996, Jin and Howe 1999). It is worth noting that AP-1 is a family of nuclear transcription factors composed of either homodimeric Jun or heterodimeric Fos-Jun complexes that interact with the AP-1 binding site to regulate gene expression in transcriptional level. The Jun family includes c-Jun, Jun B, and Jun D, while Fos gene family members include c-Fos, Fos B, Fra-1, and Fra-2. The differential expression among these gene products could certainly affect the composition of the AP-1 and their action (Hai and Curran 1991).

In summary, we have demonstrated a decrease of the human GnRHR promoter activity after GnRHa treatment in αT3-1 cells. The activation of GnRHR and the PKC pathway are shown to be important for transcriptional down-regulation of the human GnRHR gene. In addition, a putative AP-1 binding site within the human GnRHR 5' flanking region (-1000 to -994) has been functionally identified to be involved in the molecular mechanism of this down-regulation.

APPENDIX II. FUTURE STUDIES

The results obtained in the present study advanced our understanding in regulating the human GnRHR gene transcription. However, the complete mechanism in controlling the expression of this gene is still far from fully understood. Further studies are certainly needed to improve our knowledge in disclosing the regulation of this gene. These include:

1) The localization of hGR-AP-1, hGR-CRE, hGR-AP/CRE-1 and hGR-AP/CRE-2 in the human GnRHR 5'-flanking region suggests a potential regulation of this gene by both PKC and PKA pathways. Activation of these pathways in the pituitary and placental cells by various hormones, including GnRH, provides a complex hormonal network to control the expression of this gene. Hence, the identification of different hormonal pathways in the pituitary and placenta would certainly improve our understanding in regulating human GnRHR gene expression by the interaction between different hormones.

2) It is now known that both AP-1 and CREB family consist of multiple subunits, and the ability of dimerization within the family provide a complicate network in controlling the expression of the human GnRHR gene. It will be interesting to identify the individual subunit in each family that expressed in the pituitary and placenta and elucidate their role in controlling the human GnRHR gene expression.

3) The differential role of PRA and PRB in mediating the P-induced effect, and the opposite effect in mutating hGR-PRE in the expression of human GnRHR gene in the pituitary and placenta cells suggests the presence of different mediator(s) or co-activator(s) in conducting the

P-action. The identification of this cell-specific factor(s) will improve our comprehension in cell-specific regulation of human GnRHR gene by P. In addition, examine the regulation of PR gene expression in pituitary and placenta cells provide an extra mechanism in controlling the human GnRHR gene expression.

4) Although the role of hGR-PRE and hGR-AP/CREs in conducting the P and cAMP are confirmed, mutations of these elements dose not completely eliminate the effect induced by P or forskolin treatment. These results suggest the presence of additional regulatory mechanism or transcription factor in mediating the full responsiveness. The identification of other transcription factor binding site(s) and their co-operative action in mediating the full effect need to be explored.

5). Using 5'- and 3'-deletion human GnRHR promoter luciferase constructs, a strong negative regulatory region has been located between –1018 and –771 (Chapter 4, Fig. 4.2). To examine the role of this region, the DNA fragment between –1018 and –771 was subcloned into a luciferase vector with a minimal TK promoter and transient transfected into αT3-1, IEVT and JEG-3 cells. Our preliminary study indicates that this region contains an extremely strong negative regulatory element, as the presence of this region eliminate the promoter activity (Fig. A2.1). It will be interesting to elucidate the transcription factor(s) that binds to this region.

6) It is well demonstrated that estradiol plays an active role in regulating the expression of GnRHR gene in animal (see Chapter 1; section 1.4.2 for review). However, no significant change in human GnRHR promoter activity is observed after estradiol treatment in both αT3-1 and JEG-3 cells (Fig. A2.2). Western blot analysis shows that the endogenous level of estrogen receptor α (ERα) is very low in JEG-3 and αT3-1 cells when compared to human breast MCF-7

cells (Saceda et al. 1988) or human granulose-luteal cells (Chiang et al. 2000), which has been demonstrated to express ER (Fig. A2.3). Hence, the lack of responsiveness to estradiol treatment in αT3-1 and JEG-3 cells may due to the low levels of ER. The role of estradiol in regulating human GnRHR gene expression in transcription level can be studied by over-expression of ERα in these cells. Despite of ERα, it is now known that the estradiol action may also be mediated by ERβ. It will be interesting to elucidate the role of ERα and ERβ in mediating the estradiol action.

7) Using *in vitro* transfection system, several transcription factors have been identified in the present study in controlling the expression of human GnRHR gene. The role of these transcription factors can be further examined *in vivo* using transgenic model. The results from these works will not only confirm our data *in vitro*, but also reveal the potential physiological relevance/role of these transcription factors *in vivo*.

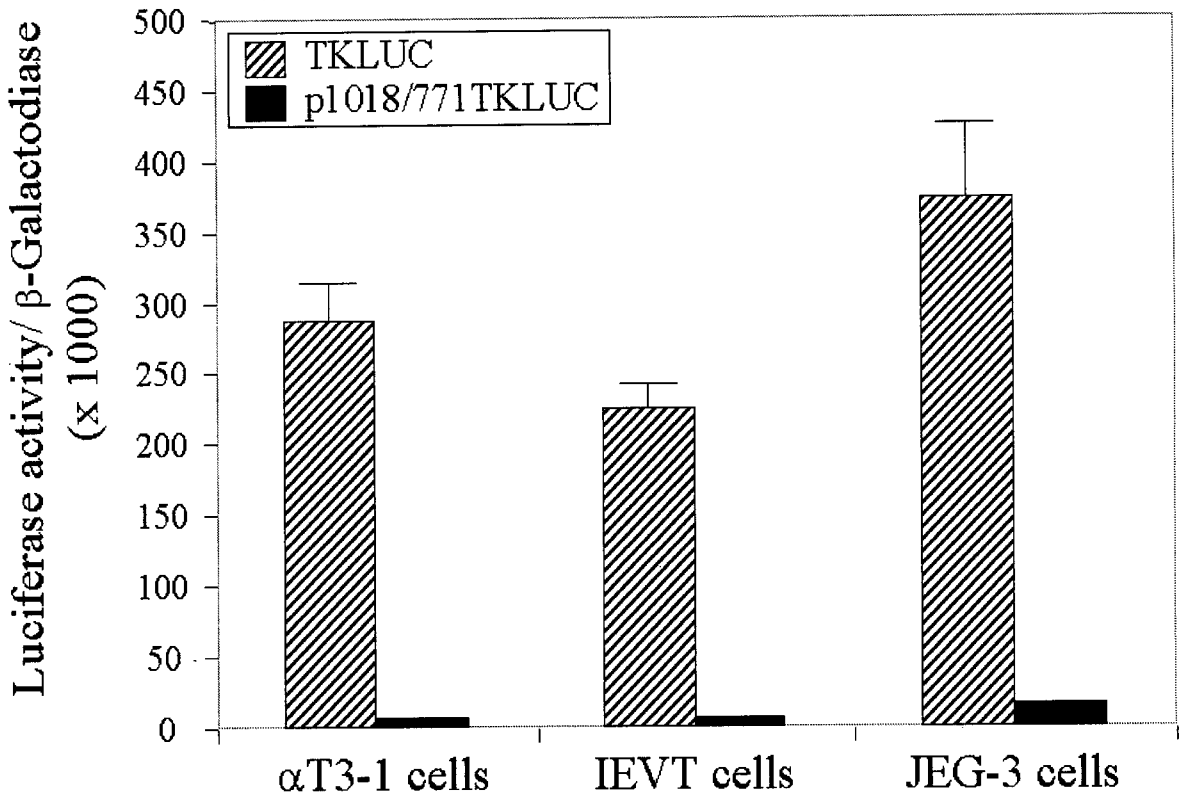


FIG. A2.1 Confirmation of transcription repressor in human GnRHR promoter. The negative regulatory region (-1018 to –771) located was subcloned to a luciferase vector drive by TK minimal promoter and transiently transfected into αT3-1, IEVT and JEG-3 cells. The RSV-LacZ vector was co-transfected to normalize for varying transfection efficiencies. Luciferase units were calculated as luciferase activity/ β-galactosidase activity and presented as means +/- SD from triplicate assays in three separate experiments.