國立臺灣大學生命科學院漁業科學研究所

碩士論文

Institute of Fisheries Science

College of Life Science

National Taiwan University

Master Thesis

日本鰻之促性腺激素釋放素啟動子分析

Gonadotropin-releasing hormone (GnRH) promoter analysis of *Anguilla japonica*

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中華民國 99 年 7 月

July, 2010

謝辭

首先感謝指導教授韓 玉山老師這兩年來的辛勤教導，且孜孜不倦地與學生討論並解決實驗上所遇到的問題，此外傳授我許多研究中的精隨，使我了解許多在實驗室中待人接物的觀念，尤其是老師對於科學研究上探索真相之專注與執著，不僅是最佳身教，同時也是鞭策學生在求學路上不敢鬆懈的重要助力，謹此致上衷心之謝忱。

感謝口試委員鄭 達智老師及蕭 仁傑老師在百忙之中撥冗口試，斧正論文，在口試中，對於論文寫作及報告上的指教評點，更讓學生受益匪淺，使論文更臻完善。

在碩士班學習期間中，我要感謝實驗室全體成員與同學們的協助與幫忙。謝謝怡誠學長的指導，以及每次出國都會很貼心的幫我們選購紀念品，我會努力學會讓回力標飛回來的!還有感謝家芸學姊與佳伶學姊在百忙之中聽我預講專題報告，並幫我訂購實驗上所需的器材，且帶我們發掘還不錯的餐廳，讓我不用兩年都只吃後街的餐館，此外那瓶蜆精也讓我的肝在開夜車的地獄中恢復不少。謝謝黃偉不厭其煩地陪我探討研究的方法與方向，並提供我一些實驗設備上的資源，雖然我的籃球生命很短暫，但那兩個月下午的籃球時光真的很讚，很熱血!感謝永昇提供並與我討論分生實驗上的可行方案，且陪我揪團衝夜唱，讓我在台北的生活精彩了許多。謝謝博智在實驗室扮演開心果的角色，還有不吝惜你的 B 群維他命。感謝桂香與我們分享的人聞軼事，增添了生活中的趣味。謝謝鴻鈞的台南小吃與何敏的點心讓我有不少口福。也謝謝于溶的烏龍茶，讓我在深夜有精神繼續趕論文。並感謝所有幫助過我的人，謝謝你們。

最後我要感謝我的家人，因為你們的支持和鼓勵，讓我得以專注於論文研究，尤其秉潔的打氣和陪伴，使我充實且愉快地度過在台大的時光。僅以此論文感謝與祝福所有身邊的人健康快樂，並願大家與我一同分享論文完成的喜悅，謝謝!

Contents

中文摘要

日本鰻為一降海洄游性魚類，擁有複雜的生活史。在亞洲，日本鰻是重要的經濟養殖魚種之一，然而其養殖產業所需的魚苗(玻璃鰻)，卻只能依靠捕撈天然的玻璃鰻供應之。但氣候變遷、環境汙染與日漸嚴重之過漁的影響，日本鰻苗的自然資源量逐漸稀少，導致養鰻產業的發展受到阻礙，而日本鰻人工繁殖成為解決此瓶頸的重要關鍵。由於現存之鰻魚人工繁殖技術無法成功應用在產業上，我們需要更進一步了解其生殖調控機制，以找出日本鰻自然性成熟所需的條件。鰻魚與其他硬骨魚的生殖週期同樣是由下視丘-腦下垂體-生殖腺所組成的神經內分泌軸線所控制，能夠整合外部環境之刺激以誘發青春期的活化，而促性腺激素釋放素（GnRH）即為此軸線之關鍵調控者。因此本研究針對 GnRH 基因進行啟動子位置的分析，將其基因轉錄起始位（start condon, +1）上游可能為啟動子的區域，以 PCR 增幅放大後，構築到 pGL3-basic 載體中，再轉殖進 GT1-7 細胞株中，以冷光強度分析這些區域轉錄表現情況，確定 GnRH 基因啟動子的關鍵區域以及其轉錄調控情形。研究結果顯示，GnRH 基因轉錄起始位上游 -388 至 -499 為此啟動子重要的區域；當移除此位置，啟動子之活性會受到明顯的抑制。針對此啟動子區域進行轉錄因子分析，在 -488 與 -432 位置上分別發現有 Oct-1 和 Pbx-1a 轉錄因子的結合位，且此兩種轉錄因子已被證實與 GnRH 轉錄之啟動有極大的關係，顯示此區域（ -388 ~ -499 ）為具有調控 GnRH 基因表現的關鍵位置。而在外源性荷爾蒙刺激 GnRH 表現的實驗中，發現雄性素與雌性素的刺激，同樣會受到啟動子中此位置（ -388 ~ -499 ）的調控，此外不論是雄性素或雌性素，在濃度的變化下對GnRH表現均無顯著作用,但若縮短處理的時間,則會失去促進GnRH轉錄的能力，表示性類固醇在 GnRH 轉錄的作用裡，可能以間接的方式調控其基因表現。

關鍵字:促性腺激素釋放素、啟動子、轉錄因子、性類固醇刺激

Abstract

Anguilla japonica is one of important species in Asia aquaculture. However, glass eels production is continuously declining, resulting in great impact on the development of eel aquaculture industry. In order to reach a sustainable eel production, we should develop complete artificial propagation. For this reason we need to know the mechanisms of eel nature maturation. Like other teleost, the center of eel reproduction endocrine control system is hypothalamus-pituitary-gonad (HPG) axis, and GnRH is the major regulator in HPG axis. For this reason, this study focuses on promoter analysis of eel GnRH gene. In the promoter assay, the upstream sequences before GnRH start coding will be available from 5'-Genome Walking. Difference of GnRH promoter area is zonated by specific primers and PCR. After that, PCR product is constructed into pGL3-basic vector then transfect in GT1-7 cell line. Promoter region of GnRH is determinated by assaying the firefly luciferase activity. The results indicate that the critical region for GnRH transcription locates in -388 to -499 that contain the important transcription factors Oct-1 and Pbx-1a for GnRH expression. Moreover, sex-steroids maybe indirectly activate GnRH promoter via this region from -388 to -499.

Key words: Gonadotropin-releasing hormone, promoter, transcription factors, stimulation of sex-steroids

Introduction

1. The life of Japanese eel

Anguilla japonica called Japanese eel is a kind of temperate and catadromous fish with a long migratory loop and lengthy leptocephalus stage. *Anguilla japonica's* life cycle is complex and special. The spawnings live around western Mariana Islands near 14°-16°N 142°E (Tsukamoto, 2006), 2000-3500 km away from the East Asia continent, such as Taiwan, China, Korea, and Japan (Tesch, 2003).The leptocephalus larvae are born between Apil and November (Tsukamoto et al., 2003) ,and they are drifted by the North Equatorial Current (NEC). It takes 4-6 months for them to reach the coasts of East Asia. (Cheng and Tzeng, 1996) When they get the estuaries which are near continental shelf, they are metamorphosed into glass eel. The eels live in rivers for more than 4 years before metamorphosing into silver eels (onset of puberty) in autumn and winter (Han et al., 2003). They migrate back to their birthplace to spawn and eventually die, to complete eel life cycle (Tsukamoto, 1992; Tesch, 2003).

2. The condition of Japanese eel aquaculture

Anguilla japonica is one of the most important species in Taiwan aquaculture. The value of exportation even exceeds ten billion dollars (Liao, 2001). Because the technique of Japanese eel's artificial reproduction is not available, the elvers (glass eels)

must be caught in wild that is the only eel source for aquaculture. Dues to environmental pollution, climate change, and over-fishing, glass eels' production is continuously declining, it makes a great impact on the development of eel aquaculture industry. In order to solve this problem, the development of artificial propagation has become more and more important.

3. The artificial propagation research of Japanese eel

Because the researchers never discover the mature eel in the wild, we could only understand eel procreative system by the test of artificial reproduction. The eel artificial propagation research was started in European eel, using carp pituitary extracts to induce spawning (Fontaine et al., 1964). The study of Japanese eel artificial propagation was beginning in 1970. Yamamoto and Yamauchi inject eels with Salmon pituitary extracts (SPE) to obtain zygote, and incubate larvas which are growing up to 7 mm preleptocephalus stage (Yamauchi et al., 1976). Although SPE can stimulate the vitellogenesis of eel eggs, it couldn't form mature stage alone. It causes ova degenerate that brings unstable fertilization and hatching rates of eel eggs. Since 1990, the artificial propagation of Japanese eel has improved by using 17α, 20β-dihydroxy-4-pregnen-3-one (DHP) to induce the maturation of eggs after SPE generated vitellogensis. This method successfully leaded eel to ovulate, that induced

fertilization and hatching rate at the same time. However, current methods are not suitable for glass eel production due to high cost; moreover, DHP induces inconsistent quality of egg that cause high mortality and dysplasia of the larva (Ijiri et al., 1995). In other teleost, we wait for the fish natural maturation before using high dose external hormone to induce ovulation. In this situation, the quality of the egg is stable. Therefore, finding the environmental conditions of eel maturation becomes a key point in artificial propagation.

4. Eel silvering

Puberty comprises the transition from an immature juvenile to a mature adult state of the reproductive system, i.e. the individual becomes capable of reproducing sexually for the first time, which implies functional competence of the brain–pituitary–gonad (BPG) axis. The characteristics that puberty start in the fish are spermatogenesis and vitellogenesis (Schulz and Miura, 2002; Patino and Sullivan, 2002). In the puberty, fish will undergo morphological and physiological change controlled by Hypothalamus-Pituitary-Gonad axis (HPG axis; Okuzawa, 2002) In Japanese eel the characteristic of puberty activation is eel silvering. When eels start silvering the morphological modifications includes a change in skin color from yellow to silver/bronze, integumental thickness, increased eye size, and changes in the shape of

pectoral fins and snout (Tesch, 1977; Pankhurst, 1982a; Pankhurst, 1988; Sorensen and Rohr *et al*., 2001; Han *et al*., 2003a). The physiological changes include degeneration of digestive tract, changes of visual pigments, modification in the composition and function of skeletal muscle and bone, more developed swim bladder, higher density of chloride cells in the gill, higher muscle fat contents, lower nonesterified fatty acids, and more developed gonad (Lewander *et al*., 1974; Kleckner, 1980; Pankhurst, 1982b; Pankhurst, 1982b; Colombo *et al*., 1984; Pankhurst and Sorensen, 1984; Fontaine *et al*., 1995; Lokman *et al*., 1998; Lokman and Young, 1998; Cottrill *et al*., 2001; Ellerby *et al*., 2001; Han *et al*., 2001; Yamada *et al*., 2001; Yamada *et al*., 2002). The changes in endocrine profiles include the increase of sex steroids and thyroid hormone, in addition the decrease of growth hormone, cortisol and prolactin (Lewander *et al*., 1974; Marchelidon et al., 1996; Han *et al*., 2003b, 2003c, 2003d, 2004).

5. Relation between Hypothalamus-Pituitary-Gonad axis (HPG axis) and puberty

The center of reproductive endocrine control system in Japanese eel is like other teleost, which is regulated by HPG axis. The basic organization of this physiological axis is highly conserved and underlies reproductive competence in a wide variety of diverse vertebrate species including birds, reptiles, fish, and mammals (AI-Kindi et al., 2001). This HPG axis consists of a small subset of hypothalamic neurons that express

the decapeptide hormone GnRH, the gonadotrope cells of the anterior pituitary, and the gonads. Activation of this endocrine axis commences with the pulsatile secretion of GnRH from the hypothalamus. GnRH is delivered to the anterior pituitary via the hypophyseal portal circulation where it binds to the GnRH receptor on the surface of gonadotropes triggering the synthesis and secretion of the gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH), which are responsible for gonadal steroidogenesis and gametogenesis. Subsequently, gonadal steroid hormones which are created by gonads play an important role in feedback regulation of HPG axis (Salisbury et al., 2008; Fig. 1). In addition fish lacks a hypophyseal blood portal system, GnRH is produced by hypothalamus that directly innervates the pituitary and stimulates the secretion of GtHs.

6. Gonadotropin-releasing hormone, GnRH

To achieve appropriate reproductive function, vertebrate have precise regulatory in HPG axis; control by GnRH synthesis and release (Foran and Bass, 1999). Furthermore, the vast dopamine repress GnRH expression and inhibit gonad growth in European eel, when separate the spawning migration (Se ́bert et al., 2008). For these reasons, GnRH becomes the key to study the mechanism of eel maturation. In teleost, GnRH neurons produce in the olfactory placode and then migrate into the brain to several regions,

including the preoptic area (POA), telencephalon, diencephalon, hypothalamus, and terminal nerve ganglion (Zandbergen et al., 1995). According to different position, it has different function, too. The GnRH neurons which locate in POA, hypothalamus, and pituitary are classified to GnRH Ⅰ (Fernald and White, 1999). It's a species-specific GnRH, such as mGnRH and sbGnRH, which mediate pituitary release GtHs and other pituitary hormones. Position in telencephalon is classed to GnRH Ⅱ, likes cGnRH-Ⅱ. The *in vitro* experiment showed that cGnRH-Ⅱ could stimulate GtHs release but its functions are still unclear (Bosma et al., 2000). GnRH is a peptide hormone composed of ten amino acids. According to the differences in amino acid sequences, localizations and embryonic origins, 24 GnRHs have been identified in the nervous tissues, from vertebrates to protochordates (Millar, 2005). Despite the above divergences, all of these variants decapeptides share highly similar structures. Generally, two or three forms GnRHs can be found in one species of vertebrate at the same time (Sherwood et al., 1991；Robinson et al., 2000). In European eel found two different types GnRH, i.e., mGnRH and cGnRH Ⅱ (King et al., 1990), and mGnRH is main type in hypothalamus, to regulate HPG axis (Montero et al., 1995).

7. Research purpose

Japanese eel is an important aquaculture species in Asia, but wild glass eel

production is reducing year by year. It cause in great impact on the development of eel aquaculture industry. In order to solve the problem, we should improve eel artificial propagation via studying the conditions for natural maturation. Because GnRH neuron has ability to join the information of the environment and activation of HPG axis, analysis GnRH transcriptional regulation becomes a method to find conditions of eel natural maturation. However the GnRH neuron in the brain is sparse and dispersive, which makes it difficult to study *in vivo*. Therefore we use a mouse hypothalamus cell line (Gonadotropin-Releasing Hormone Neurons cell; GT1-7 cell) that retain the characteristic of GnRH neuron physiology as the experiment model. The process of transcription begins when the RNA polymerase recognizes a promoter sequence, that promoter is a major regulator for gene expression. Promoters commonly consist of two separate sequence features: the core element, near the transcription start site, where general transcription factors (GTFs) bind, and more distantly located regulatory elements, known variously as enhancers (or silencers). These regulatory sequences are recognized by specific DNA-binding proteins that activate transcription above basal levels (enhancers bind transcriptional activators) or repress transcription (silencers bind repressors). The core region consists of a TATA box indicating the transcription start site. Other regulatory elements include short nucleotide sequences (sometimes called response elements) found near the promoter (the promoter-proximal region) that can

bind certain specific transcription factors, such as proteins that trigger expression of a related set of genes in response to some physiological signal (hormone) or challenge (temperature shock). Therefore researching the transcription factors is necessary for mGnRH expression. It becomes a method to find out the natural conditions for eel maturation. Before transfect the plasmid into GT1-7 cells, we recombine the different region of mGnRH promoter with a luminescence vector (PGL3-Basic vector). Luminescence value was used to find out the critical promoter site for GnRH activation. In addition, Gonadal steroid hormones play a particularly important role in feedback regulation of GnRH expression (Glidewell-Kenney et al., 2007), which has different effect in different fish species and GnRH type. For this reason, we also analyze estradiol-17β (E2) and 17α-Methyltestosterone (MT) effect on GnRH transcription in Japanese eel.

Materials and methods

1. Biological Materials

1.1 *Sample of Japanese eel*

The wild Japanese eels fished from an outfall of Kaoping River.

1.2 *Competent cell*

Escherichia coli DH5α	RBC Bioscience
GT1-7 cell line	Bioresource Collection and Research Center

1.3 *Plasmid*

pGEM-T Easy Vector	Promega
pGL3-Basic Vector	Promega
pGL3-Control Vector	Promega
pRL-TK Vector	Promega

2. Reaction reagents and Mediums

2.1 *Enzyme*

A. DNA polymerase

BioTaq DNA polymerase	Bioman
Pfu DNA polymerase	Bioman

B. Rstriction endonucleases

EcoRI	Fermentas

MluI	Fermentas
XhoI	Fermentas

C. Modifying enzyme

T4 DNA ligase	Promega

2.2 *Sex steroids*

17β-estradiol(E2)	Promega
Methyltestosterone	a gift from Dr. Lo

2.3 *Cell culture*

Dulbecco's Modified Eagle Medium (DMEM)	GIBCO™
Fetal bovine serum (FBS)	HyClone
Penicillic-Streptomycin	GIBCO™
Trypsin-EDTA	HyClone

Phosphate Buffered Saline;PBS

NaCl	137 mM	ZYMESET
KCl	5 mM	BioShop
NaH_2PO_4	1.5 mM	BioShop
Na_2HPO_4	8.1 mM	BioShop

LB-ampicillin broth (per liter)

NaCl	10 g	ZYMESET

Tryptone	10 g	ZYMESET
Yeast extract	5 g	ZYMESET

To dissolve in 1 ml primary water, than auto clave. After cooling add 50 mg ampicillin.

LB-ampicillin agar (per liter)

NaCl	10 g	ZYMESET
Tryptone	10 g	ZYMESET
Yeast extract	5 g	ZYMESET
Agar	15 g	ZYMESET

To dissolve in 1 ml primary water, than auto clave. After cooling add 50 mg ampicillin and place in dishes.

2.3 *Experimental kit*

APAgene™ Genome Walking Kit	BIO S&T
Dual-Glo® Luciferase Assy System, 10ml	Promega
EasyPure Genomic DNA spin kit	Bioman
EasyPure PCR/Gel Extraction kit	Bioman
EasyPure Plasmid DNA miniprep kit	Bioman
QIAfilter™ Plasmid Midi kit	QIAGEN

3. Experimental methods

3.1 *Genomic DNA extraction*

Using BIOMAN EasyPure Genomic DNA Spin Kit purify *Anguilla japonica* genomic DNA. Cut up 20mg of *Anguilla japonica* muscle tissue and transfer to a microcentrifuge tube. Add 200µl GT buffer into the tube and continue to homogenize the sample with grinding. Add 20µl Proteinase K (10mg/ml) to the tube and mix by vortexing. Incubate at 60℃ for 30min to lyse the sample. During incubation, invert the tube every 5min. Add 200µl GB Buffer and vortex for 5sec to mix sample. Incubate at 70℃ for 20min until the sample lysate is clear. During incubation, invert the tube every 5min. At this time, preheat required Elution Buffer (ddH_2O, 50µl per sample) in a 60℃ for DNA elution.Add 200µl of 100% EtOH to the sample lysate and vortex immediately for 10sec to mix sample. Place a GD Column on a 2ml Collection Tube. Apply the total mixture from previous step to the GD Column. Close the cap and centrifuge at 13,000 rpm for 5min. Discard the flow-through and return the GD column to 2ml Collection Tube. Add 400ul of W1 Buffer in the GD Column, and centrifuge at 13,000rpm for 30sec. Discard the flow-through and return the GD column to 2ml Collection Tube. Add 600ul of Wash Buffer to GD column, and centrifuge at 13,000rpm for 30sec. Discard the flow-through and return the GD column to 2ml Collection Tube. Centrifuge again at 13,000rpm for 3min to dry the column matrix.Transfer dried GD Column into a new microcentrifuge tube. Add 50µl of preheat Elution Buffer to the center of the column

matrix. Allow to stand for 3-5min until Elution Buffer is absorbed by the matrix. Centrifuge for 1min at 13,000rpm to elute purified DNA.

3.2. *Genome Walking*

Using BIO S&T APAgene™ Genome Walking Kit acquire GnRH promoter sequence. To design GnRH gene specific primer: GnRH P6, GnRH P4, and GnRH P5 (Table. 3) by *Anguilla japonica* prepro-mGnRH complete cds sequence (GenBank Accession AB026991.1; Fig. 2). First-round PCR prescription

APAgene™ Buffer	5.0 ul
DRT Primer/dNTP Mix D1	7.0 ul
APAgene™ Enzyme Mix	0.6 ul
GnRH P6 primer (20pmol/ul)	1.0 ul
Anguilla japonica Genomic DNA	0.8 ul
Sterile ddH_2O	0.6 ul

Mix gently and briefly spin down in a microcentrifuge. And run Program 1 Polymerase chain reaction, PCR (Table.1). At the end of the first-round PCR, analyze the PCR results by electrophoresis (5ul on a 1% agarose gel in 1x TAE buffer). Smears or multiple bands should be observed. Dilute 1ul of PCR mixture in 49ul sterile H_2O and used as templates for the second PCR. Proceed with the second round PCR. Second-round PCR prescription

APAgene^{TM} Buffer	5.0 ul
Tagging Primer/dNTP Mix T1*	7.0 ul
APAgene^{TM} Enzyme Mix	0.4 ul
GnRH P4 primer (20pmol/ul)	1.0 ul
First-run PCR mixture	1.0 ul
Sterile ddH_2O	0.6 ul

Mix gently and spin down briefly. Then run Program 2 PCR (Table.2). At the end of the second-round PCR, dilute 1ul of PCR mixture in 49ul sterile H_2O and used as templates for the third-round PCR. Third-round PCR prescription

APAgene^{TM} Buffer	5.0 ul
Tagging Primer/dNTP Mix T1*	7.0 ul
APAgene^{TM} Enzyme Mix	0.4 ul
GnRH P5 primer (20pmol/ul)	1.5 ul
Second-run PCR mixture	1.0 ul
Sterile ddH_2O	0.1 ul

Mix gently and spin down briefly. Then run Program 2 PCR (Table.2). At the end of the third PCR, analyze the PCR results by electrophoresis on a 1% agrose gel in 1x TAE buffer. To observe one to two dominant bands ranging from 200-4000 bp. Excise the specific band and purify it use BIOMAN EasyPure PCR/Gel Extraction Kit, and use

TA clone to recombine the purified products, then be sequenced using T7 and SP6 primers.

3.3 *Gel Extraction*

Excise the agarose gel slice containing DNA fragments and removing extra agarose to minimize the size of the gel slice. Transfer up to 300mg of the gel slice into a microcentrifuge tube. Add 500ul of PG Bufferto the sample and mix by vortexing. Incubate at 55℃ for 15min until the gel slice has been completely dissolved. During incubate, invert the tube every 2-3min. Place a Spin Column in a Collection Tube. Apply 800ul of the sample mixture from previous step into the Spin Column. Centrifuge at 13,000rpm for 30sec. Discard the flow-through and place the Spin Column back in the Collection Tube. Add 500 Wash Buffer to the Spin Column. Centrifuge at 13,000rpm for 30sec. Discard the flow-through and place the Spin Column back in the Collection Tube. Centrifuge again for 2min at 13,000rpm to dry the column matrix. Transfer dried spin Column into a new microcentrifuge tube. Add 30ul ddH_2O to the center of the column matrix. Allow to stand for 2min until ddH_2O is absorbed by the matrix. Centrifuge for 2min at 13,000rpm to elute purified DNA.

3.4 *TA clone*

Using Promege pGEM® -T and pGEM® -T Easy Vector Systems (Fig. 3) to perform TA clone. Mix the ligation reagent by pipetting, and then incubate the reactions 1 hour

at room temperature.

Ligation reagent

2X Rapid Ligation Buffer	5 ul
pGEM® -T Easy Vector	1 ul
Insert DNA	3 ul
T4 DNA Ligase	1 ul

Prepare LB/ampicillin/IPTG/X-Gal plates. Centrifuge the ligation reactions briefly. Add 2.5ul of ligation reaction to 50ul DH5α Competent Cells, and vortex 1sec. Place on ice for 8min. Heat-shock the cells for 45sec in a water bath at exactly 42℃. Immediately return the tubes to ice 2min. Transfer into selection plate. Incubate plate at 37℃ for 16-18 hours.

3.5 *Plasmid Extraction*

To select white colonies transfer into 4ml LB medium, incubate at 37℃ for 16-18 hours. Using BIOMAN EasyPure Plamid DNA miniprep kit purified plasmid. Transfer 1.5ml of bacterial culture to a microcentrifuge tube. Centrifuge for 1min at 13,000rpm and discard supernatant. Add 200ul of PD1 Buffer and resuspend the cell pellet by vortexing or pipetting. Add 200ul of PD2 Buffer and mix gently by inverting the tube 10 times. Do not vortex, avoid shearing genomic DNA. Allow mixture to stand for 2min at room temperature until lysate clears. Add 300ul PD3 Buffer and mix immediately by

inverting the tube 10 times. Centrifuge for 2min at 13,000rpm. Place a PD Column in a 2ml Collection Tube. Apply the supernatant to PD Column. Centrifuge at 13,000rpm for 30sec. Discard the flow-through and return the PD Column to the 2ml Collection Tube. Add 400ul of W1 Buffer in the PD Column. Centrifuge at 13,000rpm for 30sec. Discard the flow-through and return the PD Column to the 2ml Collection Tube. Add 600ul of Wash Buffer to PD Column. Centrifuge at 13,000rpm for 30sec. Discard the flow-through and return the PD Column to the 2ml Collection Tube. Centrifuge again for 3min at 13,000rpm to dry the column matrix. Transfer the dried PD column to a clean 1.5ml microcentrifuge tube. Add 30ul ddH2O directly onto the centre of the membrane. Allow to stand for 2min until the liquid is absorbed. Centrifuge for 2min at 13,000rpm to elute purified DNA. To analyze the ligation results of plasmid by restriction enzyme and electrophoresis on a 1% agrose gel in 0.5x TAE buffer. To confirm the right DNA fragment size and sequencing the DNA sequence.

3.6 *GnRH Promoter Truncation assay*

According sequencing result of *Anguilla japonica* GnRH promoter design a specially primers, which contain the restriction enzyme cutting sites of *Mlu I* and *Xho I* (Table.4). Using PCR and specially primers to separate the GnRH promoter of *Anguilla japonica* into 12 parts (Fig.7).

3.7 *Luciferase assay vector prepare*

These specific promoter fragments were clone into pGL3-Luciferase Reporter Vector: pGL3-basic (Fig. 4). After that, quantity production Luciferase Vector and purify plasmid use QIAGEN QIAfilter^{TM} Plasmid Midi Kit. Pick a single colony from a freshly streaked selective plate and inoculate a starter culture of 3ml LB medium containing the appropriate selective antibiotic. Incubate for approx. 8hr at 37℃ with vigorous shaking. Dilute the starter culture 200ul into 100ml LB medium. Grow at 37℃ for 16hr with vigorous shaking. Harvest the bacterial cells by centrifugation at 6000 x g for 15min at 4℃. Resuspend the bacterial pellet in 4ml Buffer P1. Add 4ml Buffer P2, mix thoroughly by vigorously inverting the sealed tube 4-6 times, and incubate at room temperature for 5min. Add 4ml chilled Buffer P3 to the lysate, and mix immediately and thoroughly by vigorously inverting 4-6 times. Pour the lysate into the barrel of the QIAfilter Cartridge. Incubate at room temperature for 10min. Equilibrate a QIAGEN-tip 100 by applying 4ml Buffer QBT, and allow the column to empty by gravity flow. Remove the cap from the QIAfilter Cartridge outlet nozzle. Gently insert the plunger into the QIAfilter Midi Cartridge and filter the cell lysate into the previously equilibrated QIAGEN-tip. Allow the cleared lysate to enter the resin by gravity flow. Wash the QIAGEN-tip with 2 x 10ml Buffer QC. Elute DNA with 5ml Buffer QF. Precipitate DNA by adding 3.5ml room temperature isopropanol to the eluted DNA. Mix and centrifuge immediately at 15,000 x g for 30min at 4℃. Carefully decant the

supernatant. To wash DNA pellet with 2ml of room temperature 70% EtOH, and centrifuge at 15,000 x g for 10min. Carefully decant the supernatant without disturbing pellet. Air-dry the pellet for 10min, and redissolve the DNA in 100ul ddH_2O.

3.8 *Cell culture and transfections*

GT1-7 were cultured in DMEM containing 10% fetal bovine serum, and 1% penicillin/streptomycin in a humidified 5% CO_2 incubator at 37℃. Cells were seeded into 96-well plates (4 x 10^5 cells in 100ul medium for each well) and incubated overnight at 37℃ before being transiently transfected using Lipofectamine reagent. Solution 200ng of luciferase reporter vector and 200ng of the internal-control pRL-TK vector (Fig. 5) into 25ul DMEM mix gently. Mix Lipofectamine™ 2000 gently before use, then dilutes 0.4ul of Lipofectamine into 24.6ul DMEM. Incubate 5min at room temperature. After the 5min incubation, combine the diluted DNA with diluted Lipofectamine™ 2000. Mix gently and incubate 20min at room temperature. Removing the normal medium and washing with PBS for each well. Add the 50ul of complexes to each well containing cells. Mix gently by rocking the plate back and forth. After 6 hours for transfection, changed from transfected reagent to normal medium and continuously incubated 36 hours. In sex steroid stimulation test, a medium contain 100nM or 10nM sex steroid (E2 or MT) was used before 24 or 6 hours for Luciferase assay. In addition, use pGL3-Control (Fig. 6) as a positive control and pGL3-Basic (Fig. 4) as a negative

control for every Luciferase test.

3.9 *Luciferase assay of GnRH expression*

Using Promega Dual-Glo™ Luciferase Assay System detect luciferase values. Add equal culture medium volume (50ul) of Dual-Glo™ Luciferase Reagent to each well and mix. Wait 20min, then transfer the mixture to NUNC DENMARK 96-well plate and measure the firefly luminescence. Add 50ul Dual-Glo™ Stop & Glo® Reagent to each well and mix. Wait 20min, then measure luminescence. Calculate the ratio of luminescence from the experimental reporter to luminescence from the control reporter.

3.10 *Data analysis*

Luminescent value was normalized by formula of relative response ratio: (experimental sample – negative control)/(positive control - negative control) x 100%. Data were analyzed by one-way ANOVA followed by Tukey HSD and Duncan using the computer software SPSS. Data were considered significantly different from each other at $P < 0.05$. The presume transcription factor in GnRH promoter was searched by transcription factor database Transcription Element Search System (TESS; http://www.cbil.upenn.edu/cgi-bin/tess/tess?RQ=WELCOME) and The high-quality transcription factor binding profile database (JASPAR; http://jaspar.genereg.net/).

Results

GnRH Genome Walking in Japanese eel

Because promoter-proximal region can bind certain specific transcription factors, that trigger expression of a related set of genes in response to some physiological signal or challenge. Researching the transcription factors is necessary for GnRH expression. It becomes a method to find out the natural conditions for eel maturation. In addition, mGnRH is a major type GnRH in Japanese eel hypothalamus to regulate HPG axis. In order to understand the mechanisms of mGnRH transcription, we use 5'-Genome Walking to obtain mGnRH promoter sequence of Japanese eel. The third PCR with primer GnRH P5 result in the appearance of a major band of about 1.8 kb. The sequence of the PCR product revealed that mGnRH contained first intron and a novel sequence of 1857 bps added at the 5'-end of first exon (Fig. 2). A five prime untranslated region (5' UTR) was present within the mGnRH promoter sequence at position -942 bps.

Basal GnRH promoter activity assay

To investigate the critical region on promoter for GnRH gene transcription, specific ranges were created within the -1,785 bps eel mGnRH promoter by special primer (Table. 4). To compare with other groups GnRH1 has an intron that analyses the

influence of intron on mGnRH promoter activity. Besides, there has a 5' UTR in the eel mGnRH promoter. Although this region is not translated, several regulatory sequences may be found in the 5' UTR to promote the initiation of translation. To examine the 5’ UTR whether affects mGnRH promoter activity. We remove this region in GnRH2 to form GnRH4, and then contrast to GnRH3 which retain the 5’ UTR (Fig. 2). Figure 8 illustrates the location of difference group on the mGnRH promoter. The GnRH2 and GnRH3 region increased basal transcription compare to wild type GnRH1 (9 and 10 fold respectively), while the GnRH4 decreased basal activity by 1 fold. The results that luminescence value of GnRH1 was lower than GnRH2 might be due to inhibition of an intron, or the frameshift of luciferase gene caused by the starting code within GnRH1. These data suggest that the region GnRH3 which contain the 5’ UTR play a critical role in mGnRH transcription.

Since the enhanced of mGnRH gene expression by the region GnRH3, we used truncation analysis to identify the necessary regions. Truncation to GnRH7 (-740 bps) resulted in slightly increased of luminescence value (Fig. 9). It hinted that the “AC” repeats microsatellite between GnRH6 and GnRH7 had inhibiting ability to mGnRH transcription. To investigate the hypothesis, we created a new group GnRH11 using specific mutations to kick out microsatellite within GnRH2. Figure 10 illustrates GnRH11 which causes mutation of the microsatellite slightly but not significant

increased basal transcription compared to GnRH2. After that, we use Transcription Element Search System (TESS) and the high-quality transcription factor binding profile database (JASPAR) to analysis this location. These database both found a high similarity binding site (CCCCCCAACACACACACACA) for Ras responsive element binding protein 1 (RREB1) in the microsatellite, which is a transcriptional repressor protein to inhibit gene expression in the brain (Thiagalingam et al., 1996). These data indicate RREB1 maybe the reason that cause microsatellite inhibition of mGnRH expression.

Furthermore, truncation to GnRH 10 (-388 bps) resulted in significant reductions in basal activity of mGnRH expression (Fig. 9). These results demonstrate that, transcription factors crucial for basal gene expression probably activate mGnRH transcription by binding the region between GnRH 9 and 10 (from -388 to -499 bps).

Effects of Sex-steroids on GnRH expression

Incubate with 100nM E2 & MT for 24hr

The gonadal steroid hormones, which are key regulators of reproduction, in turn act tightly to regulate GnRH synthesis and release through a negative feedback system between the gonads and brain. To test whether regulation of sex-steroids on mGnRH activity involved with promoter, GT1-7 cells were treated with 100nM MT or E2 and

incubation for 24 hours before to detect the luminescence value. The GnRH2 and GnRH3 region improved transcription compares to wild type GnRH1, while the GnRH4 decreased luminescence levels (Fig. 11). These data indicate GnRH3 is more important than other group to regulate mGnRH expression, when treatment with sex-steroids.

Since sex-steroids caused the slightly elevation of mGnRH transcription at the region GnRH3, we used truncation analysis to identify the necessary regions to interact with sex-steroids. Truncation to GnRH10 (-388 bps) resulted in significant reductions in hormone activity of mGnRH expression (Fig. 12A and 12B). These results suggest that MT and E2 induces mGnRH transcription probably interaction with the region between GnRH9 and 10 (from -388 to -499 bps).

Incubate with 100nM E2 & MT for 6hr

In the ovary, different GnRH mRNA levels were created by a long-term E2 treatment or a short-term treatment (Nathwani et al., 2000). To test whether mGnRH expression induced by sex-steroids was influenced by incubation time, GT1-7 cells were treated with 100nM MT or E2, and culture time decreased from 24 to 6 hours. In these experiments, reduced treatment time generally abolished both MT/E2 stimulation on mGnRH transcription (Fig. 13A and 13B).

Incubate with 10nM E2 & MT for 24hr

Since reduced treatment time abolished sex-steroids stimulation on mGnRH expression, as a contrast we decreased concentration of both MT/E2 from 100nM to 10nM and maintain incubation time. These results indicate both E2/MT significant increase mGnRH transcriptions after 24 hours incubation in GnRH9, even though decreased concentration of sex-steroids (Fig. 13A and 13B).

Incubate with 10nM E2 & MT for 6hr

Because long-term culture in GT1-7 with low concentration sex-steroids could advance luminescence value in GnRH9, this stimulation seemed to be regulated by incubation time. To investigate the hypothesis, we reduce concentration and incubation time in both MT/E2. Fig. 15A and 15B illustrates this treatment generally abolished the enhancement of mGnRH transcription in GnRH9. According to these results the mechanisms of sex-steroids to induce mGnRH activation are regulated via the incubation time but not concentration. For this reason, both E2/MT might indirectly interact with GnRH9 to stimulate mGnRH expression.

Discussion

GnRH Genome Walking in Japanese eel

Since current methods of eel artificial propagation are not suitable for glass eel production, detection of the conditions of eel's natural maturation becomes important. Because the GnRH neuron has ability to join the information of the environment and activation of HPG, analysis of GnRH transcriptional regulation become a method to find conditions of eel natural maturation. However there are seldom studies regarding the transcriptional regulation of the GnRH gene in Japanese eel. The proximal promoter is the proximal sequence upstream of gene, which tends to contain primary regulatory elements in response to some physiological signal. In general, the proximal promoter locates approximately -250 bp upstream of the start site. We cloned about -1857 bps upstream of the eel mGnRH start site, which contain the potential proximal promoter. And research the eel mGnRH characterization of transcriptional regulation. In order to determine its core functional promoter, several deletions of 5'-regulatory region were constructed into pGL3-Basic vector and transferred into GT1-7 cells.

Basal GnRH promoter activity assay

GnRH is essential for reproductive processes, therefore it is important to

understand the control of its synthesis and release. However, it is difficult to study the regulation of GnRH gene transcription in vivo, due to the scarcity and scattered distribution of the GnRH neurons. For this reason, an immortalized GnRH-expressing neuronal cell lines have been the effective and manageable resources that explore mechanisms regulating the expression, synthesis and release of GnRH. Studies on transcriptional regulation of the GnRH have been performed largely in GnRH secreting cell lines, such as GT1, GT1-7, and human granulosa-luteal cells (hGLCs) (Cheng and Leung, 2005). The GT1-7 cell is recognized as a good model for studying neuron specific expression of the GnRH gene, as GT1-7 cells retain many characteristics of in vivo GnRH neurons. Although GT1-7 is not teleostean cell line, it is a good model to research GnRH promoter interaction with conservative transcription factors; for instance Pur proteins are GnRH transcription factor existed in *A. burtoni* and mouse, which have different molecular weight in each other, but the Pur protein of mouse still has function to regulate GnRH expression in *A. burtoni*. This finding to imply different molecular weight of transcription factor is caused by different post-translational modifications, for example N-glycosylation or phosphorylation, but the functional structure binding with GnRH promoter is conservative in different species (Sheng, 2009). Therefore, GT1-7 cell line can be the model in analysis crucial element on the GnRH promoter of Japanese eel.

Results of luciferase reporter assay showed that the GnRH10 caused significant reduction of transcriptional activity than any other group (Fig. 9). It indicated that the upstream region, between GnRH9 and GnRH10 was a core functional promoter and there was at least one activator binding site from nucleotides -388 to -499 bps. To investigate the hypothesis that there contain activator binding sites between GnRH9 and GnRH10, we used TESS and JASPAR to analysis this region and found multiple potential transcription factor binding sites, including Oct-1, Pbx-1a, C/EBPbeta, SRY, FOXI1, FOXJ2 and NF-IL6 (Fig. 16). Several factors have been shown to regulate GnRH cell-specific transcription, including Oct-1 and Prep/Pbx, the POU homeodomain protein Oct-1 was identified as an essential factor regulating basal and hormone-induced transcription of the GnRH gene by binding functional elements in both distal enhancer and promoter-proximal conserved region (in mouse; Bendall et al., 1993). Mutation of Oct-1-binding sites resulted in a significant reduction in GnRH transcription and blocked the pulsatile of GnRH activity in GT1–7 cells (in mouse; Vazquez-Martinez, 2002). However Oct-1 is not a strong transcription factor sufficient for functioning enhancer by itself. It conjugated with other proteins, called coactivators to promote potent transcription of target genes. Thus, the function of Oct-1 in tissue-specific expression of GnRH might involve interactions with the other proteins binding the promoter with specific coactivators (in mouse; Clark et al., 1995). Pbx1/Prep1 has been

shown that interplay with Oct-1 contributes to transcriptional activation of the GnRH gene in hypothalamic neurons (GT1-7) through specifically binding to functional elements within the GnRH promoter regions (Rave-Harel, 2004).

These data support the critical elements for GnRH transcription location from -388 to -499 bps, by interaction with anticipative transcription factors Oct-1 and Pbx-1a in Japanese eel. Furthermore, the results thus far confirm that GnRH promoter as researchable platform for *Anguilla japonica* had been successful established for analyzing GnRH expression.

Effects of Sex-steroids on GnRH expression

Gonadal steroid hormones play a particularly important role in feedback regulation of hypothalamic secretion of GnRH. We speculate whether the feedback regulation of sex-steroids would be affected if interacted with GnRH promoter. Although in GT1-7 cells E2 was shown to repress expression of GnRH gene, our results indicated that long-term E2 treatment could stimulate GnRH promoter activity. The different effect maybe cause by species, that an *in vivo* study in female *Anguilla anguilla* observed treatment only E2 or combination androgens also can induce increases of mGnRH levels in brain and pituitary (Montero et al., 1995).

Our sex-steroids tests revealed that E2 action on GnRH was indirectly controlled

via other neurons, due to the fact that E2 induced activation of GnRH transcription was not restricted to concentration but influenced by incubation time (Fig. 12, 13, 14 and 15.). Besides in the sheep, E2 could stimulate hypothalamic production of GnRH by kisspeptin-positive neurons that increasing estrogen levels leads to enhanced synthesis of kisspeptin. Kisspeptin stimulation of hypothalamic GnRH neurons was mediated through the kisspeptin receptor (KISS1R; GPR54) then leads in turn to rapid and significant increases in hypothalamic GnRH secretion (Herbison, 2008; Oka, 2009). And the expression of kisspeptin and GPR54 mRNAs were also significantly increased (8- and 6-fold, respectively) after 24 hours treatment of GT1-7 cell with E2 (Jaboci et al., 2007). Although estrogen-responsive element-like motifs have been demonstrated on the GnRH promoter of the Atlantic salmon (Klungland *et al.*, 1993), estrogen receptors have not been demonstrated on GnRH neurons in teleosts (Navas *et al.*, 1995). For these reason, E2 probable indirectly stimulate GnRH expression via kisspeptin neurons in Japanese eel. In addition, the GnRH promoter region between -388 to -499 bps might contain KISS1R binding elements, where truncation caused a significant down-regulating of GnRH.

The up-regulation of GnRH by MT shown here is inconsistent with the previous study performed by Belsham et al. (1998) that androgen downregulation of GnRH mRNA expression in GT1-7. The difference in results may be due to the species. Like

the current result, testosterone indeed has the positive effect on GnRH in juvenile male Japanese eel and other fish including masu salmon, African catfish (Okubo et al., 2002; Amano et al., 1994; Dubois et al., 1998).

In our experiments, MT resulted similar expression of GnRH with E2 restricted to incubation time but not concentration (Fig. 12, 13, 14 and 15.). It revealing MT acts on GnRH neurons by an indirect pathway connected with E2. Moreover, androgen receptors in GnRH neurons or androgen-responsive elements within the promoter region of the GnRH gene have not been reported in teleosts. It has been suggested by a number of investigators that testosterone must be aromatized to E2 before it can perform the function (in fish and mouse; Breton and Sambroni, 1996; Montero *et al.*, 1995; Spratt and Herbison, 1997). And aromatase exist in many E2 target tissues, in particular of the brain. In European sea bass, aromatase highly activity was found in the olfactory bulb, telencephalon, and hypothalamus which conform the place of GnRH neuron (Gonzalez and Piferrer, 2003). These results shows GT1-7 contain enough aromatase to convert MT into E2 and probably through kisspeptin neurons product which is identical result with E2 treatment. To determine whether testosterone acts directly on the GnRH neurons, without the need of aromatization, further experiments with nonaromatizable androgens, such as dihydrotestosterone and aromatase inhibitors, should be done in the future.

In summary, we have cloned mGnRH promoter to establish GnRH promoter as a researchable platform of Japanese eel, and demonstrated a critical region on mGnRH promoter from -388 to -499 bps for basal transcription, which contain some potential transcription factor binding elements, like Otx-1 and Pbx-1a (Fig. 16). In addition, E2 probable stimulate mGnRH expression via kisspeptin neurons in *Anguilla japonica* and there might contain KISS1R elements on mGnRH promoter from -388 to -499 bps. MT may converse to E2 through aromatase, and caused the same effect in GnRH expression like E2. Our studies provide an important basis for further understanding of GnRH regulation and function in Japanese eel.

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Tables

Table1. Program 1 PCR procedure of Genome Walking

Cycle number	Temperature	Hold Time
1	95℃	4 min
2	95℃	30 sec
3	60℃	30 sec
4	63℃	30 sec
5	65℃	30 sec
6	Go To 3. 2 more	
7	Go To 2. 3 more	
8	95℃	30 sec
9	30℃	30 sec
10	Ramp 0.1℃/sec to 60℃	
11	60℃	30 sec
12	63℃	30 sec
13	65℃	30 sec
14	Go to 11. 2 more	
15	95℃	30 sec
16	67℃	4 min 30 sec
17	Go to 15. 1 more	
18	95℃	30 sec
19	40℃	30 sec
20	Ramp 0.5℃/sec to 60℃	
21	60℃	30 sec
22	63℃	30 sec
23	65℃	30 sec
24	Go to 21. 2 more	
25	Go to 15. 5 more	
26	95℃	30 sec

27	67℃	4 min 48 sec
28	Go to 26. 1 more	
29	95℃	30 sec
30	40℃	30 sec
31	Ramp 0.5℃/sec to 60℃	
32	60℃	32 sec
33	63℃	32 sec
34	65℃	32 sec
35	Go to 32. 2 more	
36	Go to 26. 7 more	
37	68℃	10 min
38	4℃	Indefinitely
39	End	

Table 2. Program 2 PCR procedure of Genome Walking

Cycle number	Temperature	Hold Time
1	95℃	3 min
2	94℃	30 sec
3	67℃	4 min 30 sec
4	Go To 2. 1 more	
5	94℃	30 sec
6	40℃	30 sec
7	60℃	30 sec
8	63℃	30 sec
9	65℃	30 sec
10	Go To 7. 2 more	
11	Go To 2. 4 more	
12	94℃	30 sec
13	67℃	4 min 48 sec
14	Go to 12. 1 more	
15	94℃	30 sec
16	40℃	30 sec
17	60℃	32 sec
18	63℃	32 sec
19	65℃	32 sec
20	Go to 17. 2 more	
21	Go to 12. 7 more	
22	68℃	10 min
23	4℃	Indefinitely
24	End	

Table 3. Primers for Japanese eel GnRH Genome Walking. Primers design by *Anguilla japonica* gene for prepro-mGnRH complete cds.

Primer name	Primer sequence
GnRH P4	**5'-CAA AAT CTA CTg CTg TTT ggg AAg CAC TAg-3'**
GnRH P5	**5'-CCT TAg gAC TgA ATg TTg ATA ggC AC-3'**
GnRH P6	**5'-CAC AgT CCA gCA gTg ATA gTT ATC CTT CC-3'**

Table.4. Primers for GnRH Promoter Truncation assay. To show the groups difference by the different combination of primers, and the size of it range.

Group	Forward primer	Reverse primer	Size of product (bp)
GnRH 1	GnRH P19 5'-ACgCgTgCCACAggAAAgCCATCAC-3'	GnRH P25 5'-CTCgAgAgggCgCTCTTATCTgCC-3'	1805 bp
GnRH 2	GnRH P19 5'-ACgCgTgCCACAggAAAgCCATCAC-3'	GnRH P24 5'-CTCgAgATggACACTgggCggCAg-3'	1578 bp
GnRH 3	GnRH P20 5'-ACgCgTCATgAggTgTggATgAggC-3'	GnRH P24 5'-CTCgAgATggACACTgggCggCAg-3'	1083 bp
GnRH 4	GnRH P19 5'-ACgCgTgCCACAggAAAgCCATCAC-3'	GnRH P22 5'-CTCgAgAACTgggTgAAAgTgTAggg-3'	893 bp
GnRH 5	GnRH P21 5'-ACgCgTCCCTACACTTTCACCCAgTTC-3'	GnRH P24 5'-CTCgAgATggACACTgggCggCAg-3'	746 bp
GnRH 6	GnRH P28 5'-ACgCgTTgTCTTTgAgTATTgATgACCC-3'	GnRH P24 5'-CTCgAgATggACACTgggCggCAg-3'	739 bp
GnRH 7	GnRH P29 5'-ACgCgTgTCTgCCTATTgAAgACCAC-3'	GnRH P24 5'-CTCgAgATggACACTgggCggCAg-3'	533 bp
GnRH 8	GnRH P30 5'-ACgCgTATgAgTTCCTTgTATAgACAC-3'	GnRH P24 5'-CTCgAgATggACACTgggCggCAg-3'	405 bp
GnRH 9	GnRH P31 5'-ACgCgTTTgggAAAgTggTgCAAAg-3'	GnRH P24 5'-CTCgAgATggACACTgggCggCAg-3'	292 bp
GnRH 10	GnRH P23 5'-ACgCgTCgAgAAgAgggATgAgTgC-3'	GnRH P24 5'-CTCgAgATggACACTgggCggCAg-3'	181 bp
GnRH 11	GnRH P19 5'-ACgCgTgCCACAggAAAgCCATCAC-3'	GnRH P24 5'-CTCgAgATggACACTgggCggCAg-3'	1530 bp
GnRH 12	GnRH P20 5'-ACgCgTCATgAggTgTggATgAggC-3'	GnRH P32 5'-CTCgAgCCCAGCCCGGATCCTGTGT-3'	963 bp

Figures

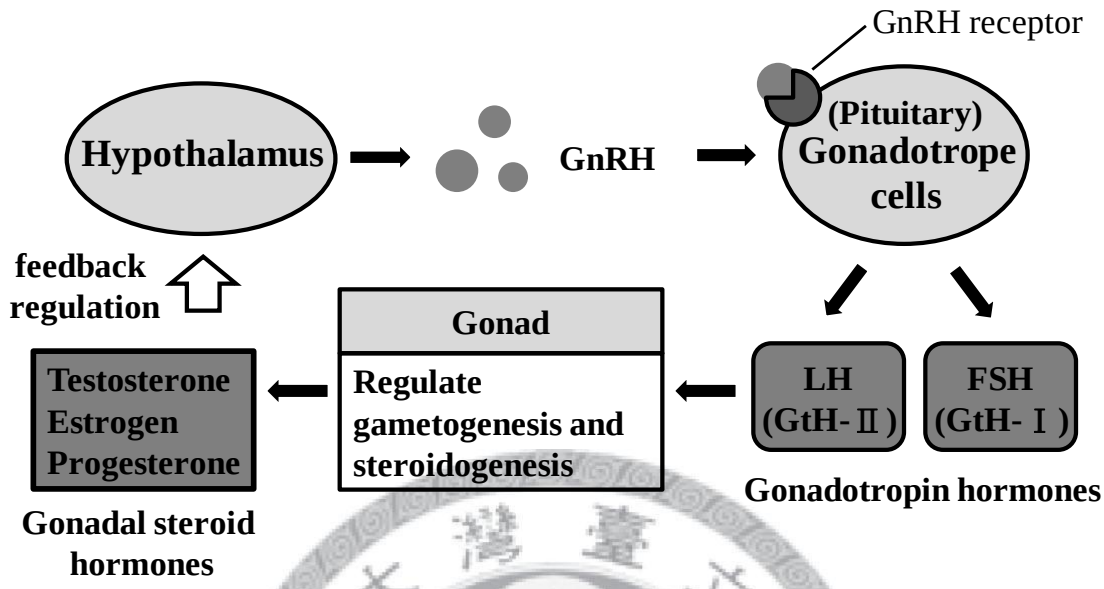

Fig. 1. GnRH is a central regulator in the HPG axis of the reproductive hormonal cascade. It is expressed in a discrete population of neurosecretory cells located throughout the basal hypothalamus of the brain, and is released into the hypothalamo-hypophyseal portal circulation in a pulsatile manner and in surges during the female preovulatory period. The released GnRH is transported to the anterior pituitary gland, where the hormone binds to its receptor on the gonadotropes. This triggers the synthesis and release of the LH and FSH, which are responsible for gonadal steroidogenesis and gametogenesis.

```
  1. TAGACCTCCA TTATGAGCCA TTTTCTGACT GTGTGTATGT TGCTTGCGTT
                                                GnRH19 →
 51. TTGTTCATAG CTGTGTATGT TGCCTCTAAT GGGCTTGCCA CAGGAAAGCC
101. ATCACTCATA CGCATAGATG AGTATTGCTG CTTCTAATCT TTGGATTTGA
151. GGTATGAGGG CAAGAAATGC ATGGAAATAT GAATATGTAC CAAATATTGT
201. TACACGGTCG ACATGAAAGT TGTCTGTCTT GCACCAAATA GTAAGTGGAG
251. TACGGTTGTA AAACAACACT CCCCATAGGA TAACAAAATT ATAAGCTGGA
301. TTTATCTTTT TAATTTATTC TGTCTATGCT TGGCACCAAA ACACAATCCA
351. CACCCTTGTG TACTTGTATG CTTATTCCAA TAGGCCCATT ACTAATGGTC
401. AAACTCAAAT CATGTTTATA TCCCTAGAAT GCTTCATGAA GCAAATAATC
451. TCTAATTGAC TGTAGTGCAT ATCAACATTC AGTCCTAAGG TATTTAAAAT
501. CTAGTGCTTC CCAAACAGTA GATTTTATTT TGATACTGGA AGGATAACTA
551. TCACTGCTGG ACTGTGTTTT TTGTATTTTT TGTTTATTTT AACTTATCTG
                    GnRH-P20 →
601. TTGTCAGACT GTAAATTCAT GAGGTGTGGA TGAGGCTTTG CATTCTTACG
651. TTATTGAATT TAACTATGGT TAGCAGCATT GGTCAGGACA ATAACTGTGG
701. AAGAAACTGC TTTTGAAATG TAGGTGTAGC AATTACTTTC CCAAGGTACT
751. CAGTCTGTCT TTGTTATGGC AATCAGAAAA ATAATTTTGC ACTCTGAGTG
801. CGTTGTGCTT CATATATTAC AACCTTGAAA AAAGGAGGAA GGTTATTTAT
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Fig. 2. The GnRH gene and promoter sequence of Japanes eel generated from Genome Walking

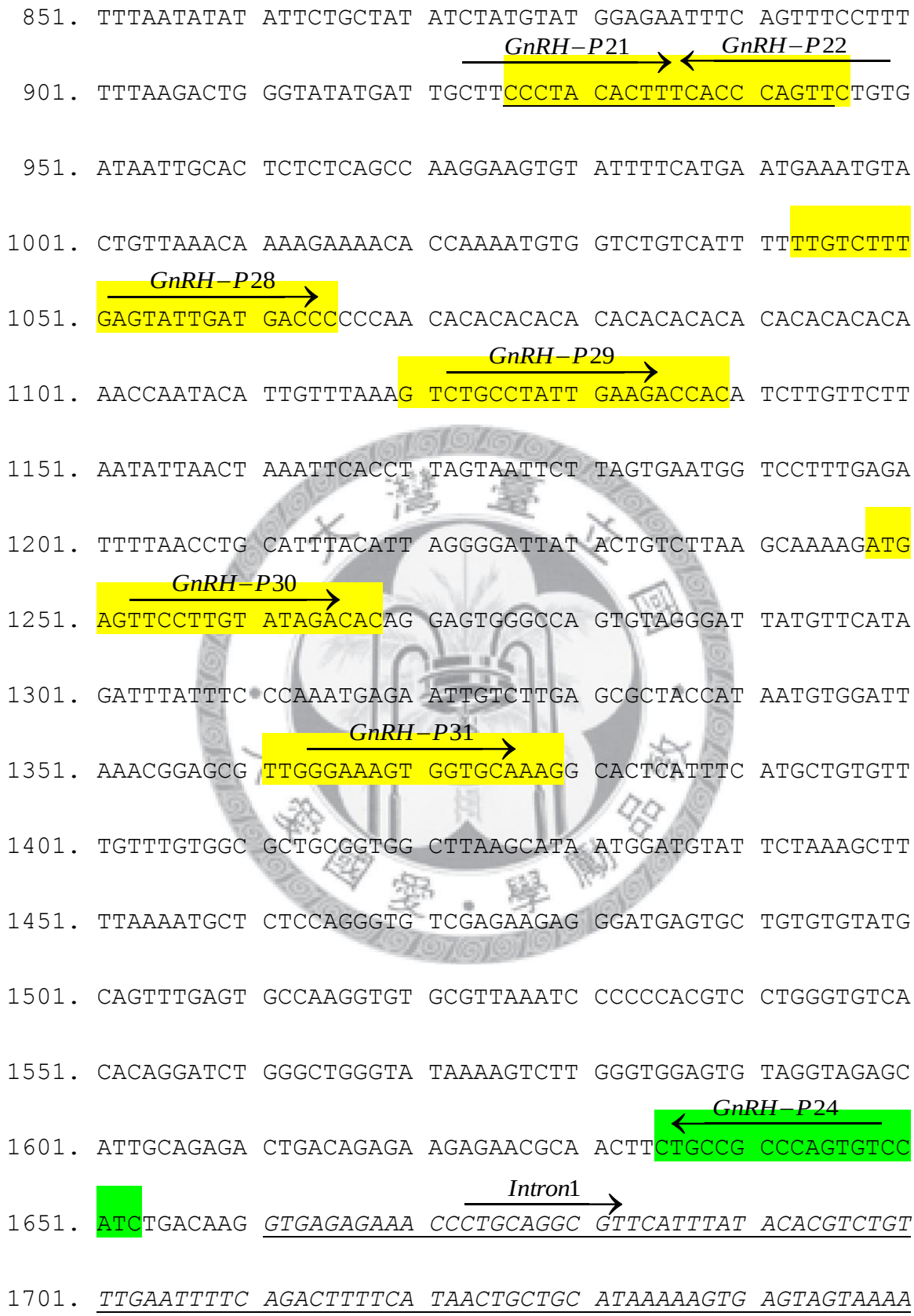

Fig. 2. continued

1751. *GCTTTTAGTG GTAAATGTGA CTCAATTCAA GCAAGTGTAA TCTGAGGTAC*

1801. *AATCTAAGAA AGGCGTATTT CCACTTCAAA ATGTGCTAAG AGCGGTGTGT*

Start−code *GnRH−P25*

1851. *GGTTTCAG***AA TGGCAGATAA GAGCGCCCTC TTGTGGCTGG GGCTGGCTGT**

1901. **GGCACTGGTG TGTCAGGGAT GCTGTCAGCA CTGGTCTTAC GGCCTGAGAC**

1951. **CCGGGGGCAA GAGAGGAGCA GATAGTCTAC AGGACACGCT GCAAGAT***GTA*

Intron2

2001. *CGTGATGCAT GAACTACACC TCCCAGGGTT CTTTGACTAT TCAGCAAAAT*

2051. *GCATGTGCAT AGCACAGCAA TTGGCCATGG GAAACTTTTA ACTGATATGT*

2101. *GATCATAGGC AAAAGAAGTA TGTCAAAACT GTTGAGCACC TACTTTCAAT*

2151. *TTATAATCAT ATCTCAAAAC TACAGCTGTA GTGTTTATTC ATTTTATTTA*

2201. *TATGCTTATG ATTGCTTATT ATATATTTCA GATTGTGAAA ATTGGTGCAC*

2251. *AGATCTGTAT TGCTACAACT GAGATATTTT GCTCACAAAC AAAACAAGTT*

2301. *ATGTTTTTGA GAGAAACTGA TTTGTAATTC CTAGCCACTC AAGACATTAT*

2351. *TGATGAAAAT GCCAACATGA CAATAAATCA TGTGTCTAAA TATCAAAGTA*

2401. *GGGAGGACTT GACTGTAAAT GGTAATTGGA GCTGTGGGAT TGGTTAAAAG*

2451. *TAATCGGGGC TATGGGATTG GTTCCAAGTA ATCAGGGCTA TGGGATTGGT*

2501. *TCTAAGTAAT CGGGGATATG GGATTGGTTC CACAG***ATTAT AGAGGAGCTG**

2551. **CAGAAGCTGG ACACCTCCAG TTTGCCCAGC TGCAATGACC TCTCCCCACA**

Intron3

2601. **TATTACACTT TCCAGCCTGA AGGAAATACT G***GTGAGAACA GTAGCTTTGA*

Fig. 2. continued

2651. *GTGTTTCGCA ATCCCTCTTC GTCACTCTGG TTTAACCTGT GTTTGTGAAC*

2701. *TCACCCATCT GTGTGTAACA TATGACATTC TTCCCATCTT TCTGGTACAG*

2751. *TTCTTTATTT GATGCTGTTT TATGACCTGA TACTTCTCTT CTGACTCATT*

2801. *ATTTTTATGT ATGTGCATGT CATATTAACT GTGCATGGGG CATTTCTCCT*

2851. *GAAAGTGCCT TTTTTCTTTC TTACAG***GCAA ATCTGGCTGA CAGAGAAACT**

Stop−code →

2901. **GGACGGAAAA ATATATAGAA GATATTATGC ATGCATTCCT TCAATAAAAC**

2951. **ATGACTGTGC**

Fig. 2. continued

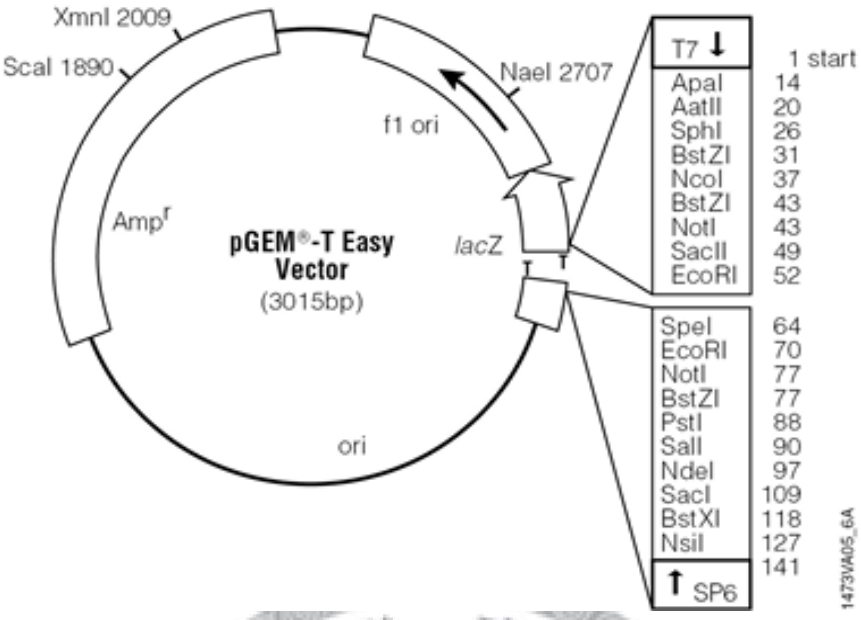

Fig. 3. pGEM® -T Easy Vector circle map and sequence reference points.

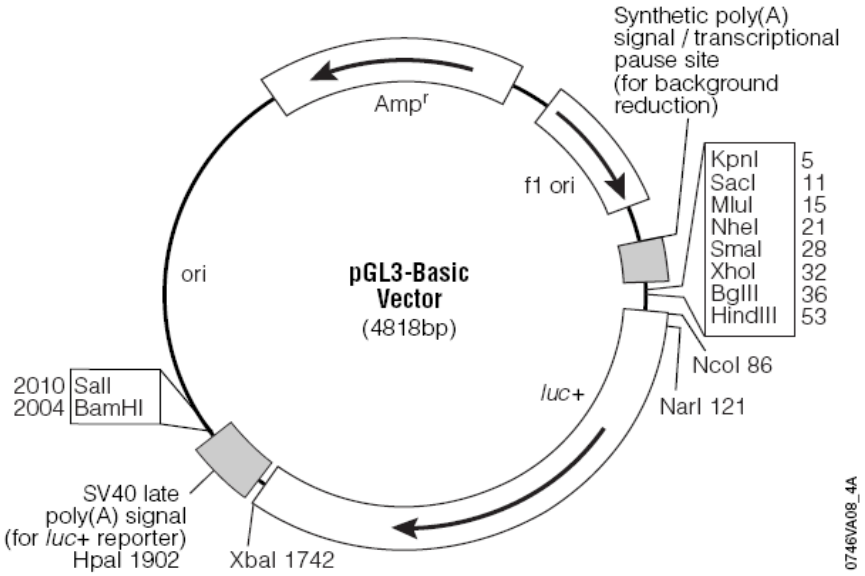

Fig. 4. pGL3-Basic Vector circle map. Additional description: *luc+, cDNA* encoding the modified firefly luciferase; Ampr, gene conferring ampicillin resistance in *E. coli;* f1 ori, origin of replication derived from filamentous phage; ori, origin of replication in *E. coli.* Arrows within *luc+* and the Ampr gene indicate the direction of transcription; the arrow in the f1 ori indicates the direction of ssDNA strand synthesis.

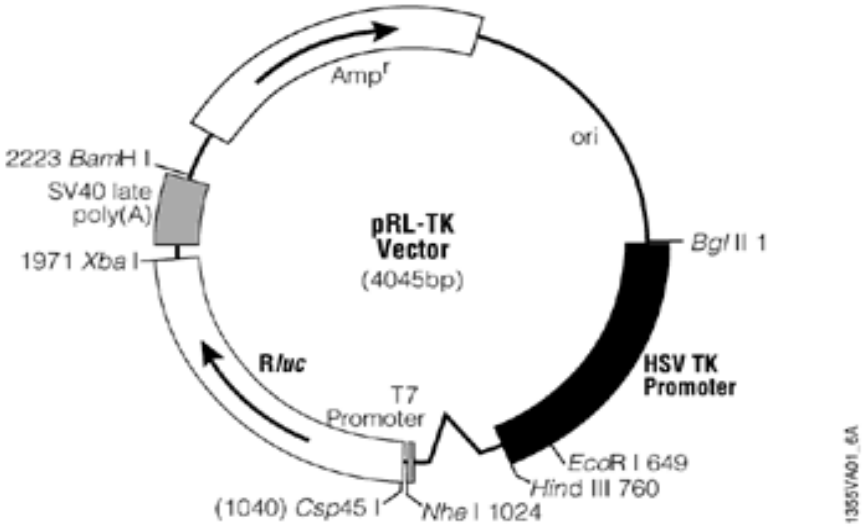

Fig. 5. Circle map of the pRL-TK Vector. Additional description: _^_, position of intron; *Rluc*, *cDN*A encoding the Renilla luciferase enzyme; Ampr, gene conferring ampicillin resistance in *E. coli;* ori, origin of plasmid replication in *E.coli.* Arrows within the *Rluc* and *Ampr* gene indicate the direction of transcription.

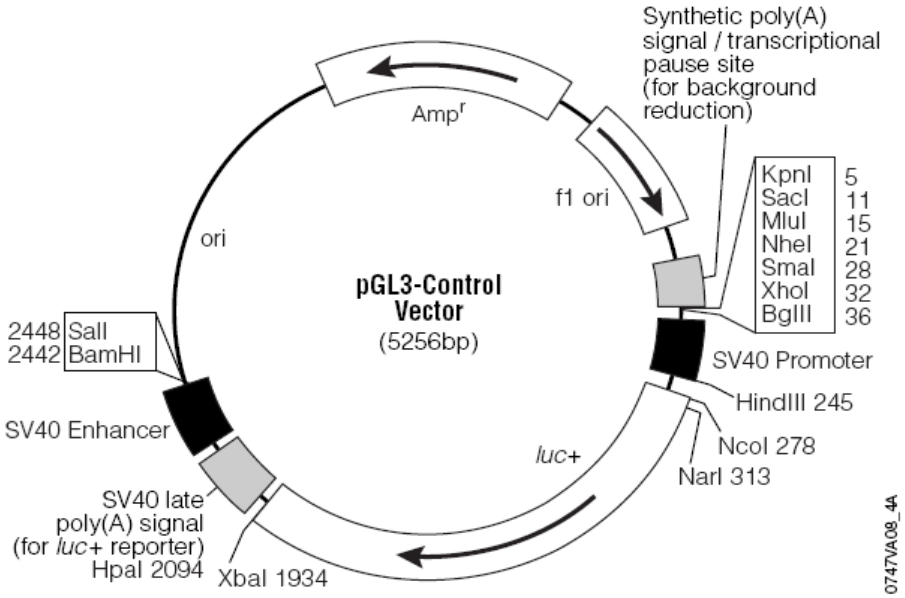

Fig. 6. pGL3-Control Vector circle map. Additional description: *luc+, cDNA* encoding the modified firefly luciferase; Ampr, gene conferring ampicillin resistance in *E. coli;* f1 ori, origin of replication derived from filamentous phage; ori, origin of plasmid replication in *E. coli.* Arrows within luc+ and the Ampr gene indicate the direction of transcription; the arrow in f1 ori indicates the direction of ssDNA strand synthesis.

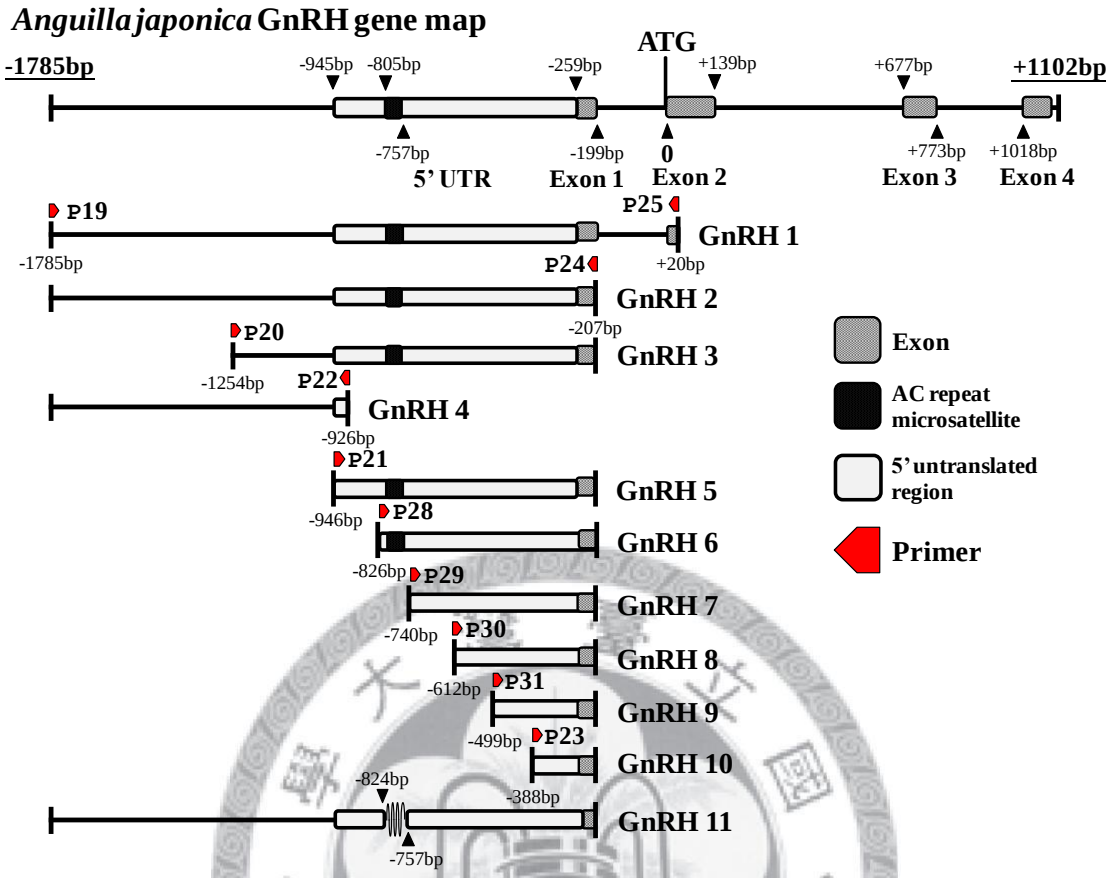

Fig. 7. The size and location of the fragments in GnRH gen Map of *Anguilla japonica*, which was selected from GnRH promoter Truncation assay. The genome annotation is reference by GenBank: AB026991.1.

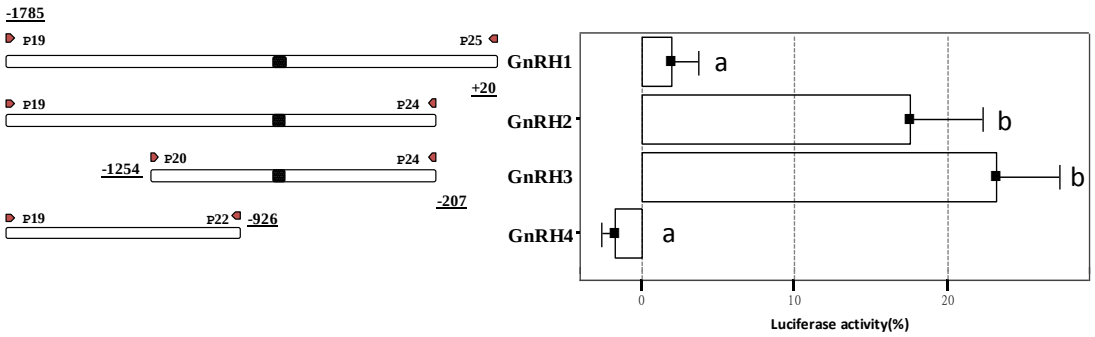

Fig. 8. The Luminescence expression value (right) cause by difference GnRH promoter region of GnRH1, GnRH2, GnRH3, GnRH4 and GnRH5 (left). GnRH2 and 3 has a significant increase of Luminescence value compare to GnRH1 and 4. Each bar represents the *M*±1.0S.E. of triplicate wells from a representative experiment that was repeated at least three times with similar results. Values with superscripts that have different letters are significantly different from each other, $p < 0.05$, ANOVA and Tukey HSD test.

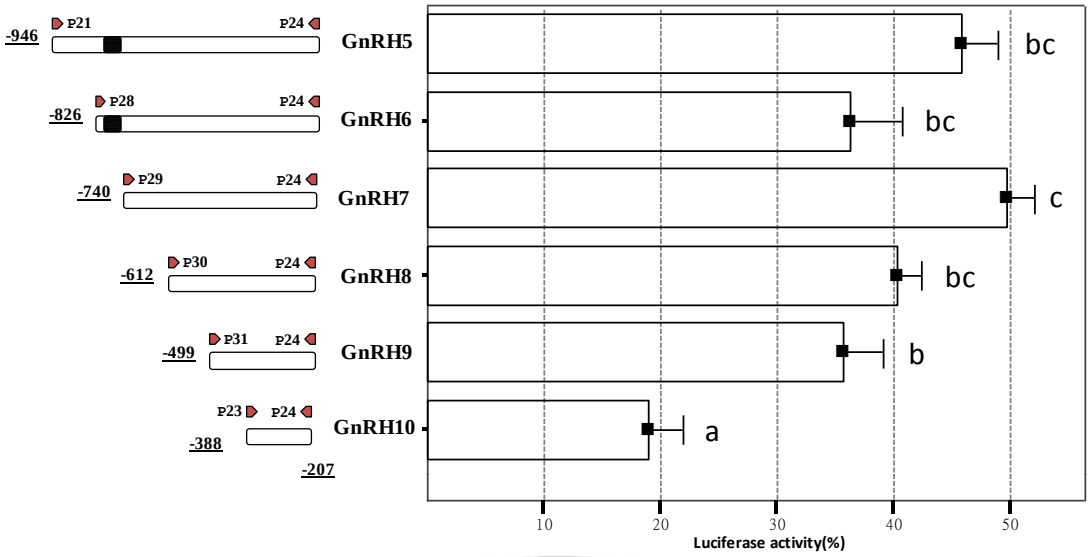

Fig. 9. The Luminescence expression value (right) causes by different GnRH promoter region of GnRH5, 6, 7, 8, 9 and 10 (left). GnRH10 has the lowest luminescence value in this test contrast to other. Each bar represents the *M*±1.0S.E. of triplicate wells from a representative experiment that was repeated at least three times with similar results. Values with superscripts that have different letters are significantly different from each other, $p < 0.05$, ANOVA and Tukey HSD test.

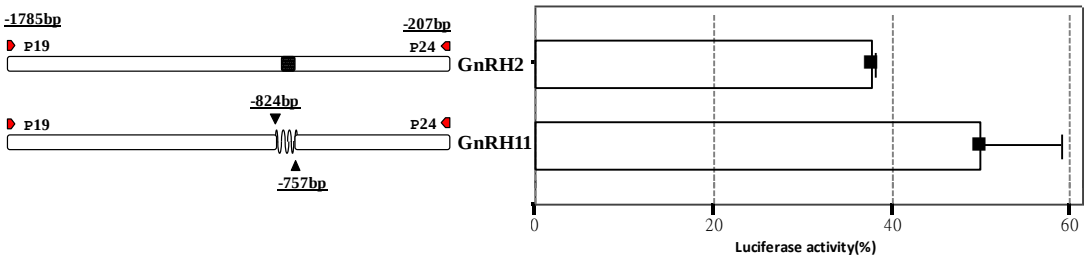

Fig. 10. The luminescence expression value (Right) cause by difference GnRH promoter region of GnRH2 and 11 (Left). GnRH11 has slight increase of luminescence value compare to GnRH2. Each bar represents the *M*±1.0S.E. of triplicate wells from a representative experiment that was repeated at least three times with similar results.

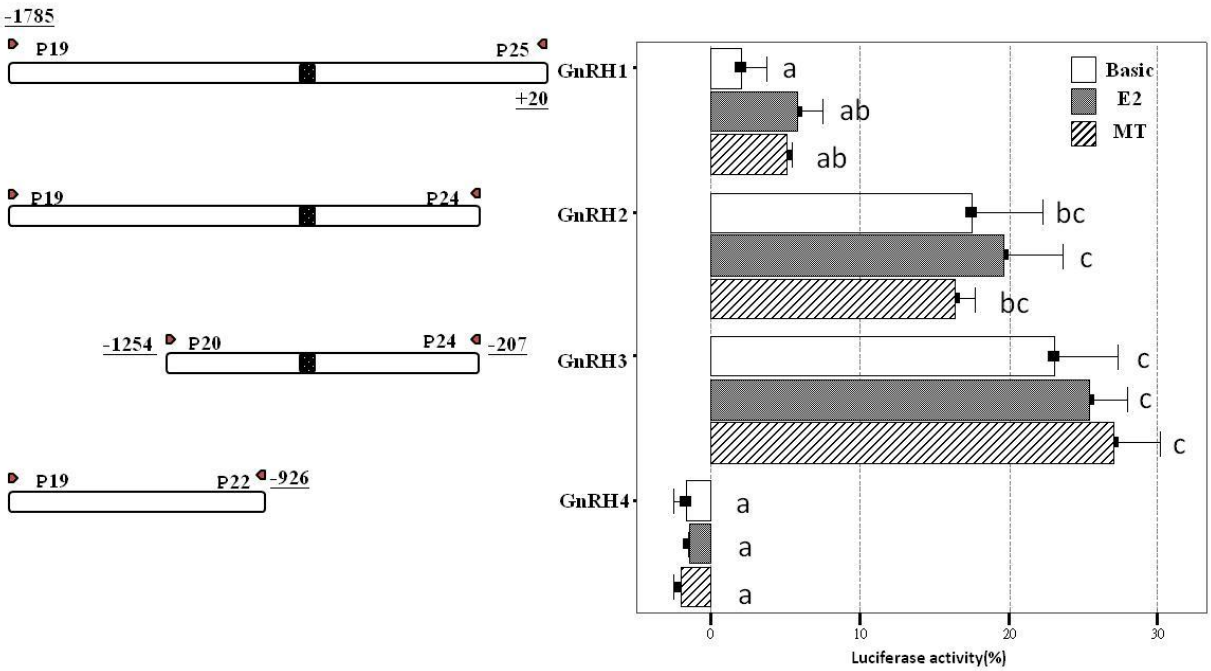

Fig. 11. Infuence of luminescence expression value (Right) cause by difference GnRH promoter region of GnRH1, 2, 3, and 4 (Left) stimulation with 100nM E2 or MT for 24 hours. GnRH2 and 3 has a significant increase of luminescence value compared to GnRH1 and 4. Results shown are average ± SEM from a representative experiment that was repeated at least two times with similar results. Values with superscripts that have different letters are significantly different from each other, $p < 0.05$, one-way ANOVA and Tukey HSD test.

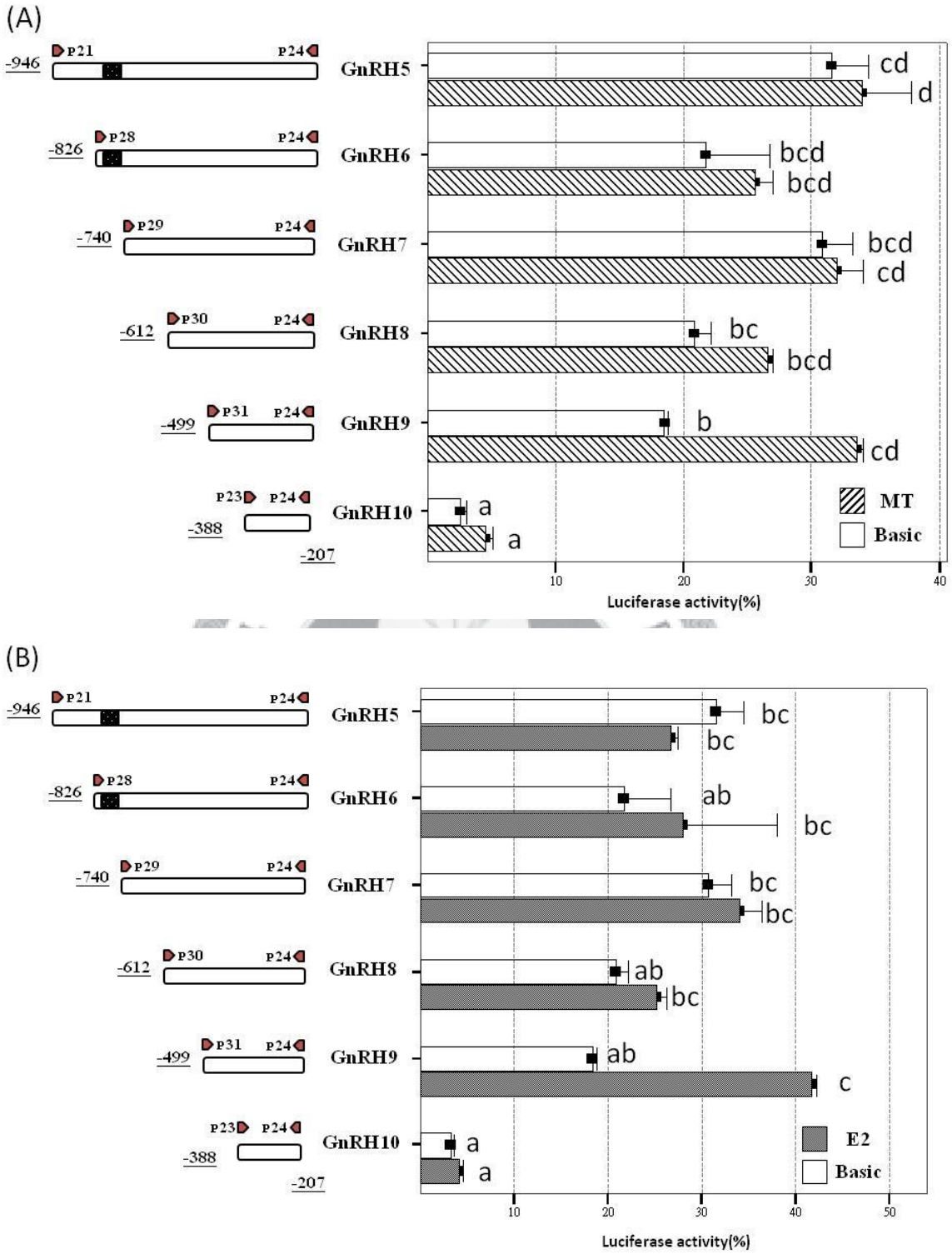

Fig. 12. Infuence of luminescence expression value (Right) cause by difference GnRH promoter region of GnRH5, 6, 7, 8, 9 and 10 stimulation with 100nM MT(A) or E2 for 24 hours (Left). GnRH19 has significant increase luminescence value when treatment both 100nM MT/E2 for 24 hours. Results shown are average ± SEM from a representative experiment that was repeated at least two times with similar results. Values with superscripts that have different letters are significantly different from each other, $p < 0.05$, one-way ANOVA and Tukey HSD test.

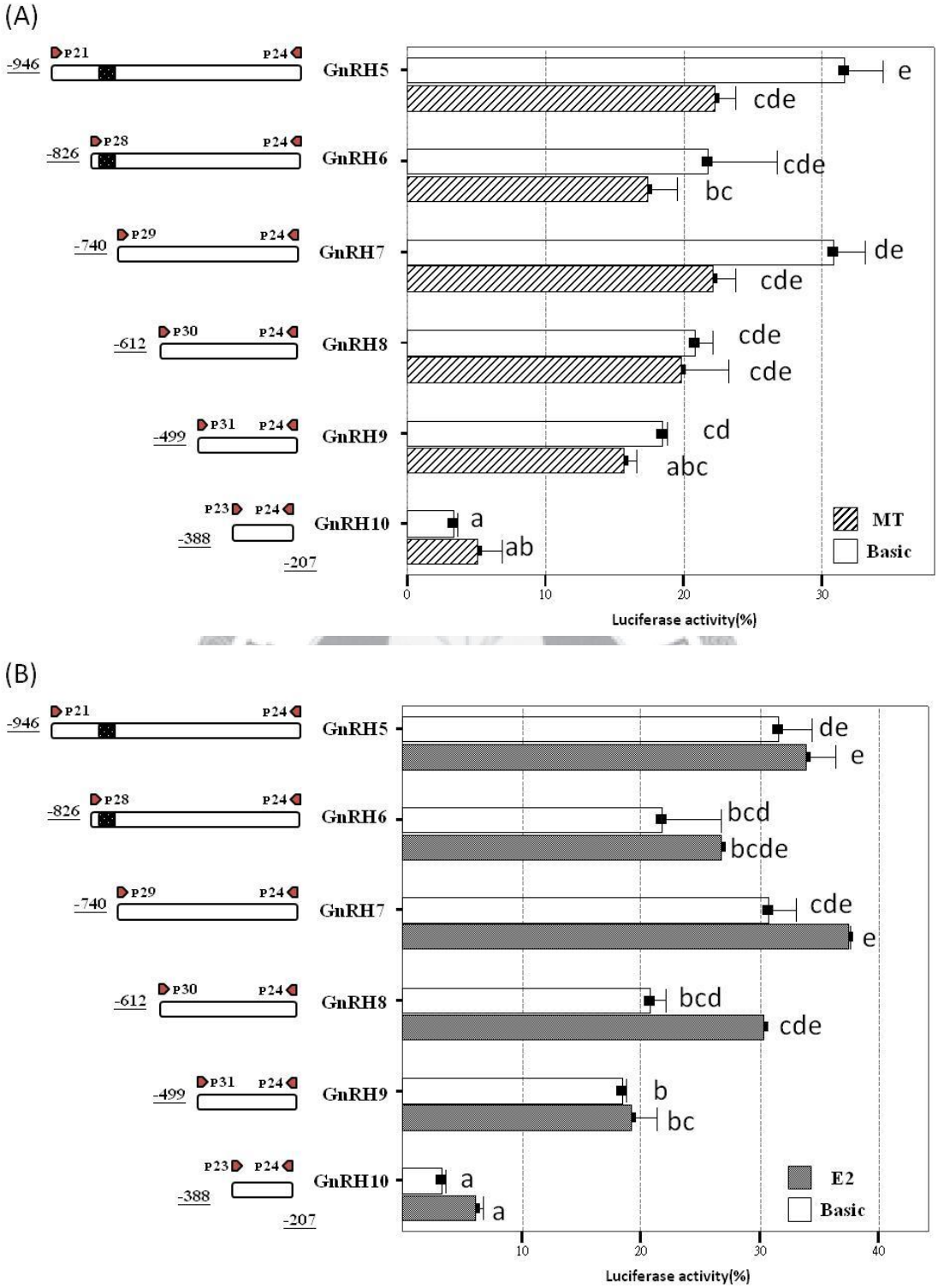

Fig. 13. Influence of luminescence expression value (Right) cause by difference GnRH promoter region of GnRH5, 6, 7, 8, 9 and 10 stimulation with 100nM MT (A) or E2 (B) for 6 hours (Left). Sex-steroids abolished ability for stimulation GnRH expression when reduce time. Results shown are average ± SEM from a representative experiment that was repeated at least two times with similar results. Values with superscripts that have different letters are significantly different from each other, $p < 0.05$, one-way ANOVA and Tukey HSD test.

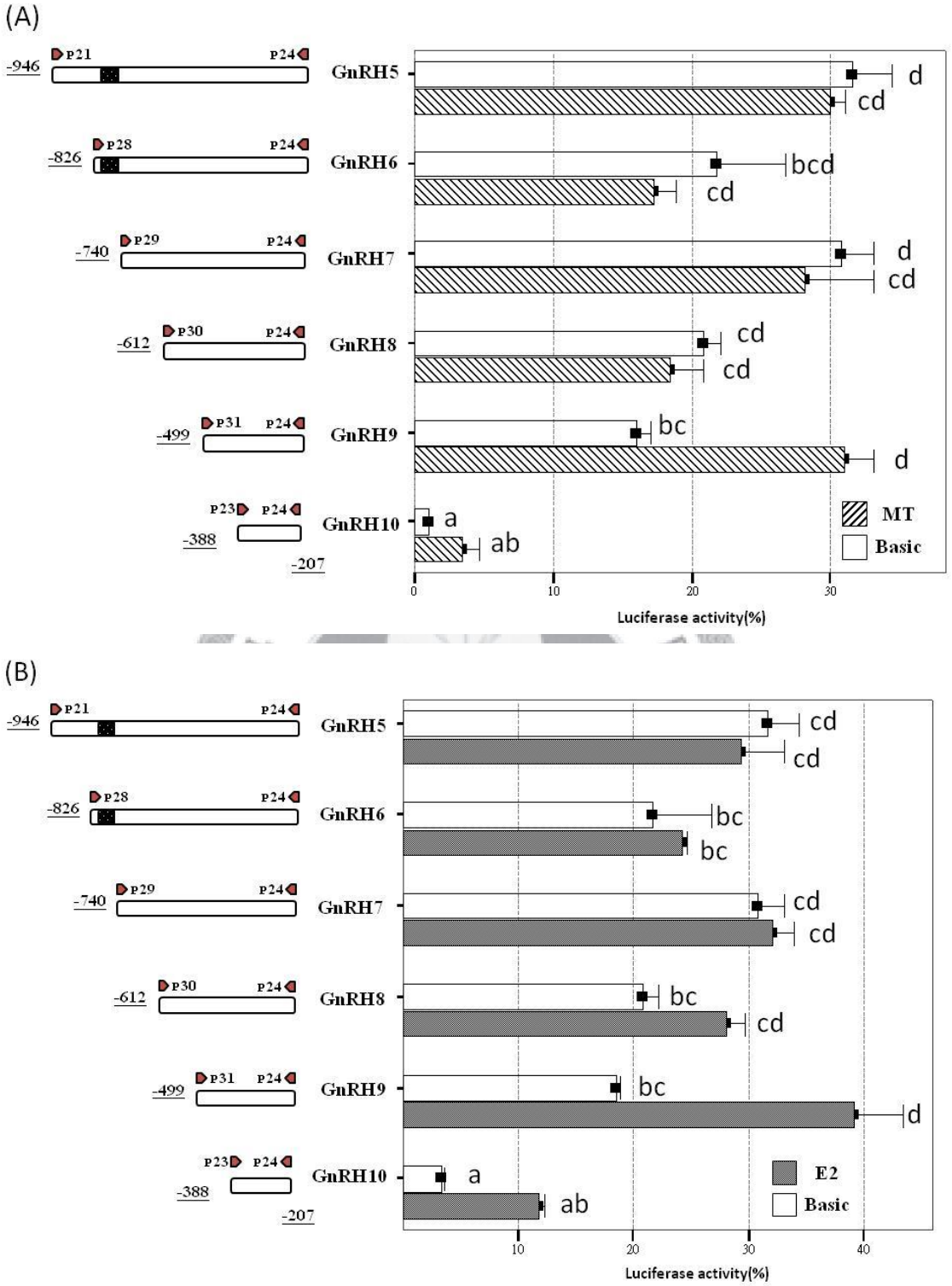

Fig. 14. Influence of luminescence expression value (Right) cause by difference GnRH promoter region of GnRH5, 6, 7, 8, 9 and 10 incubation with 10nM MT (A) or E2 (B) for 24 hours (Left). Both MT/E2 significantly increase GnRH9 expression after 24 hours incubation. Results shown are average ± SEM from a representative experiment that was repeated at least two times with similar results. Values with superscripts that have different letters are significantly different from each other, $p < 0.05$, one-way ANOVA and Duncan test.

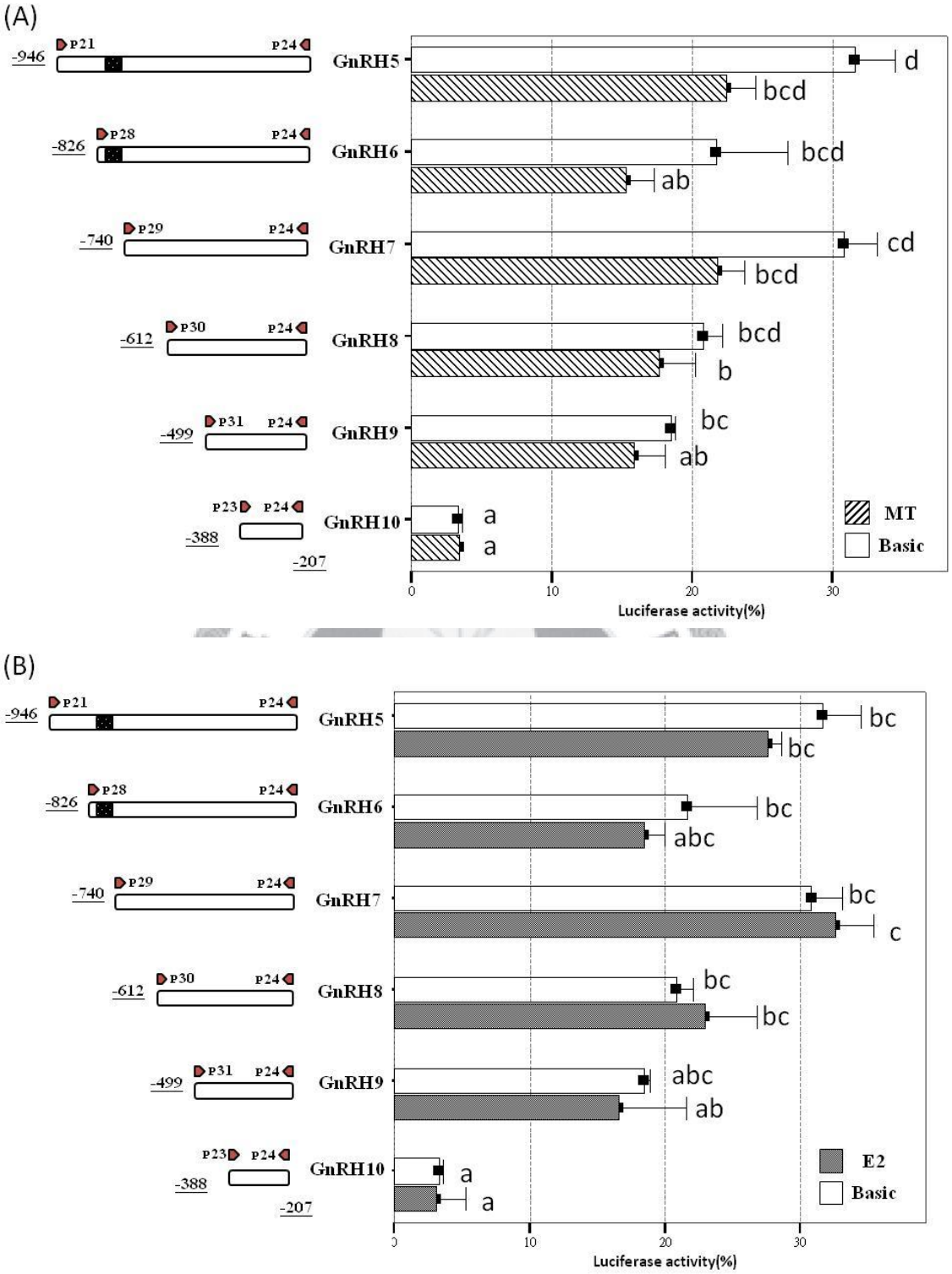

Fig. 15. Influence of luminescence expression value (Right) cause by difference GnRH promoter region of GnRH5, 6, 7, 8, 9 and 10 stimulation with 10nM MT (A) or E2 (B) for 6 hours (Left). Sex-steroids abolished ability for stimulation GnRH expression when reduce time and concentration. Results shown are average ± SEM from a representative experiment that was repeated at least two times with similar results. Values with superscripts that have different letters are significantly different from each other, $p < 0.05$, one-way ANOVA and Tukey HSD test.

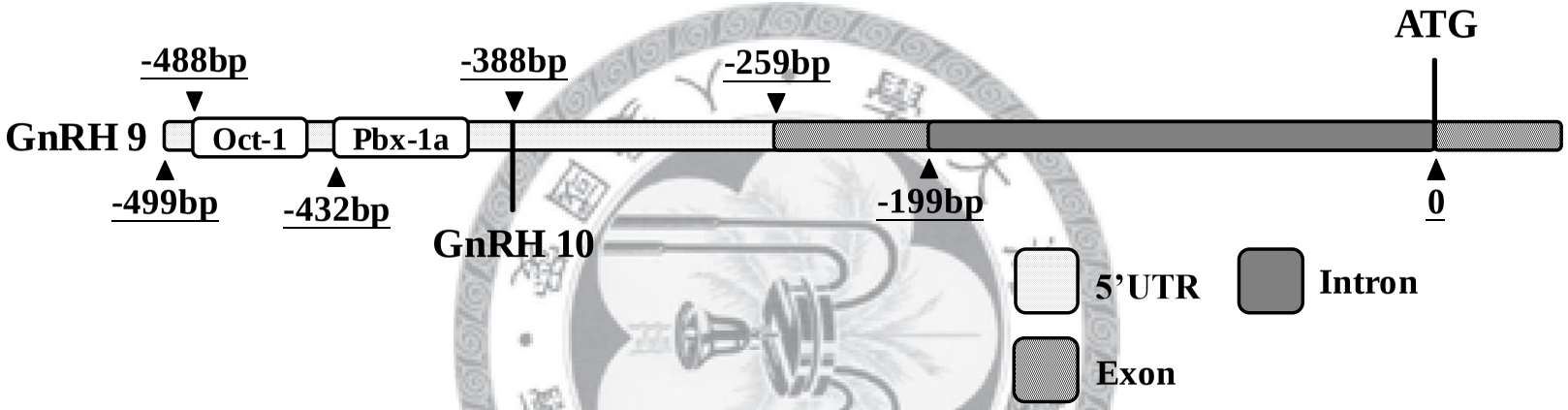

Fig. 16. Potential transcription factor binding sites was found by TESS and JASPAR location between -388 bps and -499 bps. Oct-1 binds the element 5'-GTGCAAAG-3' form -488 to -480 in GnRH promoter. Pbx-1a binds the element 5'-ATAATGGAT-3' form -432 to -423 in GnRH promoter.