Cloning and Characterisation of the Thyrotrophin-Releasing Hormone Receptor and Gonadotrophin-Releasing Hormone Receptor from Chicken Pituitary Gland

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SUMMARY

The hypothalamic hormones, thyrotrophin-releasing hormone (TRH) and gonadotrophin-releasing hormone (GnRH), play pivotal roles in the growth and sexual maturation of chickens. In chickens, TRH regulates the release and synthesis of thyrotrophin (TSH) and also acts as a growth hormone-releasing factor. GnRH stimulates the release and synthesis of gonadotrophins (LH and FSH).

TRH and GnRH are released and stored in the median eminence, and both hormones are transported into the pituitary gland via the hypophysial portal circulation. TRH and GnRH exert their physiological functions by binding to their specific receptors (TRH receptor and GnRH receptor, respectively) on the surface of cells in the pituitary gland. The activated receptors couple to guanine nucleotide-binding regulatory proteins (G proteins), G_q and/or G_{11}, which in turn triggers the secondary messenger [1,2-diacylglycerol (DAG) and inositoltrisphosphate (IP_3)] signalling cascade. The signalling generates the physiological effects of the hormones. The TRH-R and GnRH-R are members of G-protein coupled receptor (GPCR) family.

The objective of this thesis was to clone and characterise the chicken TRH and GnRH receptors as useful tools for investigating the regulatory roles of TRH and GnRH receptors in the growth and sexual maturation of chickens. In addition, sequence information of the receptors would potentially assist in elucidating the binding sites and the molecular nature of the processes involved in receptor activation.

Although the TRH and GnRH receptors have been cloned from several mammalian species, none had been cloned from non-mammalian vertebrates at the commencement of this study. In view of the difficulties of cloning these receptors, a combined strategy has been used to clone the TRH and GnRH receptors from the chicken pituitary gland. The chicken TRH receptor cDNAs have been cloned from a pituitary cDNA library, using the mouse TRH receptor, and a partial gene of the chicken TRH receptor, as probes. However, they are all incomplete cDNA clones. Consequentially, PCR has been applied to amplify the full-length chicken TRH receptor. Various "mutated" sequences (differences in the sequences of the cDNA clones isolated from a cDNA library) were noted in the full-length clone. Therefore, a "wild-type" chicken TRH receptor has been constructed. On the other hand, the chicken GnRH receptor cDNA could not be isolated from the cDNA libraries, constructed from intact or castrated chicken pituitary glands, either by using a

partial gene of the chicken GnRH receptor, or the mouse GnRH receptor cDNA as a probe. A combined strategy (including PCR, 5′-RACE, and screening of a chicken genomic library) has been applied to obtain the 5′un-translated and 3′un-translated regions of the chicken GnRH receptor from the chicken pituitary cDNA and genomic DNAs, respectively. The sequence data of both regions has been used to design two pairs of nested primers, which flank an entire open reading frame. A full-length chicken GnRH receptor has been amplified by PCR with two pairs of the nested primers. However, the PCR application fails to amplify up "wild type" sequences from the receptor gene. Therefore, a "wild type" chicken GnRH receptor was constructed.

The chicken TRH receptor shares over 80 % identity of amino acid sequences with mammalian TRH receptors. The putative binding sites of the mammalian TRH receptor, Tyr^{106}, Asn^{110}, Tyr^{282}, and Arg^{306}, are all conserved in the chicken receptor. Pharmacological studies employing several TRH analogs reveal that there are no significant differences between the chicken and mouse TRH receptors on ligand binding and inositol phosphate production. Several truncated chicken TRH receptors have been isolated from a chicken pituitary cDNA library. They contain a stop codon (TAA) in the putative third intracellular loop. The 20 amino acids that precede the stop codon in these clones have no homology with the mammalian counterpart. The divergent region has been verified as an intron by southern blot and PCR analysis.

The chicken GnRH receptor comprises several unique features, compared to the mammalian GnRH receptor. The chicken GnRH receptor contains a longer N-terminus, a strikingly short intracellular loop 1, and an intracellular C-terminal tail that is absent in the mammalian GnRH receptor. Additionally, the chicken receptor possesses two Asp (D) residues in TM 2 and TM 7 (D/D), whereas Asn (N) and Asp residues present in these domains in mammal receptors and D/N in the majority of GPCRs. The amino acid sequences of the chicken GnRH receptor shows only about 40 % identity to those of the mammalian homologues. However, the putative binding sites Asn^{102}, Lys^{121}, and Glu^{301}, which have been proposed in the mouse GnRH receptor, are all conserved in the chicken GnRH receptor. Intriguingly, the chicken GnRH receptor exhibits distinct pharmacological properties. For example, the chicken GnRH receptor displays higher affinity for chicken GnRH II analogues than for mammalian GnRH or chicken GnRH I, while the mammalian receptor favours mammalian GnRH. Although several mammalian antagonists behaved as pure antagonists in their interaction with the chicken receptor,

three mammalian GnRH antagonists generated an agonistic effect to the chicken receptor. This feature was apparently due to the presence of a D-Lys residue in position 6 of these analogues.

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To Stephen and my parents

for their endless love and support

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1 INTRODUCTION

The growth and sexual maturation of chickens is controlled by an intricate mechanism which involves endocrine, paracrine and autocrine factors. Growth hormone (GH), thyroid hormones (T_3 and T_4), and sex hormones (testosterone, estradiol, and progesterone) are three major classes of factors involved in the mechanism (Scanes, *et al.*,1990). These hormones are controlled and regulated by hypothalamic hormones, thyrotrophin-releasing hormone (TRH) and gonadotrophin-releasing hormone (GnRH), and are also regulated by the interactive network between systems of the thyroid and the gonad. It is well established that TRH and GnRH initiate their physiological functions by interacting with their specific receptors (i.e. TRH-R and GnRH-R) on the surfaces of cells and that these receptors couple to guanine nucleotide-binding regulatory proteins (G proteins) (Hinkle, 1989; review Sealfon *et al.*, 1997) which in turn trigger the signalling cascades. The signalling promotes the biological effects of the hormones. This study has been undertaken to clone and pharmacologically characterise the TRH and GnRH receptors. The cloned receptors will provide useful tools to unveil the molecular basis of action of the TRH and GnRH in avian growth and sexual maturation. Furthermore, the amino acid sequences of the receptors will give an insight into the structural conformation and molecular functioning of the TRH and GnRH receptors.

The present chapter will briefly explain the physiological roles of GH and gonadotrophins in the growth and maturation of the chicken. This will be followed by a discussion of the physiological functions of TRH and GnRH on regulating GH and gonadotrophin release. Finally, the structure and function of the TRH and GnRH receptors will be considered.

The growth and sexual maturation of chickens are under complex hormonal control. GH is the most important hormone in the control of growth in poultry (Scanes and Lauterio, 1984; Scanes *et al.*, 1990). In physiological conditions, plasma concentrations of GH are first detectable on day 17 of chicken embryonic development, and increase during the week following hatching. The concentrations of GH are high between 1 to 8 weeks of age. Thereafter, the concentrations decline to reach a low, relatively stable level prior to puberty (Scanes *et al.*, 1990). In addition, before the end of the rapid growth phase, concentrations of plasma gonadotrophins begin to increase slowly, reaching maximum

values at the onset of puberty, between 17-21 weeks of age. In puberty, GH regulates gonadal functions in birds, by means of the somatomedins' actions on ovarian follicular development. The release of somatomedins is stimulated by GH from liver. They exhibit an action on the proliferation of granulosa and theca cells, and inhibit LH-induced aromatase activity in the theca cells (Peddie *et al.*, 1993). Somatomedins increase basal progesterone production and enhances LH-stimulated progesterone production (Peddie *et al.*, 1993). During the follicular growth period of the hen, in addition to accumulation of yolk, the number of granulosa and theca cells also increases rapidly (Etches *et al.*, 1983). There are different productions of steroid hormones by the follicles. For example, a dramatic increase in basal and LH stimulated progesterone production (Hammond *et al.*, 1981), a marked reduction in progesterone metabolism to androstenedione by theca cells (Marrone and Hertelendy, 1985), and a decline in aromatase activity in theca cells, 48 h before ovulation (Armstrong, 1984).

GH secretion is under the control of both stimulatory and inhibitory hypothalamo-hypophysiotropic factors in mammals and birds. These factors are released from nerve terminals of the median eminence into the anterior pituitary gland via the hypophyseal portal circulation. The system in birds seems to differ from that of mammals. In birds, the factors involved in GH secretion are TRH (stimulatory), growth hormone-releasing factors (GRF, stimulatory), and somatostatin (SRIF, inhibitory) (Scanes and Lauterio, 1984). TRH is more potent than human GRF in increasing plasma concentrations of GH in chicken *in vivo* and *in vitro* (Harvey *et al.*, 1984; 1985), whereas, in mammals, TRH shows contradictory results (stimulation or inhibition) on the synthesis of GH in physiological condition (see review Harvey 1990a). The differential role of TRH in chicken may further be elucidated by intricate interactions between the hypothalamo-pituitary-thyroid axis and GH. In chicken, GH stimulates the conversion of thyroxine (T_4) to tri-iodothyronine (T_3) by enhancing hepatic monodeiodinase activity (Kühn *et al.*, 1987), but suppresses thyroidal iodide uptake (Chandola and Thapliyal, 1992). Conversely, T_3 depresses plasma concentrations of GH (Harvey, 1983), attenuates the TRH-induced peak of GH in a dose-dependent manner (Tixier-Boichard *et al.*, 1991), and inhibits both TRH- and GRF-induced GH release from pituitary tissue in vitro (Scanes and Harvey, 1989). Taken together, TRH acts as a growth hormone-releasing factor in the chicken.

In sexual maturation of the chicken, it has been well established that the hypothalamic peptide, GnRH, plays a pivotal role in the hypothalamo-pituitary-gonad reproductive axis. The pulsatile secretion of GnRH stimulates luteinising hormone (LH) and follicle-stimulating hormone (FSH) release from the pituitary gland in a pulsatile manner, which in turn regulates gonadal functions. Interestingly, there is evidence indicating that gonadal conditions affect the pituitary-thyroid system. The plasma concentrations of TSH and T_4 are significantly decreased in orchidectomised rats (Chen and Walfish, 1978). On the other hand, the concentrations of TRH and TRH-Gly (a precursor) in the posterior pituitary gland increase significantly with orchidectomy, and are returned to the normal range by testosterone replacement (Pekary *et al.*, 1990). Furthermore, TRH in the posterior pituitary gland is increased by 25 % in the castrated rat, but shows no effect on the level of TRH-Gly mRNA in the hypothalamus (Rondeel *et al.*, 1995).

The isolation and synthesis of TRH and GnRH over 2 decades ago (Bøler *et al.* 1969; Burgus *et al.*, 1969; Matsuo *et al.*, 1971; Burgus *et al.*, 1972), lead to the understanding of their distribution and biological functions. Both peptides are ubiquitous in the hypothalamus and in extra-hypothalamic regions, and exert biological roles as hormones, neurotransmitters, and neuromodulators.

1.1 Distribution and physiological roles of TRH and GnRH

TRH

TRH was the first hypophysiotrophic peptide to be isolated from pig hypothalamus and is a tripeptide consisting of pGlu-His-Pro-NH_2 (Burgus *et al.*, 1969; Bøler *et al.*, 1969). The structure of TRH is identical in all vertebrates classes and is active in all mammals, birds and amphibians. Structure-function activity studies of a large number of analogues have demonstrated that the side chains of all three amino acid residues of TRH are involved in receptor binding (Vale *et al.*, 1973; Gershengorn *et al.*, 1996)).

Distribution : It has been postulated that the TRH-producing cells are derivatives of the embryonic neuroectoderm in vertebrates. Immunoreactive (ir)-TRH neurons are present in many regions of the hypothalamus, extending from rostral portions of the

preoptic nucleus to the mammillary complex (Lechan and Jackson, 1982). Regions include the paraventricular nucleus, the dorsal and ventral portions of the dorsomedial nucleus, the arcuate nucleus, the perifornical region, the periventricular nucleus throughout the extent of the third ventricle, the median eminence, the dorsal and ventral premammillary nuclei. The ir-TRH neurones are also found in extra-hypothalamic regions of the brain (including the medulla, the cerebrum, the cerebellum, and the spinal cord) and gastrointestinal tissues (including the pancreas, the stomach, the duodenum, and the colon) (Leppäluoto *et al.*, 1978). TRH has also been isolated from frog skin and retina (Jackson and Reichlin, 1977). The wide distribution of TRH in and outside of the CNS reflects the diversity of its physiological roles.

Physiological roles : TRH is released by neurones in the hypothalamus and is stored in the median eminence. It is transported to the pituitary gland by the hypophyseal portal circulation. TRH displays different physiological functions on different cell types of the pituitary gland in different vertebrates. TRH stimulates the biosynthesis and release of thyrotrophin (TSH) from thyrotrophs in the mammals and aves, but has no effect on TSH release in the amphibian (Nelson, 1982). It also stimulates prolactin release from lactotrophs in most mammals and aves (Fink *et al.*, 1983; Shimada *et al.*, 1991). It has been shown that TRH plays an important role in increasing the transcription of prolactin mRNA in the chicken. The prolactin is responsible for the behaviours of incubation and brooding in hens (Shimada *et al.*, 1991; Zadworny *et al.*, 1989).

An important physiological role of TRH is to stimulate GH release from somatotrophs in the pituitary in birds (see review, Harvey 1990a). TRH and its analogues rapidly (within 5 min) increase GH release from somatotrophs *in vivo* and *in vitro* in chickens, turkeys, pigeons, and ducks. The activity of TRH on stimulating GH release is more potent than that of mammalian GRF preparations in chickens (Harvey *et al.*, 1984). It has been suggested that TRH and GRF exerted synergistic effects on GH release (Perez *et al.*, 1989), indicating that the pathway of TRH-induced GH release differs from that of the GRF effect. The physiological function of TRH in GH regulation is inhibited by SRIF *in vivo* (Harvey and Scanes, 1987) and *in vitro* (Leung and Taylor, 1983). Moreover, thyroid hormone may provide a negative feedback pathway in the control of TRH-induced GH secretion (Harvey, 1990b). In addition to its hypophysiotropic effects, TRH has roles in

neurotransmission, neuromodulation, and peripheral hormone actions in vertebrates (Metcalf and Jackson , 1989).

GnRH

Mammalian GnRH (mGnRH: pGlu^{1}-His^{2}-Trp^{3}-Ser^{4}-Tyr^{5}-Gly^{6}-Leu^{7}-Arg^{8}-Pro^{9}-Gly^{10} amide) was first purified from the hypothalami of pigs (Matsuo *et al.*, 1971) and sheep (Burgus *et al.*, 1972). To date, ten additional isoforms of GnRH have been isolated from other vertebrates and protochordates: chicken GnRH I (cGnRH I, King and Millar, 1982; Miyamoto *et al.*, 1982), salmon GnRH (Sherwood *et al.*, 1983), chicken GnRH II (cGnRH II , Miyamoto *et al.*, 1984), lamprey GnRH I (Sherwood *et al.*, 1986), catfish GnRH (Ngamvongchon *et al.*, 1992), dogfish GnRH (Lovejoy *et al.*, 1992), lamprey GnRH III (Sower *et al.*, 1993), seabream GnRH (Powell *et al.*, 1994), tunicate GnRH I (Powell *et al.*, 1996), and tunicate GnRH II (Powell *et al.*, 1996) (Fig. 1.1).

	1	2	3	4	5	6	7	8	9	10
mammal	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	$GlyNH_2$
chicken I	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Gln	Pro	$GlyNH_2$
seabream	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Ser	Pro	$GlyNH_2$
Salmon	pGlu	His	Trp	Ser	Tyr	Gly	Trp	Leu	Pro	$GlyNH_2$
catfish	pGlu	His	Trp	Ser	His	Gly	Leu	Asn	Pro	$GlyNH_2$
dogfish	pGlu	His	Trp	Ser	His	Gly	Trp	Leu	Pro	$GlyNH_2$
chicken II	pGlu	His	Trp	Ser	His	Gly	Trp	Tyr	Pro	$GlyNH_2$
lamprey III	pGlu	His	Trp	Ser	His	Asp	Trp	Lys	Pro	$GlyNH_2$
lamprey I	pGlu	His	Tyr	Ser	Leu	Glu	Trp	Lys	Pro	$GlyNH_2$
tunicate I	pGlu	His	Trp	Ser	Asp	Tyr	Phe	Lys	Pro	$GlyNH_2$
tunicate II	pGlu	His	Trp	Ser	Leu	Cys	His	Ala	Pro	$GlyNH_2$

Fig. 1.1 ***Comparison of the primary structures between eleven types of GnRHs.*** The amino acids differing from those of mammalian GnRH are indicated in red letters.

The GnRH isoforms are all decapeptides, which have been highly conserved through several hundred million years of evolution. Residues 1, 2, 4, 9, and 10 of the peptides are 100 % conserved. Trp at position 3 is conserved in all forms, except for lamprey I. The amino acid at position 8 is not conserved. The NH_2 - and COOH-terminal sequences of the peptides are highly conserved, and it has been proposed that both termini are important for receptor binding. The NH_2 terminus is also responsible for receptor activation in mammals and chicken (Millar *et al.*, 1989; Davidson *et al.*, 1990; Sealfon *et al.*, 1997). In most

species, at least two different GnRH isoforms are found: chicken II, and a second / third form which varies in the different species (King and Millar, 1997). Two isoforms of GnRH, cGnRH I and cGnRH II, have been isolated from the hypothalamus and brain of chickens. Their structures differ from that of the mammalian counterpart, in that positions 5, 7, and 8 of the peptides are replaced by Gln^8, and His^5-Trp^7-Tyr^8 , respectively (Fig. 1.1).

Distribution : The embryonic development of the GnRH neuronal system has been studied extensively in many species by using immunocytochemical methods. In the chicken, the neurones expressing cGnRH I originate within nasal structures and migrate into the brain (Norgren and Lehman, 1991). In days 4 and 5 of the chicken embryo, a small cluster of cells of immunoreactive (ir)-GnRH neurones are found in the epithelium of the ventral olfactory pit. In E7, most of the ir-GnRH neurones are found in the medial half of the olfactory nerve and along the medial half of the telencephalon. The final destinations of ir-cGnRH I neurones are distributed in the medial preoptic area, the medial septum, the nucleus dorsomedialis anterior thalamus, the nucleus supraopticus, and the nucleus accumbens (Fig. 1.2). In addition, the ir-cGnRH I fibres occur throughout the preoptic area and in ventral portions of the septal region, and terminate in the palisade layer of the median eminence (Millam, *et al.*, 1993). A similar origin and migration have also been observed in mammals (Schwanzel-Fukuda and Pfaff, 1989; 1990; Ronnekleiv and Resko, 1990; Quanbeck *et al.*, 1997) and in amphibians (Sétáló, 1996).

The neurones expressing cGnRH II do not appear to differentiate from the olfactory placode, because the neurones are still found in the fetal brains of chickens after bilateral olfactory placode ablation during an early stage of embryogenesis (Akutsu *et al.*, 1992; Norgren and Gao, 1994). The ir-cGnRH II neurones are located along a dorsoventral line extending from the ventral border of the substantia grisea centralis to the dorsal border of the nucleus ruber, and also observed slightly medial to the nucleus of the basal optic root (Fig. 1.2). The fibres of cGnRH II are prominent in the nucleus preopticus medialis, and no fibres are detected in the median eminence (Mikami *et al.*, 1988; Sharp *et al.*, 1990; Millam, *et al.*, 1993).

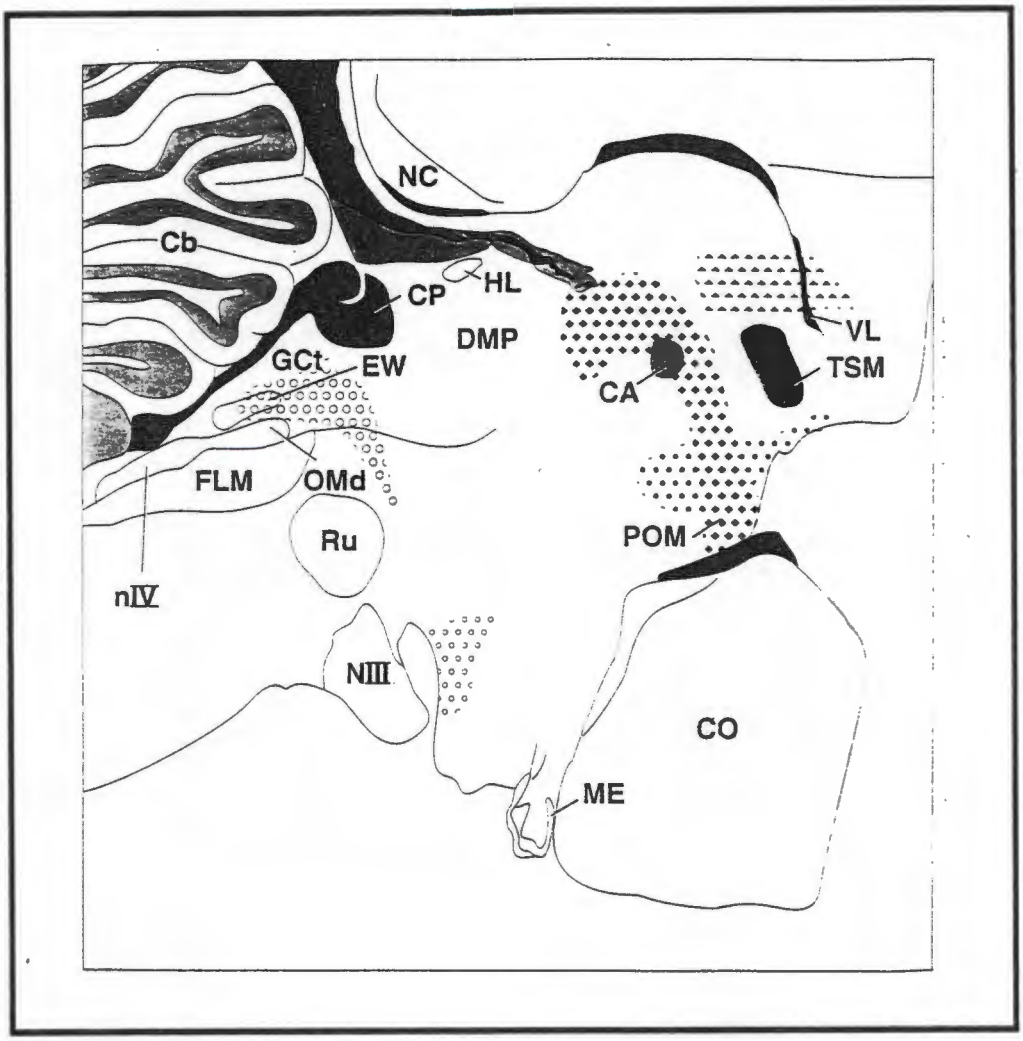


Fig. 1.2 ***Diagram of the distribution of ir-cGnRH I and ir-cGnRH II neurones in turkey hen brain*** (Millam et al., 1993). Three major groups of ir-cGnRH I neurons are indicated and they are lateral ventricular group (triangles), basotelencephalic group (closed circles), midline group (diamonds). Two groups of ir-cGnRH II neurons are magno-cellular group (large open circles) and parvicellular group (small open circles).

Cb: cerebellum; NC: neostriatum caudale; Gct: substantia grisea centralis; CP: commissura posterior; HL: nucleus habenularis lateralis; EW: nucleus of Edinger-Westphal; DMP: nucleus dorsomedialis posterior thalami; nIV: nervus trochlearis; FLM: fasciculus longitudinalis medialis; OMd: nucleus nervi oculomotorii, pars dorsalis; Ru: nucleus ruber; NIII: Nervus oculomotorius; CA: commissura anterior; VL: ventriculus lateralis; TSM: Tractus opticus; POM: nucleus preopticus medialis; CO: chiasma opticum; ME: median eminence.

Physiological roles : As to the biological functions of cGnRH I and II in chicken, it has been suggested that according to the regional brain distribution of both hormones, cGnRH I, but not cGnRH II, plays an important role in regulating the pituitary-gonadal axis.

It has been shown that both cGnRH I and II stimulate LH and FSH release *in vitro* in chickens (Millar *et al.*, 1986; Hattori *et al.*, 1986). cGnRH II is approximately 6-fold and 13-fold more potent than cGnRH I in releasing LH and FSH, respectively (Millar *et al.*, 1986). On the other hand, some studies have shown that cGnRH I and II are equipotent in releasing LH from anterior pituitary glands (Hattori *et al.*, 1986; Chou *et al.*, 1985). The conflicting effects of cGnRH I and II might be due to sex differences in the LH responses. cGnRH I and II show more rapid and greater effects in cockerels than in hens (Sharp *et al.*, 1987).

Although cGnRH II stimulates LH and FSH release in chickens, it does not have a physiological role on regulating the pituitary-gonadal axis. As mentioned earlier, cGnRH II is undetectable in the medium eminence. Several other points of evidence support this conclusion. The amounts of hypothalamic cGnRH I show changes that correlate with the different reproductive states of chickens (Sharp *et al.*, 1990). In this study, the amount of cGnRH I in the median eminence is higher in laying than in out-of-lay hens. Passive immunisation with anti-cGnRH I, but not anti-cGnRH II, disrupts egg production. Moreover, the content of cGnRH I in the hypothalamus is increased in somatically immature birds after castration and in cockerels at the onset of puberty. There are no correlated changes in the amounts of hypothalamic cGnRH II in the same experimental samples. The expression of the cGnRH I gene is closely associated with the changes of plasma LH and ovary weight in laying and incubating hens (Dunn *et al.*, 1996). In European starlings, the changes of cGnRH I neurones in number and size are associated with the photoperiodically-induced reproductive cycle (Parry *et al.*, 1997).

The physiological importance of cGnRH II remains elusive. The peptide might play a role as a neurotransmitter or neuromodulator in extra-hypothalamic areas of the brain. It has been suggested that cGnRH II might play a specific role in the medulla, an area which contains the highest concentration of cGnRH-II in non-mammalian vertebrates (King and Millar, 1997). In ring doves, cGnRH II impacts on the reproductive behaviour after being

injected into the third ventricle (Besmer *et al.*, unpublished). In amphibians, brain concentrations of cGnRH II exhibit significant reproductive status-related fluctuations in *Rana esculenta* (Rastogi *et al.*, 1997). In the sympathetic ganglia of bullfrogs, cGnRH II is the most potent peptide in inhibiting M current among GnRH analogues (Jones, 1987). Recently, cGnRH II was identified in sympathetic ganglia by a cGnRH II antiserum and co-eluted with synthetic cGnRH II on a HPLC gradient (Troskie *et al.*, 1997). Additionally, its specific receptor has been detected by radio-ligand binding assay (Troskie *et al.*, 1997). cGnRH II may have other functions. Moreover, cGnRH II has been found in the goldfish pituitary, and it plays a role in stimulating GH secretion (Chang *et al.*, 1990; Cook *et al.*, 1991).

1.2 The TRH and GnRH receptors

TRH and GnRH exert their hypophysiotropic effects through binding to their specific receptors on the surfaces of cells in the pituitary gland. These interactions result in conformational changes in the receptors and allow them to interact with the heterotrimeric G-proteins in the cell membranes which in turn promote second messenger signalling cascades to trigger biological actions of the hormones (see review, Sealfon *et al.*, 1997; Gershengorn *et al.*, 1996). The structures of the cloned TRH and GnRH receptors reveal that both receptors are members of the G protein-coupled receptor (GPCR) family.

1.2.1 Features of G protein-coupled receptors

Members of the GPCRs family (approaching 1000 members) are characterised by seven hydrophobic stretches of 20-30 amino acids, which are predicted to form transmembrane (TM) α helices. These helices are connected by alternating hydrophilic extracellular loop (EL) and intracellular loop (IL), and an extracellular N-terminal, and an intracellular C-terminal domain (Strader *et al.*, 1994). The TM domains are the most conserved , whereas the N- and C-terminal domains and IL 3 can be quite divergent (Dohlman *et al.*, 1987).

GPCRs have a wide variety of agonists (e.g. cations, small biogenic amines, peptides, proteolytic enzymes, glycoprotein hormones) that stimulate the diverse second-messenger pathways. However, these receptors share considerable structural homology.

The primary sequence identity in the TMs of these receptors ranges from 85-95 % for species homologues of a given receptor, to 60-80 % for related subtypes of the same receptor, and 35-45 % for other members of the same family, down to 20-25 % for unrelated GPCRs (Strader *et al.*, 1994).

All of the GPCRs have at least one consensus sequence for N-linked glycosylation (Asn-X-Ser/Thr) in ELs. It has been shown that glycosylation plays a role in receptor expression or stability in the β_2-adrenergic receptor (β_2AR), rhodopsin, and mammalian GnRH receptors (Rands, *et al.*, 1990; Liu *et al.*, 1993; Davidson *et al.*, 1995).

GPCRs contain a number of conserved cystein residues. There are two highly conserved Cys residues in EL 1 and 2, which have been shown to form a disulphide bond to maintain the active conformation of the receptor in β_2AR, rhodopsin (Dixon *et al.*, 1987a; Dohlman *et al.*, 1990), mouse TRH receptor (Perlman *et al.*, 1995), and mammalian GnRH receptors (Davidson *et al.*, 1997; Cook and Eidne, 1997). Additionally, many GPCRs possess a Cys residue in the C-terminal tail. This residue has been shown to be palmitoylated in α-, β-ARs (O'Dowd *et al.*, 1989; Kennedy and Limbird, 1993), and rhodopsin (Ovchinnikov *et al.*, 1988; Papac *et al.*, 1992). It has been speculated that the palmityl group may anchor part of the cytoplasmic tail of the receptor to the plasma membrane, which is important for interactions with G protein. Intriguingly, the homologous Cys residues in the mouse TRH receptor are not necessary for G protein coupling, but may play a role in restraining the receptor in an inactive conformation that is optimal for rapid internalisation of the receptor (Nussenzveig *et al.*, 1993a).

The Asp-Arg-Tyr motif (DRY) adjacent to TM 3 is a highly conserved intracellular sequence in GPCRs. The DRY motif has been implicated in signal transduction (see review, Probst *et al.*, 1992). The highly conserved residue Asp in TM 2 plays a role in ligand binding and G protein activation in many GPCRs (Strader *et al.*, 1987; Horstman *et al.*, 1990; Wang *et al.*, 1993). In addition, the membrane proximal regions of the third intracellular loop (IL) are necessary for G protein coupling in a number of GPCRs (Cotecchia *et al.*, 1990; Baldwin, 1994). There are a series of conserved helix-breaking Pro residues in each of TMs 4, 5, 6, and 7, whose role is proposed to transfer the energy of agonist binding to conformational changes in ILs that allow association with G proteins (Millar and King, 1994).

1.2.2 The putative binding domains of GPCRs

Structural interactions between ligands and receptors have been extensively studied for many members of GPCRs by a variety of approaches (e.g. mutagenesis, computer modelling, biophysical analysis). Experimental data has revealed that different subfamilies of GPCRs (biogenic amine receptors, peptide receptors, and glycoprotein hormone receptors) possess different key elements in receptor binding. In general, the ligand binding sites of biogenic amine receptors reside within the TM domains, whereas the binding sites of peptide and glycoprotein hormone receptors are present in TMs, ELs, and the N-terminus.

The binding domain of biogenic amine receptors:

The biogenic amine receptors [e.g. α-, ß-adrenergic receptor (α-, ß-AR), muscarinic receptor, dopamine receptor (DAR), and serotonin receptor (5HT-R)] contain an acidic group in the binding pocket to provide a counterion for the protonated amine of the ligand. Asp^{113} in the TM 3 domain has been identified in ß-AR by mutagenesis as the counterion for the amine groups of the ligands (Strader *et al.*, 1987). This Asp also has been found at homologous position in α-AR (Wang *et al.*, 1991) and muscarinic receptors (Fraser *et al.*, 1989). Asp^{79} in TM 2 also plays a role in receptor binding and transduction in biogenic amine receptors (Strader *et al.*, 1987; Horstman *et al.*, 1990; Wang *et al.*, 1993). Additionally, Ser^{204} and Ser^{207} in TM 5 (Strader *et al.*, 1989) and Phe^{290} in TM 6 (Dixon *et al.*, 1988) of the ß-AR form hydrogen bonds with the aromatic catechol moiety of ß-adrenergic agonists. It has been demonstrated that the hydrophilic loop regions of the receptors are not important for receptor binding (Dixon *et al.*, 1987b).

The binding domain of peptide receptors:

The peptide receptors of GPCRs include TRH, GnRH, neurokinin (NK), substance P, angiotension (AT), neuropeptide and glucagon receptors, whose natural ligands range from 3-40 amino acids in size. It has been proposed that the peptide-binding sites of GPCRs contain a larger surface area than that of biogenic amine receptors. Mutagenesis studies have suggested that the extracellular domains of receptors may provide additional contact sites for peptide ligands. For example, in the NK 1 receptor, Asn^{23}, Gln^{24}, and Phe^{25} in the N-terminus and Asn^{96} and His^{108} in EL 1 are required for the high-affinity

binding of NK (Fong *et al.*, 1992). In the AT receptor, the N-terminus (His^{24}, Y^{26}, and I^{27}), EL 1 (Y^{92} and K^{102}), EL 2 (V^{179}), and part of EL 3 are important for ligand binding (Hjorth, *et al.*, 1994). In addition to residues in the extracellular domain, the TM regions are also critical for peptide binding for many GPCRs. For example, the residues Asn^{85}, Asn^{89}, Tyr^{92}, Asn^{96} (in TM 2), and Tyr^{287} (in TM 7) of the NK 1 receptor (Huang *et al.*, 1994) or the residues Lys^{113} and Tyr^{129} (in TM 2) of the endothelin receptor (Zhu *et al.*, 1992) take part in receptor binding.

The binding domain of glycoprotein hormone receptors:

The extracellular domains, especially the N-terminal region, are also involved in ligand binding for the glycoprotein hormone receptors. The glycoprotein hormone receptors contain a very large N-terminus ranged at 300-400 amino acids. The N-terminal region of the lutropin receptor alone displays high affinity and specificity for LH (Tsai-Morris *et al.*, 1990). The N-terminus of the TSH receptor plays a pivotal role in the high-affinity binding of its ligand (Nagayama *et al.*, 1991).

1.2.3 TRH receptor (TRH-R)

General structural features : The TRH-R DNAs have been cloned from 3 mammalian species: two isoforms from mouse (Straub *et al.*, 1990; Duthie *et al.*, 1993a), two isoforms from rat (De La Peña *et al.*, 1992; Sellar *et al.*, 1993; Zhao *et al.*, 1992), and human (Duthie *et al.*, 1993b; Matre *et al.*, 1993; Yamada *et al.*, 1993), but not from any other vertebrate classes. The two variant forms of the TRH-R, from mouse or from rat, are characterised by different amino acid sequences in their respective C-terminal tails. The amino acid sequences of the TRH-R share over 90 % identity amongst the 5 cloned receptors.

The structure of the TRH-R reveals that the receptor contains several residues which are highly conserved in GPCRs. Three potential sites for N-linked glycosylation are present in the N-terminal region (Asn^{3} and Asn^{10}) and in EL 2 (Asn^{167}). Two Cys residues (Cys^{98} and Cys^{179}) in EL 1 and 2 of the receptor appear to form a disulphide bridge (Perlman *et al.*, 1995). Two additional Cys residues, Cys^{335} and Cys^{337} , have been found in the C-terminal tail, but the palmitoylation in both loci is yet to be determined. Moreover, there are Ser and Thr residues in IL3 and in the C-terminal tail, which could

serve as sites for regulatory phosphorylation (Lefkowitz, 1993). In the TRH-R, several additional residues in the proximal and distal juxtamembrane regions of IL 3 are important for coupling to $G_{q/11}$, whereas a large part of the middle portion of the loop can be deleted without any effect on the coupling (Nussenzveig *et al.*, 1993b).

Ligand binding sites

The putative binding sites of the mammalian TRH-R have been identified by mutagenesis and computer modelling. The ligand binding sites include Tyr^{106}, Asn^{110}, Tyr^{282}, and Arg^{306}. The findings reveal that the interaction between TRH and its receptor is non-ionic, unlike the binding of biogenic amine receptors.

Tyr^{106}: The Tyr residue in TM 3 of the muscarinic receptor is involved in ligand binding by forming an hydrogen-bonding connection with ligand. Amongst mutations of several residues that could form an hydrogen bond with TRH, Tyr^{106} of the TRH-R has been verified as the candidate residue in the mouse. Mutation of Tyr^{106} to Phe, which abolishs the hydroxyl group of tyrosine, causes an unmeasurable level of affinity for TRH (Perlman *et al.*, 1994a). Further study has revealed that the hydroxyl group of Tyr^{106} binds the carbonyl group of the $pGlu^1$ moiety of TRH.

Asn^{110}: According to the interactions between the pGlu moiety of TRH and TM 3 of the TRH-R by computer modelling, it shows that the ring N-H of Glu interacts with the side chain C=O of Asn^{110}. Mutagenesis of Asn^{110} has confirmed the importance of Asn^{110} in ligand binding (Perlman *et al.*, 1994b).

Tyr^{282} and Arg^{306}: A three-dimensional model of the TRH-R reveals that Tyr^{282} in TM 6 forms an hydrophobic interaction with the imidazole of the His residue of TRH and Arg^{306} forms hydrogen bonds with C=O of pGlu and C=O of $ProNH_2$ of TRH (see review, Gershengorn *et al.*, 1996).

Ligand-selectivity of TRH receptor :

Structure-function activity studies of many TRH analogues have established that the side chains of all three amino acid residues of TRH are involved in binding to the TRH-R. Most TRH analogues bind the receptor with low affinity. Only one TRH analogue, N^{Γ}-[*methyl*]His-TRH, shows about an 8- and 2-fold increase in stimulating TSH release *in vivo* and *in vitro*, respectively (Vale *et al.*, 1971). This can be attributed to

a greater binding affinity for the receptor (Perlman *et al.*, 1994b) and/or an increased efficacy to trigger the secondary messenger.

G protein coupling : The TRH-R has been shown to couple to G_q and G_{11}, which activate phophoinositide-specific phospholipase C (PPI-PLC) in GH_3 cells and in HEK-293 cells expressing mammalian TRH receptors (Aragay *et al.*, 1992; Hsieh and Martin, 1992; Hepler and Gilman, 1992; Kim *et al.*, 1994). Some findings suggest that the TRH-R couples to other G proteins. Gollasch *et al.* (1993) have provided evidence that G_{i-2} and G_{i-3} are involved in the TRH stimulation of voltage-sensitive calcium channels in GH_3 cells. There are controversial findings in the TRH-R coupling to G_s that is known to activate adenylyl cyclase and calcium channels and to inhibit sodium channels (Hepler and Gilman, 1992). One investigator group has suggested that the TRH-R couples to G_s and stimulates adenylyl cyclase in GH_3 cells (Paulssen *et al.*, 1991; 1992). However, most findings do not support the evidence for TRH stimulation of adenylyl cyclase in GH_3 cells or in other cell types expressing mammalian TRH receptors (Heinflink *et al.*, 1994; Kim *et al.*, 1994; Quick *et al.*, 1994). In chickens, cAMP may participate in the effect of TRH-induced GH release (Perez *et al.*, 1989). Some findings indicate that a G_s-like protein is involved in TRH stimulation of a rise in intracellular free calcium in GH_3B_6 cells (Bauer *et al.*, 1994) and in TRH stimulation of PPI-PLC in *Xenopus* oocytes expressing the mammalian TRH-Rs (De la Peña *et al.*, 1995). However, a finding suggests that coexpression of the TRH-R and G_s does not affect signalling through PPI-PLC in *Xenopus* oocytes (Quick *et al.*, 1994).

1.2.4 GnRH receptor (GnRH-R)

General structural features : The GnRH-R has been cloned from several mammalian and a non-mammalian species: mouse (Tsutsumi *et al.*, 1992), rat (Eidne *et al.*, 1992; Kaiser *et al.*, 1992; Perrin *et al.*, 1993), human (Kakar *et al.*, 1992; Chi *et al.*, 1993), sheep (Brooks *et al.*, 1993; Illing *et al.*, 1993), cow (Kakar *et al.*, 1993), pig (Weesner *et al.*, 1994), and catfish (Tensen *et al.*, 1997). In the comparison of amino acid sequences, the mammalian GnRH-Rs share over 85 % amino acid identity. However, the catfish GnRH-R, the only non-mammalian receptor that has been cloned, shares only 40 % identity with the human GnRH-R. As compared to other GPCRs, the mammalian GnRH-Rs

possess several unusual or unique features. These include a complete absence of the intracellular C-terminal tail, the change of the highly conserved Asp-Arg-Tyr (DRY) sequence to Asp-Arg-Ser (DRS) at the end of TM 3, and the reciprocal exchange of the conserved TM 2 Asp and TM 7 Asn. However, the catfish GnRH-R has a C-terminal tail. The C-terminal tail has been demonstrated to play a role in receptor palmitoylation (Ovchinnikov *et al.*, 1988), homologous desensitisation in GPCR receptors (Bouvier *et al.*, 1988; Widmann *et al.*, 1997)), and internalisation (Widmann *et al.*, 1997). Mutagenesis studies of the mammalian GnRH-Rs have identified glycosylation sites in the N-terminal domain (Asn^4 and Asn^8) in the mouse GnRH-R (Davidson *et al.*, 1995). Mutation of the Asn^4 or Asn^8 to Gln results in lower expression of the receptor than that of the wild type receptor. However, the ligand-binding affinities of the mutated receptors are unchanged. Additionally, disulphide bonds in the mammalain GnRH receptors have been verified by photo-affinity cross-link and mutagenesis studies (Davidson *et al.*, 1997; Cook and Eidne, 1997). The bonds are located at two different places: between Cys^{14} (in N-terminus) and Cys^{200} (in EL 2) and between Cys^{114} (EL 1) and Cys^{196} (EL 2) in the human GnRH receptor, whereas only residues Cys^{114} and Cys^{195} form a disulphide bridge in the rat receptor. The disulphide bridges are essential for receptor binding and activation. Furthermore, the residues Asn^{87} (TM 2) and Asp^{318} (TM 7) of the mammalian GnRH-Rs, in which the analogous positions are TM 2 Asp and TM 7 Asn in most GPCRs, have been shown to interact with each other (Zhou *et al.*, 1994). Substitution of Asn^{87} to Asp abolishes receptor function (binding and IP formation). Interestingly, the reciprocal mutation (Asp^{87} and Asn^{318}) restores the receptor binding; however, the mutant receptor exhibits very poor IP formation. The finding indicates that the two residues might be involved in the active conformation of receptor.

Ligand binding sites

Several putative ligand interacting sites have been identified in the GnRH receptor by site-directed mutagenesis studies. The putative binding sites contain Glu^{301} (or Asp^{302} in human GnRH-R), Lys^{121}, and Asn^{102} in mouse GnRH-R.

Glu^{301} (or Asp^{302}): The findings of the GnRH-R binding studies reveal that the Arg^8 residue of mGnRH is critical for high affinity binding of the mammalian

GnRH-R. Therefore, it has been postulated that the positively-charged Arg^8 of mGnRH might interact with a negatively-charged acidic amino acid residue or sialic acid residue in the receptor. However, the polysaccharide sialic acid residues do not affect ligand binding (Davidson *et al.*, 1995). Amongst mutations of all acidic amino acids in ELs, Glu^{301} of the mouse GnRH-R (or Asp^{302} of the human receptor) in EL 3 has been identified as the candidate for interacting with the Arg^8 residue of mGnRH (Flanagan *et al.*, 1994). In this study, the substitution of Glu^{301} with Gln^{301} results in a 100-fold reduction in affinity for [Arg^8]-GnRH; however, the mutant receptor exhibits unchanged affinity for [Gln^8]-GnRH and increased affinity for [Glu^8]-GnRH. The finding indicates that the Glu^{301} plays a critical role in identifying the positive charge of [Arg^8]-mGnRH.

Lys^{121}: It has been demonstrated that the highly conserved Asp residue in TM 3 of biogenic amine receptors acts as the counterion for amine ligands (Strader *et al.*, 1987). Therefore, the homologous residue of the GnRH-R, Lys^{121}, might take part in the receptor binding. The Lys^{121} residue was mutated to Asp, Ala, Leu, or Gln and the mutations cause more than 1000-fold reduction in affinity of agonist binding, whereas the mutant receptor (Lys121Gln) exhibits normal antagonist binding (Zhou *et al.*, 1995). Moreover, the Lys^{121} might interact with His^2 or $pGlu^1$ of GnRH (Zhou *et al.*, 1995).

Ans^{102}: Another putative binding site of the GnRH-R has been located in the junction between TM 2 and EL 1. Mutation of Asn^{121} to Ala leads to a 100 ~ 1000-fold reduction in affinity for GnRH analogues which contain the $GlyNH_2$ carboxy-terminal residue, but shows only a slight effect on that of ligands containing N-ethylamide at the carboxyl-terminus (Davidson *et al.*, 1996). The result suggests that the Asn^{121} residue of the GnRH-R might directly interact with Gly^{10} NH_2 of GnRH analogues.

<u>Ligand-selectivity of GnRH receptor :</u> Through 500 million years of evolution, all the vertebrate GnRHs are active in all vertebrates, but differences in ligand-selectivity exist amongst vertebrate GnRH-Rs. Non-mammalian vertebrate GnRHs have been shown to have relatively poor activities of receptor binding and gonadotrophin release in mammalian systems, whereas all of these GnRHs (with the exception of

lamprey GnRH II) have relatively high activity in birds, reptiles, amphibians, bony fish, and cartilaginous fish (see review, King and Millar, 1997).

Previous studies have shown that the chicken receptor has dramatic differences in ligand-selectivity as compared with that of the mammalian receptor. The chicken receptor can not discriminate basic or neutral amino acids in the position 8 residue of GnRHs, while the mammalian receptor has a specific preference for Arg in this position (Millar *et al.*, 1989). In addition, His^5 accompanying Arg^8 in analogues markedly diminishes activity in the chicken, whereas gonadotrophin-releasing activity is retained in the sheep pituitary. Interestingly, 2 out of 10 selected mammalian GnRH antagonists exhibit agonist effects in the chicken (Jacobs *et al.*, 1995). There are differences in ligand-selectivity amongst other non-mammalian GnRH receptors. Signalling studies (measuring inositol phosphate production) reveal that all of the cloned non-mammalian GnRH-Rs (the chicken, frog, goldfish, and catfish receptors) show a striking preference for cGnRH II (20-1000 times more active than any other ligand), whereas cGnRH II has relatively low activity in mammalian receptors (Millar *et al.*, 1997; Tensen *et al.*, 1997). The chicken receptor does not distinguish between mGnRH and cGnRH I, whereas mGnRH exhibits 200-fold more activity than cGnRH I for the frog receptor and 1000-fold more activity than seabream GnRH for the goldfish receptor (Millar *et al.*, 1997).

G protein coupling : The mechanism of GnRH stimulation of gonadotrophin secretion from anterior pituitary gonadotrope cell may be divided into three steps: i) GnRH binding and activation of the receptor; ii) intracellular signal transduction; iii) exocytosis of gonadotrophin-containing secretory granules. GnRH exerts its physiological role, firstly, via binding to its specific receptor (GnRH-R) in the membrane of gonadotrophs; then, coupling to G-proteins (G_q and/or G_{11}) which in turn promotes secondary messengers [1,2-diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3)]. IP_3 activates a calcium release channel in the endoplasmic reticulum membrane, there by allowed calcium to flood the cytosol, while DAG activates protein kinase C. This signalling cascade triggers gonadotrophins (LH and FSH) release (Hsieh and Martin, 1992). In chicken pituitary cells, GnRH causes a rapid initial spike of LH release from 0-5 min, followed by a prolonged plateau phase of release. The spike phase of GnRH-induced

LH release was partially dependent on extracellular calcium. In contrast, the plateau phase of LH release was completely dependent on extracellular calcium.

1.3 Aims of this study

GnRH analogues are extensively used as therapeutic agents in several disorders, such as precocious puberty (Comite *et al.*, 1981), endometriosis (Meldrum *et al.*, 1982), prostate and breast cancers (Borgmann *et al.*, 1982) and infertility (Conn *et al.*, 1984). Therefore, the molecular based knowledge of GnRH action, including interacting with its receptor (the binding domain) and signal coupling, are important in the development of novel analogues; especially non-peptide orally-active analogues. The chicken GnRH-R appears to have marked differences from the mammalian GnRH-Rs in ligand-selectivity and some mammalian GnRH antagonists appear to act as agonists. The molecular cloning and characterisation of the chicken GnRH-R would therefore provide the means for potentially identifying domains and residues involved in ligand-selectivity. The comparative sequences of the receptor may also assist in understanding molecular features associated with the configuration of the receptors, agonist-mediated activation, coupling, and desensitisation. Additionally, the chicken GnRH receptor will provide further insight into the development and reproductive physiology of chickens.

The TRH-R will provide a useful tool for further studies on the roles of TRH in avian growth and thyrotrophin regulation. Moreover, the information of comparative amino acid sequences might give an insight into the knowledge of the structural conformation and regulation of the TRH-R receptor.

Chapter 2

Cloning and Characterisation of chicken TRH Receptor (TRH-R)

2.1 Abstract

2.2 Introduction

2.3 Materials and Methods

2.4 Results

2.5 Discussion

2.1 ABSTRACT

The chicken TRH-R cDNA has been cloned in this study, and shares about 80% amino acid homology with the mouse TRH-R. The ligand binding pocket, which is situated in the transmembrane domains of the mouse TRH-R, is completely conserved in the chicken TRH-R. Pharmacological studies (receptor binding and inositol phosphate production) employing several TRH analogues, reveal that there are no significant differences between the chicken and mouse TRH-R. These findings show that there have been considerable evolutionary constraints on the TRH-R structure and function.

In addition to the full-length TRH-R cDNA, two isoforms of the TRH-R were identified while screening a cDNA library. Both isoforms had a stop codon at nucleotides +1006 (downstream), resulting in a receptor that prematurely terminated in the putative intracellular loop III. The 20 amino acids that precede the stop codon in these clones have no homology with mammalian TRH-Rs. The consensus 5′-end sequence of the exon-intron junction, AGgtag, is found at the point of homology divergence. The divergent region has been verified as an intron.

2.2 INTRODUCTION

As covered in the general introduction (chapter 1), thyrotrophin-releasing hormone (TRH: pGlu-His-ProNH_2) is synthesised in the hypothalamus, and is transported via the hypophysial portal circulation into the pituitary gland to regulate the biosynthesis and release of thyrotrophin (TSH). It also functions as a paracrine regulatory factor and a neurotransmitter/ neuromodulator in central and peripheral nervous systems (Metcalf and Jackson *et al.*, 1989). In addition to these physiological roles, TRH is well documented as a potent growth hormone-releasing factor (GRF) in birds (Harvey, 1990a). In the chicken, TRH stimulates growth hormone (GH) release *in vivo* and *in vitro*, and the effect is even more potent than that of mammalian GRF (Harvey *et al.*, 1984; Harvey *et al.*, 1985). TRH exerts these physiological roles by binding to its specific receptor (TRH-R) on the membranes of somatotrophs and thyrotrophs (Dannies *et al.*, 1976; Harvey and Baidwan, 1989; Sharif, 1989).

To date, five types of TRH-R cDNAs have been cloned from 3 species of the mammal (as indicated in "introduction"). The structure of the TRH-Rs reveals that the receptor is a member of G-protein coupled receptor family, consisting of seven transmembrane (TM) helixes. The receptor has been shown to couple to several G-proteins (e.g. G_q,G_{11},$G_{i\text{-}2}$, $G_{i\text{-}3}$, and a G_s-like protein that does not activate adenylyl cyclase) which in turn promotes secondary messengers (DAG, IP_3, and Ca^{++}) signalling cascade after being bound by its ligand (see review Gershengorn and Osman *et al.*, 1996). On the contrary, some findings suggest that TRH-R couples to G_s and stimulates adenylyl cyclase in GH_3 cells (Paulssen *et al.*, 1991; Paulssen *et al.*, 1992). In chickens, cAMP may participate in the effect of TRH-induced GH release (Perez *et al.*, 1989).

This study has been undertaken to clone the TRH-R gene from chickens and pharmacologically characterise the receptor by measuring inositol phosphate and cAMP formation. This study provides a useful tool for further studies on the roles of TRH in avian growth and thyrotrophin regulation. Furthermore, the cloning of the TRH-R cDNA shows that this receptor has been highly conserved through millions of years of evolution, as it retains a large degree of homology with mammalian receptors.

2.3 MATERIALS AND METHODS

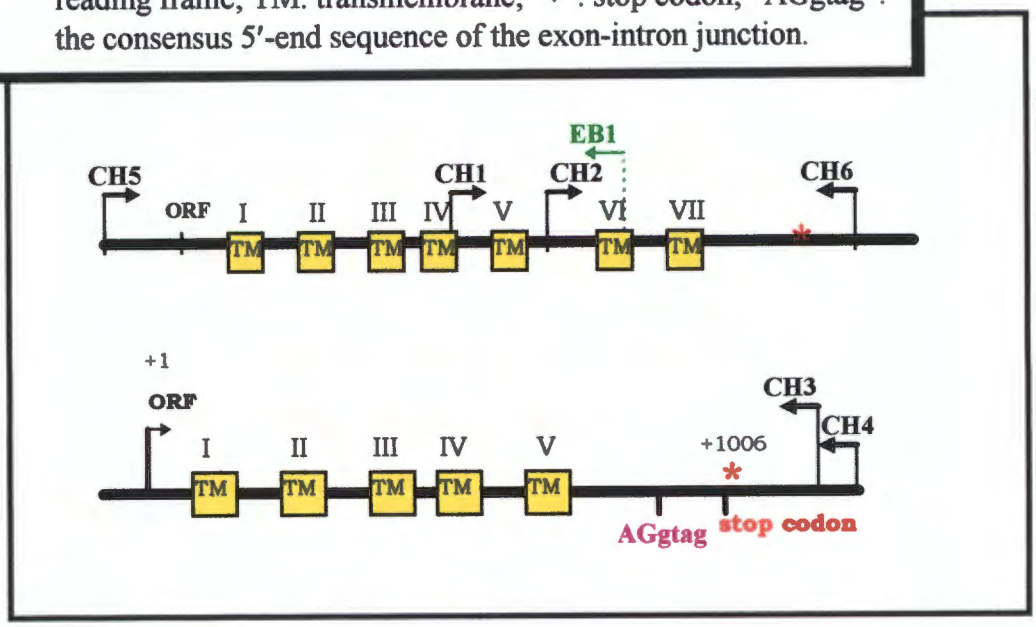
Materials

The oligo-nucleotides (primers) used in the cloning of the TRH-R cDNA are summarised in Table 2.1, and their positions on the chicken TRH-R cDNA are illustrated in Fig. 2.1.

Table 2.1 ***The nucleotide sequences of the primers used in this study***

Name of primers	Nucleotide sequences
CH1	5′-ACAAGGTGTCCAGGAGCT-3′
CH2	5′-GGAGAAATGACATGGCTC-3′
CH3	5′-ACAAGGTGTCCAGGAGCT-3′
CH4	5′-CTAGCCCTAGACTAGGT-3′
CH5	5′-CTGCTCCACTATTCCTTCTGCAGG-3′
CH6	5′-ATATCAAAAGAGAGAACA-3′
EB1	5′-CACAAGAGTCAGGAGGGCATC-3′
adaptorA	5′-GGATCCAAAGCTTGAATTCGAGCTC-3′
adaptorB	5′-ATCGATGGTCGACGCATGCGGATCC-3′

Fig. 2.1 ***The locations of the primers used in this study.***
The nucleotide sequences of the primers were derived from the chicken TRH-R cDNA (top panel) and a truncated TRH-R cDNA (bottom panel). EB1: a primer of the mouse TRH-R; ORF: open reading frame; TM: transmembrane; "*": stop codon; "AGgtag": the consensus 5′-end sequence of the exon-intron junction.



The primary structure of TRH analogues that were used in this study and their source are presented in the following Table (2.2).

Analogues	Structure 1	2	3	Source
TRH	pyroGlu	His	Pro-NH_2	Sigma Chemical Co.
Desaza[1]TRH	pyrolidone	His	Pro-NH_2	a gift from the late Dr. L.A. Cohen (Bethesda, MD, USA)
Val[2]TRH	pyroGlu	Val	Pro-NH_2	Peninsula Laboratories (Belmont CA, USA)
Phe[2]TRH	pyroGlu	Phe	Pro-NH_2	
MeTRH	pyroGlu	N^{τ}-[*methyl*]His	Pro-NH_2	Sigma Chemical Co.
Pyr[3]TRH	pyroGlu	His	Pyrrolidine	Dr. T.K. Sawyer (Parke-Davis Pharmaceutical Research)

Methods

2.3.1 Cloning chicken TRH-R gene

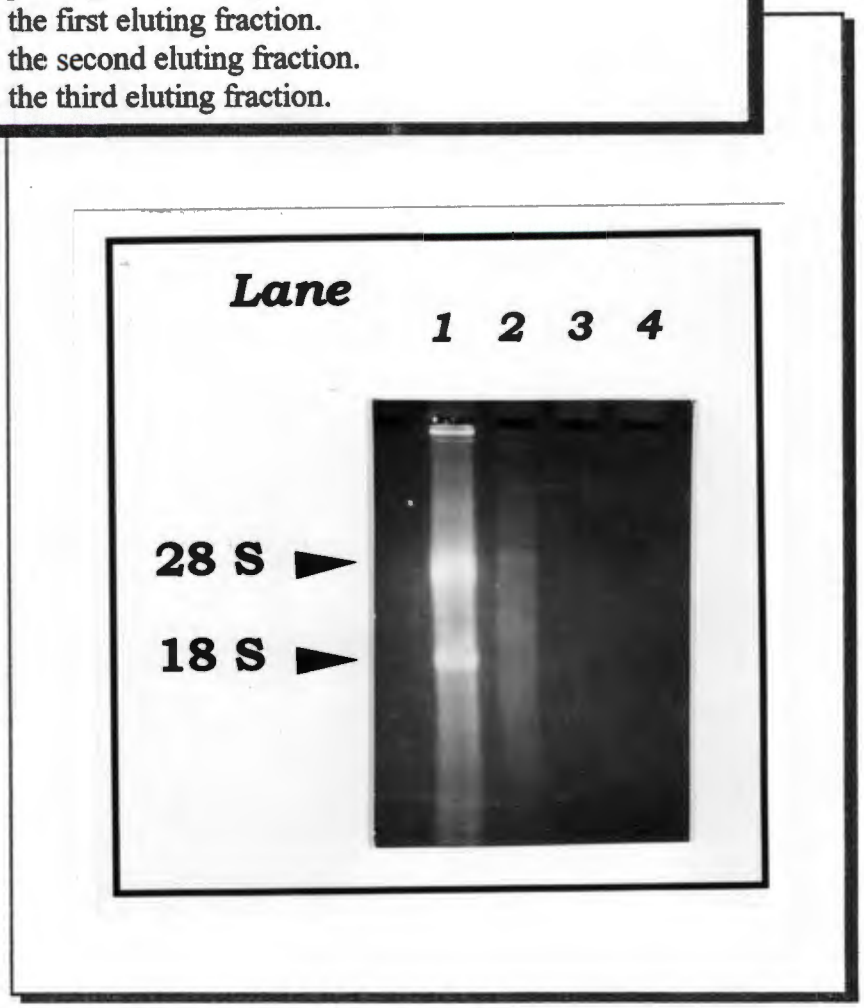
Constructing the cDNA library from the chicken pituitary gland

In order to clone the chicken TRH-R gene, the cDNA library of the chicken pituitary gland was constructed. The first step for constructing a cDNA library was to prepare pure poly(A)$^+$ RNA (about 5 μg) of the chicken pituitary gland, since 5 μg of poly(A)$^+$ RNA is optimal for constructing a good library.

Total RNA was isolated by the guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski et al., 1987) from 70 chicken pituitary glands (Golden Grove Poultry Company, Cape Town, South Africa). 6.6 μg of poly(A)$^+$ RNA of the chicken pituitary was purified with affinity chromatography using an oligo (dT)-cellulose column (made by myself). The column was made by means of a 1ml pipette tip, plugged with glass wool (about 0.5 mm) at the neck. 1ml slurry of oligo (dT)-cellulose (25mg) was poured into the column. The column was rinsed with 10 ml d.d.H_2O, and equilibrated with 30 ml of 1x loading buffer [containing 0.5 M LiCl, 10 mM Tris-Cl (pH: 7.5), 1 mM EDTA, 0.1 % of SDS, and 0.1 % diethylpyrocarbonate].

1 μg of poly(A)$^+$RNA was analysed on an RNA gel to evaluate the quality, and compared with the total RNA (Fig. 2.2). There was very little 28S and 18S of rRNAs existing in the sample of the first eluting fraction, suggesting that the first eluting sample contained very pure poly(A)$^+$ RNAs. There was no poly(A)$^+$ RNA eluted at the second and third fractions, indicating that the "home-made" oligo(dT) column worked very well.

Fig. 2.2 ***Electrophoresis of the poly(A)$^+$ RNA of the chicken pituitary gland,*** eluted consecutively from an oligo(dT) column on an 1% agarose formaldehyde denaturing gel. 18S and 28S of the rRNAs are indicated.
Lane 1: Total RNA isolated from the chicken pituitary (before passing the column).
Lane 2: the first eluting fraction.
Lane 3: the second eluting fraction.
Lane 4: the third eluting fraction.



5 μg of the poly(A)$^+$ RNA of the chicken pituitary gland were used to construct a cDNA library in the Uni-ZAP XR vector digested with EcoR I / Xho I, according to the instruction of the ZAP-cDNA synthesis kit (Stratagene, 1994). The procedure is summarised in Fig. 2.3.

Fig. 2.3 *The procedures for constructing the cDNA library from the chicken pituitary gland.*

A. ***Synthesis of first-strand cDNAs***

5 μg poly(A)$^+$ RNA extracted from chicken pituitary glands was used to synthesise first-strand cDNA, initiated by a 50-base oligo which contained an oligo $(dT)_{18}$ and a Xho I recognition site.

B. ***Synthesis of second-strand cDNAs***

The second-strand cDNAs were synthesised at 16 °C for 2.5 h, using the first-strand cDNAs as templates and primed by a multitude of RNA fragments which was nicked by RNase H.

C. ***Ligating EcoR I adapters onto the double-strand cDNAs***

The EcoR I adaptors were ligated at both ends of cDNAs.

D. ***Ligating cDNAs into the Uni-ZAP XR vector arms***

The double-stranded cDNAs were size-selected using Sephacryl S-400 Columns and subcloned into the Uni-ZAP XR λ vector (digested with EcoR I/Xho I).

E. ***Packaging***

The lambda library was packaged into λ phage heads.

F. ***Titration and amplification of the cDNA library***

The cDNA library was plated out *E. coli (XL1-Blue MRF')*.

Firstly, first-strand cDNAs were synthesised by M-MuLV Reverse Transcriptase from 5 μg poly(A)$^+$ RNAs with a linker-primer which contained an oligo(dT)$_{18}$ and a Xho I recognition site. This step was carried out at 37 °C for 1 h. A control for first-strand cDNA synthesis was included by incorporating α ^{32}P-labelled dCTP (800 Ci/mmol, Amersham) into 5 μl of the first-strand cDNA reaction mixture. After 1 h, the reaction was stopped by putting the tube containing the mixture on ice. Second-strand cDNAs were synthesised using the first-strand cDNA as a template with DNA polymerase I and were initiated by a multitude of RNA fragments which were nicked by RNase H from the RNA-DNA chimerical strands. A trace amount of α ^{32}P-labelled dCTP (800 Ci/mmol, Amersham) was included in the second strand reaction to monitor the synthesis. The reaction of second-strand cDNA synthesis was incubated at 16 °C for 2.5 h. After synthesis for 2.5 h at 16 °C, the reaction was terminated by putting the reaction on ice. Double-stranded cDNAs were purified by phenol (pH:8.0):chloroform [1:1 (v/v)] and precipitated by 1/10 Vol. of 3 M sodium acetate and 2.5 Vol. of 100 % ethanol. The quality of the first-strand and second-strand cDNAs of the library was analysed on a gel (Fig.2.4). A lot of high molecular weight products (> 1 kb) were produced in the cDNA synthesis, suggesting that the cDNA synthesis was successful.

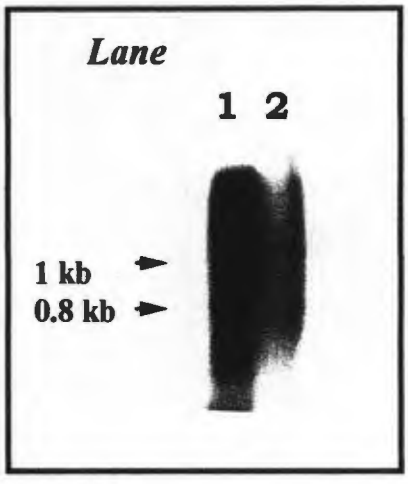
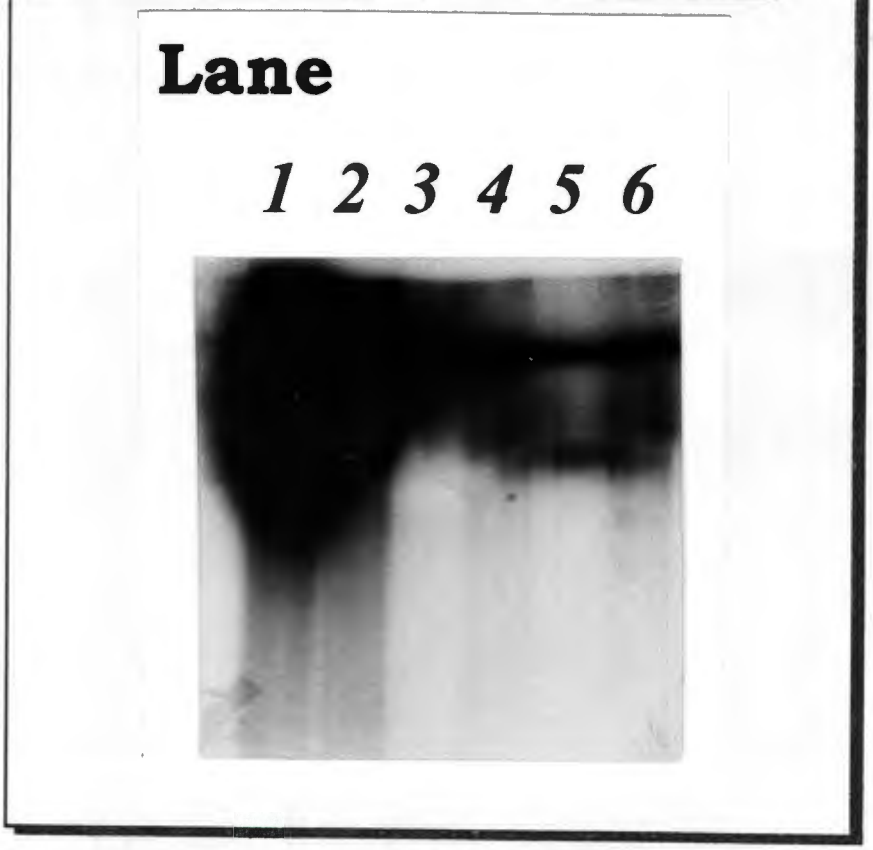


Fig. 2.4 ***Autoradiograph of the first-strand and second-strand cDNAs synthesised during the construction of the chicken pituitary cDNA library.*** The samples, which were incorporated with α ^{32}P-labelled dCTP, were analysed on 1% alkaline agarose gel. 0.8 and 1 kb sizes are indicated.
Lane 1: the first-strand cDNA.
Lane 2: the second-strand cDNA.

Then, the uneven termini of the double-stranded cDNAs were blunt-ended with Klenow at 37 °C for 30 min and dephosphorylated EcoR I adaptors were ligated onto the ends of double-stranded cDNAs by T_4 DNA ligase at 4 °C overnight. The EcoR I ends were treated with T_4 kinase to enable their ligation into the dephosphorylated vector arms, and were digested with Xho I to release the EcoR I adaptor and residual linker-primer from 3′ end of the cDNAs.

Before ligating the double-stranded cDNAs onto the ZAP XR λ vector arms, the digested cDNAs were purified and sized using a Sephacryl spin column. Six cDNA fractions, were collected and analysed on a sequencing gel to choose the fractions with large transcripts (Fig. 2.5). The first fraction from the spin column contained the largest cDNAs.

Fig. 2.5 ***Size fractionation of the double-stranded cDNAs of the chicken pituitary gland.*** Six cDNA fractions were collected consecutively after passing through a Sephacryl spin column and analysed on a sequencing gel.



The concentration of the six fractions was quantified on an ethidium bromide plate, which contained known concentrations of DNA as standards (200 ng to 10 ng) (Fig. 2.6).

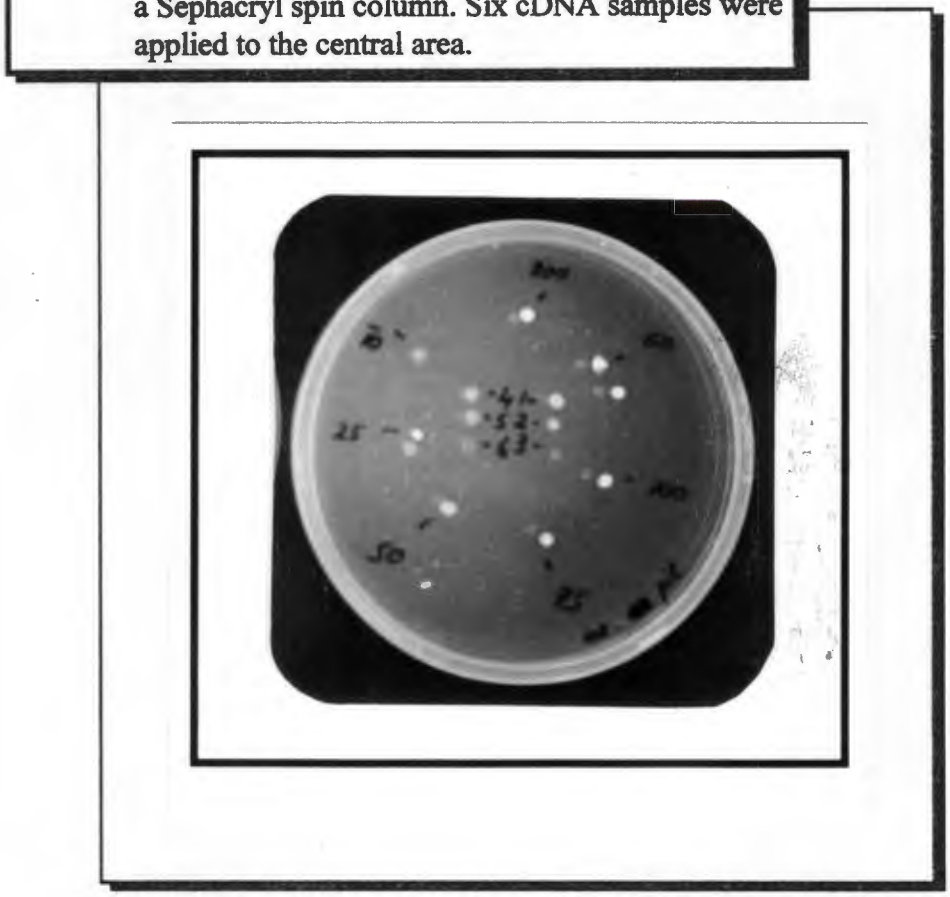


Fig. 2.6 ***Quantification of the six cDNA fractions of the chicken pituitary gland,*** sized by passing through a Sephacryl spin column. Six cDNA samples were applied to the central area.

On the basis of these results (Fig. 2.5 and 2.6), the fraction 1 was chosen to be subcloned into the Uni-ZAP λ vector, which was digested with EcoR I and Xho I. Finally, the vector was packed into λ phage heads. The cDNA library was titred and amplified using *E. coli* (XL_1-Blue) as the host. The phage lysate was collected from the 1x 10^6 plaques, and was used to re-infect E.coli to amplify the cDNA library to a titre of 2.5x 10^9 plaques/ml.

Screening of the cDNA library of the chicken pituitary gland

Approximately $1x10^6$ recombinants were screened in 20 (150 mm) petri plates according to the protocol of Stratagene (1994). Duplicated nylon filters were lifted sequentially (1 min absorption for the first filter; 2min for the second), denatured in the solution containing 0.5 M NaOH and 1.5 M NaCl, neutralised in the solution [1.5 M NaCl, 0.5 M Tris-HCl (pH: 7.2), and 0.001 M EDTA (pH: 8.0)] and baked at 80 °C for 20 min. A 2.3 kb fragment of the mouse TRH-R cDNA (a gift from Prof. Gershengorn, Cornell University Medical College, New York, USA) was labelled with α ^{32}P-dCTP (3000 Ci/mmol, Amersham) by random hexamer priming using Megaprime labelling kit (Amersham). The filters were pre-hybridised at 42 °C in 50 % formamide solution (containing 0.8 M NaCl, 0.02 M PIPES, 50 % De-ionised formamide, and 0.5 % Sodium dodecyl sulphate). After 3 h pre-hybridisation, the labelled probe was added to the membranes and the hybridisation was carried out at 42 °C overnight. The filters were washed with 1x SSC, 0.1 % SDS , at room temperature, for 15 min ; 0.5x SSC, 0.1 % SDS, 50 °C, for 30 min, and 0.2x SSC, 0.1 % SDS, 50 °C, for 20 min. Secondary and tertiary screenings for the positive clones were performed by the same procedures.

Rescuing chicken TRH-R cDNA from the Uni-ZAP vector

The positive clones, which were obtained from screening the cDNA library, were rescued from the arms of the Uni-ZAP vector by the ExAssist™/ SOLR™ system (Stratagene, 1994) for further characterisation. Positive clones from the tertiary screening were isolated from agar plates and were transferred to a sterile microfuge tube containing 500 ml of SM buffer [0.1 M NaCl, 0.02 M $MgSO_4$, 50 ml of 1 M Tris-HCl (pH: 7.5), and 5 ml 2% gelatine] and were incubated at 4 °C, overnight, to release the phage particles into the SM buffer. 200 µl of the SM buffer with 200 µl of XL_1-Blue cells and 1 µl of R_{408} helper phage was cultured in 3 ml of 2x YT media (0.15 M of NaCl, 10 g of Yeast extract, and 16 g of Bacto-tryptone) for 2.5 h at 37 °C. The cells were shocked by heating to 70 °C for 20 min, then the cells were spun down at 4000 g for 5 min. The supernatant (containing the pBluecript phagemid with the chicken TRH-R cDNA) was decanted into a sterile tube and stored at 4 °C.

3′RACE (Rapid Amplification of the 3′end)

The first-strand cDNA of the chicken pituitary gland was synthesised by Reverse transcriptase (Strategene) with an oligo($dT)_{16}$-adaptorB-adaptorA primer. Then, the second- strand cDNA was synthesised by the same procedure as described in "constructing cDNA library" section. PCR (polymerase chain reaction) was applied to amplify the 3′-end region of the chicken TRH-R gene by Taq polymerase (Amersham) with a pair of primers, adaptor B and CH2, which were shown in Table 2.1 and Fig. 2.1. The PCR samples were denatured at 93 °C for 4 min, and were subjected to 35 cycles (93 °C for 1 min, 60 °C for 2 min, and 72 °C for 3 min). The samples were elongated at 72 °C for 6 min and were cooled down at 4 °C for 10 min.

Amplification of the full-length chicken TRH-R cDNA

The cDNA fragment of the chicken TRH-R, which encodes a region from EL 2 to TM 6, was amplified from the chicken pituitary cDNA by PCR with a pair of primers, i.e. a chicken specific primer CH1 (sense) and a mutated mTRH-R primer EB_1 (antisense) (a gift from Prof. Gershengorn) (shown in Table 2.1 and Fig. 2.1). Using this cloned fragment as a probe to screen the chicken cDNA library, 25 positive clones were isolated. Of the 25 clones, 9 clones (Trun-4 to Trun-12) were identified by partial sequencing and by restriction enzyme mapping as incomplete receptor cDNAs, but contained an entire 3′-end region. Primer CH6 was designed, based on the sequences of the 3′ end of TRH-R gene. The full-length cDNA of the chicken TRH-R was amplified by PCR with a pair of primers, CH5 and CH6, which flanked the entire open reading frame of the TRH-R (shown in Table 2.1 and Fig. 2.1). The cycles of PCR were described above.

Sequencing

Plasmid DNAs of 12 clones and full-length chicken TRH-R cDNA clones (amplified by PCR) and the constructed full-length clone (the chicken TRH-R cDNA) were purified by Wizard maxi preps column (Promega, USA) and sequenced manually, using a sequenase kit (U.S. Biochemical Corp.). To confirm the nucleotide sequences of the chicken TRH-R cDNA, the plasmid DNA was sequenced in both directions.

Identification of the divergent region of the truncated chicken TRH-R

The truncated chicken TRH-R cDNA, Trun-1, -2, and -3 clones, were isolated from a cDNA library. They were identified by sequencing and all contained a premature stop codon (TAA) in the putative third IL 3. The 20 amino acids that precede the stop codon in these clones had no homology with the mammalian TRH-R. The region from the 20 amino acids onwards, was called a divergent region. To analyse the divergent region, firstly, the chicken TRH-R gene was isolated from a chicken genomic library constructed in bacteriophage λ Charon 4A (EcoR I digested) (Dodgson et al., 1979) by hybridising with α^{32}P-labelled Trun-3 cDNA. Restriction enzyme digests of the chicken TRH-R genomic clone were subjected to Southern blot analysis by hybridising with probe A, which contained the coding region of TM 1 to most of IL 3; then stripped of the probe and re-hybridised with probe B, containing an entire divergent region. PCR was applied to re-confirm the location of the divergent region, by using two pairs of gene specific primers, CH1 & CH4 and CH2 & CH3 (shown in Table 2.1 and Fig. 2.1). The cycles of PCR were described above.

Northern blot analysis

Total RNAs from the chicken and sheep pituitary glands, were isolated by the method as described (Chomczynski *et al.*, 1987). The total RNAs (30 μg) from the two species, were analysed on a formaldehyde denaturing gel, and were transferred into the Hybond N membrane (Amersham). The samples were fixed in the membrane using a UV crosslinker (Amersham). The membrane was hybridised with the full-length chicken TRH-R cDNA, which was labelled with α^{32}P-dCTP (Amersham) using Megaprimer labelling kit (Amersham). The autoradiograph of the membrane was exposed for 5 days.

2.3.2 Characterisation of the chicken TRH-R

Cell culture and transfection

COS-1 cells were grown at 37 °C in an humidified atmosphere containing 5 % CO_2. One to two days before transfection, the COS-1 cells were harvested with trypsin,

and plated out at 0.6 ~ 0.8 x 10^5 cells/ 25mm well for inositol phosphate (IP) and cyclic AMP (cAMP) formation assays. The cells were transfected for 3.5 h with the chicken TRH-R cDNA, or the mouse TRH-R cDNA, which were subcloned into the pcDNA I/Amp vector (Invitrogen Corp., CA), using a modification of the DEAE-dextran method (Cullen, 1987). For IP formation assay, the cells were labelled with *myo*-[^{3}H]-inositol (1μCi/ml, Amersham) in Dulbecco's modified Eagle's medium (DMEM supplemented by 2 % fetal calf serum and penicillin / streptomycin) for 16 h to 22 h in a 37 °C incubator (5 % CO_2), 48 h after transfection.

Inositol phosphate (IP) formation assay

IP formation was measured as previously described (Millar et al., 1995). Briefly, the *myo*-[^{3}H]-inositol labelled cells were washed twice with buffer A (140 mM NaCl, 4mM KCl, 20mM HEPES, 0.1% BSA, 8 mM D-glucose, pH 7.4, 1mM $CaCl_2$ and 1 mM $MgCl_2$) at 37 °C for 5 min. Then, the cells were incubated with buffer A (containing 10 mM LiCl) in the presence or absence of TRH analogues for 1 h at 37 °C. All experiments were performed in duplicate and were repeated at least twice.

cAMP formation assay

The cells were washed twice with buffer A and incubated with the buffer (containing 10 mM LiCl) in the presence or absence of TRH (1 nM ~ 10 μM) for 60 min with 0.25 mM IBMX (a cyclic nucleotide phosphodiesterase inhibitor) at 37 °C, 48 h after transfection. COS-1 cells, transfected with human β_2-adrenergic receptor cDNA, were incubated with 1 μM of epinephrine (in the presence of 0.25 mM IBMX) as a positive control. cAMP was extracted using 4 mM EDTA, and measured by radioimmunoassay (Amersham kit, TKR 342). Individual experiments were repeated twice in duplicate.

Receptor binding assay

The receptor binding of the chicken TRH-R was carried out at Prof. Gershengorn's lab, and was done by Dr. H. Ho (Dept. of Medicine, Cornell University Medical College, New York, USA).

Data analysis

The values of K*i* (receptor affinity), and EC_{50} (IP formation) were estimated by non-linear regression analysis using the PRISM program (GraphPad Inc.). Dose-response curves (each data point is a mean of all experiments) of IP and cAMP formation assays and competition binding assay were drawn using the same program.

2.4 RESULTS

Summary of the work in cloning chicken TRH-R cDNA

The work in cloning the chicken TRH-R gene is summarised in Fig. 2.7. In order to elucidate the multiple steps of the cloning work, methods which were applied to the work are presented step by step in Figure 2.7. The result of each step is included to indicate the problem which I faced at the time, and the result became a useful tool to tackle the next problem.

Firstly, the cDNA library of the chicken pituitary gland was constructed and it was a good library. Three chicken TRH-R cDNA clones (Trun-1, -2, and -3) were isolated from the cDNA library of the chicken pituitary gland, using a mouse TRH receptor cDNA probe. They were identified by sequencing and they all contained a stop codon (TAA) within the putative intra-cellular (IL) loop 3. Five primers, CH1 to CH5, were designed.

In order to get more 3′-end sequences of the receptor, two strategies were used: 3′-RACE (3′-end rapid amplification) and PCR (polymerase chain reaction). In the first step, 3′-RACE was applied to amplify the 3′-untranslated region of the receptor from chicken pituitary cDNAs using a gene specific primer (CH2) and an oligo (dT) primer/adaptorB. The PCR-products were subcloned and were identified by sequencing. However, the three clones that were analysed all had the same stop codon in the putative IL 3, and a poly (A_{50}) tail. In the second approach, the cDNA library was screened with a chicken TRH-R probe, which extended from EL 2 to TM 6. This probe was isolated using a PCR strategy with a chicken primer (CH1) and a primer designed against TM 6 of the mouse TRH-R. None of the 9 clones, which were analysed by restriction enzyme mapping and partial sequencing, was a full-length receptor. They were all incomplete receptors, but contained an entire 3′ un-translated region and a poly$(A)^+$ tail. Therefore, one primer (CH6), which was located at the 3′ un-translated region just after the stop codon, was designed according to the sequence data derived from the incomplete cDNA clone.

A full-length chicken TRH-R cDNA was amplified by PCR with a pair of gene specific primers (CH5 and CH6), which flanked the entire coding region of the chicken TRH-R. However, various mutated amino acids were verified in full-length cDNA clones, as compared to amino acid sequences of the cDNA clones (eg. Trun-1 and Trun-4), which were isolated by screening the cDNA library. Therefore, a "wild type" chicken TRH-R cDNA was constructed by ligating two parts of the chicken receptor gene from Trun-3

and Trun-4 clones. The detailed information for each step will be addressed in the following sections.

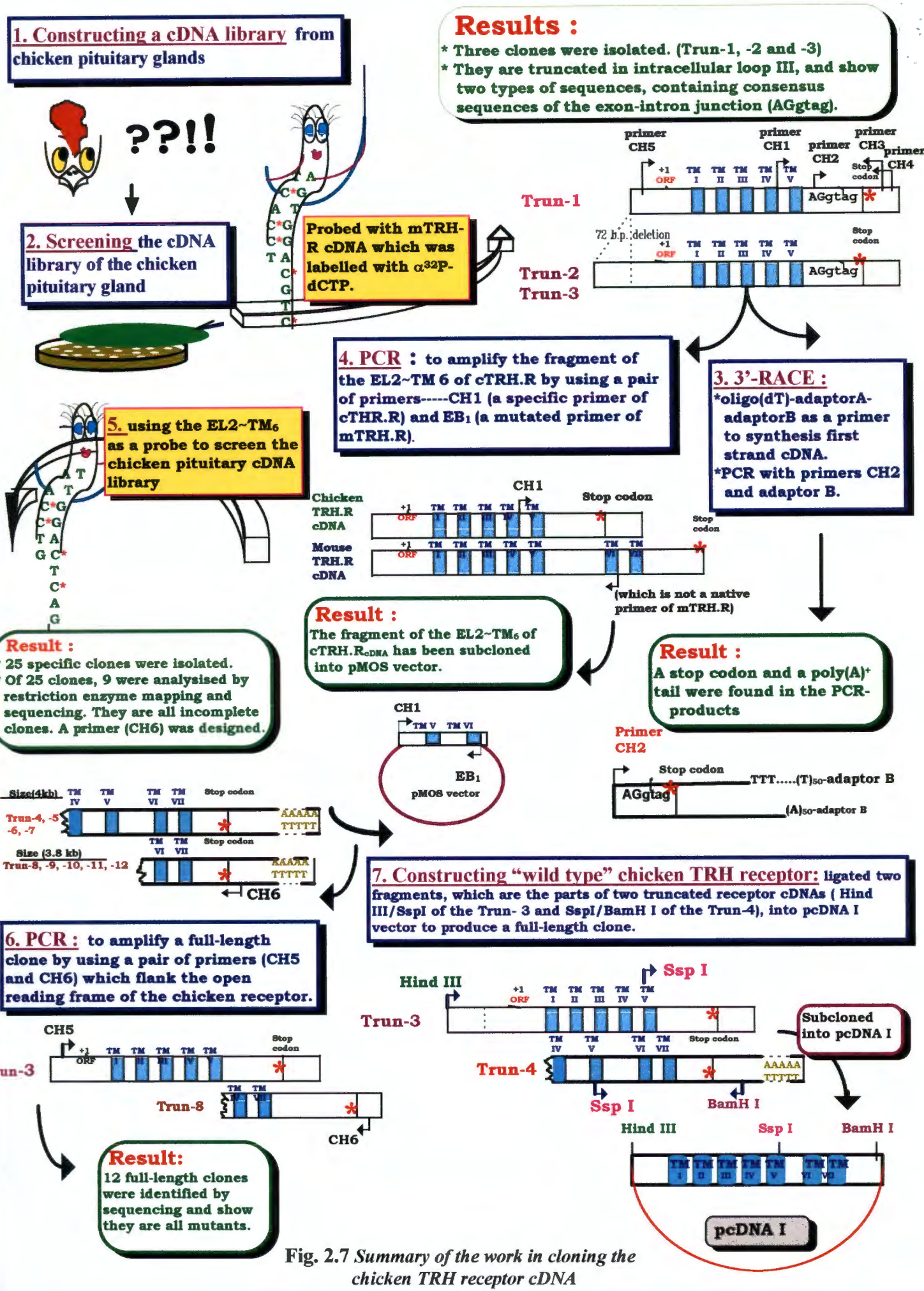


Fig. 2.7 *Summary of the work in cloning the chicken TRH receptor cDNA*

The cDNA library of the chicken pituitary gland

The cDNA library of the chicken pituitary was titered at $2x10^6$ pfu before amplification, and $1.2x10^{10}$ pfu/ml phage lysate after amplification. The ratio of the cDNAs inserted into the Uni-ZAP XR λ vector arms, at the primary construction, was more than 100 white:blue ratio selected by IPTG/X-Gal. White plaques indicate that the vector contains cDNA; blue ones have no insertion. After cDNA amplification, the white:blue ratio was 30. In addition, the chicken library contained 1.4% of β-actin recombinants identified by screening 10^5 plaques hybridised with α ^{32}P-labelled mouse β-actin cDNA. (β-actin is highly expressed and is usually used as an "internal control". If one library contains more than 1 % of β-actin, the library is qualified as a good library.)

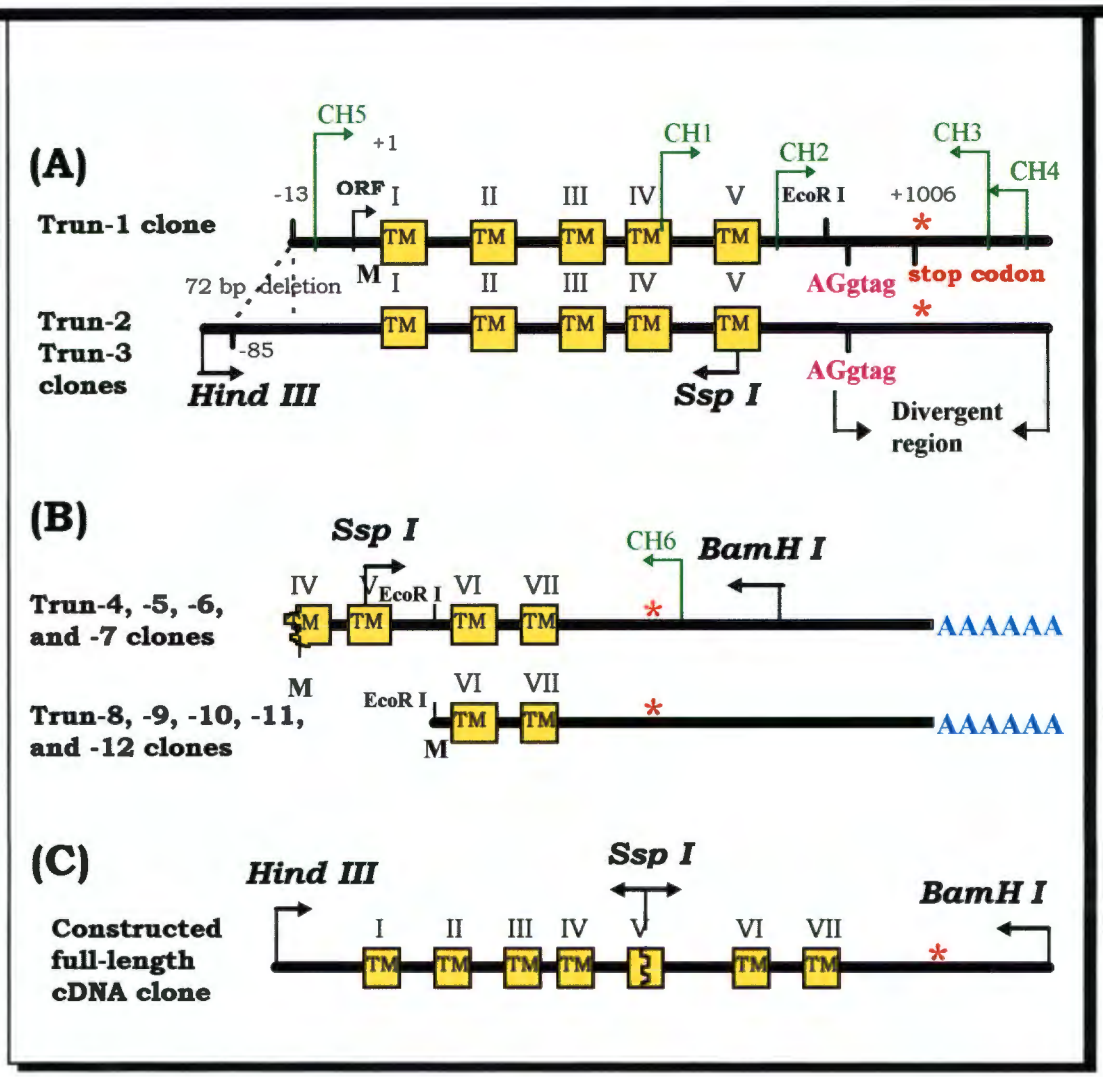
The chicken TRH-R cDNA clones

Twelve truncated chicken TRH-R cDNAs were isolated from a cDNA library of the chicken pituitary gland by using the mouse TRH-R or the chicken TRH-R probes (Fig. 2.8). Trun-1,-2, and -3 (size: about 1.4 kb), which were cloned using the full-length mouse THR-R cDNA as a probe, were identified by sequencing in both directions (Fig. 2.8, panel A). The nucleotide sequence data of the three clones revealed that they all had a stop codon (TAA) at the nucleotide +1006 (downstream) that resulted in a receptor that prematurely terminates in the putative IL 3. The downstream region, from a point 20 amino acid sequences before the stop codon, had no homology with the mammalian TRH receptor and was termed the divergent region. The consensus 5′-end sequence of the exon-intron junction, AGgtag , was found at the point of homology divergence. Trun-2 and -3 were identical. However, Trun-1 showed slightly different nucleotide sequences from the other two clones in its lack of 72 bp at 13 nucleotides (-13) upstream from the start of the open reading frame (+1). Five primers, CH1 to CH5, were designed (Fig. 2.8, panel A).

In order to obtain the 3′-end sequence data of the chicken TRH-R gene, 3′-RACE was applied to amplify up the 3′-untranslated region of the receptor gene with primers CH2 and adaptorB. Three of the cloned PCR-products were identified by sequencing and they showed that they all were comprised of a premature stop codon at the nucleotide +1006 and a poly$(A)_{50}$ tail. Therefore, another strategy (PCR) was used to amplify the EL

2 ~ TM 6 coding region of the chicken TRH-R gene with a gene specific primer CH1 and a mouse

Fig. 2.8 ***A diagram illustrating the different types of cTRH-R cDNAs isolated from the cDNA library of the chicken pituitary gland.*** **(A)** Three truncated clones were isolated by screening the chicken cDNA library hybridised with α^{32}P-labelled mouse TRH-R cDNA. **(B)** Nine truncated clones were isolated by using α^{32}P-labelled chicken TRH-R cDNA probe. **(C)** The constructed full-length chicken THR-R. Three restriction enzyme sites are indicated. A Hind III restriction site is in the pSK+ vector and Ssp I and BamH I sites are in the chicken TRH-R. ORF: open reading frame; M: methionine; CH1~6: primers; TM: transmembrane ; * : stop codon; "-" and "+" : up- and down-stream of the open reading frame (+ 1); numbers: a positional number of nucleotide sequence. AAA: poly(A)$^+$ tail.



TRH-R primer. This PCR-product was subcloned into pMOS-Blue vector (Amersham) and verified by sequencing. This PCR clone was used to screen the cDNA library of the chicken pituitary gland. 25 positive clones were isolated. Of the 25 clones, 9 clones (Trun-4 to Trun-12, sized: 3.8 Kb ~ 4.1 Kb) were identified by partial sequencing and by restriction enzyme mapping (Fig. 2.8, panel B). They appeared to be two types of incomplete cDNA clones, encoding either from half of TM 4, or from half of IL 3, to the poly(A)$^+$ tail. Interestingly, they all contained a methionine at the beginning of the clones. Since these incomplete forms all contained a large 3′-untranslated region (> 3 Kb), they might result from a limited efficiency of the Reverse Transcriptase. Based on the nucleotide sequence data of the 3′-untranslated region, a primer (CH6) was designed against the region following after the stop codon.

PCR was applied to amplify the full-length TRH-R cDNA with a pair of primers, CH5 and CH6, from the chicken pituitary cDNA. The PCR-products were subcloned into the pMOS-Blue vector. Six of the full-length TRH receptor cDNA clones were verified by sequencing, and they all contained randomly mutated sequences as compared to those of the cDNA clones, which were isolated from the chicken cDNA library. Therefore, a full length chicken TRH receptor cDNA was constructed by ligating two fragments from truncated clones. The Hind III/Ssp I fragment from Trun-3, was ligated to the Ssp I/ BamH I fragment from Trun-4, and cloned into the pcDNA I/Amp vector digested with Hind III/BamH I restriction enzymes (Fig. 2.8, panel C).

The chicken TRH receptor

The constructed full-length chicken TRH-R (size about 1.2 Kb) showed 76 and 84 % identity to the nucleotide and amino acid sequences of the mouse TRH-R, respectively (Fig. 2.9A and B). The nucleotide sequences of the chicken TRH-R has been submitted to the EMBL and its accession number is Y18244. Completely conserved regions in both receptors are IL 1 and 2, EL 3, TM 2, and TM 7. The divergent parts are the extracellular N-terminus, the middle part and the C-terminal part of IL 3 (if the loop is divided into three parts), as these parts only share 55, 53, and 60 % homology, respectively. There are two additional amino acids (Thr^{23} and Ala^{24}) in the chicken TRH-R. The chicken TRH-R contains two potential sites for N-linked glycosylation (Asn-X-Ser/Thr) in the N-terminus, also conserved in the mouse counterpart.

A)

```
atg gag aat ggc aca ggc gac gag cag aac cat act ggg ctg ctg ctg tca agc cag
 M   E   N   G   T   G   D   E   Q   N   H   T   G   L   L   L   S   S   Q
gag ttc gtt aca gct gaa tac caa gtg gtt acc atc ctc ttg gtc ctc ctc atc tgt
 E   F   V   T   A   E   Y   Q   V   V   T   I   L   L   V   L   L   I   C
gga ctg ggc atc gtg ggc aac atc atg gtg gtc ttg gtg gtc ctc aga acc aaa cac
 G   L   G   I   V   G   N   I   M   V   V   L   V   V   L   R   T   K   H
atg aga act ccc act aac tgc tat ctg gtt agt ctg gct gtg gca gat ctc atg gtg
 M   R   T   P   T   N   C   Y   L   V   S   L   A   V   A   D   L   M   V
ctt gtg gct gca gga cta ccc aat atc aca gaa agt ctg tac aaa tcc tgg gtc tat
 L   V   A   A   G   L   P   N   I   T   E   S   L   Y   K   S   W   V   Y
ggc tat gtg ggg tgc ctc tgc atc act tat ctc cag tac cta ggg atc aat gct tct
 G   Y   V   G   C   L   C   I   T   Y   L   Q   Y   L   G   I   N   A   S
tct ttt tcc atc act gct ttc acc atc gag aga tat ata gct atc tgc cac cca atc
 S   F   S   I   T   A   F   T   I   E   R   Y   I   A   I   C   H   P   I
aaa gct caa ttc cta tgc aca ttt tca aga gct aag aag atc att att ttt gtc tgg
 K   A   Q   F   L   C   T   F   S   R   A   K   K   I   I   I   F   V   W
tct ttc gcc tca gta tac tgt atg ctc tgg ttt ttc cta tta gat ctc aat ata gca
 S   F   A   S   V   Y   C   M   L   W   F   F   L   L   D   L   N   I   A
gtc tac aaa gac act acg gtt gtg tct tgt gga tac aag gtg tcc agg agc tat tac
 V   Y   K   D   T   T   V   V   S   C   G   Y   K   V   S   R   S   Y   Y
tct cct atc tac atg atg gac ttc gga ata ttt tat gtt ttg cca atg gta ttg gca
 S   P   I   Y   M   M   D   F   G   I   F   Y   V   L   P   M   V   L   A
act gtc ctc tat ggc ctg att gct aga ata ctg ttc ctg aat ccc atc cct tcg gac
 T   V   L   Y   G   L   I   A   R   I   L   F   L   N   P   I   P   S   D
cca aaa gaa aac tct aac acg tgg aaa aat gac atg gct caa caa aac aag act gtg
 P   K   E   N   S   N   T   W   K   N   D   M   A   Q   Q   N   K   T   V
aat tcc aag atg act aac aag agt ttc aat agc act att gct tct aga aga cag gtt
 N   S   K   M   T   N   K   S   F   N   S   T   I   A   S   R   R   Q   V
acc aag atg ttg gct gtg gtg gta gtc ctc ttt gca ttt ctg tgg atg ccc tat cga
 T   K   M   L   A   V   V   V   V   L   F   A   F   L   W   M   P   Y   R
aca ctg gtg gtt gtc aat tcc ttt ctc tcc agt ccc ttc caa gaa aac tgg ttc ctg
 T   L   V   V   V   N   S   F   L   S   S   P   F   Q   E   N   W   F   L
cta ttt tgc aga atc tgt att tat tta aat agt gcc atc aat cct gta att tac aat
 L   F   C   R   I   C   I   Y   L   N   S   A   I   N   P   V   I   Y   N
ctc atg tcc cag aaa ttc aga gca gcc ttc agg aaa ctc tgc aac tgc cat cta aaa
 L   M   S   Q   K   F   R   A   A   F   R   K   L   C   N   C   H   L   K
cgg gac aag aaa cct gcc aat tac agt gtg gcc cta aat tat aat gtc atc aaa gag
 R   D   K   K   P   A   N   Y   S   V   A   L   N   Y   N   V   I   K   E
tct gat cac ttc agc agt gaa ata gaa gat att act gtc acc aat acc tat ttg tcc
 S   D   H   F   S   S   E   I   E   D   I   T   V   T   N   T   Y   L   S
tct gca aaa aca tcc att ggt gac aca tgt ttg tct tct gag gcc tga
 S   A   K   T   S   I   G   D   T   C   L   S   S   E   A   *
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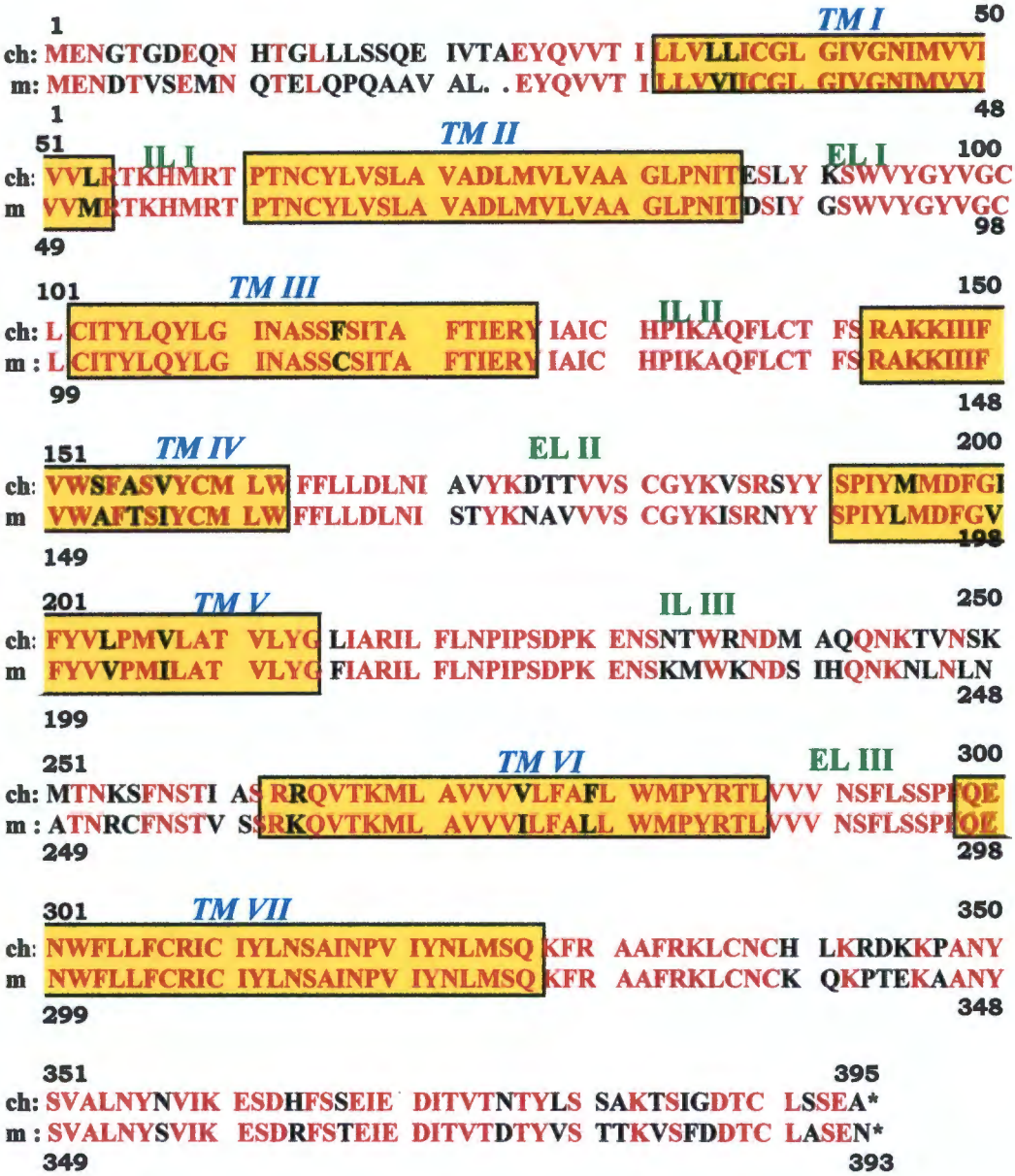
Fig. 2.9 **(A)** ***The nucleotide and amino acid sequences of the chicken TRH-R.*** **(B)** ***Alignment of the deduced amino acid sequences of the chicken TRH-R (ch) and the mouse TRH-R (m).*** Putative transmembrane (TM) helixes for the chicken TRH receptor were assigned based on those of the mouse receptor (Gershengorn *et al.*, 1996). Identical residues between both species are shown in red letters. IL: intracellular loop, EL: extracellular loop, "..." : space, " * ": stop codon, and the numbers: the positions of amino acids (top number for the chicken receptor; bottom one for the mouse receptor).

B)


TM I
TM II
IL I
EL I
TM III
IL II
TM IV
EL II
TM V
IL III
TM VI
EL III
TM VII
ch: MENGTGDEQN HTGLLLSSQE IVTAEYQVVT I LLVLLICGL GIVGNIMVVI
m: MENDTVSEMN QTELQPQAAV AL. . EYQVVT I LLVVIICGL GIVGNIMVVI
ch: VVLRTKHMRT PTNCYLVSLA VADLMVLVAA GLPNITESLY KSWVYGYVGC
m VVMRTKHMRT PTNCYLVSLA VADLMVLVAA GLPNITDSIY GSWVYGYVGC
ch: L CITYLQYLG INASSFSITA FTIERY IAIC HPIKAQFLCT FS RAKKIIIF
m : L CITYLQYLG INASSCSITA FTIERY IAIC HPIKAQFLCT FS RAKKIIIF
ch: VWSFASVYCM LW FFLLDLNI AVYKDTTVVS CGYKVSRSYY SPIYMMDFGI
m VWAFTSIYCM LW FFLLDLNI STYKNAVVVS CGYKISRNYY SPIYLMDFGV
ch: FYVLPMVLAT VLYG LIARIL FLNPIPSDPK ENSNTWRNDM AQQNKTVNSK
m FYVVPMILAT VLYG FIARIL FLNPIPSDPK ENSKMWKNDS IHQNKNLNLN
ch: MTNKSFNSTI AS RRQVTKML AVVVVLFAFL WMPYRTLVVV NSFLSSPFQE
m : ATNRCFNSTV SSRKQVTKML AVVVILFALL WMPYRTLVVV NSFLSSPFQE
ch: NWFLLFCRIC IYLNSAINPV IYNLMSQ KFR AAFRKLCNCH LKRDKKPANY
m NWFLLFCRIC IYLNSAINPV IYNLMSQ KFR AAFRKLCNCK QKPTEKAANY
ch: SVALNYNVIK ESDHFSSEIE DITVTNTYLS SAKTSIGDTC LSSEA*
m : SVALNYSVIK ESDRFSTEIE DITVTDTYVS TTKVSFDDTC LASEN*

However, there is an additional glycosylation site in the EL 2 of the mammalian receptors, but it is absent in the chicken receptor (Fig. 2.9). The primary structural details of the chicken receptor will be addressed in the discussion section.

Identification of the divergent region of the truncated chicken TRH-R

In order to analyse the divergent region of the Trun-1, -2, and -3 clones, the chicken TRH-R genomic clones were isolated from $7.5x10^5$ plaques of a chicken genomic library by hybridising with α ^{32}P-labelled Trun-3 cDNA. Two positive clones, YS1 and YS2, were isolated after tertiary screening (Fig. 2.10, 2.11, and 2.12). The position of the divergent region of the Trun-1, -2, and -3 clones in the TRH-R gene, was identified by analysing the TRH-R genomic clone (YS1) (Fig. 2.13) using Southern blot (Fig. 2.14, panel A) and PCR analysis (Fig. 2.14, panel B).

Nucleotide sequence analysis of the truncated chicken TRH-Rs (Trun-1, -2 and -3) identified the presence of an EcoR I site and a Xba I site, proceeding the divergent region. The Trun-3 clone (subcloned into the pSK- vector digested with Hind III and BamH I) was used to make two probes (A and B) by digesting with Pst I, Xba I, and BamH I (Fig. 2.13). Probe A contained the 5'un-translated region to IL 3 of the receptor, and probe B was the divergent region. The Southern blot analysis revealed that the YS1 contained the probe A region, i.e. exon 1 (in the 1.2 Kb EcoR I fragment), and the probe B region in the 1.5 Kb EcoR I/Hind III or EcoR I/ BamH I fragments (Fig. 2.14, panel A). The exon 1 and the divergent region were located in an 8.0 Kb Hind III (or BamH I) digest which retained a 5.3 Kb fragment of the λ charon 4A vector (Fig. 2.13, bottom panel; Fig. 2.14, panel A); that is, the band of the chicken receptor gene that was hybridised with the probes A and B had a size of 2.7 Kb. It indicated that exon 1 adjoined the divergent region.

This was re-confirmed by PCR using the cDNA and genomic clones as templates. Same size PCR-products from the templates, Trun-3 and genomic clones, were amplified with two pairs of primers (Fig. 2.14, panel B), whereas the negative control (H_2O) was blank. In addition, there were no PCR-products shown in the Trun-4 cDNA clone (a negative control), in which the intron had been spliced out. The finding suggested that the divergent region of Trun-3 is part of an intron (Fig. 2.13).

Fig. 2.10 ***One of two positives (shown in duplicate), YS1 (the chicken genomic clone), hybridised with α^{32}P-labelled Trun-3 in 7.5x10^5 plaques primary screening.*** The autoradiographs were exposed for 4 days.

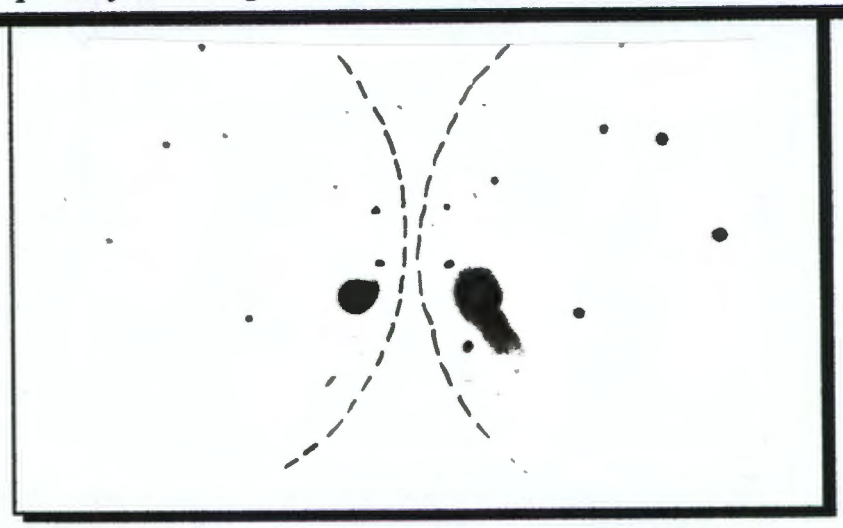


Fig. 2.11 ***Ample positives, hybridised with α^{32}P-labelled Trun-3 in the secondary screening (duplicate membranes) of the YS1 clone.*** The autoradiographs were exposed for 5 hr.

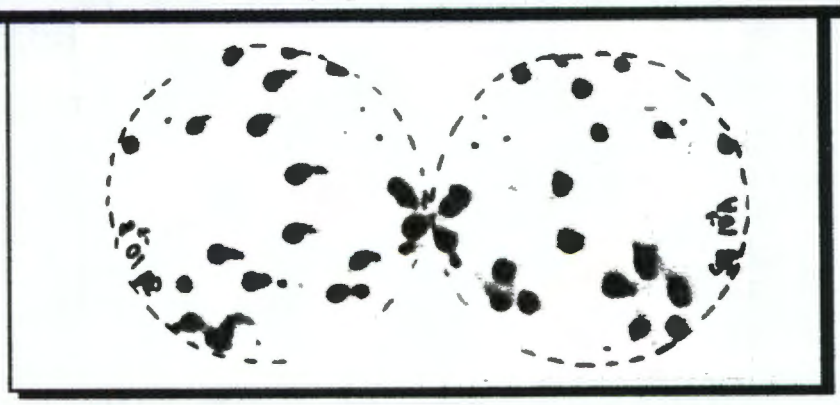


Fig. 2.12 ***Ample positives, hybridised with α^{32}P-labelled Trun-3 in the tertiary screening (duplicate membranes) of the YS1 clone.*** The autoradiographs were exposed for 5 hr.

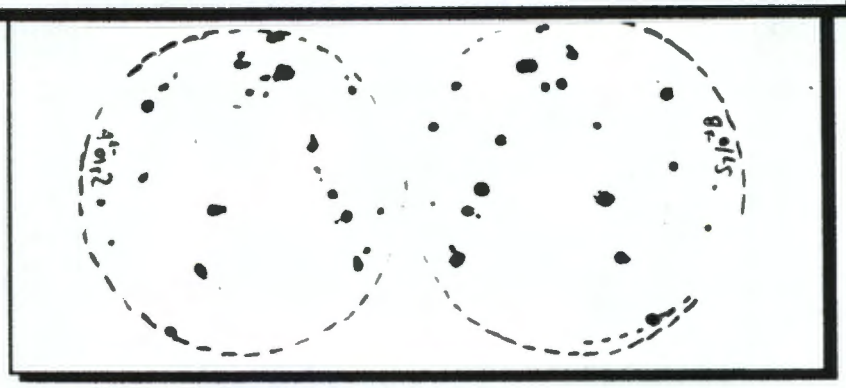


Fig. 2.13 ***Schematic representation of the chicken TRH-R genomic and truncated cDNA clones.*** The divergent region of the truncated cDNA was verified as an intron by Southern blot analysis and PCR (shown in Fig. 2.14). The intron follows on from exon 1 where the consensus 5′-end sequence of the exon-intron junction (AGgtag) is shown. Exon 1 encodes the 5′-untranslated region through most of intracellular loop 3. The probes A and B, and 4 primers, which are indicated in Fig. 2.14, are shown at the corresponding regions of the cDNA clone. ORF: open reading frame; TM: transmembrane; "-" or "+" : up- or down-stream of the open reading frame (+ 1); numbers: a positional number of nucleotide sequence. " // ": region not shown.

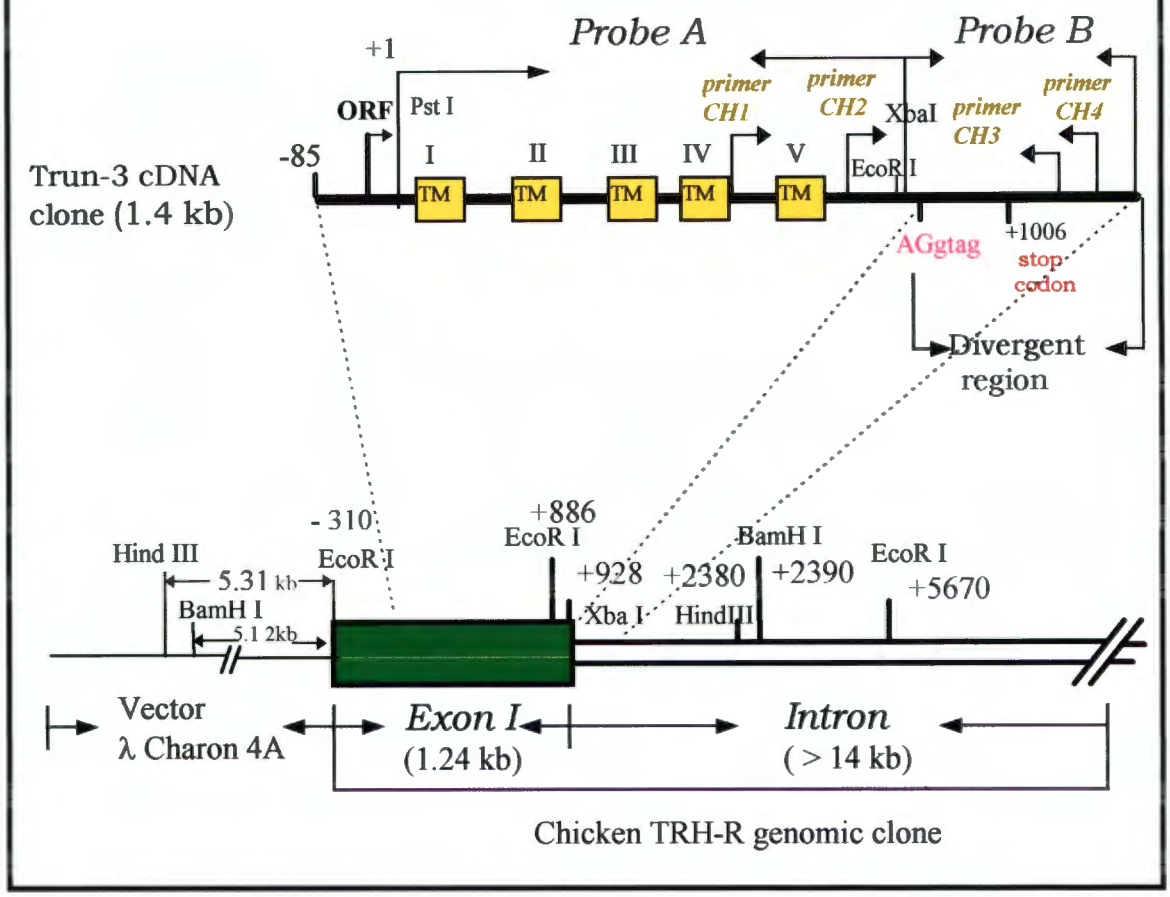
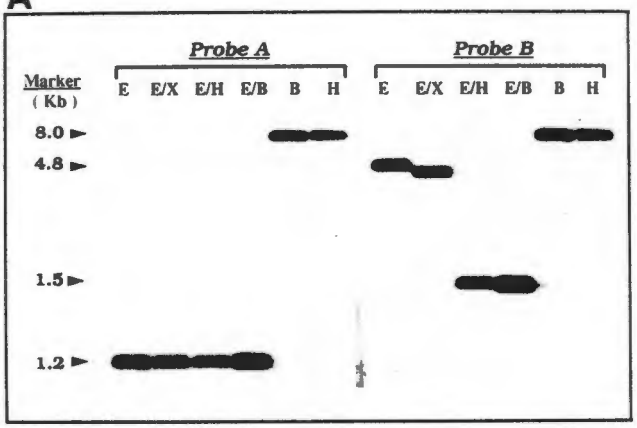


Fig. 2.14 ***Identification of the divergent region of the truncated chicken TRH-R.***

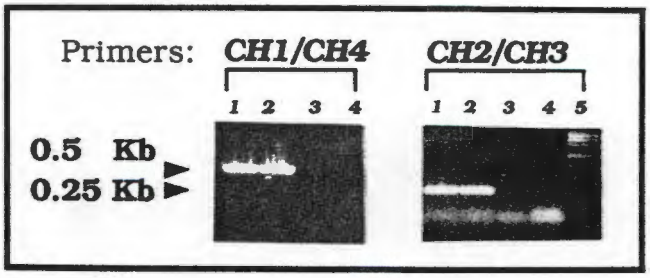
(A) Southern blot analysis of the chicken TRH receptor genomic clone (YS1 clone in the λ charon 4A vector digested with EcoR I) digested with EcoR I (E), Xba I (X), Hind III (H), and BamH I (B) or combinations, and hybridised with α^{32}P-labelled probes (A and B) (shown in Fig. 2.13).

(B) Polymerase chain reaction was applied to identify the divergent region using the gene specific primers of the chicken TRH-R (shown in Fig. 2.13). The 0.5 and 0.25 Kb of PCR-products were amplified with two pairs of primers, CH1 & CH4 and CH2 & CH3, respectively, from two templates, which are Trun-3 clone (lane 1) and genomic clone (lane 2), while the negative controls, Trun-4 clone (lane 3), and water (lane 4) are blank. Marker presents in lane 5.

A



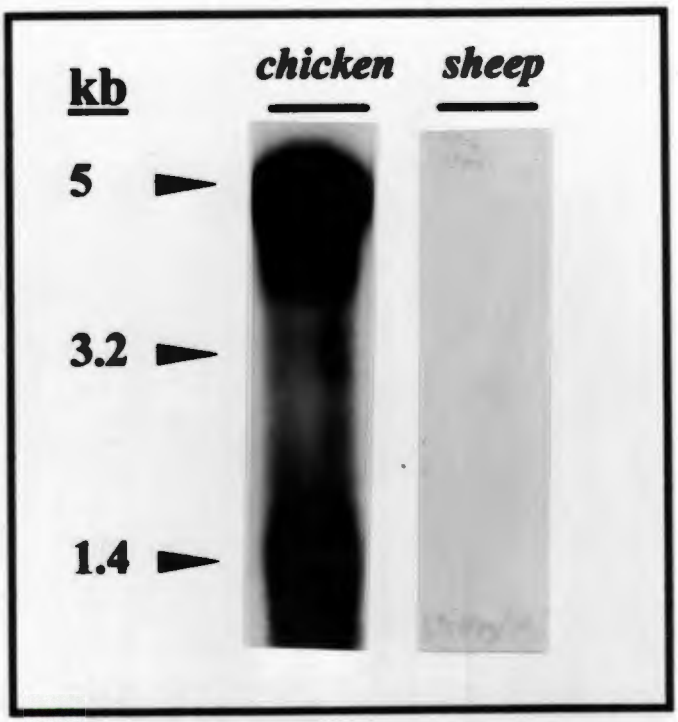
B



The expression of the chicken TRH-R gene in pituitary glands

The expression of the chicken TRH-R gene was detected by Northern blot, hybridised with α^{32}P-labelled full-length chicken TRH receptor cDNA. Three mRNA transcripts of the chicken receptor (sizes at about 5, 3.2, and 1.4 kb), were detected in the chicken pituitary, whereas no signal was detected in the negative control (the sheep pituitary) (Fig. 2.15). The major species of the transcript was sized at 5 kb.

Fig. 2.15 ***The Northern blot analysis of the chicken TRH-R gene in the pituitary gland.*** Three mRNA transcripts of the chicken receptor, 5 kb, 3.2 kb, and 1.4 kb, were detected in the chicken pituitary gland (chicken), by autoradiograph, after 5 days exposure. Sheep: total RNAs from the sheep pituitary gland.



Pharmacological properties of the chicken TRH-R

To characterise the constructed full-length chicken TRH-R, the receptor binding and IP formation assays were performed in COS-1 cells expressing the chicken TRH-R or the mouse TRH-R (as a comparison). The assays were determined by employing several TRH analogues, which have different substitutions at the residues 1, 2, and 3 of TRH, since it has been proposed that the side chains of all three amino acid residues of TRH are involved in binding to TRH-R.

In binding experiments that were completed at Prof. Gershengorn's lab (Cornell U.), the affinity (K_i = 8.1 nM) of TRH to the chicken receptor was 500-, 1500-, and 1850-fold higher than that of Desaza^{1}TRH, Val^{2}TRH, and Pyr^{3}TRH, respectively. The mouse receptor showed a similar pattern of decreased affinity for these analogues. There was no significant difference in affinity, between chicken and mouse receptors, for any analog. The EC_{50} (10.2 nM) for stimulation of IP formation by TRH in the chicken receptor was about 40-fold lower than those of Desaza^{1}TRH and Phe^{2}TRH, and 10-fold higher than that of MeTRH (Fig. 2.16; Table 2.3). The mouse receptor displayed a similar pattern of potency for stimulating IP formation by these analogues. There was no significant difference in this pharmacological aspect between the chicken and mouse receptors. Figure 2.17 shows that there is no cAMP formation in the presence of various doses of TRH (up to 10 μM) in COS-1 cells transfected with the chicken or the mouse TRH-R cDNAs. However, the positive control (COS-1 cell transfected with β_2-adrenergic receptor cDNA) showed cAMP formation in the presence of 1 μM epinephrine.

In order to test whether the truncated receptors were functional, IP formation assays were performed to evaluate their responses to TRH. The truncated isoforms were expressed in COS-1 cells alone, or co-expressed in the cells [e.g. Trun-3 and -4, or Trun-3 and the constructed full-length receptor (wild type)]. They were characterised by measuring IP formation in the presence of various doses of TRH. No truncated isoforms (neither individual nor co-transfection) exhibited IP formation (Fig. 2.18). Additionally, there was no significant difference in the EC_{50} values in COS-1 cells expressing the wild type receptor, alone or with the Trun-3 receptor. This finding indicated that the truncated receptor did not exhibit a negative effect on the wild type receptor.

Table 2.3 ***Receptor binding affinity and stimulation of inositol phosphate (IP) formation*** by TRH analogues in COS-1 cells expressing the chicken or mouse TRH-Rs

Analogue	Receptor binding (K*i*)[a] (nM)		IP assay (EC_{50}) (nM)	
	chicken	*mouse*	*chicken*	*mouse*
TRH	**8.1 (4.3 - 15)[b]**	**11 (7.9 - 16)**	**10.2 (5.2 - 20.1)**	**11.1 (7.9 - 15.5)**
Desaza[1]TRH	**4200 (2200-8000)**	**4800 (1900-12000)**	**453.3 (251-817)**	**341.6 (299-494)**
Phe[2]TRH	**ND**	**ND**	**425.5 (133-536)**	**158.7 (88.4-285)**
MeTRH	**ND**	**ND**	**0.93 (0.5 - 1.6)**	**1.91 (0.9 -3.8)**
Val[2]TRH	**12000 (6800-22000)**	**5600 (3800-8400)**	**ND**	**ND**
Pyr[3]TRH	**15000 (7700-28000)**	**14000 (8600-23000)**	**ND**	**ND**

[a]: The data were obtained from Prof. Gershengorn's lab.

[b]: 95 % confidence interval

ND: undetermined

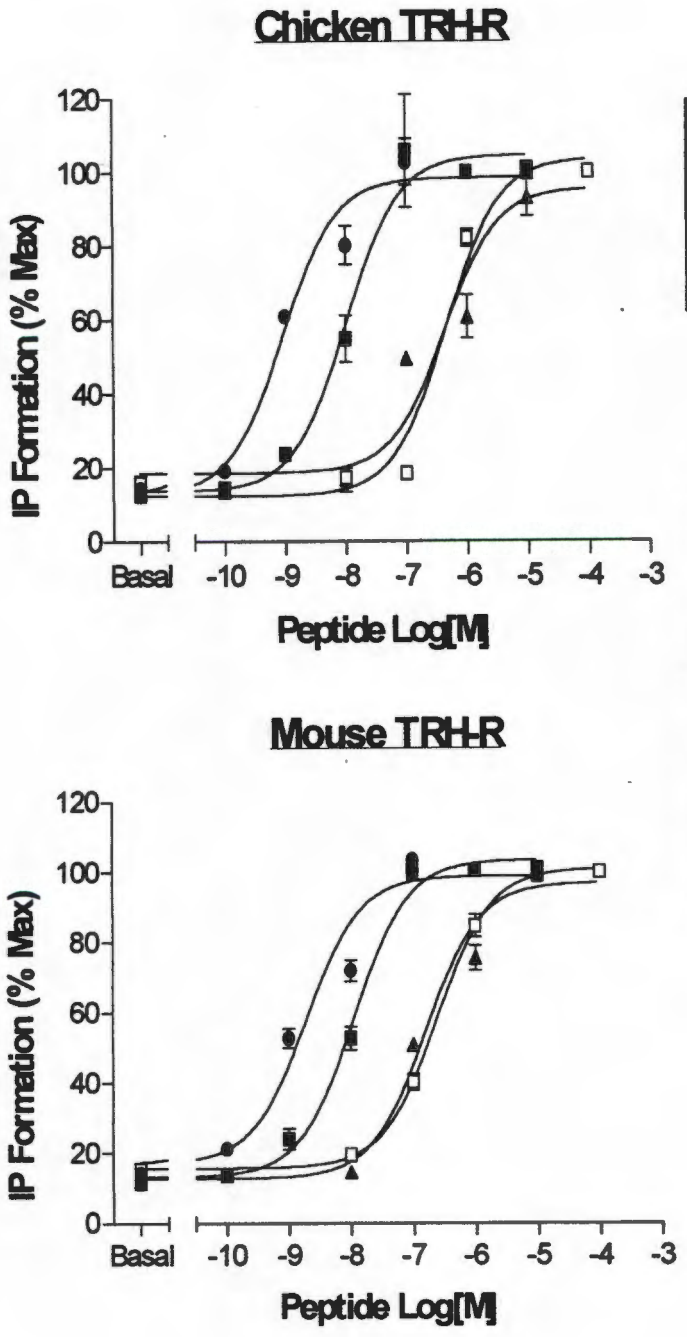


Fig. 2.16 ***Dose-response curves for inositol phosphate formation*** stimulated by TRH analogs in COS-1 cells expressing chicken or mouse TRH-Rs. Data shown are the mean ± S.E.. Each curve is representative of three experiments in duplicate.

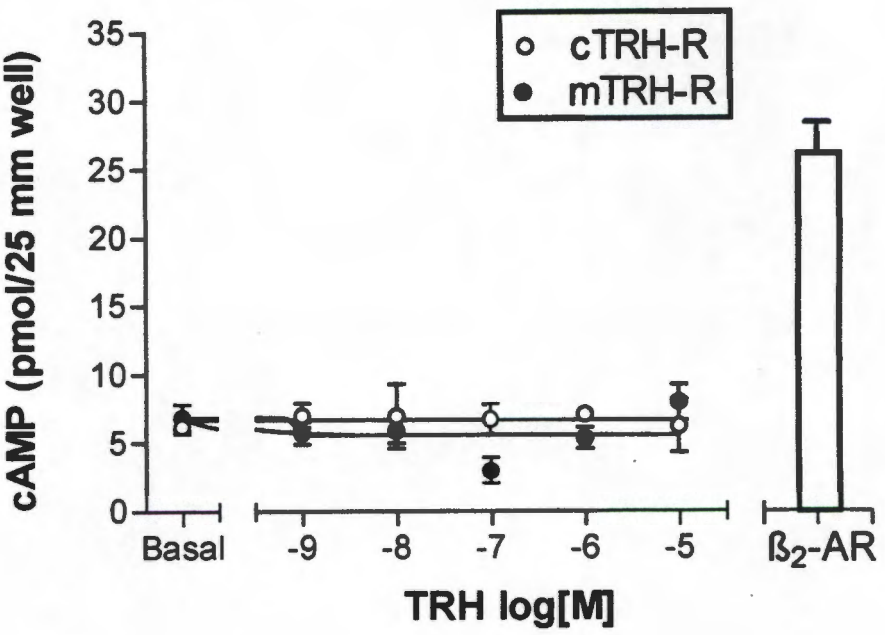


Fig. 2.17 ***Lack of stimulation of cAMP formation by TRH in COS-1 cells expressing chicken TRH-R (cTRH-R) or mouse TRH-R (mTRH-R).*** The COS-1 cells expressing β_2-adrenergic receptor (β_2-AR) were stimulated with 1 μM epinephrine as a positive control. The levels of cAMP were measured in the presence of 0.25 mM IBMX. Each curve is representative of two experiments in duplicate.

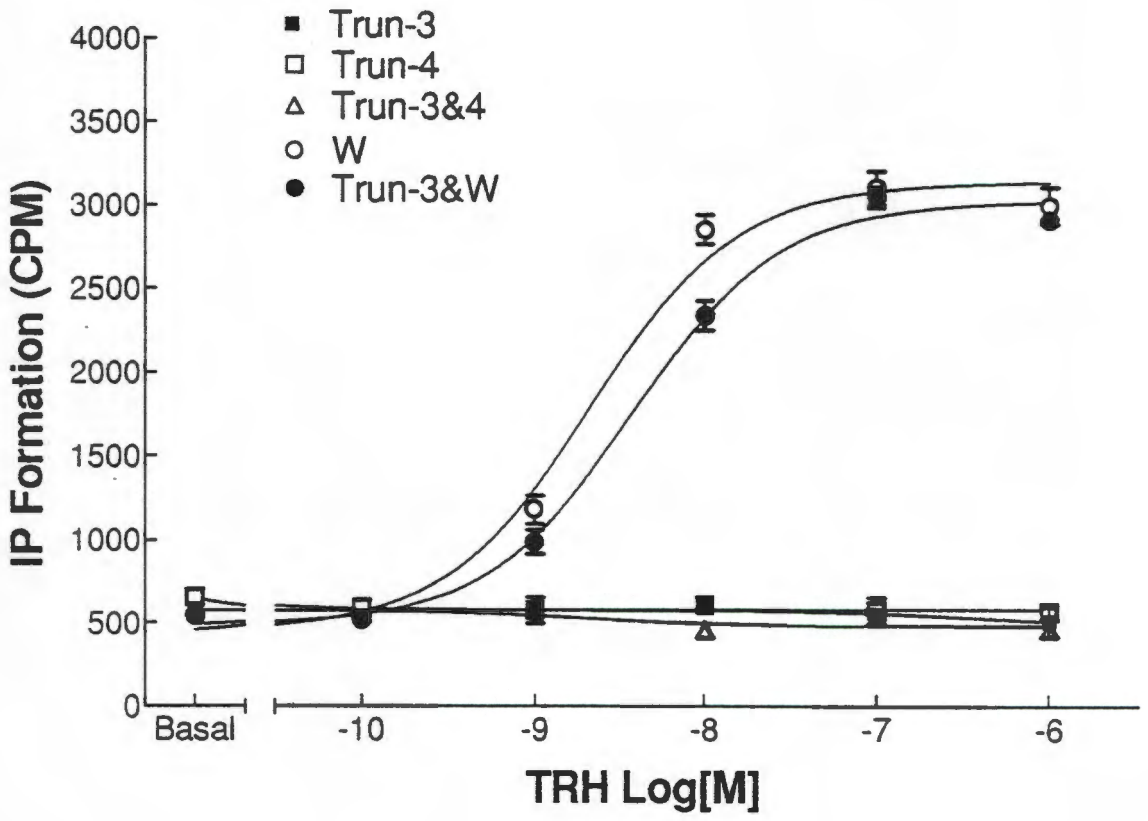


Fig. 2.18 ***Dose-response curves for inositol phosphate formation stimulated by TRH in COS-1 cells expressing the chicken TRH-R isoforms, alone, or co-expression.*** W: the constructed wild type receptor; Trun-3&-4: co-transfecting the Trun-3 and -4 clones; Trun-3&W: co-transfecting the Trun-3 and the wild type receptor.

2.5 DISCUSSION

The deduced amino acid sequence of the chicken TRH-R exhibits about 80 % identity to the mammalian counterparts (excluding two isoforms of rodents). It is of interest to note that the related chicken gonadotrophin-releasing hormone (GnRH) receptor shares only 40 % identity with mammalian GnRH-Rs (Troskie *et al.*, 1997). It appears that there has been considerable evolutionary constraint on the TRH-R structure for interaction with the smaller ligand. This is consistent with the finding that there is high conservation of primary amino acid sequences between receptors for small ligands amongst different species in the biogenic amino receptors (Cascieri *et al.*, 1995).

Using a combination of mutagenesis and computer simulation studies, a putative binding pocket of mouse TRH-R has been identified (Perlman *et al.*, 1992). All of these candidate ligand interacting sites are conserved in the chicken receptor. It has been proposed that the binding of TRH to its receptor involves hydrogen bonding interactions and hydrophobic interactions rather than ionic interactions. The putative binding pocket includes Tyr^{106} (Tyr^{108} in the chicken receptor) in TM_3 , whose OH group is suggested to form an H bond with the C=O of the pyroGlu residue of TRH, because the substitution of Tyr^{106} with Phe^{106} (abolishing OH group) impaired the receptor affinity for TRH by 100,000-fold (Perlman *et al.*, 1994a). Using the same approach, the Asn^{110} residue (Asn^{112} in the chicken receptor) was shown to form an H bond with the NH group of pyroGlu of TRH at its C=O group (Perlman *et al.*, 1994b). Following computer simulation studies, a three-dimensional model of the TRH-R was developed (Gershengorn *et al.*, 1996). It identified that the Tyr^{282} (Tyr^{284} in chicken) in TM 6 forms a hydrophobic interaction with the imidazole of the His residue of TRH. In addition, the Arg^{306} (Arg^{308} in chicken) in TM 7 interacts with the $ProNH_2$ of TRH. Other residues have been identified (e.g. Gln^{105} and Tyr^{282}) that are involved in inter-helical H-bonding interactions but do not directly interact with TRH (Perlman *et al.*, 1994b). These residues are all conserved in the chicken receptor. It has been suggested that TRH binds to the TRH-R by interacting initially with residues in ELs and is then guided into the TM binding pocket (Perlman *et al.*, in press). Asn^{289} is one of the EL interacting sites, and it is also present at an homologous position in the chicken receptor.

Apart from the binding pocket, the chicken receptor is likely to share similarities in the mechanism of receptor activation with the mammalian receptors. For example, two residues, Asp^{71} in TM 2 and Arg^{283} in TM 6, which play a role in G-protein coupling in the mouse receptor (Perlman *et al.*, 1992, Perlman *et al.*, 1995) are conserved in the chicken receptor. On the other hand, the chicken receptor displays some features that are different from the mammalian homologues. The chicken receptor lacks a potential N-glycosylation site in EL 2, which is present in the mammalian receptors (Asn^{167}). It has been shown that glycosylation may play a role in receptor expression or stability (Davidson *et al.*, 1995). Additionally, a difference in the distribution of putative phosphorylation sites (Kennelly and Krebs, 1991) exists in the chicken and mammalian receptors. The chicken receptor contains 4 additional putative phosphorylation sites in the C-terminal part of IL 3, whereas the mammalian receptors consist of four extra putative phosphorylation sites in the C-terminal tail. Phosphorylation may play a part in desensitisation of receptors. The chicken receptor exhibits a difference in down-regulation from that of the mammalian counterpart (Harvey *et al.*, 1990b). Down-regulation of the chicken TRH-R occurs within 30 min after administration of TRH and is maintained for at least 60 min in *in vivo* and *in vitro* studies. On the contrary, the down-regulation of the mammalian receptors occurs after several days of TRH challenge *in vivo* (Gershengorn *et al.*, 1978; Simasko and Horita, 1985) or after 12-24 h of TRH treatment *in vitro* (Hinkle and Tashjian, 1975). It remains to be further investigated whether or not the putative phosphorylation sites are involved in the regulation of receptor down-regulation.

The chicken TRH-R, like mammalian TRH-Rs, has retained several amino acids which are highly conserved in GPCRs. These include TM_2-Asp^{73} and TM_7-Asn^{318} (Asp^{71} and Asn^{316} in the mammalian receptors), in which the Asp residue is involved in receptor activation in many GPCRs (Baldwin, 1994) including the mouse receptor (Perlman *et al.*, 1992). The highly conserved Cys residues at 100 and 181 (98 and 179 in mammals) have been shown to form a disulphide bond between EL 1 and EL 2 to stabilise the structure of functional receptor in GPCRs (Baldwin, 1994) and the mouse TRH-R (Perlman *et al.*, 1995). Two other Cys residues, Cys^{337} and Cys^{339} (335 and 337 in the mammalian receptors) are present in the C-terminal tail. Homologous Cys residues have been shown to be palmitoylated and may anchor the tail in the plasma membrane which is proposed to be important for interactions with G protein (Ovchinnikov *et al.*, 1988; O'Dowd *et al.*, 1989).

In contrast, the Cys residues in the mammalian TRH-R are not necessary for G-protein coupling, but may play a role in restraining the receptor in an inactive conformation that is optimal for rapid internalisation of the receptor (Nussenzveig *et al.*, 1993a) because a truncation mutation (C335Stop) at this locus of the mouse TRH-R exhibits constitutive (or agonist-independent) activity (Matus-Leibovitch *et al.*, 1995). The highly conserved Asp-Arg-Tyr (DRY) motif at the C-terminal part of TM 3, which is involved in G-protein coupling and receptor internalisation (Arora *et al.*, 1997), is present as ERY in the chicken and mammalian receptors as in several other receptors. A substitution of the Asp residue with Glu (E) in a mammalian GnRH-R results in increasing binding affinity, activation, and the rate of internalisation (Arora *et al.*, 1995). Following the DRY motif, the XXI/VXXPL/I motif, in which the Pro and Leu residues have been established to play a role in G-protein coupling (Arora *et al.*, 1995; Sealfon *et al.*, 1997), is also conserved in the chicken and mammalian receptors. Moreover, Asn^{43} (TM 1), Trp^{150} (TM 4), Pro^{203} (TM 5), FXXXWXP motif (TM 6), and NPXXY motif (TM 7), which are conserved GPCR residues present in the mammalian TRH-Rs, are also conserved in the chicken TRH-R.

The pharmacological properties of the chicken receptor were evaluated by applying different TRH (pGlu-His-Pro-NH_2) analogues, in which residues 1, 2, and 3 were substituted individually with different amino acids, to measure their receptor binding activities and/ or G-protein coupling. These experiments confirmed that the side chains of all three amino acid residues of TRH are involved in receptor binding. $Desaza^1$ TRH, in which the NH ring of pyroGlu was substituted by a CH_2 group, exhibits an approximately 500-fold decrease in affinity for the chicken and mouse receptors. Modifications in the histidine residue of TRH can lead to marked effects on the binding affinity that in turn affects the potency for IP formation and for secretion of thyrotrophin (TSH). One of these TRH analogues, Val^2 TRH, in which the histidine residue of TRH was replaced by valine, showed an over 500-fold decrease in affinity for the chicken and mouse receptors. A portion of the decrease may be due to the loss of a stacking interaction between the ligand and receptor, because it was postulated that the imidazole of the His residue forms hydrophobic interactions with a pocket formed by Tyr^{282}, Tyr^{188}, Tyr^{192}, Phe^{196}, and Phe^{199} (Gershengorn *et al.*, 1996; Han and Tashjian, 1995). This may also explain why when histidine was substituted with a strongly hydrophobic phenylalanine there was less than an 100-fold decrease in the binding affinity (Perlman *et al.*, 1994b) and in stimulating IP

formation in this study. In addition, a portion of the decrease in affinity of Val^2 TRH or Phe^2 TRH may result from the loss of an H bond interaction, because residue Ser^{113} of the receptor was suggested to form an H bond with the histidine (Gershengorn *et al.*, 1996). Furthermore, the binding affinity of the chicken receptor for Pyr^3 TRH, in which the $ProNH_2$ residue is substituted by a pyrrolidine ring, is 1,800-fold lower than for TRH, as with the mouse receptor. It was suggested that the C=O group of Pro residue of TRH forms an H bond with the guanidino group of Arg^{306} residue in the receptor.

It has been suggested that the adenylyl cyclase-cyclic adenosine 3′,5′-monophosphate (cAMP) pathway plays a role as a secondary messenger in TRH-induced physiological functions, in addition to the phospholipase C-inositol 1,4,5-trisphosphate - 1,2-diacylglycerol -Ca^{++} pathway. Evidence has been presented that cAMP is involved in TRH-stimulated prolactin secretion in rat pituitary tumour cell line (GH cells) (Gautvik *et al.*, 1983), and may participate in TRH-induced growth hormone release in chicken somatotrophs (Perez *et al.*, 1989). However, we demonstrated here that TRH does not elevate cAMP levels in the COS-1 cells transiently expressing chicken or mouse TRH-Rs. The result is consistent with the finding that TRH did not stimulate cAMP formation in the mouse TRH-R expressed in 5 different cell types (Heinflink *et al.*, 1994).

The truncated isoforms of the chicken TRH-R cDNA that we isolated are not functional receptors. Trun-1, -2, and -3 clones that contain a premature stop codon (TAA) in IL 3, are due to the retention of an un-spliced intron. Intriguingly, these truncated receptor clones were shown by PCR and sequencing studies to contain a poly $(A)_{49}$ tail and an AATAAA consensus sequence of the polyadenylation signal site upstream of the tail (unpublished data). Additionally, the sizes of these truncated cDNA clones (about 1.4 kb) are correlated with one of the mRNA transcripts of the chicken receptor expressed in the pituitary gland. This indicates that these receptor are fully processed mRNA transcripts and are not cloning artifacts. It is noteworthy that the human TRH-R gene also contains a large intron (25 kb) at an homologous position (Iwasaki, *et al.*, 1996). In addition, two alternatively spliced forms of TRH-R have each been cloned, from mouse (Duthie *et al.*, 1993b) and from rat (De La Pena *et al.*, 1992). These isoforms are characterised by different amino acid sequences in their respective C-terminal tails and are functional receptors. The isoforms of the rat receptor are produced by alternative splicing of a retained intron, whereas those of the mouse receptor may be formed by alternative splicing

of two exons. Other truncated forms of the cTRH-R , Trun-4 to Trun-12, might be due to inefficiency of the reverse transcriptase (M-MuLVRT), which was used in first strand cDNA synthesis, because these cDNA clones all contain a large 3′ un-translated region (> 3kb).

In conclusion, we have cloned a cDNA for the chicken TRH-R and found it to be highly homologous and pharmacologically indistinguishable from the previously cloned receptors from mouse, rat and human species. This degree of evolutionary conservation is unusual and may be related to the small number of contacts that must be maintained between a tripeptide and its receptor to achieve high affinity binding and optimal activation.

Chapter 3

Cloning and Characterisation of chicken GnRH Receptor (GnRH-R)

3.1 Abstract

3.2 Introduction

3.3 Materials and Methods

3.4 Results

3.5 Discussion

3.1 ABSTRACT

The chicken GnRH-R cDNA has been cloned from the chicken pituitary gland. As compared to the cloned mammalian receptors, the chicken receptor consists of some unique features: a C-terminal tail containing 56 amino acids, a longer N-terminus (7 amino acids longer), a short intracellular loop 1 (only 6 amino acids), Asp (D) residues in TM 2 and TM 7 (N/D in the mammalian receptors and D/N in most of GPCRs), an Asp-Arg-His (DRH) motif at the intracellular junction of TM 3 (DRY in the mammalian receptor), and two additional Cys (Cys^2 and Cys^{29}) in the N-terminal tail. Moreover, the chicken GnRH-R only shares about 40 % identity of amino acid sequences with its mammalian counterparts. The N-terminus and intracellular loops are more divergent (about 20 % identity), while having a comparatively higher level of amino acid sequence identity within the transmembrane domains and the extracellular loops. However, the putative binding sites Asn^{102}, Lys^{121}, and Glu^{301} for GnRH that have been proposed in the mouse GnRH-R, are all conserved in the chicken receptor. Additionally, two Cys residues in EL 1 and EL 2 that are predicted to form a disulphide bridge, and the putative glycosylation sites, are found in homologous residues in the chicken receptor.

Given the strikingly different structural features from the mammalian receptors, the chicken receptor possesses some unique pharmacological properties. For example, the chicken receptor exhibits over 10-fold higher affinity for cGnRH II and [D-Arg^6]GnRH II than for cGnRH I and mGnRH, whereas the mammalian receptors prefer mGnRH to cGnRH I, II, and [D-Arg^6]GnRH II. Moreover, of six mammalian antagonists studied, three antagonists (135-18, 135-25, and 26) display an agonistic behaviour for the chicken receptor, while they act as full antagonists in the mammals.

3.2 INTRODUCTION

As covered in the general introduction (chapter 1), the hypothalamic decapeptide GnRH (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2), plays a vital role in regulating the hypothalamo-pituitary-gonad reproductive axis. To date, twelve structural forms of GnRH have been identified in vertebrates. Chicken GnRH I (cGnRH I: [Gln^8]-GnRH) and II (cGnRH II: [His^5, Trp^7, Tyr^8]-GnRH) have been isolated from the chicken hypothalamus and brain (King *et al.*, 1982; Miyamoto *et al.*, 1984). The distribution of the two hormones is not the same. One of the most important differences is that immunoreactive (ir)-cGnRH I fibres have been detected in the median eminence, but not ir-cGnRH II fibres (Mikami *et al.*, 1988). Moreover, the amount of cGnRH I in the median eminence of the chicken is regulated by the physiological reproductive state, however, no cGnRH II is detected in this region (Sharp *et al.*, 1990). These findings suggest that cGnRH II may not be important for the pituitary-gonad axis, but it may serve a neurotransmitter or neuromodulator role.

It is well established that GnRH exerts its physiological role, firstly, via binding to its specific receptor (GnRH-R) in the membrane of gonadotrophs; then, coupling to G_q and/or G_{11} which in turn promotes secondary messengers (DAG and IP_3) signalling cascade (Hsieh *et al.*, 1992). The GnRH-R, a member of the G-protein coupled receptor (GPCR) family, has been cloned from several mammalian species and one non-mammalian class: mouse (Tsutsumi *et al.*, 1992), rat (Eidne *et al.*, 1992), human (Chi *et al.*, 1993), sheep (Illing *et al.*, 1993), cattle (Kakar *et al.*, 1993), pig (Weesner *et al.*, 1994), and catfish (Tensen *et al.*, 1997). As compared to other GPCRs, the mammalian GnRH-Rs possess several unusual or unique features. These include a complete absence of intracellular C-terminal tail, the change of the highly conserved Asp-Arg-Tyr (DRY) motif to Asp-Arg-Ser (DRS) at the end of the transmembrane (TM) domain 3, and the reciprocal exchange of the conserved TM 2 Asp and TM 7 Asn.

Mutagenesis studies of the GnRH-R have identified glycosylation sites (Davidson *et al.*, 1995), location of disulphide bridge between extracellular loop I and II (Davidson et al., 1997; Cook et al., 1997), intermolecular transmembrane interactions (Zhou *et al.*, 1994), and several putative ligand interacting sites which are Glu^{301} (Flanagan *et al.*, 1994), Lys^{121} (Zhou *et al.*, 1995), and Asn^{102} (Davidson *et al.*, 1996). Since understanding the role of the GnRH-R in reproductive functions is crucial, knowledge of the structural conformation of

the GnRH-R interacting with GnRH may assist in the design of new GnRH analogues for therapeutic purposes. While there have been considerable evolutionary constraints on the structure and function of the mammalian GnRH-Rs, the far greater antiquity of the chicken GnRH-R provides an opportunity for unique amino acid substitutions to occur. There are different pharmacological properties between the mammalian and chicken GnRH-Rs. For example, the mammalian GnRH-R has a high affinity for [Arg^8]-GnRH, whereas the chicken receptor has no selectivity for basic or neutral amino acids at this position (Millar *et al.*, 1989). D-Trp^6 incorporation does not enhance activity in the chicken receptor, but restored activity to analogues lacking Arg^8 in the mammalian receptor. Moreover, some GnRH antagonists exhibit an agonist effect in the chicken pituitary gland, while they show full antagonism in the sheep (Jacobs *et al.*, 1995). Therefore, the advantages of the present study will offer an approach tool, and useful information, to unravel the functional binding domain of the GnRH-R. It will also give an insight into the molecular basis of GnRH actions on the reproduction of chickens.

3.3 MATERIALS AND METHODS

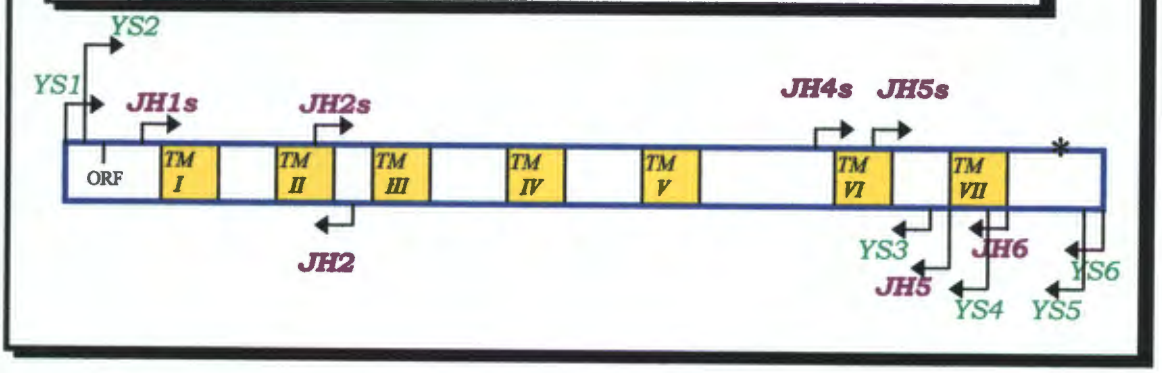
Materials

The oligo-nucleotides used in the cloning of the GnRH-R cDNA are summarised in Table (3.1), and their positions on the chicken GnRH-R cDNA are illustrated in Fig. 3.1.

Table 3.1 ***The nucleotide sequences of the primers, used in this study.*** N: A, C, G, and T; G(A/T): G or A or T.

Name of primer	Nucleotide sequences
Degenerate primer	
JH1s	5′-CTCGAATTCGGNAAG(A)ATC(A/T)C(A)GNGT-3′
JH2s	5′-CTCGAATTCGAT(C)GGNATGTGGAAC(T)ATA(T/C)AC-3′
JH4s	5′-CTCGAATTCAA(G)ATGACNGTNGCNTTT(C)GC-3′
JH5s	5′-CTCGAATTAGNATT(A/C)TGGTAT(C)TGGTT-3′
JH2a	5′-ACACTCGAGTGNACNGTA(T/G)ATG(A)TTCCACAT-3′
JH5a	5′-ACACTCGAGAACCAATACCAA(T/G)ATNCC-3′
JH6a	5′-CACTCGAGCCA(G)TAG(T/A)ATTNTG(A)NGGATC-3′
Specific primer	
YS1	5’-GCTGAGCACTTGTGCTGCCT-3’
YS2	5’-CACTTGTGC TGCCTGACTTGCTG-3’
YS3	5′-GGGCATCCTCTGGATCATGGC-3′
YS4	5′-GGTGCATGTGTGCAGCAAACC-3′
YS5	5′-TGGGTACATCTCTTCAGC ACACCGT-3′
YS6	5′-TCTAGTCTCCTTTTGGGTACATCTCTTC-3′
Adaptor primer	
AP1	5′-CCATCCTAATACGACTCACTATAGGGC-3′
AP2	5′-ACTCACTATAGGGCTCGAGCGGC-3′

Fig. 3.1 ***The positions of the primers used in this study corresponding to the chicken GnRH-R cDNA.*** The degenerate primers (in purple) and the specific primers (in green) are shown. "*" : stop codon; ORF: open reading frame; TM: transmembrane, "→": sense; "←": antisense.



The sequence of GnRH analogues used in the study are shown in Table 3.2. All agonists were synthesised by conventional solid-phase methodology and were purified to more than 95 % homogeneity, by preparative C-18 reversed-phase chromatography (Millar *et al.*, 1989). Antagonist 26 was a gift from Dr. D. H. Coy (Tulane University, Medical Centre, New Orleans, LA, USA) and antagonists 27, 135-18, 135-25, 134-53, and 134-46 were from Prof. R. W. Roeske (Indiana University, Indianapolis, IN, USA).

Table 3.2 The primary structure of GnRH agonists and antagonists

position of a.a. / Peptides	1	2	3	4	5	6	7	8	9	10
agonists :										
mGnRH	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly-NH_2
cGnRH I	_	_	_	_	_	_	_	Gln	_	_
cGnRH II	_	_	_	_	His	_	Trp	Tyr	_	_
[D-Arg^6] cGnRH II	_	_	_	_	His	Arg	Trp	Tyr	_	_
[D-Ala^6] GnRH	_	_	_	_	_	D-Ala	N-Me-Leu	_	Pro-NHEt	
antagonists :										
27	Ac-D-Nal(2)	D-α-Me-4-Cl-Phe	D-Trp	_	Ipr-Lys	D-Tyr	_	_	_	D-Ala -NH_2
134-46	Ac-D-Nal(2)	D-4-Cl-Phe	D-Pal(3)	_	Ipr-Lys	D-Trp	_	Ipr-Lys	_	D-Ala -NH_2
134-53	Ac-D-Nal(2)	D-4-Cl-Phe	D-Pal(3)	_	1-MePal	D-Trp	_	Ipr-Lys	_	D-Ala -NH_2
26	Ac-D-p-Cl-Phe	D-4-Cl-Phe	D-Trp	_	_	D-Lys	_	_	_	D-Ala -NH_2
135-25	Ac-D-Nal(2)	D-4-Cl-Phe	D-Pal(3)	_	1-MePal	D-Ipr-Lys	_	Ipr-Lys	_	D-Ala -NH_2
135-18	Ac-D-Nal(2)	D-4-Cl-Phe	D-Pal(3)	_	Ile	D-Ipr-Ly	_	Ipr-Lys	_	D-Ala -NH_2

Amino acids (a.a.) in common with mGnRH are indicated by "_". Nal(2) : 3-(2-naphthyl)alanine; 4-Cl-Phe: 3-(4-chlorophenyl) alanine; α-Me-4-Cl-Phe: 2-methyl-3-(-chlorophenyl)alanine; Pal(3): 3-(3-pyridyl)alanine; Nic-Lys: N^{ε}-nicotinoyllysine; Ipr-Lys: N^{ε}-isopropyllysine

Methods

3.3.1 Cloning the chicken GnRH-R gene

Cloning a partial GnRH-R gene from chicken by PCR

A series of degenerate primers designed against conserved amino acid residues of mammalian GnRH-R by Dr. Hapgood (Table 3.1 and Fig. 3.1), were tested to amplify up different regions of the chicken GnRH-R gene from chicken total genomic DNA by PCR. The primer combinations used in PCR were JH1s/JH2a, JH2s/JH2a, JH4s/JH5a, and JH5s/JH6a. PCR was carried out in DNA thermal cycler (Perkin Elmer Cetus). The cycling

parameters of PCR were at 94 °C for 4 min, 35 amplification cycles (1 min at 93 °C, 2 min at 55 °C, and 3 min at 72 °C), 10 min at 72 °C, and 10 min at 4 °C.

Screening a chicken genomic library

$7.5x10^5$ individual recombinants of a chicken genomic library, constructed in bacteriophage Lambda Charon 4A (Dodgson et al., 1979), were screened in 20 (150 mm) petri plates. Duplicated nylon filters were lifted as described in the "TRH-R" section. The pCH 1 that is a partial gene of the chicken GnRH-R, encoding a region from the C-terminal part of TM 6 to TM 7 of the chicken GnRH-R, was isolated by PCR with degenerate primers JH5s and JH6a (Fig. 3.2 and 3.3). The pCH 1 was used as a probe, and was labelled with α^{32}P-dCTP by random hexamer priming (Megaprimer labelling kit, Amersham). The filters were pre-hybridised for 3 hr at 42 °C in a solution containing 50% formamide, 2x PIPES, 1 % SDS and 100 μg/ml herring sperm DNA. After at least 3 h pre-hybridisation, the labelled probe was added to the membranes and the hybridisation was carried out at 42 °C overnight. The filters were washed with 1xSSC, 0.1% SDS at room temperature for 15 min, in 0.5xSSC, 0.1% SDS at 50 °C for 20 min, and 0.2xSSC, 0.1% SDS at 50 °C for 20 min.

5'RACE (Rapid Amplification of the 5'end) and nested PCR

Four anti-sense primers to the chicken GnRH-R cDNA, YS3 to YS6, were designed from the nucleotide sequence data derived from the exon 3 of the receptor (pCH2) (Fig. 3.2 and 3.3). The nucleotide sequences and positions of the primers were shown in Table 3.1 and Fig. 3.1.

cDNAs were synthesised by using the Marathon cDNA synthesis kit (ClonTech), from 2 μg total RNA of the castrated chicken pituitary (a gift from Prof. Sharp). The YS6 primer was used to initiate first strand synthesis. Marathon cDNA adaptors consisting of the sequence for the AP1 and AP2 primers (Table 3.1), were ligated onto the ends of the cDNAs. The 5'-end of the chicken GnRH-R cDNA was amplified up by three rounds of nested PCR with KlenTaq polymerase (ClonTech), using the following combination of primers; round 1 (AP1 / YS5), round 2 (AP2 / YS4), and round 3 (AP2 / YS3). The

samples were subjected to the following condition: 94 °C for 2 min, 30 amplification cycles (30 sec at 94 °C, 30 sec at 66 °C, and 3 min at 68 °C), and 5 min at 4 °C.

Amplification of the full-length chicken GnRH-R cDNA

Two sense primers, YS1 and YS2, were designed to the region 5' before the start codon of the chicken receptor (Table 3.1 and Fig. 3.1). Two rounds of nested PCR, with primer combinations (YS1/YS6 and YS2/YS5) were used to amplify up a full-length chicken GnRH-R cDNA from the castrated chicken pituitary cDNA. The cycling parameters of PCR were the same as described in 5'RACE. The PCR-products were sub-cloned into the pMOS vector and sequenced.

3.3.2 Characterisation of the chicken GnRH-R

Cell culture and transfection :

COS-1 cells were grown at 37°C in an humidified atmosphere containing 5% CO_2. One or two days before transfection, the COS-1 cells were seeded by trypsin, and plated out in 12-multiwell dishes (Corning), coated with poly-D-lysine (10 μg/ml), as mono-layer cultures (0.6 ~ 1 x 10^5 cells/ 25mm well). The cells were transfected for 3.5 hr with the chicken GnRH-R subclones (pCH5, 6, or 7) (1~ 0.5 μg/well) or the human GnRH-R subclone (1~ 0.5 μg/well) (Chi et al., 1993) which were all subcloned into the pcDNA vector, using a modified DEAE-dextran method (Cullen, 1987). For the inositol phosphate production assay, the cells were labelled with myo-[^{3}H]-inositol (1 μCi/ml, Amersham) in M_{199} medium (supplemented 2% fetal calf serum and penicillin / streptomycin) for 16 ~ 22 hr in a 37 °C incubator (10% CO_2) 48 hr after transfection. The cells were also used for radio-ligand binding assay 48 hr after transfection.

Inositol phosphate formation assay :

IP formation was measured as previously described (Millar et al., 1995). Briefly, the myo-[^{3}H]-inositol labelled cells were washed twice with buffer A (140 mM NaCl, 4mM KCl, 1mM $CaCl_2$ and 1 mM $MgCl_2$, 20mM HEPES, 0.1% BSA, and 8 mM D-

glucose, pH 7.4) at 37 °C for 5 min, incubated with buffer A (containing 10 mM LiCl) in the absence and presence of different doses of GnRH agonists, or with antagonists in the presence of 1nM or 10 nM cGnRH I (for cGnRH-R subclone), or of 1 nM mGnRH (for hGnRH-R subclone) at 37 °C for 1 hr. All experiments were performed in duplicate and repeated at least two times.

Whole cell radio-ligand binding assay :

The analogue [D-Ala^6-N-α-MeLeu^7, Pro^9-NHEt]GnRH (GnRH-A) was iodinated as described (Millar et al, 1989). Binding of ^{125}I-GnRH-A was measured in 12-well culture plates (triplicate) with transiently transfected COS-1 cells. Cells were washed for 5 ~ 10 min in ice-cold Buffer A, incubated for 3 hr at 4 °C in 0.5 ml of Buffer A containing 100,000 cpm radio-ligand, alone, and with increasing concentrations of unlabelled GnRH agonists. Non-specific binding was determined in the presence of 1μM unlabelled antagonist (A27). For the competition binding by GnRH antagonists, cells were incubated with various doses of antagonists at 37 °C for 3 hr, since antagonists are not internalised and have reduced "on" and "off" rate constants. After incubation, the cells were washed three times with ice-cold Buffer A (containing 0.5 % BSA). Cells were solubilised in 0.5 ml of 0.1 N NaOH and radioactivity counted. All experiments were repeated at least twice.

Data Analysis :

The values of Ki and ED_{50} (IP formation) were calculated by non-linear regression analysis using the PRISM program (GraphPad Inc.). Dose-response curves (each data point is a mean of all experiments) for IP formation and receptor binding assays were generated using the same program.

3.4 RESULTS

Summary of the work in cloning chicken GnRH-R cDNA

The chicken GnRH-R cDNA was cloned using a variety of strategies (Fig. 3.2), and the results of each strategy were illustrated in Fig. 3.3. As the receptor mRNA is expressed at extremely low levels, it could not be isolated by screening cDNA libraries (constructed from the intact and castrated chicken pituitary glands). It has been suggested that the number of GnRH-Rs is up-regulated in the castrated chicken. However, no positive signals were detected after screening the castrated chicken cDNA library using a mouse GnRH-R cDNA probe, or chicken gene specific probe (a part of chicken GnRH-R gene). Therefore, this thesis will only address the strategies which were applied successfully, to isolate the GnRH-R cDNA.

PCR was used to amplify a part of the chicken GnRH-R gene from the total genomic DNA, with degenerate primers JH5s and JH6a. The partial gene of the chicken GnRH-R was subcloned (termed pCH1) (Fig. 3.3) and was used as a probe to screen a chicken genomic library. This strategy succeeded in cloning the chicken receptor gene. After the Southern blot analysis of the receptor gene, the exon 3 of the receptor was obtained by sub-cloning (in the pSK- vector) and sequencing (called pCH2) (Fig. 3.2 and 3.3). Additionally, the exon 2 of the receptor was identified by partial sequencing (pCH3) (Fig. 3.2 and 3.3). Four antisense primers were designed on the basis of the nucleotide sequence data of the exon 3 (pCH2) (Fig. 3.3). In order to get more 5′-end sequences of the receptor, 5′ RACE was performed using a gene specific primer (YS6) to initiate first strands of the chicken pituitary cDNA and accompanied by three runs of nested PCR with primer combinations AP1/YS5, AP2/YS4, and AP2/YS3. The 5′ un-translated region of the chicken receptor was identified by sequencing from pCH4 clone (Fig. 3.2 and 3.3). Therefore, two primers (YS1 and YS2) were designed according to the sequence data of the 5′-untranslated regions. Finally, full-length chicken GnRH-R cDNA clones were amplified by PCR with the two pairs of nested primers (YS1/YS6 and YS2/YS5). However, the full-length cDNA clones contained various nucleotide sequence differences, which resulted in encoding different amino acid sequences from genomic sequences of the receptor. A "wild type" chicken GnRH-R was therefore constructed. The details of the results will be addressed in the following section.

Fig. 3.2 ***Summary of the work in cloning chicken GnRH-R cDNA.***

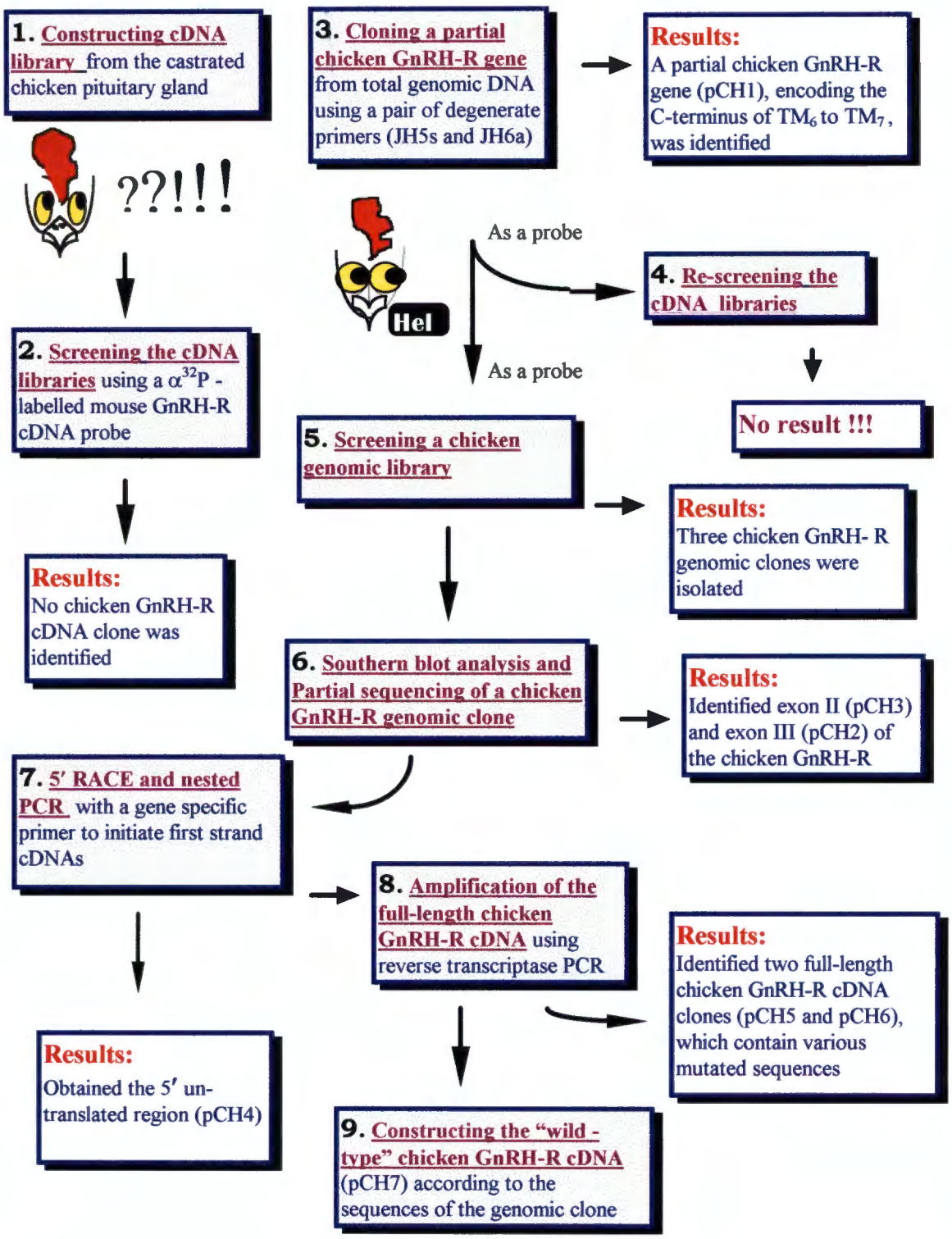
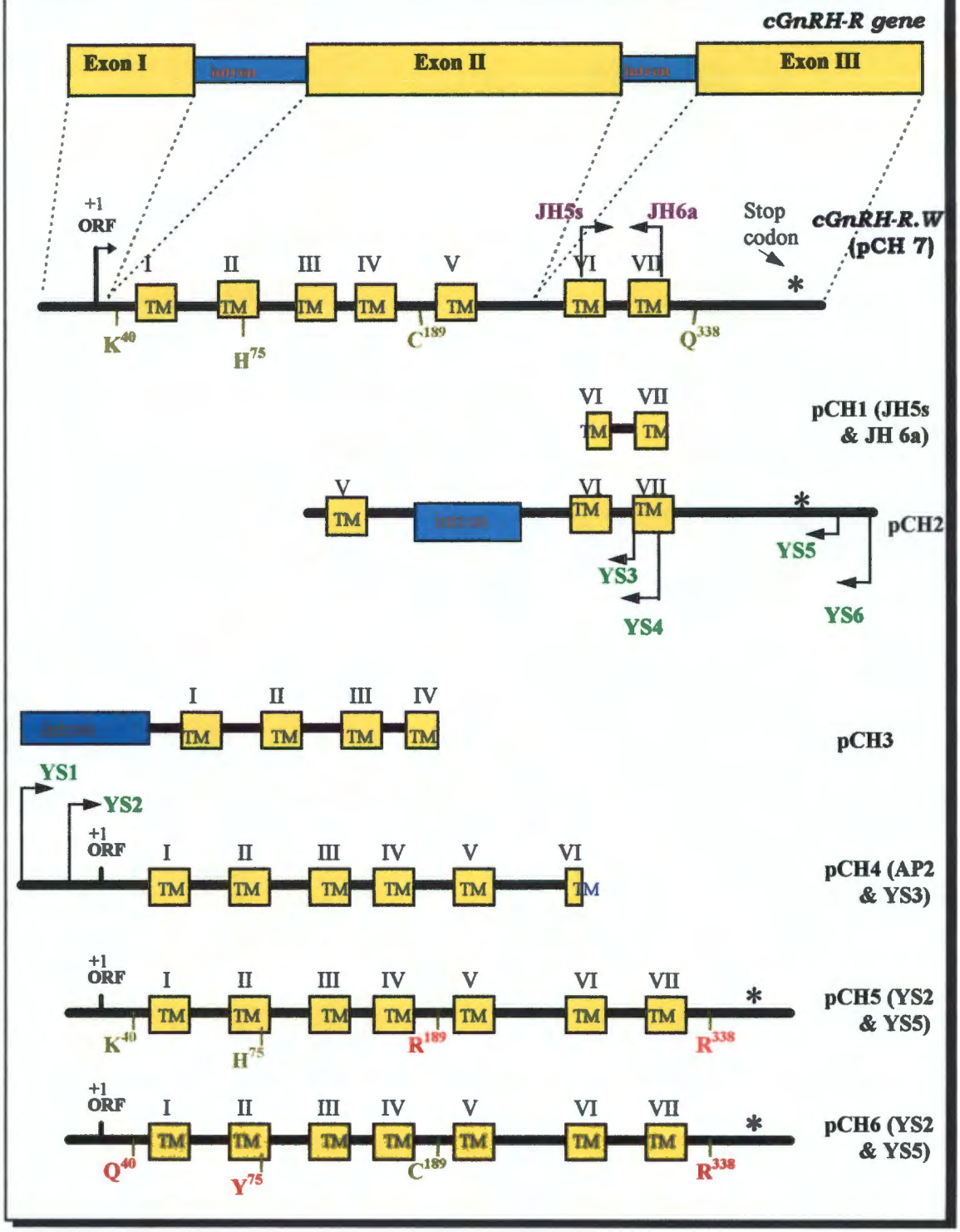


Fig. 3.3 ***Diagram of cloned cGnRH-R cDNAs and genomic clones.*** The structure of the chicken GnRH-R gene is shown. Primers are indicated in purple colour (degenerate primers), and in green (specific primers). Amino acid sequences with positional number are shown in brown (wild-type), and in red (mutation). ORF: open reading frame; TM: transmembrane; *: stop codon.



Cloning of the chicken GnRH-R gene :

As the chicken GnRH-R mRNA is of extremely low abundance, the conventional strategies for cloning, e.g. screening cDNA library, were unable to pick up positive signals at the cDNA level (data not shown). Therefore, PCR , known as the most sensitive technique, was applied to amplify a part of the chicken GnRH-R gene using degenerate primers, which were derived from the amino acid sequence data of cloned mammalian GnRH-Rs. In several sets of degenerate primers tested in PCR, the JH5s/JH6a combination was the only successful primer set to amplify a fragment of the chicken GnRH-R gene.

The part of the chicken GnRH-R gene encoding the C-terminus of TM 6 to TM 7 of the chicken GnRH-R, was amplified by PCR with JH5s and JH6a degenerate primers. The partial gene was cloned into the pMOS-Blue vector and verified by sequencing (designed pCH1) (Fig. 3.3 and 3.4). The deduced amino acid sequences of the pCH1 exhibited 70 % and 50 % identity to those of frog and mouse counterparts, respectively.

The pCH1 became a useful tool for further cloning work. A chicken genomic DNA library was screened by hybridising with α ^{32}P-labelled pCH1 probe. Three positive clones of chicken GnRH-R gene were isolated from $7.5x10^5$ recombinants in the first screening (Fig. 3.5). Of three positives, the chicken GnRH-R.genomic 22 clone was selected to carry out the secondary screening (Fig. 3.6).

Fig. 3.4 ***Schematic representative of the pCH1,*** amplified by PCR with degenerate primers JH5s and JH6a, from the chicken total genomic DNA. The deduced amino acid sequences were verified by sequencing, after being sub-cloned into the pMOS-Blue vector. TM: transmembrane domain.

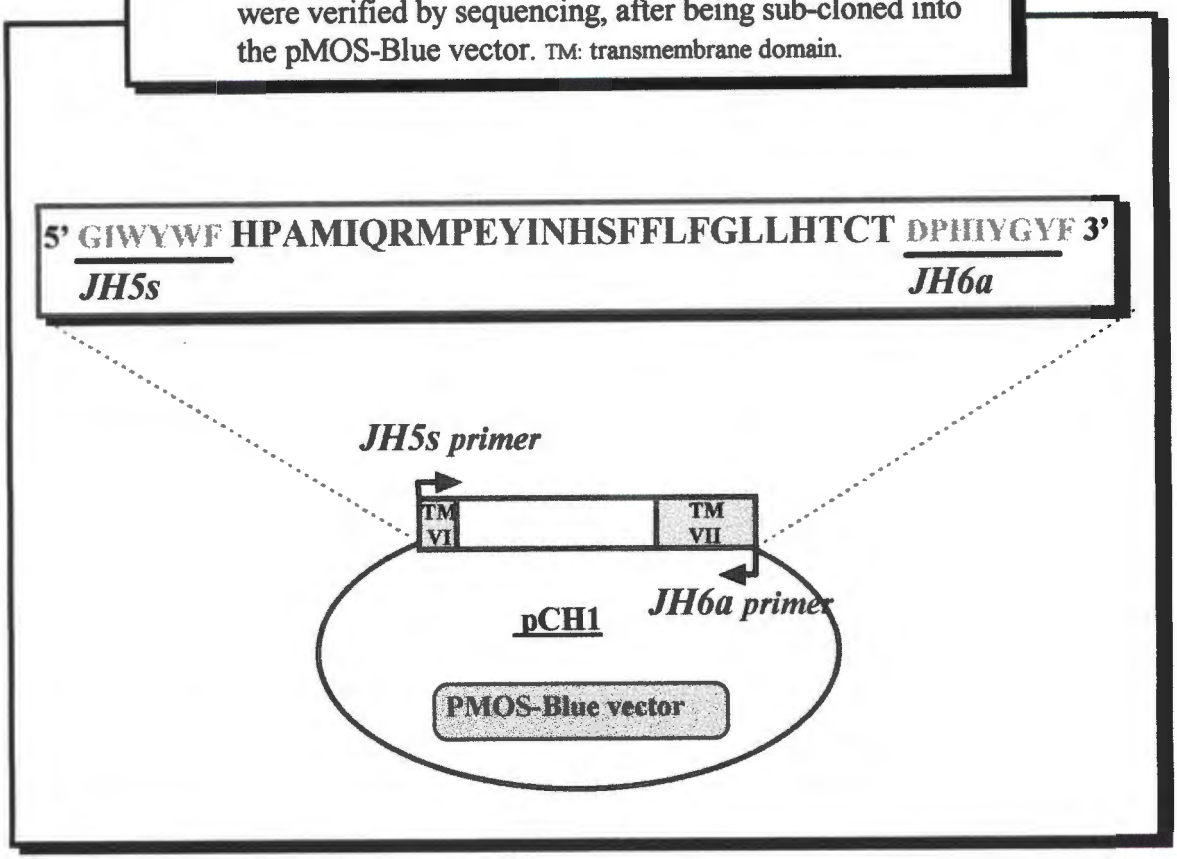


Fig. 3.5 ***One of three positives (shown in duplicate), chicken GnRH-R. genomic 22, hybridised with α-^{32}P-labelled pCH1 in 7.5x10^5 plaques primary screening.*** The autoradiographs were exposed for 4 days and orientated by marking "." ,"..", and "..." around the edge.

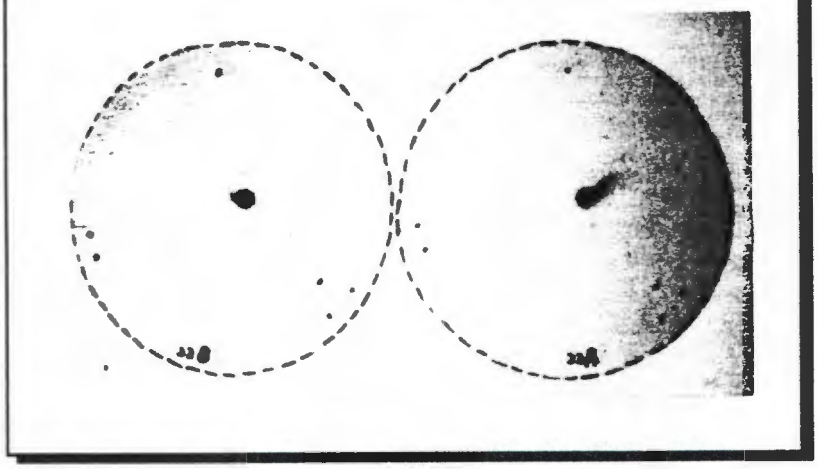
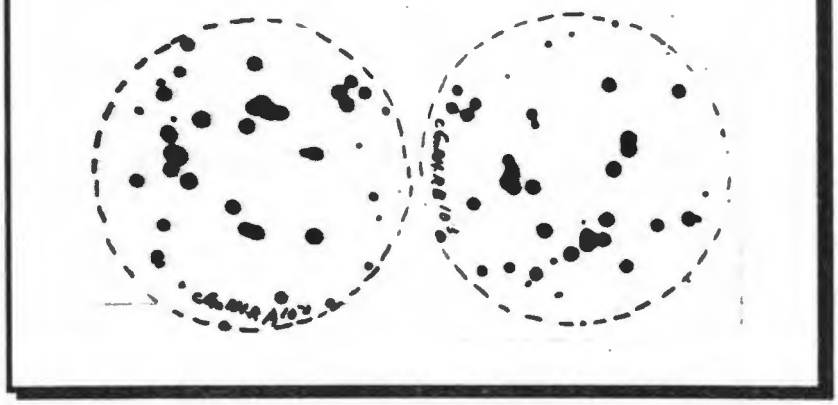


Fig. 3.6 ***Ample positives, hybridised with α-^{32}P-labelled pCH1 in the secondary screening (duplicate membranes) of the chicken GnRH-R.genomic 22.*** The autoradiographs were exposed for 5 hr and orientated by marking "." ,"..", and "..." around the edge.

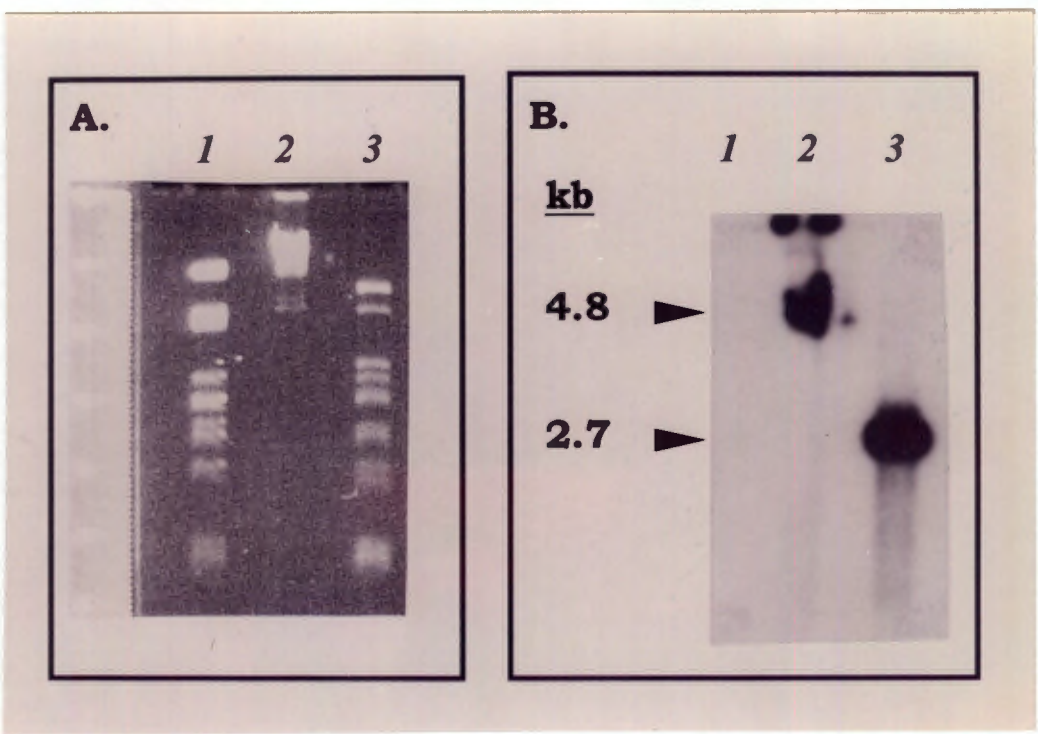


Five single plaques of the chicken GnRH-R.genomic 22 clone were isolated from the secondary screening and were amplified. 10 μg DNA each of the chicken GnRH-R.genomic 22 was digested with EcoR I and EcoR I/Pst I (Fig. 3.7, panel A), and the gel was subjected to Southern blot analysis by hybridising with α-^{32}P-labelled pCH1 that contained 3′-untranslated region of the chicken GnRH-R (Fig. 3.7, panel B). A 2.7 Kb EcoRI/Pst I (or 4.8 Kb EcoRI) digest was hybridised with the pCH1 probe, and sequenced after being subcloned into the pBluescript SK- vector (called pCH2) (Fig. 3.3).

Fig. 3.7 ***Southern blot analysis of the chicken GnRH-R.genomic 22 clone by hybridising with α^{32}P-labelled pCH1.***

(A) Electrophoresis of 10 μg each DNA of the chicken GnRH-R.genomic 22, digested with EcoR I or EcoR I/Pst I, on 1% agarose gel with a ruler at the edge of the gel.

(B) Autoradiograph of the Southern blot after overnight exposure to X-ray film. 4.8 and 2.7 Kb bands (judged by the ruler) of EcoR I and EcoR I/Pst I digested fragments, respectively, were hybridised with the probe.



The sequence data revealed that the pCH2 contained exon III, which encodes the half intracellular loop III to the C-terminal tail of the chicken GnRH-R, and part of exon II (Fig. 3.3 and 3.8). Four antisense primers, YS6, YS5, YS4, and YS3, were designed according to the sequence data of the exon 3. These four primers were used in the next strategies, 5′ RACE and nested PCR, to amplify the 5′-end region of the receptor (see the next section).

Additionally, the EcoR I/Pst I digests of the chicken GnRH-R.genomic 22 were subcloned into the pSK- vector (digested with EcoR I/ Pst I) and partially sequenced. A 0.8 kb EcoRI/Pst I digest (pCH3) contained a partial exon II (the fourth amino acid of N-terminus to half TM 4 of the chicken GnRH-R) (Fig. 3.3 and 3.9) Unfortunately, the pCH3 contained most of the N-terminal region but lacked an initiation codon (ATG), because the open reading frame was interrupted by an intron.

Fig. 3.8 ***The nucleotide and deduced amino acid sequences (in the antisense direction) of the pCH2 clone, subcloned into the pBluescript SK(-) vector digested with Pst I.*** The nucleotide sequences (capital black letters) and their deduced amino acid sequences (italic capital red letters) are displayed. Nucleotide sequences of the intron are in lowercase letters. TM : transmembrane domain [high-lighted by yellow colour, assigned according to those of the human receptor (Sealfon *et al.*, 1997)]. Four primers, YS6 to YS3, are indicated.

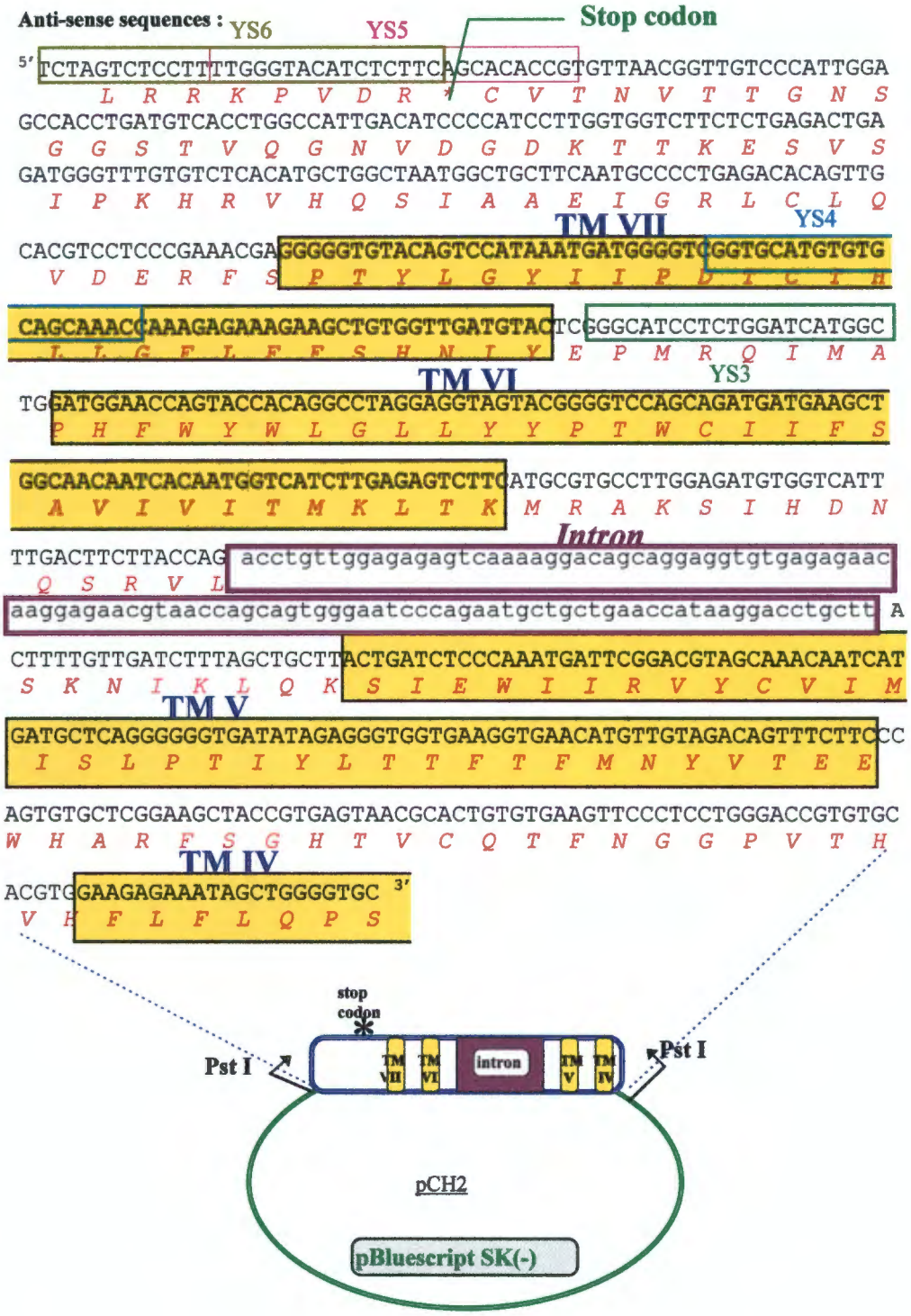
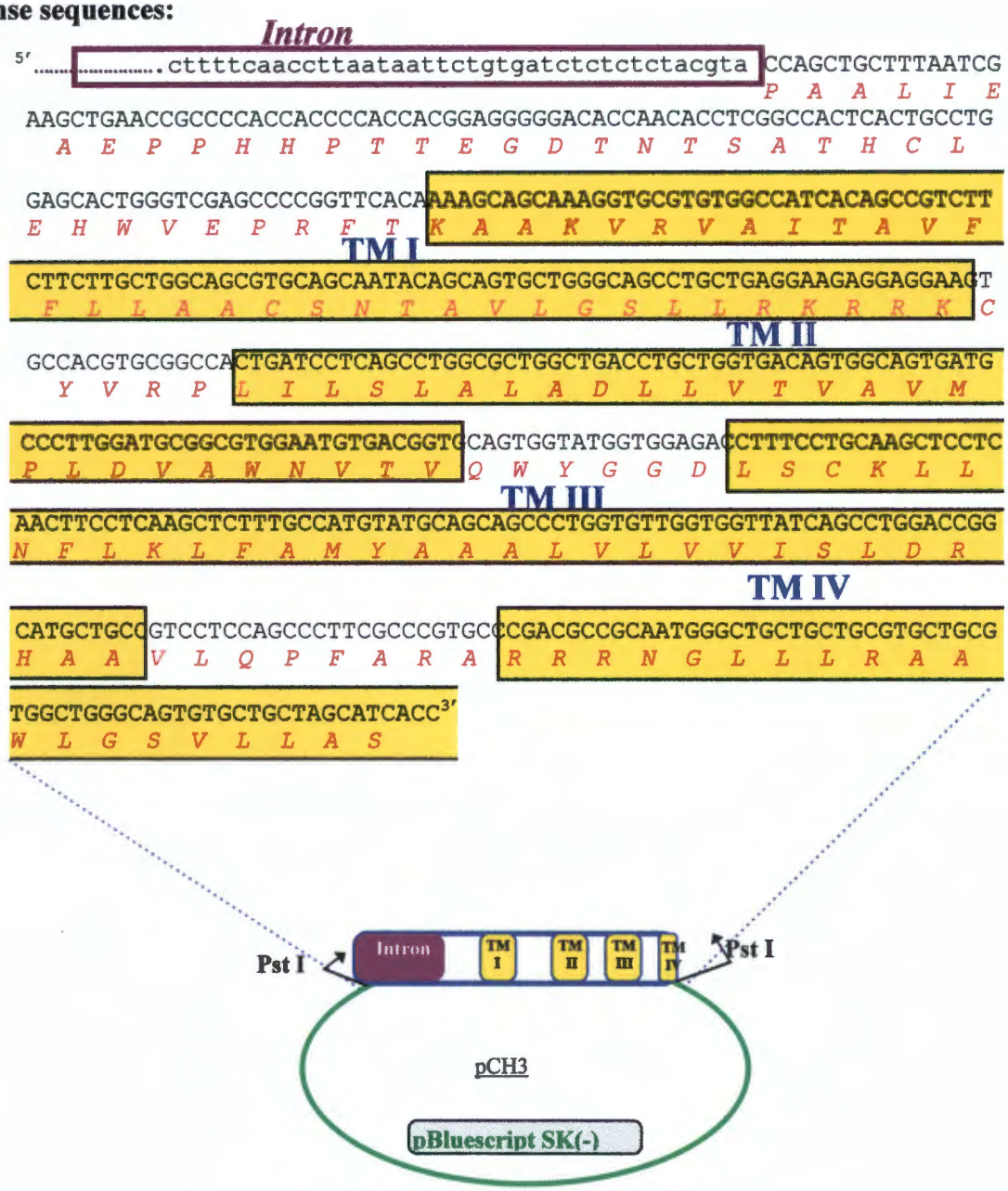


Fig. 3.9 ***Diagram of the pCH3, subcloned into the pBluescript SK(-) vector digested with Pst I.*** The nucleotide sequences (capital black letters) in the sense direction were identified by sequencing and their deduced amino acid sequences (italic capital red letters) are displayed below. Nucleotide sequences of the intron are in lower case letters. TM : transmembrane domain (high-lighted in yellow); …… : sequences not shown.

Sense sequences:



Cloning of the chicken GnRH-R cDNA :

In order to get further 5' end sequences of the chicken GnRH-R, the cDNAs of castrated chicken pituitary glands were synthesised by using a Marathon cDNA synthesis kit with a YS6 primer to initiate first-strand cDNAs. Then, the 5' end of the chicken receptor cDNA was amplified by using nested PCR with adapted primers and three gene specific antisense primers (i.e. AP1/YS5, AP2/YS4, and AP2/YS3 combinations). As the results show in Fig. 3.10, the PCR-product (panel A) was subjected to Southern blot analysis by means of hybridising with α-^{32}P- labelled pCH3 (panel B). The 0.8, 0.9, and 1.2 kb of PCR-products were hybridised α-^{32}P- labelled pCH3.

Interestingly, the positive bands 0.8, 0.9, and 1.2 kb were only produced in the PCR-products which were amplified from the first-run PCR products with primers AP_2 & YS4 or AP_2 & YS3 (Fig. 3.10), but not in that of the first-run PCR primed by nested primers AP_1 & YS5. As the first-run PCR products were amplified from the cDNAs synthesised by a gene specific primer (YS6), the finding indicated that the chicken GnRH-R mRNA is expressed at very low levels (see discussion section for the details).

The positive bands were subcloned into the pMOS-Blue vector. One positive clone (pCH4) (Fig. 3.3) was isolated from 100 colonies by colony screening, probed with α-^{32}P-labelled pCH3 (Fig. 3.11), and its sequences were further confirmed by sequencing (Fig. 3.12). Two sense primers, YS1 and YS2, were designed according to the 5' end sequences of the pCH3 (Fig. 3.12).

Fig. 3.10 ***Analysis of the PCR-products amplified by nested PCR from the cDNAs of castrated chicken pituitary glands.***

(A) Electrophoresis of the PCR-products on 1 % agarose gel. Lane 1: λ marker (Pst I); Lane 2: primed by adapted primer 1(AP_1) and YS5; Lane 3: AP_2 and YS4; Lane 4: adapted primer 2 and YS3.

(B) Southern blot analysis of the PCR-products hybridised with α-^{32}P-labelled exon II of the chicken GnRH-R gene, after 2 hr-exposure to X-ray film. Positive bands of 1.2, 0.9, and 0.8 Kb of the nested PCR-product, primed by AP_2 and YS4 or YS3, were indicated by the sizes. Interestingly, those bands were not detected in the nested PCR product, primed by AP_1 & YS5.

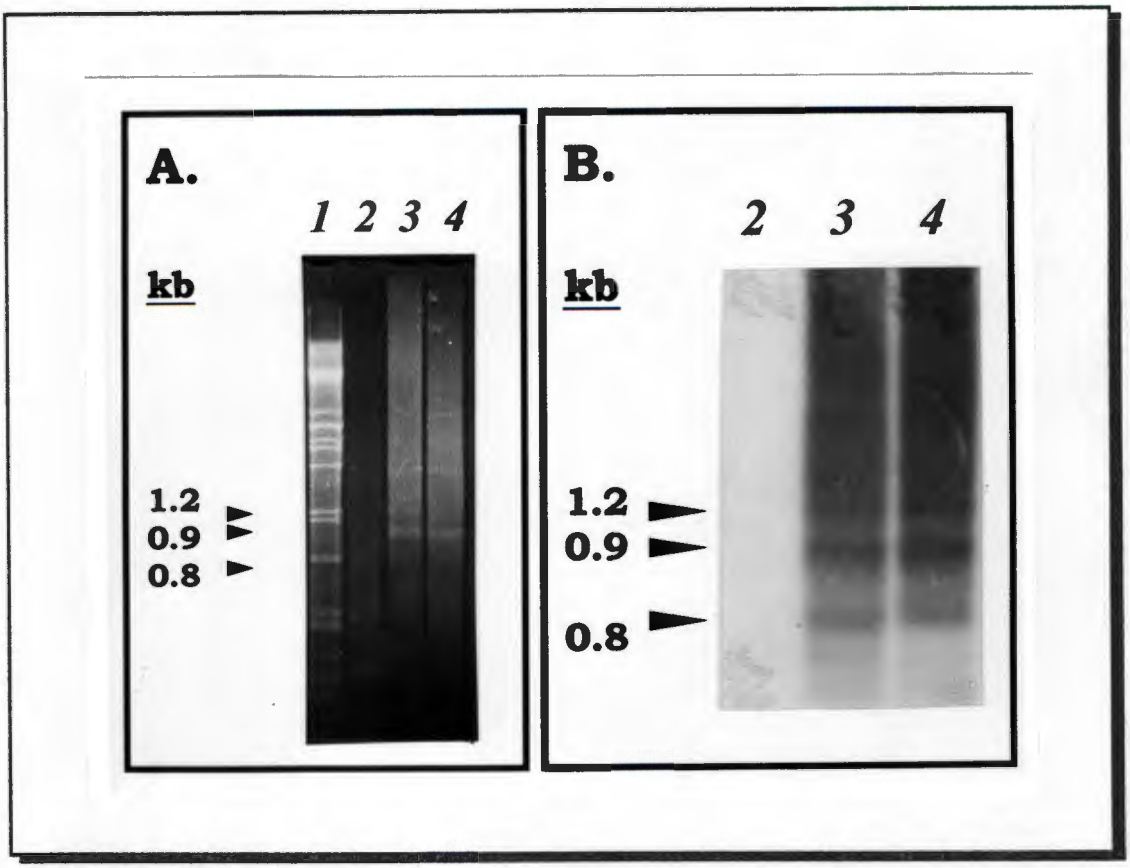


Fig. 3.11 ***Colony screening of the 0.8, 0.9, and 1.2 Kb bands of the nested PCR-products, subcloned into the pMOS-Blue vector.*** One positive colony (in duplicate) was hybridised with α-^{32}P-labelled pCH3 (exon 2).

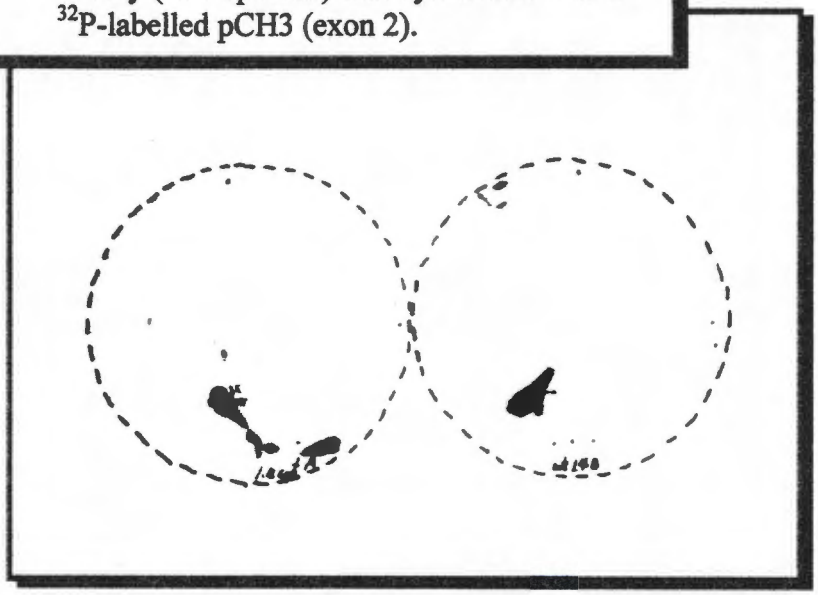
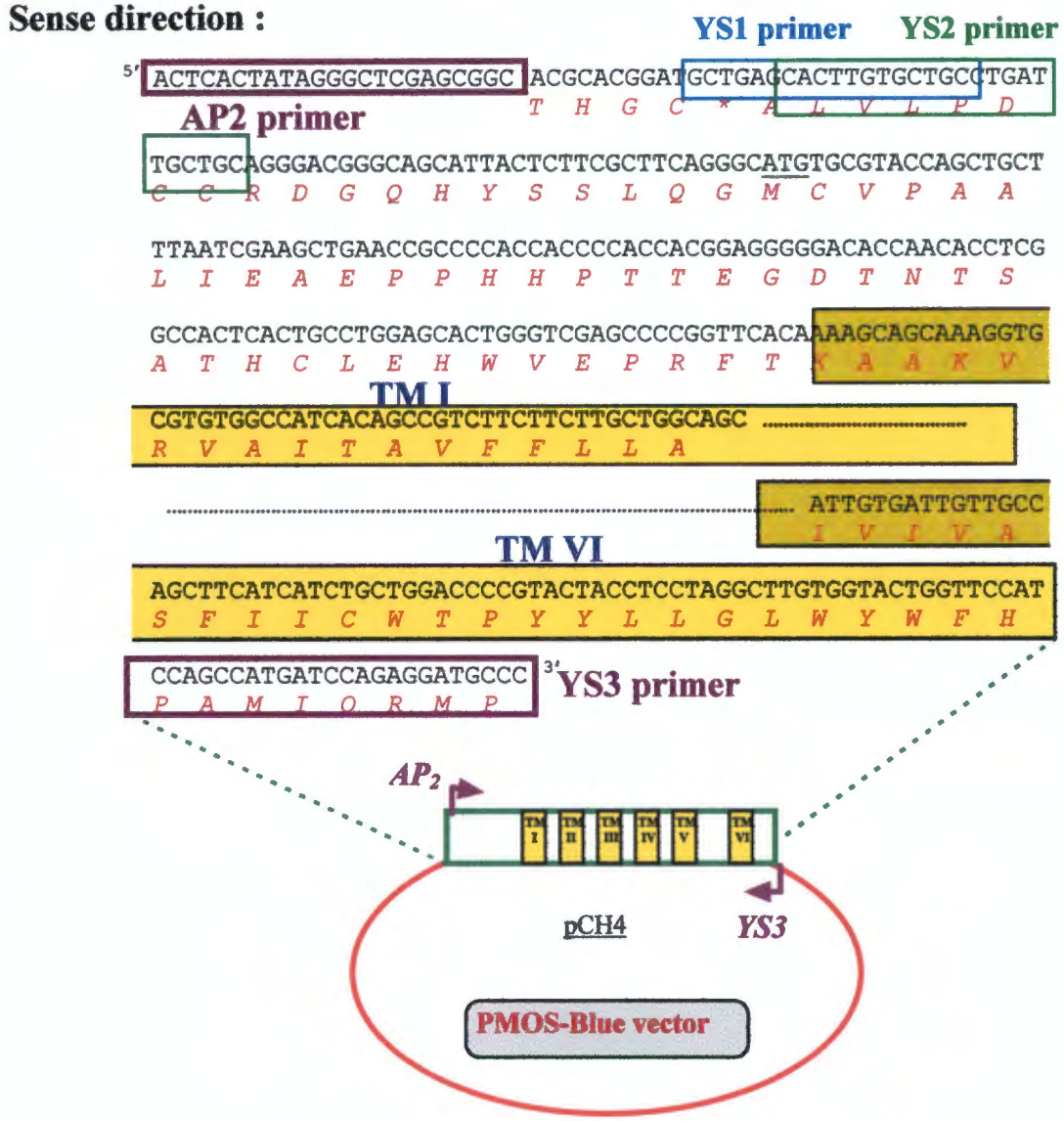


Fig. 3.12 ***Schematic representation of the pCH4, amplified by PCR with primers AP2 (adapted primer 2) & YS3 from the PCR-products of the AP2/YS4 amplification and subcloned into the pMOS-Blue vector.*** Its partial nucleotide sequences of the 5' end and the 3' end (capital black letters) were identified by sequencing (see top row) with deduced amino acid sequences (italic capital red letters). Two primers, YS1 and YS2, were designed according to the sequences of the 5' end un-translated region. An initiate codon (ATG) is underlined. TM : transmembrane domain (highlighted in yellow); …… : sequences not shown. "*" : stop codon.



The full-length chicken GnRH-R cDNA was amplified by nested PCR with primers YS1 & YS6 and YS2 & YS5 for the first and the second run PCR, respectively. The PCR-products were subcloned into the pMOS-Blue vector. Two clones, pCH5 and pCH6, were isolated. It was confirmed by sequencing that they contained an entire open reading frame of the chicken GnRH-R (Fig. 3.3 and 3.13). A comparison of the nucleotide sequence of these clones relative to the those of the genomic clones, identified a number of differences that might have arisen during the PCR reaction or represent polymorphism. The pCH5 contained an amino acid substitution of Arg^{189} compared to Cys^{189} of the genomic clone. The CH6 showed nucleotide substitutions resulting in amino acid substitutions of Gln^{40} for Lys^{40}, and Tyr^{75} for His^{75} compared with the genomic sequences. Both clones showed a substitution of Arg^{338} for Gln^{338} (Fig. 3.13). As it is uncertain whether these differences represent polymorphism or PCR errors, three different cDNA fragments from pCH5, pCH6, and pCH2, were ligated together to reconstruct a cDNA encoding identical amino acid sequences to those of the genomic clone (Fig. 3.14). The Xba I/Sph I fragment from the pCH5 was ligated to the Sph I/EcoR I fragment of the pCH6, eliminating the amino acid substitutions at positions 40, 75, and 189. In order to eliminate the substitution at position 338, the Not I/Ava I fragment from this chimerical clone was ligated to the Ava I/Pst I fragment from pCH2, and subcloned into the pSK vector (called cGnRH-R.W or pCH7) (Fig. 3.3).

The Not I/Xho I fragment of the cGnRH-R.W was sub-cloned into the mammalian expression vector pcDNA I/AMP (Invitrogen), digested with Not I/Xho I. This chicken GnRH-R/pcDNA amp subclone (cGnRH-R subclone) was sequenced in both directions using automated sequencing (Bio-Rad), and it was verified that encoded amino acid sequences are identical to those of the genomic clone.

The deduced amino acid sequences from the cGnRH-R.W clone are shown in Fig. 3.15, including those of the human and catfish GnRH-Rs. The details of comparative amino acid sequences will focus on different features of the chicken GnRH-R from the human (or mammalian) homologue, rather than from the catfish receptor, because there are distinct pharmacological properties between both receptors (see "receptor binding" and "inositol phosphate formation" sections).

Fig. 3.13 ***Schematic diagram of two full-length chicken GnRH-R cDNA clones, pCH5 and pCH6, subcloned into the pMOS-Blue vector.*** The various deduced amino acids are shown in italic capital letters with a positional number, and shown in pink colour where they differ from genomic sequences, with the genomic type in parenthesis. ORF : open reading frame; TM: transmembrane.

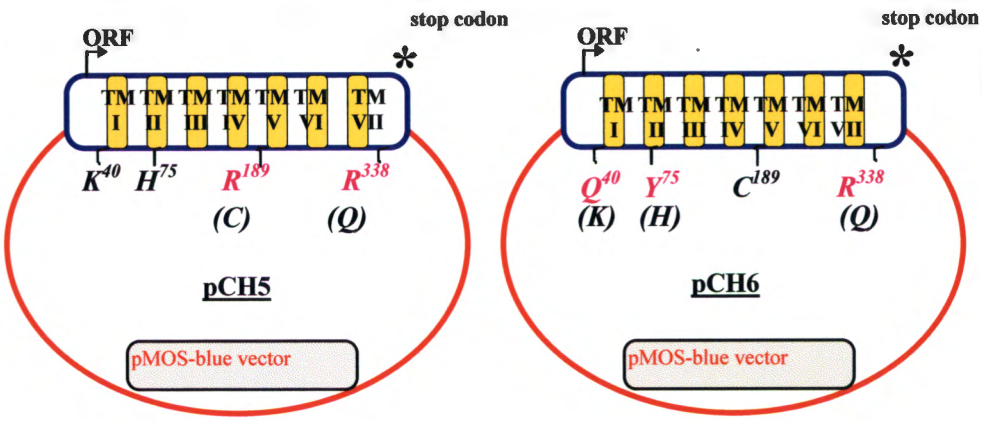
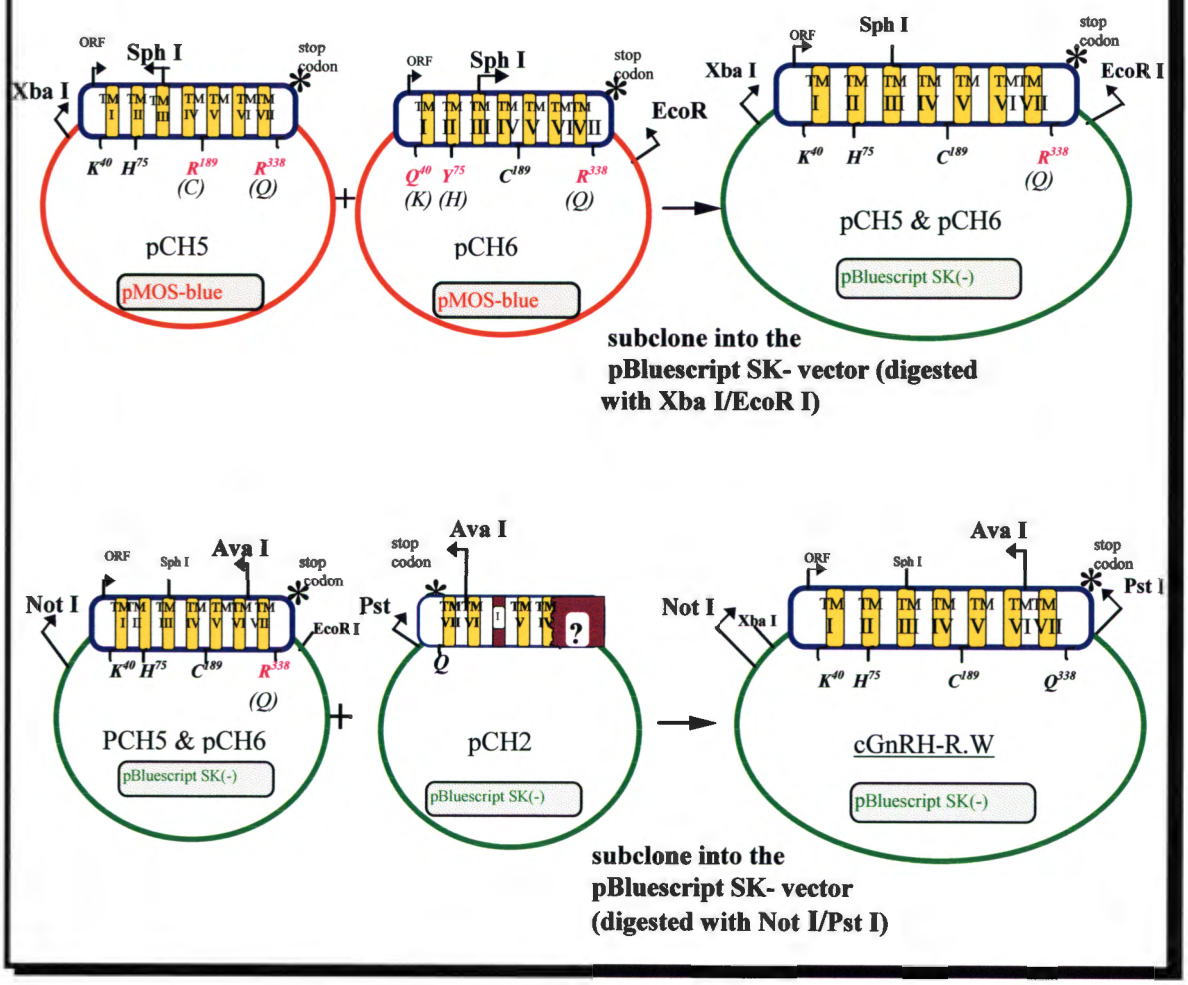


Fig. 3.14 ***Schematic diagram of constructing a wild-type cGnRH receptor cDNA.***

A chimerical clone was created by ligating two fragments, the Xba I/Sph I fragment of the pCH5 and the Sph I/EcoR I fragment of the pCH6, together. The wild-type cGnRH-R clone (cGnRH-R.W) was constructed by subcloning the Not I/Ava I fragment of the pCH5&pCH6 and the Ava I/Pst I fragment of the pCH2 into the Not I/Pst I digested pSK- vector. ORF: open reading frame; TM: transmembrane domain; I: intron; the mutated sequences (pink); the wild-type sequences (black). PMOS-Blue: a vector; "?" : un-identified region.



Comparison of structural features between the chicken and mammalian GnRH-Rs

There is a strikingly different appearance of the chicken GnRH-R from the mammalian homologues, in that it contains the C-terminal tail that is also present in the catfish GnRH-R (Tensen et al., 1997) (Fig. 3.15). Compared with the amino acid sequences of the human receptor, the chicken receptor only shares 39 % identity, excluding the C-terminal tail (Fig. 3.15). The N-terminus and intracellular loops (IL) are more divergent, while having a comparatively higher level of amino acid sequence identity within the transmembrane domains (TM) (more than 40 %, except the TM_4 , only 33 %) and the extracellular loops (EL). Moreover, based on a survey of conserved residues in a large number of G-protein-coupled receptor sequences, an arrangement of the transmembrane helices has been proposed in which TM 7 is believed to lie adjacent to TM 2, and TM 6 is adjacent to TM 3. In the amino acid sequence comparison, it appears that the percentage of amino acid identity between TM 2 and TM 7 as well as between TM 3 and TM 6 are similar (46 % vs. 52 %, and 65 % vs. 66 %, respectively). Intriguingly, the chicken GnRH-R consists of a longer N-terminus (7 amino acids longer), an intracellular C-terminal tail, and a short IL 1 (comprising only 6 amino acids) (Fig. 3.15).

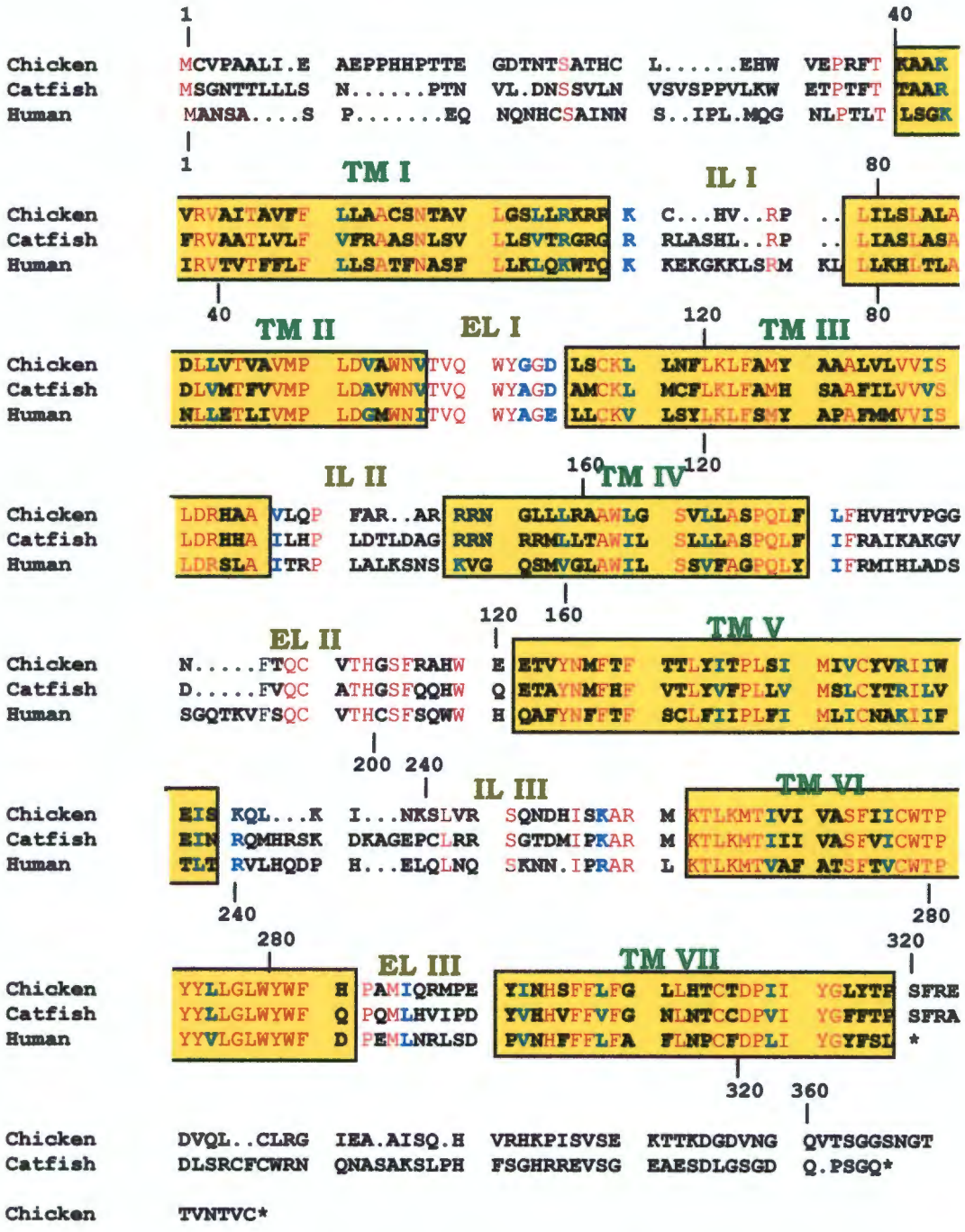
The chicken GnRH-R contains three potential sites for N-linked glycosylation (Ans-X-Ser/Thr)(Fig. 3.15). Besides two located in the N-terminus and the C-terminus of TM 2, which are the same as the mammalian counterparts, another was found within EL 2. Furthermore, most GPCRs contain a number of conserved cysteins (Cys), some of which appear to play a role in maintaining the active conformation of the receptor. There are five Cys conserved in the chicken and human receptors, which are located at TM 3, EL 2, TM 5, TM 6, and TM 7, but an additional highly conserved Cys among GPCRs was found in the C-terminal tail of the chicken receptor. Interestingly, two highly conserved GPCR residues are Asp in TM 2 and Asn in TM 7, while it appears to be interchanged at these loci in mammalian GnRH-Rs (i.e. TM 2 Asn and TM 7 Asp). Surprisingly, they are TM 2 Asp and TM 7 Asp in the chicken, also in the catfish receptor and other non-mammalian vertebrates (frog and goldfish, unpublished data). The 'DRY' (Asp-Arg-Tyr) motif in the end of TM 3 is conserved among the GPCRs and appears to play a role in the coupling of the receptors to G-proteins. While a Ser replaces the Tyr residue in the 'DRY' motif in mammalian GnRH-Rs, a His instead of Tyr in the chicken and in the catfish receptor and other non-mammalian vertebrates (unpublished data). More details of the comparative structure will be addressed in the discussion section.

A)

```
atg tgc gta cca gct gct tta atc gaa gct gaa ccg ccc cac cac ccc acc
 M   C   V   P   A   A   L   I   E   A   E   P   P   H   H   P   T
acg gag ggg gac acc aac acc tcg gcc act cac tgc ctg gag cac tgg gtc
 T   E   G   D   T   N   T   S   A   T   H   C   L   E   H   W   V
gag ccc cgg ttc aca aaa gca gca aag gtg cgt gtg gcc atc aca gcc gtc
 E   P   R   F   T   K   A   A   K   V   R   V   A   I   T   A   V
ttc ttc ttg ctg gca gcg tgc agc aat aca gca gtg ctg ggc agc ctg ctg
 F   F   L   L   A   A   C   S   N   T   A   V   L   G   S   L   L
agg aag agg agg aag tgc cac gtg cgg cca ctg atc ctc agc ctg gcg ctg
 R   K   R   R   K   C   H   V   R   P   L   I   L   S   L   A   L
gct gac ctg ctg gtg aca gtg gca gtg atg ccc ttg gat gcg gcg tgg aat
 A   D   L   L   V   T   V   A   V   M   P   L   D   A   A   W   N
gtg acg gtg cag tgg tat ggt gga gac ctt tcc tgc aag ctc ctc aac ttc
 V   T   V   Q   W   Y   G   G   D   L   S   C   K   L   L   N   F
ctc aag ctc ttt gcc atg tat gca gca gcc ctg gtg ctg gtg gtt atc agc
 L   K   L   F   A   M   Y   A   A   A   L   V   L   V   V   I   S
ctg gac cgg cat gct gcc gtc ctc cag ccc ttc gcc cgt gcc cga cgc cgc
 L   D   R   H   A   A   V   L   Q   P   F   A   R   A   R   R   R
aat ggg ctg ctg ctg cgt gct gca tgg ctg ggc agt gtg ctg cta gca tca
 N   G   L   L   L   R   A   A   W   L   G   S   V   L   L   A   S
ccc cag cta ttt ctc ttc cac gtg cac acg gtc cca gga ggg aac ttc aca
 P   Q   L   F   L   F   H   V   H   T   V   P   G   G   N   F   T
cag tgc gtt act cac ggt agc ttc cga gca cac tgg gaa gaa act gtc tac
 Q   C   V   T   H   G   S   F   R   A   H   W   E   E   T   V   Y
aac atg ttc acc ttc acc acc ctc tat atc acc ccc ctg agc atc atg att
 N   M   F   T   F   T   T   L   Y   I   T   P   L   S   I   M   I
gtt tgc tac gtc cga atc att tgg gag atc agt aag cag cta aag atc aac
 V   C   Y   V   R   I   I   W   E   I   S   K   Q   L   K   I   N
aaa agt ctg gta aga agt caa aat gac cac atc tcc aag gca cgc atg aag
 K   S   L   V   R   S   Q   N   D   H   I   S   K   A   R   M   K
act ctc aag atg acc att gtg att gtt gcc agc ttc atc atc tgc tgg acc
 T   L   K   M   T   I   V   I   V   A   S   F   I   I   C   W   T
ccg tac tac ctc cta ggc ttg tgg tac tgg ttc cat cca gcc atg atc cag
 P   Y   Y   L   L   G   L   W   Y   W   F   H   P   A   M   I   Q
agg atg ccc gag tac atc aac cac agc ttc ttt ctc ttt ggt ttg ctg cac
 R   M   P   E   Y   I   N   H   S   F   F   L   F   G   L   L   H
aca tgc acc gac ccc atc att tat gga ctg tac acc ccc tcg ttt cgg gag
 T   C   T   D   P   I   I   Y   G   L   Y   T   P   S   F   R   E
gac gtg caa ctg tgt ctc agg ggc att gaa gca gcc att agc cag cat gtg
 D   V   Q   L   C   L   R   G   I   E   A   A   I   S   Q   H   V
aga cac aaa ccc atc tca gtc tca gag aag acc acc aag gat ggg gat gtc
 R   H   K   P   I   S   V   S   E   K   T   T   K   D   G   D   V
aat ggc cag gtg aca tca ggt ggc tcc aat ggg aca acc gtt aac acg gtg
 N   G   Q   V   T   S   G   G   S   N   G   T   T   V   N   T   V
tgc tga
 C   *
```

Fig. 3.15 A) ***The nucleotide and amino acid sequences of the chicken GnRH-R***
B) Alignment of the deduced amino acid sequences with the chicken, catfish, and human GnRH receptors. The transmembrane domains (TM)(yellow shaded regions) are assigned according to those of the human receptor (Sealfon et al., 1997). Identical sequences amongst three species are shown in red letters, and conservative changes are shown in blue letters. IL: intra-cellular loop, EL: extra-cellular loop, "....": pace, "*": stop codon, and the numbers: positions of amino acids (top and bottom one for the chicken and human receptors, respectively)

B)


TM I
IL I
TM II
EL I
TM III
IL II
TM IV
EL II
TM V
IL III
TM VI
EL III
TM VII
Chicken
Catfish
Human

Whole cell radio-ligand binding assay

Agonist study:

The binding affinities of agonists were determined by competing ^{125}I-GnRH-A (Ki : 3.0 ± 0.3 nM and 1.5 ± 0.4 nM for the chicken and human receptors, respectively) with increasing doses of cold agonists in the COS-1 cells expressing the chicken receptor cDNA subclones or the human receptor subclone. The cGnRH I exhibited no significant difference in binding affinity for chicken GnRH receptor subclones (pCH5 and pCH6) and for the cGnRH-R.W subclone (data not shown), indicating that the pCH5 and pCH6 are functional transcripts; even though, they possess several amino acid sequences differing from genomic sequences of the chicken receptor.

The cGnRH-R.W were further characterised by employing several GnRH agonists to measure their binding affinities for the receptor. The dose response curves of the competition binding for the chicken and human GnRH receptors by agonists are shown in Fig. 3.16 and the affinities in Table 3.3.

In the chicken receptor (Fig. 3.16, panel A; Table 3.3), the binding affinities of cGnRH II and [D-Arg^6]cGnRH II presented 11-fold and 15-fold, respectively, higher than that of cGnRH I. There was no significant difference between cGnRH I and mGnRH, nor between cGnRH II and [D-Arg^6] cGnRH II. On the other hand, the human receptor (Fig. 3.16, panel B; Table 3.3) presented a different ligand-selectivity. The mGnRH (K*i*: 4.8 nM) had a 3-fold and 10-fold higher affinity than cGnRH II and [D-Arg^6]cGnRH II, respectively, for the human receptor. The affinity of cGnRH I exhibited 40-fold less than that of mGnRH for the human receptor.

Antagonist study:

The binding affinity of antagonists was measured by means of competing ^{125}I-GnRH-A with increasing doses of cold antagonists on the COS-1 cells expressing the chicken and human GnRH receptors. The reason that the ^{125}I-GnRH-Agonist was used to measure antagonist binding instead of ^{125}I-GnRH-Antagonist, is due to low affinities of the selected antagonists for the chicken receptor. The affinity of the antagonists for the chicken receptor was similar to the background level (data not shown).

In general, the affinity of six selected antagonists for the chicken receptor presented 10-fold less than those for the human receptor in each individual antagonist (Fig. 3.17, Table

3.3). In the chicken receptor (Fig. 3.17; panel A; Table 3.3), the antagonist 27 was found to be the most potent peptide amongst the selected antagonists. Antagonists 134-46 and 134-53 showed an un-distinguishable affinity for the chicken receptor. In addition, antagonists 135-18 and 135-25 showed very low affinity for the chicken receptor. Interestingly, the antagonists 135-18 and 135-25 are full and partial agonist for the chicken receptor, respectively (see IP assay described below).

In the human receptor (Fig. 3.17; panel B; Table 3.3), the antagonists (with the exception of antagonists 135-25 and 135-18) showed a similar affinity for the human receptor. They all acted as full antagonists.

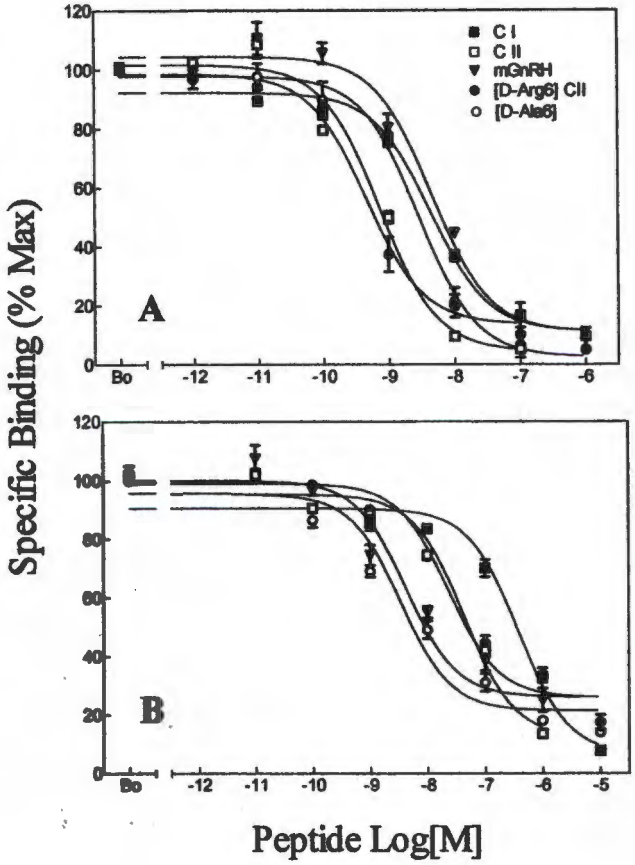


Fig. 3.16 ***Displacement of ^{125}I-[D-Ala^6, N-Me-Leu^7, Pro^{9}NHEt]GnRH (^{125}I-GnRH-A) by GnRH agonists in COS-1 cells expressing the chicken (A) and human (B) GnRH receptors.*** Binding of ^{125}I-GnRH-A in the presence of various concentrations of chicken GnRH I (C I), chicken GnRH II (C II), mammalian GnRH (mGnRH), [D-Arg^6] cGnRH II ([D-Arg^6] CII), and GnRH-A ([D-Ala]) was measured by whole-cell binding assay. Data represents the mean ± S.E. of two to three experiments performed in triplicate. (The binding of cGnRH I on the hGnRH-R was performed only once.)

Table 3.3

Receptor binding affinity and stimulation (or inhibition) of inositol phosphate production by GnRH analogues in COS-1 cells expressing chicken and human GnRH receptors

Analogue	Receptor Binding (K_i) (nM)		IP assay (ED_{50}) (nM)		Efficiency (K_i/ED_{50})	
	cGnRH-R	hGnRH-R	cGnRH-R	hGnRH-R	cGnRH-R	hGnRH-R
Agonist:						
cGnRH I	5.3 ± 0.5 [a] (1.0)	302.6 (0.02)	2.7 ± 0.6 (1.0)	0.9 ± 0.1 (0.02)	2	336
cGnRH II	0.6 ± 0.01 (11.0)	39.1 ± 8.5 (0.1)	0.04 ± 0.001 (63.2)	0.5 ± 0.2 (0.04)	15	78
mGnRH	4.1 ± 1.2 (1.6)	4.8 ± 1.2 (1.0)	0.9 ± 0.02 (3.0)	0.02 ± 0.005 (1.0)	5	240
[D-Arg^6]cGnRH II	0.6 ± 0.09 (15.1)	16.3 ± 2.5 (0.3)	0.02 ± 0.0002 (126.1)	0.3 ± 0.03 (0.07)	30	54
[D-Ala^6]GnRH	3.0 ± 0.3 (2.3)	1.5 ± 0.4 (3.2)		0.09 ± 0.02 (0.2)		17
Antagonist :						
A27	61.1± 1.3 (0.09)	4.1 ± 0.9 (1.2)	176.7 ± 43[b] (0.02)	5.9± 0.7[b] (0.003)	0.3	0.7
A134-46	310.7 ± 24.1 (0.02)	7.0 ± 1.7 (0.7)	27.3 ± 3.7[b] (0.1)	1.7 ± 0.1[b] (0.01)	11	4
A134-53	312.5 ± 19.2 (0.02)	8.1 ± 2.1 (0.6)	27.5 ± 2.1[b] (0.1)	1.8 ± 0.5[b] (0.01)	11	5
A26	125.6 ± 2.4 (0.04)	3.8 ± 0.7 (1.3)	9.7 ± 0.2[c] 79 ± 3.8[b] (0.3[c]/0.03[b])	3.8 ± 0.2[b] (0.005)	13[c]/2[b]	1
A135-25	4902.3 ± 378.2 (0.001)	55.6 ± 5.3 (0.09)	1325 ± 71[c] 4095 ± 140[b] (0.002[c]/0.001[b])	15.5 ± 1.2[b] (0.001)	4[c]/1[b]	4
A135-18	703.4 ± 153 (0.007)	22.8 ± 7.4 (0.2)	32.9 ± 2.1[c] (0.08)	2.0 ± 0.8[b] (0.03)	21	11

[a]: Mean ± S.E.M. of two to four experiments. Relative potencies, calculated as the ratio of *Ki* (binding) or ED_{50} values (IP production) of GnRH analogue relative to that of cGnRH I or mGnRH are shown in brackets.

[b]: the EC_{50} values of antagonism in competing with 1 nM cGnRH I or mGnRH for the chicken or the human GnRH receptors, respectively.

[c]: the EC_{50} values of agonism.

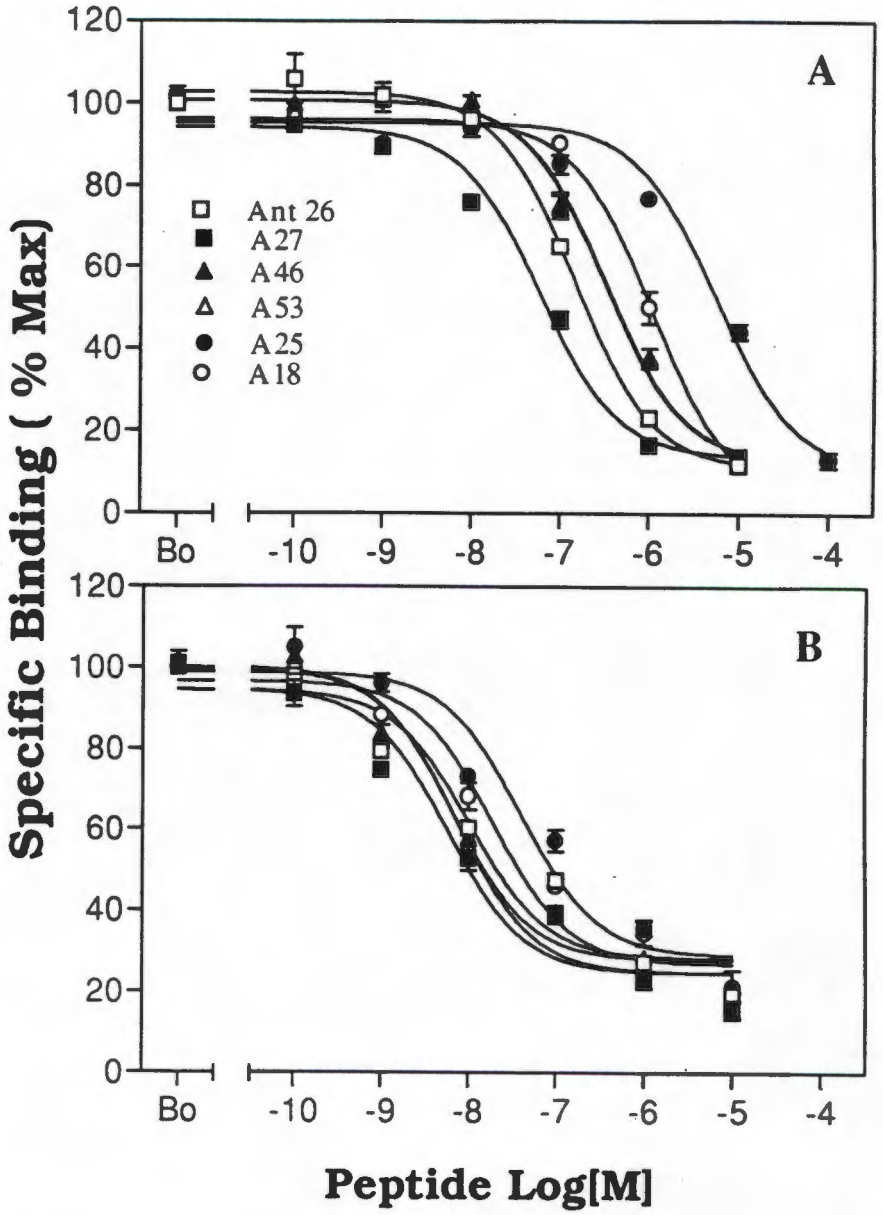


Fig. 3.17 ***Displacement of ^{125}I-[D-Ala^6, N-Me-Leu^7, Pro^{9}NHEt]GnRH (^{125}I-GnRH-A) by GnRH antagonists on COS-1 cells expressing the chicken (A) and human (B) GnRH receptors.*** Binding of ^{125}I-GnRH-A in the presence of various concentrations of antagonists 27 (A 27), 26 (A 26), 134-53 (A53), 134-46 (A46), 135-25 (A25), and 135-18 (A18) was measured by whole-cell binding assay. The experiment was performed once, in triplicate.

Inositol phosphate formation assay

Three subclones, cGnRH-R.W (pCH7), pCH5 and pCH6, were functional constructs confirmed by IP production assay (data not shown). The pharmacological properties of the cGnRH-R.W subclone (cGnRH-R cDNA) were further characterised by applying different GnRH agonists and antagonists to measure their activities on stimulating IP formation after being expressed in COS-1 cells.

Agonist study:

In the chicken receptor (Table 3.3; Fig. 3.18, panel A), cGnRH II and [D-Arg^6]cGnRH II generated profound differences in IP formation from those of cGnRH I and mGnRH , as compared with the results of receptor binding. [D-Arg^6] cGnRH II was the most potent peptide in IP formation: 2-, 42-, and 126-fold higher than cGnRH II, mGnRH, and cGnRH I, respectively. cGnRH II was the second most potent peptide: 20- and 63-fold higher than mGnRH and cGnRH I, respectively. However, cGnRH I and mGnRH both exhibited similar potency in this experiment. On the other hand, in the human receptor (Table 3.3; Fig. 3.18, panel B), mGnRH was the most potent peptide in stimulating IP production, followed by [D-Arg^6]cGnRH II and cGnRH II. cGnRH I was the least potent peptide to stimulate IP production in the human receptor.

Antagonist study:

The antagonist study was performed by measuring IP production at various doses of antagonists in the presence or absence of cGnRH I (1nM or 10 nM) and mGnRH (1nM), respectively, in COS-1 cells expressing the chicken and human receptors. Antagonists 134-53, 134-46, and 27 presented full antagonisms in both chicken and human receptors (Fig. 3.19). Antagonists 134-46 and 134-53 showed a 15-fold more potent effect in human than in chicken, while antagonist 27 exhibited 30-fold more potency in human (Table 3.3). Intriguingly, antagonists 135-18, 135-25, and 26, which have D-Lysine in position 6, exerted agonist effects in the chicken receptor (Fig. 3.20, left panel). The maximal stimulation mediated by antagonist 135-18 was equivalent to one obtained by cGnRH I, despite the agonisim being only 8 % as potent as cGnRH I (Table 3.3). Nevertheless, this antagonist did not show any antagonism in the chicken receptor, while assessed by IP production in the presence of 1 nM cGnRH I (Fig. 3.20, left panel). The results suggested

that antagonist 135-18 is a full agonist in chicken receptor. Antagonists 135-25 and 26 acted as partial agonists in the chicken receptor, by exhibiting 40 % and 15 % of maximal stimulation by cGnRH I, respectively (Fig. 3.20, left panel). In contrast, the three antagonists all showed full antagonism in human (Fig. 3.20, right panel).

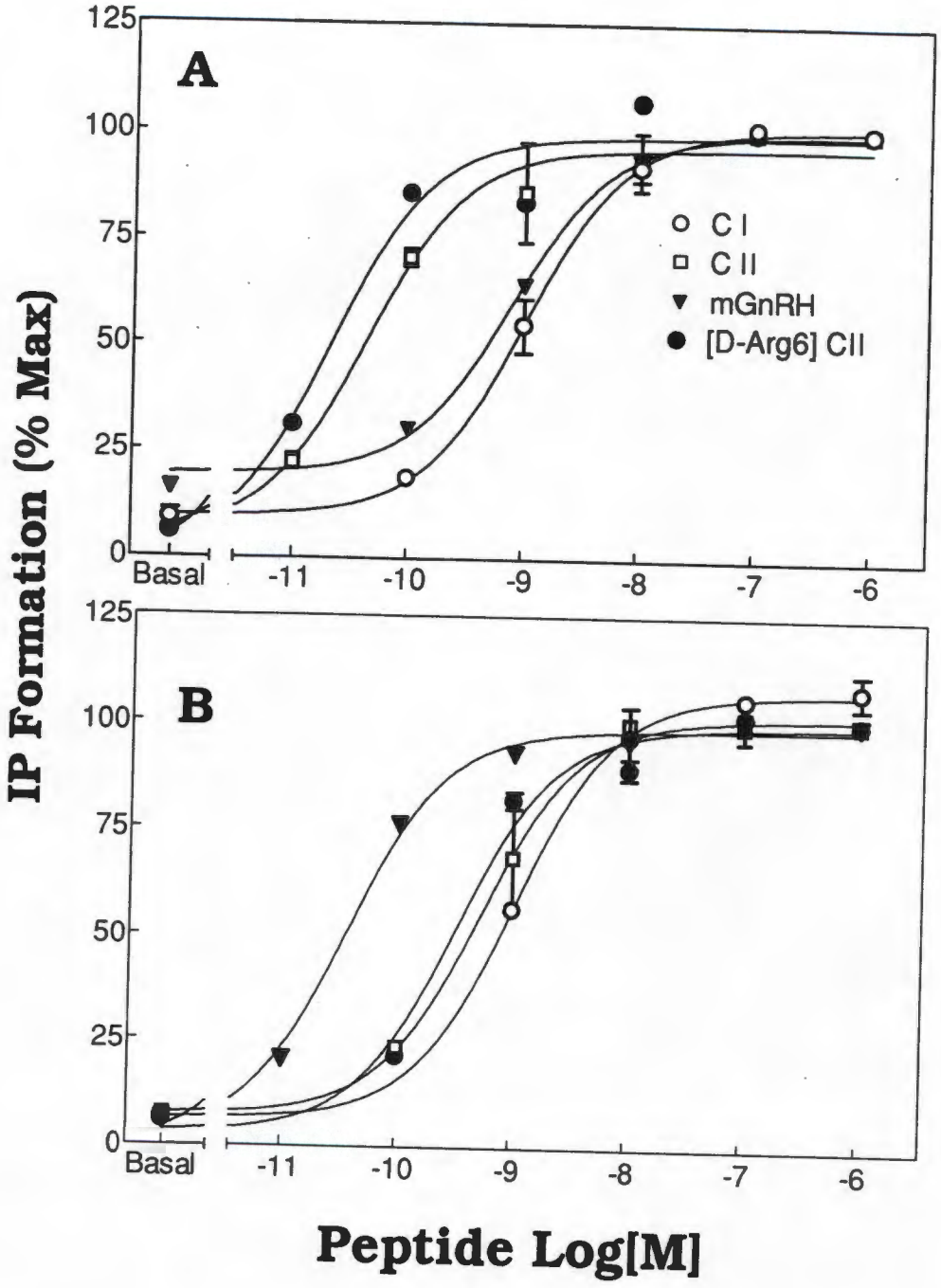


Fig. 3.18 ***Dose-response curves for inositol phosphate production stimulated by GnRH agonists in COS-1 cells expressing the chicken (A) and human (B) GnRH receptors.*** Peptides are chicken GnRH I (CI), chicken GnRH II (CII), mammalian GnRH (mGnRH), and [D-Arg^6] chicken GnRH II ([D-Arg^6] CII). Data is shown as mean ± S.E.. Each curve is representative of three to four experiments performed in duplicate.

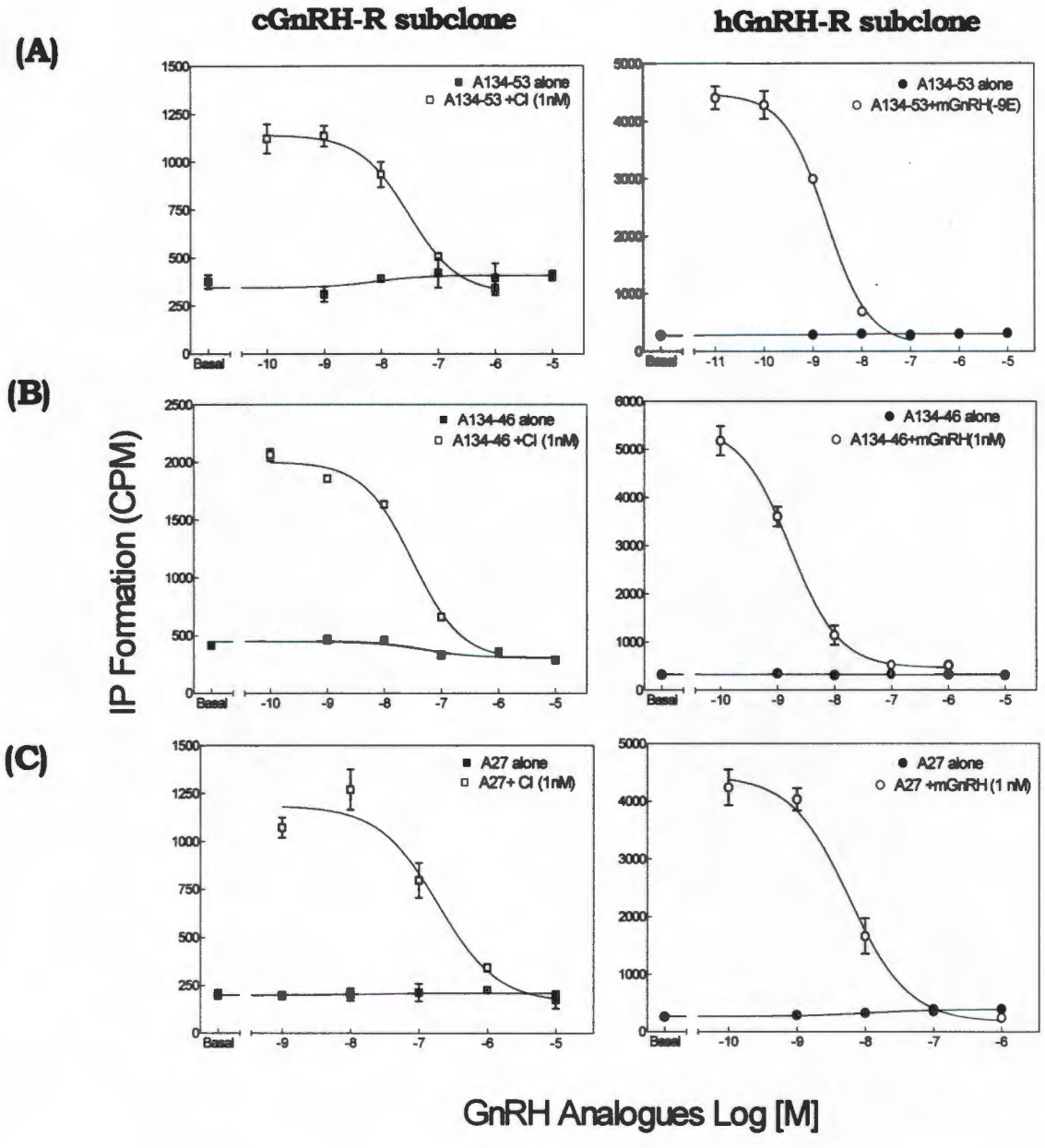


Fig. 3.19 ***Effects of antagonists on inositol phosphate production in the COS-1 cells expressing the chicken and human GnRH receptors.*** IP production was measured in the presence of various concentrations of antagonists, i.e., (A) A134-53, (B) A134-46, and (C) A27, with 1 nM of cGnRH I or without, and with 1 nM mGnRH or without in COS-1 cells transfected with chicken and human GnRH-R cDNAs, respectively. Data are the means of three or four experiments performed in duplicate.

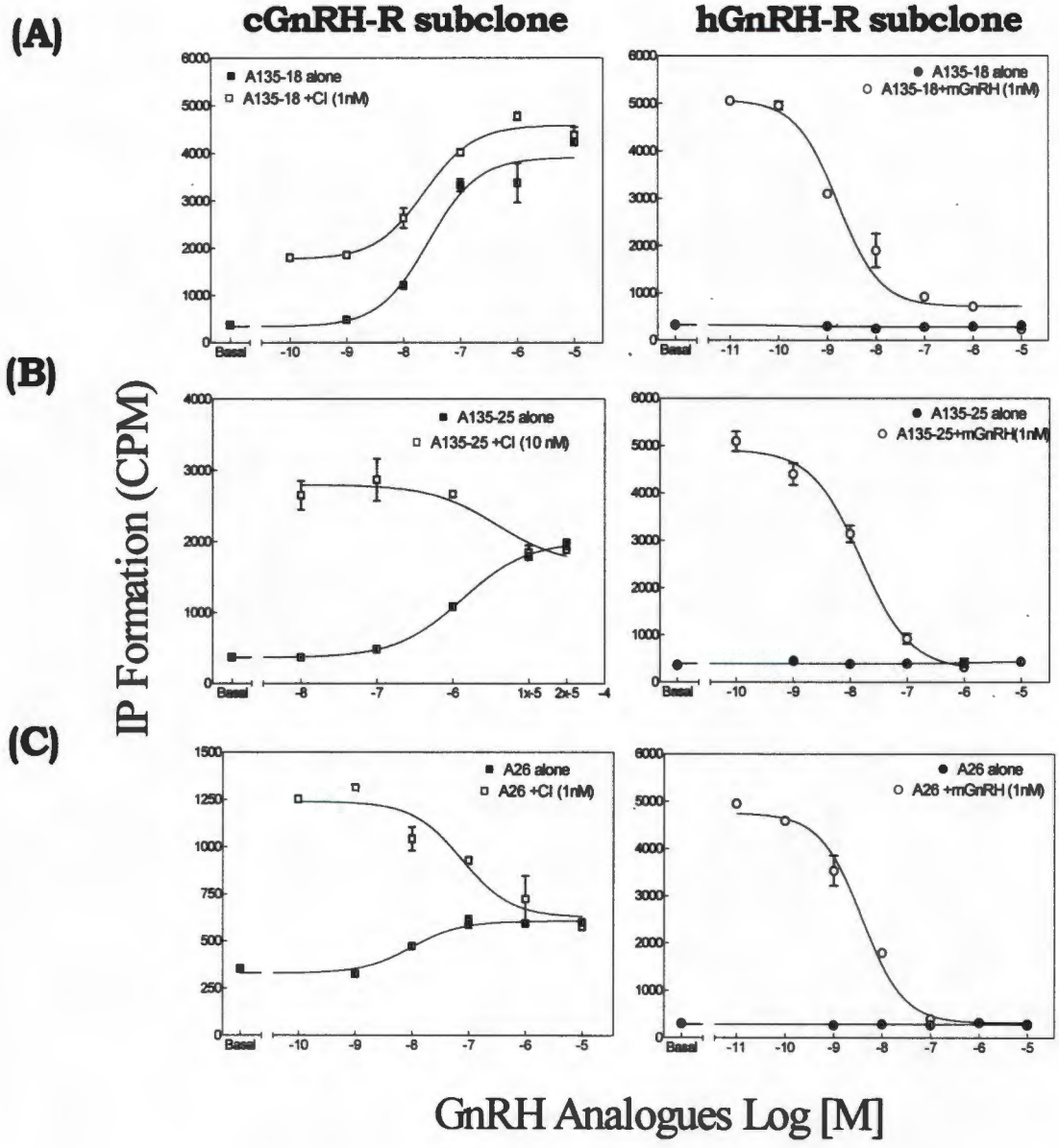


Fig. 3.20 ***Effects of antagonists on inositol phosphate production in the COS-1 cells expressing the chicken and human GnRH receptors.***
IP production was performed in the presence of various concentrations of antagonist (A) A135-18, (B) A135-25, and (C) A26, as described in the legend to Fig. 3.19. (The A135-25 experiment was performed in the presence of 10 nM cGnRH I or without.)

3.5 DISCUSSION

Conserved structural features between the chicken GnRH-R and GPCRs

The deduced amino acid sequences of the chicken GnRH receptor cDNA revealed that the chicken receptor comprises several highly conserved residues amongst GPCRs. All the GPCRs contain consensus sequence (Asn-X-Ser/Thr) for N-linked glycosylation, which may be involved in the expression and stabilisation of membrane receptors (see review Strader et al., 1994). There are two identified N-glycosylation sites within the N-terminus of the mouse GnRH receptor (Asn^{4} and Asn^{18}) and one in the human receptor (Asn^{18}), which has been shown to play a role in receptor expression or stability, but not involved in ligand binding interactions (Davidson *et al.*, 1995; Davidson *et al.*, 1996). The chicken receptor contains two potential N-glycosylation sites in the N-terminus (Asn^{23}) and EL 2 (Asn^{185}). Interestingly, the catfish receptor consists of four potential glycosylation sites in the N-terminus (Tensen et al., 1997).

Furthermore, the chicken receptor possesses highly conserved Cys residues in the junction of EL 1~TM 3 (Cys^{114}) and in EL 2 (Cys^{189}), which are also conserved in the catfish receptor (Tensen et al., 1997). The homologous two Cys residues are involved in forming a disulphide bridge that constrains a functional structure of the receptor in GPCRs (Dixon et al., 1987a; Dohlman et al., 1990; Perlman et al., 1995), and specifically in the human (Davidson et al., 1997) and the mouse GnRH receptors (Cook et al., 1997). There is an additional highly conserved Cys residue in many GPCRs. This residue has been shown to be palmitylated and the palmityl group may anchor the C-terminus in the plasma membrane and form a loop structure, which is proposed to be important for interactions with G proteins (Ovchinnikov *et al.*, 1988; O'Dowd *et al.*, 1989). Interestingly, an homologous Cys residue in the mouse TRH receptor (Cys^{335}) appears to play a role in restraining the receptor in an inactive conformation and in rapid internalisation of the receptor (Nussenzveig et al., 1993a). This Cys residue (Cys^{328}) is also conserved in the C-terminal tail of the chicken receptor, which is absent from the mammalian GnRH receptors, but is present in the catfish receptor (Tensen et al., 1997) and other vertebrate's receptors (Frog and Goldfish, unpublished data).

The highly conserved Pro residues located within the TM 4 ~ TM 7 of most GPCRs are suggested to take a part in changing the regular structure of receptors and may be important in the formation of the binding pocket (see review Probst et al., 1992). The

chicken GnRH-R contains these Pro residues, Pro^{171} (TM 4), Pro^{216} (TM 5), Pro^{273} (TM 6), and Pro^{311} (TM 7), which are also conserved in the mammalian and catfish receptors.

Conserved structural features between the chicken and mammalian GnRH-Rs

Despite the fact that the amino acid sequences of the chicken GnRH-R only share about 40 % identity to those of the mammalian counterparts, several important structural features are conserved between the chicken and mammalian GnRH-Rs over millions of years of evolution. These include the putative binding sites of the receptor and the residues for G-protein coupling.

Through mutagenesis studies, the residues of the mammalian GnRH-Rs, which are involved in ligand-interactions, were identified. Lys^{121} in TM 3 of the human receptor is important for interacting with GnRH agonist homologues, but is not involved in antagonist binding (Zhou *et al.*, 1995). The finding also suggested that the Lys residue may interact with the electron-dense aromatic rings of His^2 or Trp^3 of the ligand. Asn^{102} located in the C-terminus of TM 2 plays a role in high affinity binding of agonists and is proposed to dock the glycinamide C terminus of the ligand (Davidson *et al.*, 1996). Glu^{301} residue within EL 3 of the mouse receptor was established to be forming an electrostatic interaction with the Arg^8 residue of mGnRH (Flanagan *et al.*, 1994). These three residues are completely conserved in the chicken receptor, also in the catfish (Tensen *et al.*, 1997), frog, and goldfish receptors (unpublished data).

The conserved DRY/SXXV/IXXPL/I/F motif, which is located at the C-terminus of TM 3 to the N-terminus of IL 2, plays a pivotal role in G protein coupling. The mammalian GnRH-Rs contain a Asp-Arg-Ser (DRS) motif which is instead of the Asp-Arg-Tyr (DRY) motif amongst GPCRs. The Arg residue in the DRS motif has been shown to have a major effect on signal transduction and internalisation in the mouse GnRH-R, while the Ser residue exhibits no effect on both aspects (Arora *et al.*, 1997). The motif is present "Asp-Arg-His" (DRH) in the chicken, catfish (Tensen *et al.*, 1997), frog and goldfish receptors (unpublished data). The motif XXV/IXXPL/I/F, followed on from the DRS motif, is critical for interacting with G-protein and mediates signal transduction in other GPCRs (Probst *et al.*, 1992; Savarese and Fraser, 1992), especially in the mammalian GnRH-R (Arora *et al.*, 1995). The chicken GnRH-R contains the XXVXXPF motif on homologous

loci, and the XXIXXPL motif presents in the catfish (Tensen *et al.*, 1997), frog, and goldfish receptor (unpublished data).

The C-terminal part of the receptor IL 3 has been established to play a role in G-protein coupling in α_1-adrenergic receptor (Cotecchia *et al.*, 1990), β_2-adrenergic receptor (Samama *et al.*, 1993), and TRH-R (Nussenzveig *et al.*, 1993b). The Ala^{261} residue of the human GnRH-R was identified to be important for G-protein coupling (unpublished data). In this study, the Ala^{261} residue mutated to Lys, Glu, Phe, Leu, and Ile results in complete uncoupling. In contrast, mutations of the Ala residue, which locates in an homologous loci in α_1-adrenergic receptor (Kjelsberg *et al.*, 1992), leads to a constitutive activity. Interestingly, the chicken GnRH-R conserved the Ala residue (Ala^{252}), so does the catfish (Tensen *et al.*, 1997), frog, and goldfish (unpublished).

Different structural features between the chicken and mammalian GnRH-Rs

The general structural features of the chicken GnRH-R revealed that the chicken receptor comprises several unique features in comparison with the mammalian receptors: a longer N-terminus, a short IL 1, lack of a additional Cys residue in the junction of EL 1 ~TM 3, and a C-terminal tail.

There are additional two Cys residues in the junction of EL1~TM 3 (except porcine) and TM 5 of the mammalian receptors, but absent in chicken and catfish. It has been demonstrated that the additional Cys residues in the junction of EL 1~TM 3 forms a disulphide bond with a Cys within the N-terminus in the human (Davidson *et al.*, 1997) and mouse GnRH-R (Cook *et al.*, 1997). On the other hand, the chicken receptor possesses several Cys residues in the N-terminus and TM 1 (i.e. Cys^2 ,Cys^{29}, and Cys^{58}), which are absent in the mammalian receptor.

Two residues, an aspartic acid (Asp) in TM 2 and an asparagine (Asn) in TM 7, are conserved in over 95 % of GPCRs. The TM 2-Asp is important for agonist binding and G-protein coupling in many GPCRs (see review Probst *et al.*, 1992). Intriguingly, the mammalian GnRH-Rs appears to be interchanged at both loci (i.e. TM 2-Asn and TM 7-Asp). It was proposed that the residues in the mammalian receptor play an important role in maintaining the structural conformation of the receptor which is essential for interacting with other loci on the receptor to trigger the signalling cascade (Zhou *et al.*, 1994). The

chicken receptor consists of two Asp residues in these loci (i.e. $Asp^{87}Asp^{310}$), as does the catfish receptor.

Another feature of the chicken receptor is containing a C-terminal tail, which is absent in the cloned mammalian receptors, but is present in the catfish receptor (Tensen *et al.*, 1997). One highly conserved Cys residue present in most GPCRs, which is established to be palmitoylated, is also conserved in the chicken GnRH-R (as described above). In addition, it is well documented that the C-terminus plays a vital role in receptor's desensitisation and internalisation to regulate the receptor-G protein signalling in other GPCRs (Benovic *et al.*, 1988; Blumer and Thorner, 1991). In the regulation by heterologous desensitisation, receptors phosphorylated by protein kinases A and/or C have a reduced ability to activate G protein. Consensus sites of phosphorylation for protein kinase C [$(R/K_{1-3}, X_{2-0})$-S/T-$(X_{2-0}, R/K_{1-3})$ or S/T-$(X_{2-0}, R/K_{1-3})$ or $(R/K_{1-3}, X_{2-0})$-S/T] (Kennelly *et al.*, 1991) were located in the C-terminal tail of the chicken receptor, as well as in the C-terminal portion of IL 3, which has been shown to be important for G-protein coupling in the α_1-adrenergic receptor (Cotecchia *et al.*, 1990). Those consensus sites were also found in the catfish receptor. However, the mammalian receptors lack the C-terminal tail. Highly correlatively, the mammalian receptor does not undergo rapid desensitisation (Anderson *et al.*, 1995; McArdle *et al.*, 1995).

There are five potential phosphorylation sites that were located in IL 1, the N-terminal part of TM 2 and IL 2 of the mammalian receptor, whereas they are absolutely absent in the chicken homologue. Coincidentally, two potential sites in IL 2 are proximal to the DRSxxV/IxxPL motif, which is involved in G protein coupling and agonist-induced receptor internalisation (Arora *et al.*, 1995). No potential phosphorylation site in IL 2 was identified in non-mammalian vertebrates' receptors (the chicken and catfish receptors). It is of interest to notice that another three potential sites were located at between IL 1 and N-terminal portion of TM 2 in mammals, where the receptors have a "TM 2-Asn / TM 7-Asp" structural combination. Taken together, it is suggested that distinct regions for regulating G protein coupling might be present in non-mammalian and mammalian species.

Extraordinarily interesting, the amino acid sequences of the chicken GnRH-R shares about 40 % identity to the mammalian homologues, whereas it exhibits approximately over 60% identity amongst non-mammalian receptors (including chicken, frog, catfish, and goldfish). However, the chicken TRH-R shows over 80 % identity to the

mammalian counterparts (see the chicken TRH-R section). The drastic difference in structural features between the non-mammalian and mammalian GnRH-R is acquired by an evolutional selection over millions of years. It suggests the unique structural features of the mammalian GnRH-Rs might be required for a functional demand. Also, it is of interest to note that different receptors in the same tissue, e.g. GnRH-R and TRH-R, might have been constrained in a different way through millions of years of evolution. This is a phenomenon beyond the scope of this thesis to interpret here.

Differential agonist-selectivity between the chicken and mammalian GnRH-Rs

The pharmacological properties of the cGnRH-R were initially characterised using a selection of naturally-occurring GnRH analogues to measure their activities on receptor binding and stimulating IP production. The results revealed that cGnRH I and mGnRH had a similar binding affinity for the chicken receptor and were equipotent in stimulating IP formation, suggesting that the chicken receptor does not discriminate between a basic or a neutral amino acid at the position 8 of GnRH analogues (mGnRH : [Arg^8]; cGnRH I : [Gln^8]). On the other hand, cGnRH II [His^5,Trp^7, Tyr^8] and [D-Arg^6]cGnRH II exerted a 10-fold higher affinity than cGnRH I for the chicken receptor, as well as 63-, 126-fold potencies, respectively, on stimulating IP production. This confirms a previous study (Millar *et al.,* 1989) that the substitutions of Trp^7 and/or Tyr^8 in GnRH analogues promote higher activity on LH release in the chicken pituitary.

Conversely, mGnRH exhibited the highest potency (ED_{50}: 20 pM) to stimulate IP production in COS-1 cells expressing the human GnRH-R, whereas cGnRH II and [D-Arg^6]cGnRH II showed an intermediate effect (ED_{50} : 0.3 ~ 0.5 nM), and cGnRH I was the least potent (ED_{50} : 0.9 nM). These findings are concomitant with the results found in sheep and rat cultured pituitary cells on receptor binding and LH release (Millar *et al.,* 1989), and in COS-1 cells transiently transfected with mouse GnRH-R cDNA (Flanagan *et al.*, 1994) or transfected with human GnRH-R cDNA (Davidson *et al.,* 1996) on stimulating IP production. Taken together, the results demonstrate that the chicken receptor has a preference for GnRH analogues with substitutions of Trp^7 and/or Tyr^8; albeit less request for a basic or a neutral amino acids at position 8 in the analogues, while the mammalian receptor has a specific selection for the Arg^8 of mGnRH.

It has been suggested from mutagenesis studies that the electrostatic interactions between the Arg^{8} residue of the ligand and the Glu^{301} residue of the mouse GnRH-R alter the receptor conformation to a stable form, implying that the Glu^{301} of the receptor plays an important role in recognition of the Arg^{8} in the ligand (Flanagan *et al.*, 1994). Interestingly, the Glu is also conserved in the chicken receptor (Glu^{293}). However, the Glu in the chicken receptor does not preferentially favour the Arg^{8} of the ligand, since mGnRH and cGnRH I display an equal activity in receptor binding, stimulation of IP formation, and release of LH (Millar *et al.*, 1989). In addition, other postulated binding sites of mammalian GnRH-R, Lys^{121} and Asn^{102}, are also conserved in the chicken receptor (as described above). Both residues Asn^{102} and Glu^{301} in the mammalian receptor display differential affinities to different GnRH agonists. For example, the mutant Ala^{102} of the human GnRH-R severely impaired (over 200-fold decrease) its affinities to cGnRH I, cGnRH II and mGnRH, but only mildly affected (only 20-fold decrease) its affinity to [D-Trp^{6}]GnRH by measuring those ligands in stimulating IP formation (Davidson *et al.*, 1996). The mouse GnRH-R mutant, in which the Glu^{301} residue was mutated to Gln, exhibited a 56-fold decrease in apparent affinity for mGnRH, while its affinity for cGnRH II and cGnRH I was only decreased 2.2-fold and unchanged, respectively (Flanagan *et al.*, 1994). The mutations at the putative binding sites of the mammalian receptor give rise to different effects on the binding affinity of GnRH agonists. This indicates that the binding sites of the mammalian GnRH-Rs for mGnRH might not be the same sites for other native GnRH analogues (i.e. cGnRH I and cGnRH II), perhaps overlapping. Thus, it is anticipated that the binding domains of the chicken receptor might share some similarity to those of the mammalian counterparts, however the chicken receptor is more susceptible to activation by GnRH analogues with substitutions of Trp^{7} and/or Tyr^{8}.

An interesting question is: what factors might contribute to a receptor's preference (higher affinity) for a particular type of agonist? For example, the chicken receptor has higher affinity to cGnRH II analogues, whereas the mammalian homologue exhibits the highest affinity to mGnRH. This discrimination in both species might be due to the structural conformation of the receptor required for receptor's activation. As discussed above, there are several different structural features in both receptors which may be attributed to receptor selectivity. In particular, the chicken receptor consists of the TM 2-Asp and TM 7-Asp structural combination, instead of TM 2-Asn and TM 7-Asp in the

mammalian receptors. It has been postulated that an hydrogen-bonding network involving TM 2-Asp and TM 7-Asn forms a component of the helix:helix interactions which is required for the conformational change underlying receptor activation by agonists in the 5-HT_{2A} receptor (Sealfon *et al.*, 1995). Moreover, it was proposed that the TM 2-Asn and TM 7-Asp residues in the mammalian receptor play an important role in maintaining the normal receptor structure and receptor-G protein coupling (Zhou *et al.*, 1994). In that study, the Asp^{87} mutant ($Asp^{87}Asp^{318}$) abolished receptor binding for both agonist and antagonist, but the reciprocal mutant ($Asp^{87}Asn^{318}$) restored high affinity binding for both ligands. However, the reciprocal mutant receptor showed poor coupling to the phosphoinositol turnover. The results are consistent with those found in the 5-HT_{2A} reciprocal mutant receptor, whereas the TM 2-Asp/ TM 7-Asp mutant exhibits functions similar to the wild type receptor (Sealfon *et al.*, 1995). The importance of the two residues in TM 2 and TM 7 for receptor activation was substantiated by another group (Blomenröhr *et al.*, 1997, abstract). In the study, the TM 2-Asp and TM 7-Asp of the catfish receptor, whose C-terminal tail was truncated, were mutated into the TM 2-Asn/ TM 7-Asp, TM 2-Asp/ TM 7-Asn, and TM 2-Asn/ TM 7-Asn combinations, respectively. Only the TM 2-Asp/ TM 7-Asn mutant receptor responded to various GnRHs.

Thus, the chicken receptor may represent a natural mutation, relative to mammalian receptor, so that unique structural configuration in the chicken receptor could occur due to the interaction network between these two residues and with other loci. The different conformation of the receptor might take a part in comprising a binding domain favoured by a higher-affinity agonist (eg. cGnRH II) in the chicken receptor. The putative residues which account for this higher-affinity ligand in the chicken receptor remain to be identified.

Agonistic behaviours of mammalian antagonists in the chicken GnRH-R

The possibility of a different structural conformation between the chicken and mammalian receptors was suggested in pharmacological studies using mammalian GnRH antagonists. Of six selected mammalian GnRH antagonists, three antagonists display agonism in COS-1 cells expressing the chicken GnRH-R. A135-18 acts as a full agonist and A135-25 and A26 display partial agonistic effects. The results are in accordance with the finding that A135-18 and A26 showed agonist activity in stimulating LH release from

chicken pituitary cells, but not from sheep pituitary cells (Jacobs *et al.*, 1995). This suggests that the opposite effect of those peptides is not caused by a different cell environment (COS-1 cell or pituitary cell). Intriguingly, the structures of the antagonists reveal that D-Lys at position 6 is required for exerting partial agonist activity of these antagonists. If Lys is substituted with either Trp or Tyr (i.e. A27, A134-46, and A134-53), the peptides inhibit cGnRH I-stimulated IP formation in COS-1 cells expressing the chicken GnRH-R. Even replacing the Lys with Arg in the antagonists (i.e. A131-148 and A131-146) results in inhibition of LH release in the chicken pituitary cells (Jacobs *et al.*, 1995). These results suggest that a characteristic charge-strengthened hydrogen bond donor of Lys may account for the partial agonism, but the length of side chain might be crucial. It is of interest that the antagonist (i.e. A135-18) which contains D-Lys at position six accompanied with Ile at the fifth position behaves as a full agonist.

Little is known about the molecular mechanism of antagonists, while that of agonists has been studied extensively. In the two-state model of receptor activation (Samama *et al.*, 1993), it has been postulated that receptors are in equilibrium between the inactive conformation (R) and a spontaneously active conformation (R*) that can couple to G protein in the absence of ligand. Neutral competitive antagonists (pure antagonists) have equal affinity for R and R* and do not displace the equilibrium. However, agonists have a high affinity for R* and increase the concentration of R*. In this study, three antagonists exhibited a varying efficacy of agonism in the chicken receptor. To accommodate the findings in the two-state model, it was speculated that these antagonists might enhance a ligand-related constant (β) which represents the ability of hormone binding to overcome the receptor constrain (R), in order to drive the receptor into its active conformation which subsequently activated the G-protein (Samama *et al.*, 1993). The speculation is conceivable, because the agonism required the presence of ligand (i.e. antagonists), but how an antagonist binds to a receptor and generates "un-predicted" behaviour, that is a full or partial agonist effect. However, it lacks an explanation in the two-state model.

The mechanism underlying the agonist effects by these antagonists in the chicken receptor remains elusive. There are two possible hypothesises to delineate the action. Firstly, the antagonists (A135-18, -25, and A26) do bind to the chicken receptor, according to competition binding and IP formation experiments. The results revealed that the binding domains of the antagonists may overlap with those of agonists, but might not be identical

as suggested by earlier reports (Zhou *et al.*, 1995; Strader *et al.*, 1988). After binding to the receptor, somehow, that hormone binding disrupted a mechanism constraining the receptor (Kjelsberg *et al.*, 1992 ; Samama *et al.*, 1993), which is absent or different from that of the mammalian receptor; then, promoted the receptor into a "relaxed" form that is optimal for G-protein activation. Interestingly, the C-terminal tail of the avian β-AR appears to be involved in the constraining effect in the native receptor (Parker and Ross, 1991). In the study, the β-AR which was truncated within the C-terminal tail, displayed 2- and 3-fold increase in the basal and exhibits partial agonist effect of two full antagonists. Moreover, the C-terminal tail of the mouse TRH-R is also involved in constraining the receptor in an inactive form (as indicated above) (Matus-Leibovitch *et al.*, 1995). Coincidentally, the chicken receptor contains a C-terminal tail, which is absent in the mammalian receptor. However, the mechanism of constraining receptor in the chicken is yet to be established.

Rare expression of the chicken GnRH-R mRNA

In this study, I did not succeed in isolating the chicken GnRH-R gene by screening cDNA libraries (intact and castrated chicken pituitary libraries) using a mouse GnRH-R cDNA probe or the chicken GnRH-R genomic probe (which encodes a fragment from TM 6 to TM 7 of the receptor). The implication is that the chicken GnRH-R mRNA is very low in abundance, and/or technical problems in the screening, or that the cDNA libraries are not satisfactory. However, three cDNA clones of the chicken TRH-R were isolated from the same screening membranes (see the "cTRH-R" section), suggesting that the libraries and technique applied in this study were satisfactory. Thus, the rare GnRH-R mRNA copy appears to account for the failure in the cloning work. There is other evidence to support this. According to the analysis of the Northern blot on the chicken pituitary gland, no positive results were obtained while the blot was hybridised with ^{32}P-labelled chicken GnRH-R cDNA probe. In order to verify whether this result was due to a technical problem, the same blot membrane was re-hybridised with ^{32}P-labelled chicken TRH-R cDNA probe. Three mRNA transcripts of the chicken TRH-R were identified (see the "TRH-R " section), indicating that the failure of the Northern blot analysis can be attributed to extremely low copies of the chicken GnRH-R mRNA. Likewise, I was unable to trace a signal in the first-run nested PCR-products amplified from the castrated chicken pituitary cDNAs by means of Southern blot analysis. It appears that the chicken GnRH-R

mRNA is expressed at low levels even in the castrated condition, which has been suggested to up-regulate the receptor number (Illing *et al.*, 1993).

As indicated above, PCR was the only successful strategy for isolating a full-length chicken GnRH-R cDNA. Three full-length chicken GnRH-R cDNA clones (one clone was shown in the result section), were obtained by PCR. However, they comprised various amino acid sequence changes and at least 6 different "silent mutations" (changed nucleotide sequence, but encoding the same amino acids), as compared to those of the chicken GnRH-R genomic clone. The three subclones all contained Arg (R) at position 338 instead of the Glu (Q) in the genomic clone. Furthermore, the pCH6 subclone consisted of Lysine (K) at position 40 the same as the genomic sequence, while the pCH5 and pCH7 (data not shown) subclones shared an identical sequence (Q) at that position. Even though they are all functional constructs, according to IP assay. It remains to be further investigated whether the variation of sequences are due to the polymorphism of the gene or mis-reading by PCR polymerase.

Chapter 4

CONCLUSIONS

4 CONCLUSION

The mammalian TRH-R and GnRH-R have been cloned, and used to investigate their physiological roles in the physiological regulation of the hypothalamo-pituitary-thyroid or hypothalamo-pituitary-gonad axis in mammals. However, there is little known about physiological functions of the receptors in the endocrinology of chickens, especially in growth and sexual maturation. The chicken TRH-R and GnRH-R were therefore cloned and characterised in this study.

Understanding the molecular mechanism of ligand binding, signal propagation and G-protein coupling of the human GnRH-R, is crucial for rationally designing novel orally-active analogues for application in reproductive medicine. Considerable progress in this regard has been made through a combination of computer modelling and mutagenesis studies. The sequence information from a related but pharmacologically distinct receptor would contribute substantially in these endeavours, while the cloned mammalian receptors share over 85 % homology and exhibit similar ligand selectivity.

The TRH-R and GnRH-R are well known as a rarely expressed receptor. Although the TRH-R and GnRH-R have been cloned from several mammalian species, their nucleotide sequence data might not be a useful tool to clone these receptors from chickens, because of being millions of years apart in evolution. In this study, a multiple combined strategy was used to clone both receptors from the chicken pituitary gland. It revealed that there were differential difficulties in cloning the two receptors. Although the TRH-R is more abundant than GnRH-R in the chicken pituitary, the truncated transcripts of TRH-R mRNA were highly expressed (related to the full-length transcript) in the pituitary gland. Only truncated forms of the chicken TRH-R cDNA were isolated from a cDNA library using the mouse TRH-R cDNA and a partial gene of the chicken TRH-R as probes. Therefore, there was a challenge to clone a full-length TRH-R. In order to isolate a functional TRH-R cDNA, multiple techniques (PCR, 3'RACE, screening of a cDNA library) were applied in this regard. On the other hand, the chicken GnRH-R is extraordinarily rare in the pituitary gland and contains a strikingly low percentage of homology with the mammalian counterparts. The chicken GnRH-R cDNA cannot be cloned by screening cDNA libraries (the intact and castrated chicken pituitary gland) using

the mouse GnRH-R cDNA and a partial gene of the chicken GnRH-R as probes. A multiple combined strategy (including PCR, 5′-RACE, screening of a genomic library, nested PCR) was applied to clone a full-length chicken GnRH-R cDNA.

The amino acid sequences of both receptors show that the chicken TRH-R and GnRH-R share about 84 % and 40 % identity to those of thier mammalian counterparts, respectively. Additionally, from pharmacological studies, the chicken GnRH-R displays a different selectivity for GnRH agonist analogues from that of the mammalian homologue and exhibits agonistic behaviours from the mammalian antagonists. On the other hand, there are no discriminatory properties on the receptor binding and IP formation between the chicken and mouse TRH-Rs. The findings suggest that there has been a functional demand for structural change in the mammalian GnRH-R during its evolution from the non-mammalian ancestral receptor, whereas the TRH-R has considerable evolutionary constraints on both aspects for conserving functional importance, though several hundred millions of years of evolution.

The chicken GnRH-R possesses several characteristically unique features and distinct pharmacological properties in its interaction with GnRH agonist and antagonist analogues. The comparative amino acid data between the chicken and mammalian GnRH-Rs provides valuable information for recognising important elements in the receptors, which are involved in the structure and function. For example, our group has recently identified the EL 2 domain of the mammalian GnRH-R as a critical region for converting antagonism to agonism from one selected antagonist (i.e. antagonist 135-18). Moreover, we have shown that the chicken GnRH-R generate internalises 10-fold more rapidly than the human counterpart. Also, truncating the C-terminal tail of the chicken receptor at the S^{337} residue changes the internalisation rate to that of the human receptor (Pawson *et al.*, J. Endocrinol., in press).

Chapter 5

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