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THE GONADOTROPIN-RELEASING HORMONE GENE: CHARACTERIZATION, REGULATION AND EXPRESSION IN TWO SALMONIDS

Kristian Robert von Schalburg
B. Sc., University of Victoria, 1991

A Dissertation Submitted in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY
in the Department
of
Biology

We accept this dissertation as conforming
to the required standard

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ABSTRACT

There are currently thirteen members of the gonadotropin-releasing hormone (GnRH) family. The GnRH members that activate the synthesis and release of the pituitary gonadotropins are the best understood. These members stand central to the development and maintenance of reproductive function. The roles of GnRH that act in the brain and not in the pituitary, or that are expressed in extraneural tissues, are not well characterized.

My goal was to determine whether 1) the regulatory regions and organization of the GnRH gene is conserved between mammals and fish, 2) GnRH is expressed in tissues other than the brain of salmonids and 3) the processes that regulate the expression of GnRH are conserved between two salmonid species with different habitats and reproductive patterns (sockeye salmon, *Oncorynchus nerka*; rainbow trout, *Oncorynchus mykiss*).

To determine whether the regulatory regions and organization of the GnRH gene were conserved across the species, I isolated and characterized salmon (s)GnRH gene1 from rainbow trout and sGnRH gene2 from sockeye salmon. In salmon, which are tetraploid, each duplicated sGnRH gene encoded a different mRNA (mRNA1 or mRNA2), but the identical sGnRH peptide. A Southern blot analysis revealed that other related forms of GnRH exist in the sockeye salmon genome. Also, I determined from RT-PCR analysis that GnRH was not expressed in the heart, liver, gut, adrenal, spleen or retina, but was expressed in the gonads of sockeye salmon.

To understand the function of GnRH in the gonads, it was necessary to learn when GnRH was expressed during development and throughout the reproductive cycle. Studies using RT-PCR analysis and primer extension analysis demonstrated that the reproductive tissues of salmonids use an upstream promoter to regulate GnRH expression. Intron 1 may be retained, resulting in mRNAs containing 5'-untranslated regions longer than their brain counterparts. These sGnRH transcripts are initiated by a TATA-less promoter region from a start site at 315 basepairs upstream from that utilized in the sockeye salmon brain.

Using the same techniques, differences in the expression of GnRH in embryonic tissue and gonads of sockeye salmon and rainbow trout were noted over the first two years of their lives. First, the upstream promoter is transiently used for expression of GnRH as early as 14 days after fertilization in rainbow trout and 30 days after fertilization in sockeye salmon. Second, in sockeye salmon ovary and testis, GnRH was expressed in October of the first year and then only during May and June of the second year in precocious tissue. For rainbow trout, GnRH was expressed in the first year from May to October and in the second year only in December. Precociously mature ovary and testis expressed GnRH from June to October in the second year.

It was also important to determine whether the GnRH mRNA expressed in the developing ovary and testis was translated into protein. High pressure liquid chromatography and radioimmunoassays were used to demonstrate the presence of at least three forms of GnRH in precociously mature ovaries and testes during the second year.

The expression of sGnRH mRNA2 in the salmonid ovary and testis utilizes an alternative promoter. The resulting mRNAs have long 5'-untranslated regions that may be important in post-transcriptional control. Expression of GnRH in the brain is constant, but is intermittant in the salmonid gonad. GnRH mRNA is expressed in undifferentiated gonadal tissue in the first year and briefly in differentiated, but immature gonads. However, in precociously mature ovary and testis in the second year GnRH is transcribed and translated at the stage that precedes ovulation and spawning. Differences in pattern and longer duration of GnRH expression are shown in the ovary and testis of rainbow trout in comparison to sockeye salmon. This might indicate that GnRH is important in the regeneration of new sets of germ cells in the iteroparous rainbow trout, but not in the semelparous sockeye salmon.

A comparison of the genes that encode sGnRH mRNA1 and mRNA2 reveals significant sequence divergence in their 5'-flanking regions following tetraploidization. A large portion of the sockeye salmon gene2 is missing in comparison to the Atlantic salmon

gene2. However, the salmonid genes all share strong sequence identity in the proximal-promoter region. Although large segments of sequence identity do not exist in the regulatory regions of the GnRH-encoding genes of mammals and salmonids, some similarities exist in the positions of potential POU-homeodomain regulator and estrogen response element motifs. This suggests that some regulatory control for expression of GnRH in both the brain and gonads may be conserved.

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LIST OF ABBREVIATIONS

Gonadotropin-releasing hormone (GnRH) family:

cGnRH-I:	chicken GnRH-I
cGnRH-II:	chicken GnRH-II
cfGnRH:	catfish GnRH
dfGnRH:	dogfish GnRH
gpGnRH:	guinea pig GnRH
hGnRH:	herring GnRH
(hyp9)mGnRH:	hydroxyproline-9 mammalian GnRH
lGnRH-I:	lamprey GnRH-I
lGnRH-III:	lamprey GnRH-III
mGnRH:	mammalian GnRH
sGnRH:	salmon GnRH
sbGnRH:	seabream GnRH
tGnRH-I:	tunicate GnRH-I
tGnRH-II:	tunicate GnRH-II

Techniques and chemicals used in the isolation of GnRH:

DTT: dithiothreitol

EDTA: disodium ethylenediamine tetraacetic acid

HPLC: high pressure liquid chromatography

KCl: potassium chloride

$MgCl_2$: magnesium chloride

PCR: polymerase chain reaction

RIA: radioimmunoassay

RT-PCR: reverse transcriptase-PCR

Tris-HCl: tris(hydroxymethyl)aminomethane

Other related terms:

aa: amino acids

bps: basepairs

CNS: central nervous system

cDNA: complementary DNA

DAF: days after fertilization

DNA: deoxyribonucleic acid

dNTPs: deoxyribonucleotriphosphates

ERE: estrogen response element

FSH, GTH-I: follicle stimulating hormone, gonadotropin-I

GAP: GnRH-associated peptide

irGnRH: immunoreactive GnRH

IGF-I: insulin growth factor-I

LH, GTH-II: luteinizing hormone, gonadotropin-II

mRNA: messenger ribonucleic acid

nts: nucleotides

UTR: untranslated region

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This thesis has been like creating an artwork, not really knowing the final outcome, but beginning with one brushstroke to ultimately culminate in a beautiful painting. Actually, it was really more like a wonderful symphony because it required the playing of so many different instruments to come to fruition. First and foremost, I would like to thank Bill Harrower, Jack Nickolichuk, Kevin Nickolichuk and Jim Powell. Without their help in providing sockeye salmon or rainbow trout this project would never have been completed. Also, three important people need special mention. Sandra Krueckl and Dave Lescheid for being faithful and true friends in every measure of the word and Carol Warby whose pleasant personality and excellent technical assistance made the HPLC/RIA project a real joy.

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There will always be a special place in my heart for each one of you and I pray that the Truth you seek shall be found.

Chapter 1

General Introduction

Parts of this chapter have been modified and published as:

Sherwood, N.M., von Schalburg, K. and Lescheid, D.W. (1997). Origin and evolution of GnRH in vertebrates and invertebrates. In: GnRH Neurons: Gene to Behaviour (I.S. Parhar and Y. Sakuma, eds.), Brain Shuppan Publishers, Tokyo, pp. 3-25.

I. GnRH structure and form.

Gonadotropin-releasing hormone (GnRH) is a neuropeptide that is central to the development and maintenance of reproductive function. Synthesized and secreted from nerve cells, GnRH acts on a subset of pituitary cells known as gonadotropes. Upon binding to gonadotrope receptors, GnRH activates the synthesis and release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) in tetrapods. In fish, the comparable pituitary hormones are gonadotropin-I (GTH-I) and gonadotropin-II (GTH-II), which are biologically similar to the tetrapod FSH and LH, respectively (Swanson *et al.*, 1991). These hormones, in turn, stimulate the production of gonadal androgens and estrogens, which feedback to the brain and pituitary to complete the hypothalamic-pituitary-gonadal system and regulate sexual behaviour and reproductive cycles.

Currently eleven distinct gonadotropin-releasing hormone (GnRH) structures have been reported for vertebrates and two for invertebrates (Fig. 1-1). The first GnRH peptide structure was determined for mammals, specifically for pig (Matsuo *et al.*, 1971) and sheep (Burgus *et al.*, 1972). Since its first characterization, GnRH has now been isolated and sequenced from representatives of all seven classes of vertebrates. The structural pattern of the known GnRH peptides is clear: the length is ten amino acids and four of the amino acids are identical as shown in Fig. 1-1. Amino acid 1 (the amino terminal), amino acids 9 and 10 (carboxy terminal) and amino acid 4 are conserved, even in the two new GnRH family members identified in a protochordate, the tunicate. Amino acids 1, 2 or 3 are important for receptor activation and residues 5 to 8, the most variable region, are important for specific binding within the particular species (Sealfon *et al.*, 1997).

Fig. 1-1. Comparison of thirteen GnRH peptides. Boxed amino acids indicate changes with respect to the mammalian form.

GnRH peptide

MAMMAL	pGLU	HIS	TRP	SER	TYR	GLY	LEU	ARG	PRO GLY
GUINEA PIG	pGLU	**TYR**	TRP	SER	TYR	GLY	**VAL**	ARG	PRO GLY
CHICKEN-I	pGLU	HIS	TRP	SER	TYR	GLY	LEU	**GLN**	PRO GLY
SEA BREAM	pGLU	HIS	TRP	SER	TYR	GLY	LEU	**SER**	PRO GLY
HERRING	pGLU	HIS	TRP	SER	**HIS**	GLY	LEU	**SER**	PRO GLY
CATFISH	pGLU	HIS	TRP	SER	**HIS**	GLY	LEU	**ASN**	PRO GLY
SALMON	pGLU	HIS	TRP	SER	TYR	GLY	**TRP**	**LEU**	PRO GLY
DOGFISH	pGLU	HIS	TRP	SER	**HIS**	GLY	**TRP**	**LEU**	PRO GLY
CHICKEN-II	pGLU	HIS	TRP	SER	**HIS**	GLY	**TRP**	**TYR**	PRO GLY
LAMPREY-III	pGLU	HIS	TRP	SER	**HIS**	**ASP**	**TRP**	**LYS**	PRO GLY
LAMPREY-I	pGLU	HIS	**TYR**	SER	**LEU**	**GLU**	**TRP**	**LYS**	PRO GLY
TUNICATE-I	pGLU	HIS	TRP	SER	**ASP**	**TYR**	**PHE**	**LYS**	PRO GLY
TUNICATE-II	pGLU	HIS	TRP	SER	**LEU**	**CYS**	**HIS**	**ALA**	PRO GLY

The number (11) of distinct forms of GnRH is small in relation to the number (over 40,000) of vertebrate species. The reason is that many species have the identical forms of GnRH as shown by high pressure liquid chromatography (HPLC), radioimmunoassay (RIA) and in some cases by chemical sequencing (see Sherwood *et al.*, 1997). For example, the mammalian GnRH (mGnRH) molecule has been isolated and sequenced as a peptide from the human placenta (Tan and Rousseau, 1982), and from the brain of pig (Matsuo *et al.*, 1971), sheep (Burgus *et al.*, 1972), frog (Conlon *et al.*, 1993) and sturgeon (Lescheid *et al.*, 1995). The chicken GnRH-I (cGnRH-I) peptide was sequenced from the brain extracts of chicken (King and Millar, 1982a and b, Miyamoto *et al.*, 1982 and 1983) and alligator (Lovejoy *et al.*, 1991a).

The chicken GnRH-II (cGnRH-II) form is present in representatives of the cartilaginous fish, bony fish, amphibians, reptiles and birds. For example, cGnRH-II has been isolated and sequenced from the brain in a number of species: chicken (Miyamoto *et al.*, 1984), alligator (Lovejoy *et al.*, 1991a), frog (Conlon *et al.*, 1993), sea bream (Powell *et al.*, 1994), tilapia (Parhar, 1997), pacu (Powell *et al.*, 1997), catfish (Ngamvongchon *et al.*, 1992 and Bogerd *et al.*, 1992), dogfish (Lovejoy *et al.*, 1992) and ratfish (Lovejoy *et al.*, 1991b). Although cGnRH-II has not been isolated and sequenced as a peptide from any mammal, there is HPLC and RIA data suggesting that this form of GnRH is present in mammals including monotremes (King *et al.*, 1994), marsupials (King *et al.*, 1989, 1990, 1994), primitive placental mammals (Dellovade *et al.*, 1993) and primates (Lescheid *et al.*, 1997). However, cGnRH-II is not present in all vertebrates as it has not been detected in jawless fish such as lamprey (Sherwood *et al.*, 1986 and Sower *et al.*, 1993). To date the evidence shows that cGnRH-II has been more strongly conserved than any other GnRH peptide in vertebrates.

Other GnRH peptides that appear to be confined to various fishes include salmon GnRH (sGnRH), isolated and sequenced from salmon (Sherwood *et al.*, 1983), herring (Carolsfeld, personal communication), pacu (Powell *et al.*, 1997) and tilapia brain

(Parhar, 1997). Sea bream GnRH (sbGnRH) was sequenced from sea bream (Powell *et al.*, 1994), cichlid (Powell *et al.*, 1995), pacu (Powell *et al.*, 1997) and tilapia (Parhar, 1997). Catfish GnRH (cfGnRH) is identified in catfish only (Ngamvongchon *et al.*, 1992 and Bogerd *et al.*, 1992). Dogfish GnRH (dfGnRH) is from dogfish only (Lovejoy *et al.*, 1992). Lamprey GnRH-I (lGnRH-I) and GnRH-III (lamprey GnRH-II has not been sequenced) are identified only from lamprey (Sherwood *et al.*, 1986 and Sower *et al.*, 1993).

A modified form of GnRH was reported that did not involve a novel structure, but rather a post-translational change of one of the existing forms of GnRH. This altered form of GnRH involves mGnRH with Pro^9 replaced by hydroxyproline (Hyp^9). This form, (Hyp^9)mGnRH, was detected by HPLC and RIA in human, rodent, ovine and amphibian hypothalamic extracts (Gautron *et al.*, 1991).

The most recently characterized GnRH structures are the ones isolated from an invertebrate, the sea squirt (Protochordata; Tunicata; *Chelyosoma productum*) as shown in Fig. 1-1 (Powell *et al.*, 1996). Both tunicate GnRH-I (tGnRH-I) and tunicate GnRH-II (tGnRH-II) are 60% identical to mammalian GnRH and therefore are members of the GnRH family based on structural identity. tGnRH-I more closely resembles lGnRH-I and lGnRH-III because of the presence of Lys^8, which tGnRH-II lacks. The unusual aspect, however, is that tGnRH-II was isolated as a dimer. The two chains of the dimer are identical and linked by a cystine in position six. Tunicate GnRH-I has a structure that is distinct from the monomer of tGnRH-II and it is therefore predicted that the two peptides will have different functions.

The evidence that GnRH evolution predates chordate evolution is provided by the tunicate GnRH peptides and the presence of an immunoreactive GnRH-like molecule in the CNS neurons of the gastropod mollusc, *Helisoma trivolvis* (Goldberg *et al.*, 1993).

II. Multiple GnRH forms in single species.

Initial studies done in the 1970s indicated that only a single form of GnRH, named luteinizing hormone-releasing hormone (LHRH) or mammalian GnRH, was present in individual vertebrate brains. However, reports of GnRH structures distinct from mGnRH appeared in the early 1980s. The first indication that a species could have more than one form of GnRH came from studies showing that chicken (Miyamoto *et al.*, 1983 and 1984) and salmon (Sherwood *et al.*, 1983) each have two forms of GnRH. Chicken GnRH-I and GnRH-II were sequenced from peptides isolated from the chicken brain (Miyamoto *et al.*, 1983 and 1984); salmon GnRH (Sherwood *et al.*, 1983) and a peptide identical to chicken GnRH-II (Powell, personal communication) were sequenced from the chum salmon brain.

These three GnRH structures (cGnRH-I, cGnRH-II and sGnRH), which were distinct from mGnRH, established the two important principles that GnRH is a family of peptides and a single species can have more than one form of GnRH. However, it was not known whether individuals within the species had more than one form of GnRH in the brain because pooled brains were used for the purification procedures. At present, the best proof that single species have at least two forms of GnRH comes from studies in which two GnRH peptides have been isolated from a species and sequenced. These studies include:

lamprey (*Petromyzon marinus*)	lGnRH-I and lGnRH-III	(Sherwood *et al.*, 1986, Sower *et al.*,1993)
dogfish (*Squalus acanthias*)	dfGnRH and cGnRH-II	(Lovejoy *et al.*, 1992)
salmon (*Oncorhynchus keta*)	sGnRH and cGnRH-II	(Sherwood *et al.*, 1983; pers. commun.)
catfish (*Clarias*, two species)	cfGnRH and cGnRH-II	(Bogerd *et al.*, 1992)
		(Ngamvongchon *et al.*, 1992)
frog (*Rana ridibunda*)	mGnRH and cGnRH-II	(Conlon *et al.*, 1993)
alligator (*A. mississippiensis*)	cGnRH-I and cGnRH-II	(Lovejoy *et al.*, 1991a)
chicken (*Gallus domesticus*)	cGnRH-I and cGnRH-II	(Miyamoto *et al.*, 1983 and 1984)

In addition, the primary structure of three forms of GnRH has been determined from the brain tissue of single fish species in the orders Characiformes and Perciformes (Groups 5 and 10 in Fig. 1-2). For tilipia (Perciformes: *Oreochromis niloticus*), three GnRH forms have been sequenced as peptides: sea bream GnRH (sbGnRH), sGnRH and cGnRH-II (Weber *et al.*, 1997). For gilthead sea bream (Perciformes: *Sparus aurata*), these three forms of GnRH are also present of which sbGnRH and cGnRH-II were sequenced as peptides and sGnRH identified by HPLC-RIA (Powell *et al.*, 1994). All three GnRH structures were subsequently confirmed by comparison to cDNA sequences (Gothilf *et al.*, 1995 and 1996). For cichlids (Perciformes: *Haplochromis burtoni*), the same three GnRH forms are present as shown by HPLC-RIA data (Powell *et al.*, 1995). To confirm these identities, the sbGnRH peptide was sequenced (Powell *et al.*, 1995) and all three cDNA sequences were obtained (see White, S.A. *et al.*, 1995). In another order of fish, Characiformes, the same three GnRH peptides have been isolated and sequenced for the fish known as pacu (*Piaractus mesopotamicus)* (Powell *et al.*, 1997).

Only very recent studies indicate that two or three forms of GnRH are present in mammals. To date, HPLC-RIA or immunocytochemical data have been used to show that chicken GnRH-II is expressed as a protein in the brains of monotremes, marsupials and placental mammals including primates (Dellovade *et al.*, 1993, King *et al.*, 1989, 1990 and 1994, Lescheid *et al.*, 1997). Chicken GnRH-II cDNA has been isolated and sequenced from monkeys (Terasawa, personal communication) and humans (White *et al.*, 1998 and personal communication).

In all vertebrates the location of multiple forms of GnRH in individual brains shows a similar pattern (Fig. 1-3). Most antisera cross-react with more than one form of GnRH, but a few antisera have greater specificity for a single form of GnRH. Antisera that are specific to cGnRH-II have been used to show that GnRH-containing neurons in the midbrain contain only cGnRH-II in representatives of five classes of vertebrates (Conlon *et al.*, 1993, Dellovade *et al.*, 1993, Lepretre *et al.*, 1993, Kim *et al.*, 1995) (Fig. 1-3).

Fig. 1-2. Diagram of the major groups of living teleosts. The names of fish from which all detectable forms of GnRH have been sequenced are shown. Forms of GnRH that have been detected using only RIA-HPLC methods are not shown. sb = sea bream GnRH; s = salmon GnRH; c-II = chicken GnRH-II; cf = catfish GnRH. Numbers in each box represent teleost superorders or infradivisions: 1. Osteoglossomorpha (bony tongued fish), 2. Elopomorpha (eels), 3. Clupeomorpha (herring), 4. Protacanthopterygii (salmonids), 5. Ostariophysi (includes Characiformes), 6. Stenopterygii (e.g. lightfish and dragon fish), 7. Scopelomorpha (e.g. lantern fish and greeneyes), 8. Paracanthopterygii (e.g. cod and hake), 9. Atherinomorpha (e.g. medaka, molly), 10. Percomorpha (Order Perciformes). Diagram modified from Powell *et al.*, 1997.

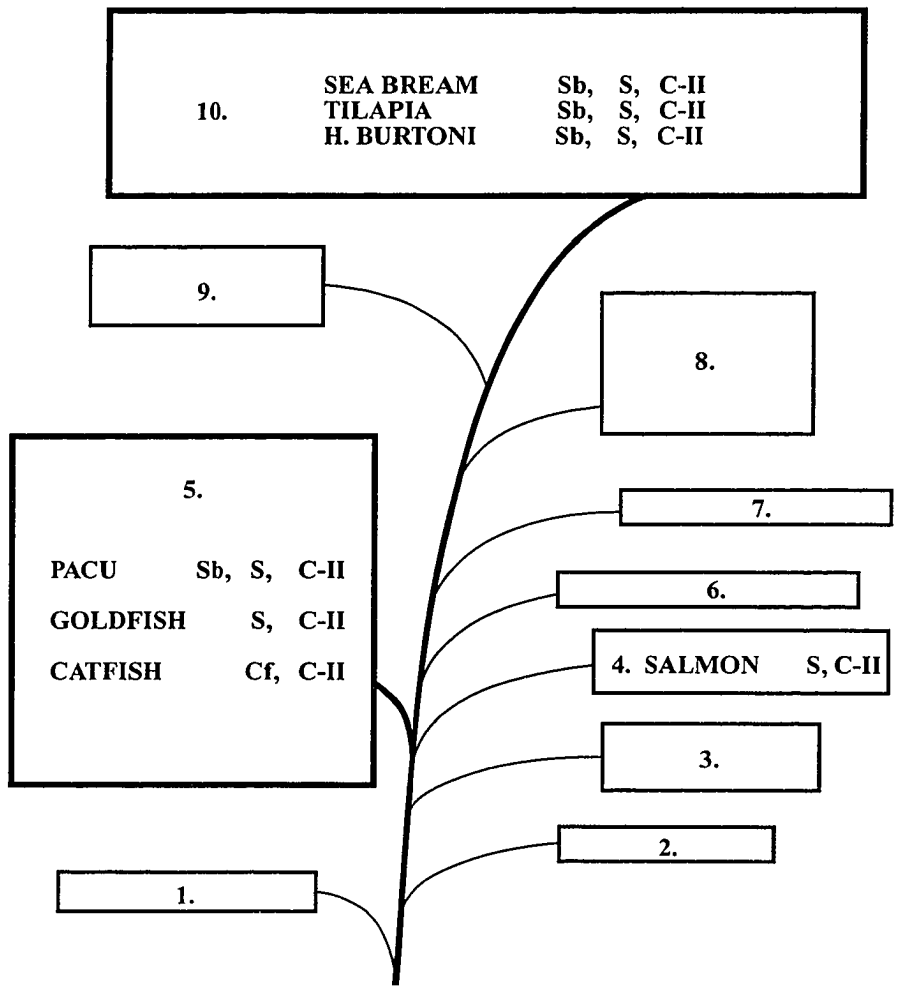
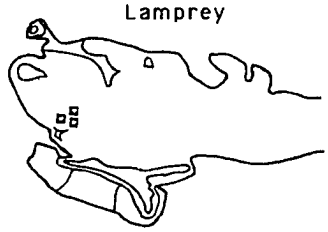

10.
SEA BREAM Sb, S, C-II
TILAPIA Sb, S, C-II
H. BURTONI Sb, S, C-II
9.
8.
5.
PACU Sb, S, C-II
GOLDFISH S, C-II
CATFISH Cf, C-II
7.
6.
4. SALMON S, C-II
3.
2.
1.

Fig. 1-3. Diagram showing location of different GnRH forms in brain sections. The modified parasaggital brain sections illustrate the location of GnRH in tunicate (Mackie,1995), lamprey (Nozaki *et al.*, 1984), shark (Nozaki *et al.*, 1984), sturgeon (Lepretre *et al.*, 1993), salmon (Amano *et al.*, 1991), cichlid (White, S.A. *et al.*, 1995), newt (Muske,1993), chicken (Millam *et al.*, 1993) and musk shrew (Rissman *et al.*, 1995). The relative location of cell bodies expressing distinct forms of GnRH for six different vertebrate classes are shown. In species in which differential location has not been reported, the same symbol is used for both forms of GnRH: tunicate GnRH-I and -II (♈), lamprey GnRH-I and -III (◘) and dogfish GnRH-I and cGnRH-II (▲) in the respective brains. In species in which differential location has been reported the symbols are mammalian GnRH (■), cGnRH-I (◆), salmon GnRH (▲), sea bream GnRH (✱), and chicken GnRH-II (●).

Tunicate

Lamprey



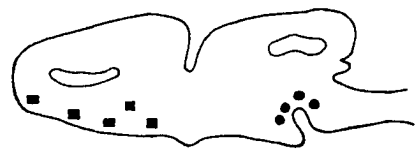
Shark

Sturgeon

Salmon

Cichlid

Newt



Chicken

Musk Shrew

The neurites, however, extend to many areas of the brain. The second form of GnRH (e.g. mGnRH, gpGnRH, cGnRH-I, sGnRH or cfGnRH) is in neurons of the preoptic-septal region, basal forebrain and para-olfactory areas. This localization pattern for two forms of GnRH within a single brain has been confirmed with specific cDNA probes in catfish (Bogerd *et al.*, 1993 and Zandbergen *et al.*, 1995).

In two cichlid species (*H. burtoni* and tilipia) with three forms of GnRH, specific cDNA probes have been used to distinguish the location of the three forms of GnRH: sGnRH is in the olfactory-forebrain areas; sbGnRH is in the preoptic region; and *cGnRH-II is in the midbrain region* (White, S.A. *et al.*, 1995, Parhar, 1997) (Fig. 1-3).

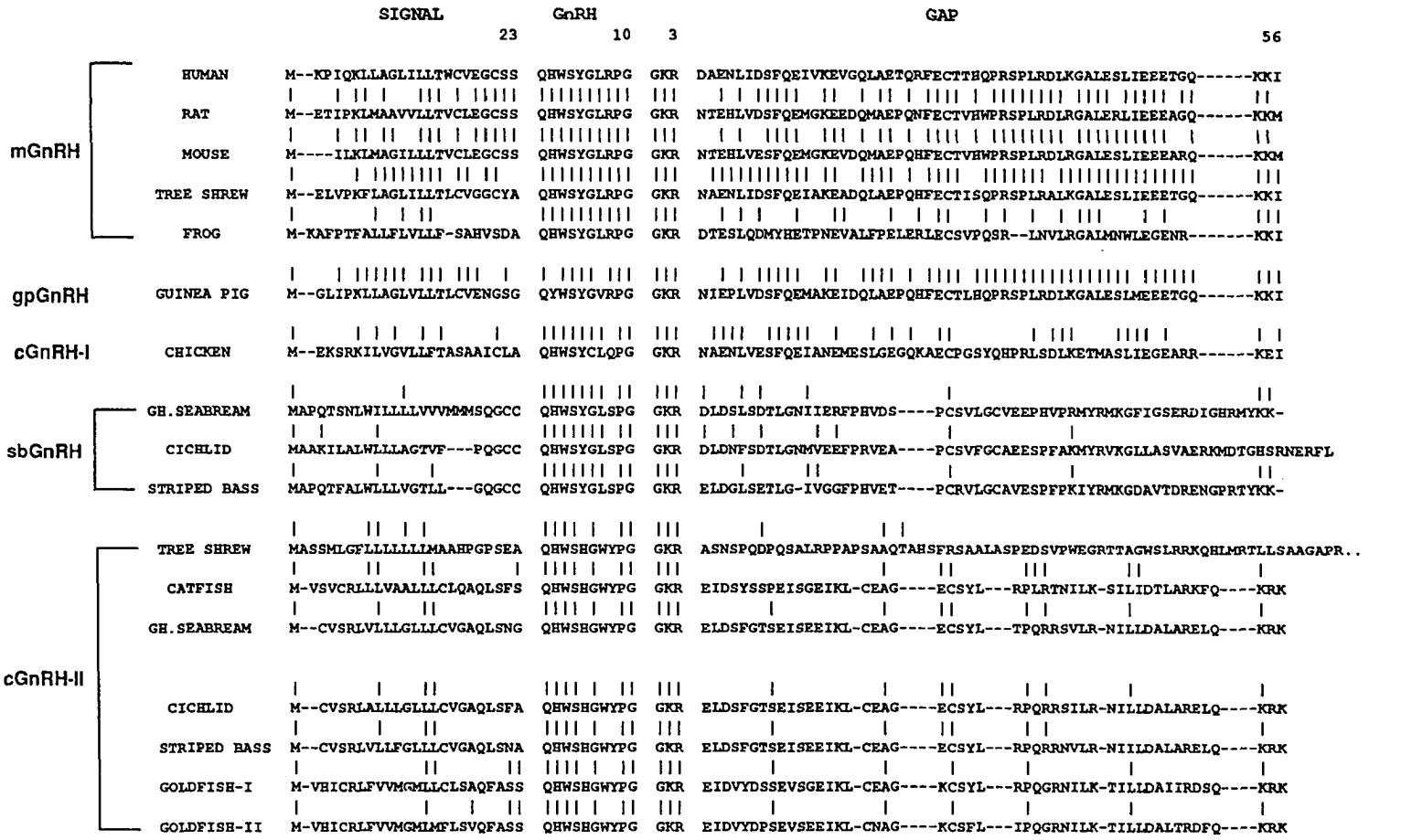
Multiple forms of GnRH in a single species is an ancient pattern in evolution as shown by the two distinct forms of GnRH in a protochordate (Fig. 1-1) (Powell *et al.*, 1996). Immunocytochemical studies of GnRH in several tunicate species show that GnRH is present in the brain (neural ganglion) (Bollner *et al.*, 1997) and in a neural plexus outside of the ganglion (Georges and Dubois, 1980, Powell *et al.*, 1996). To date, the two types of GnRH have not been distinguished in two different populations of cells in this tunicate.

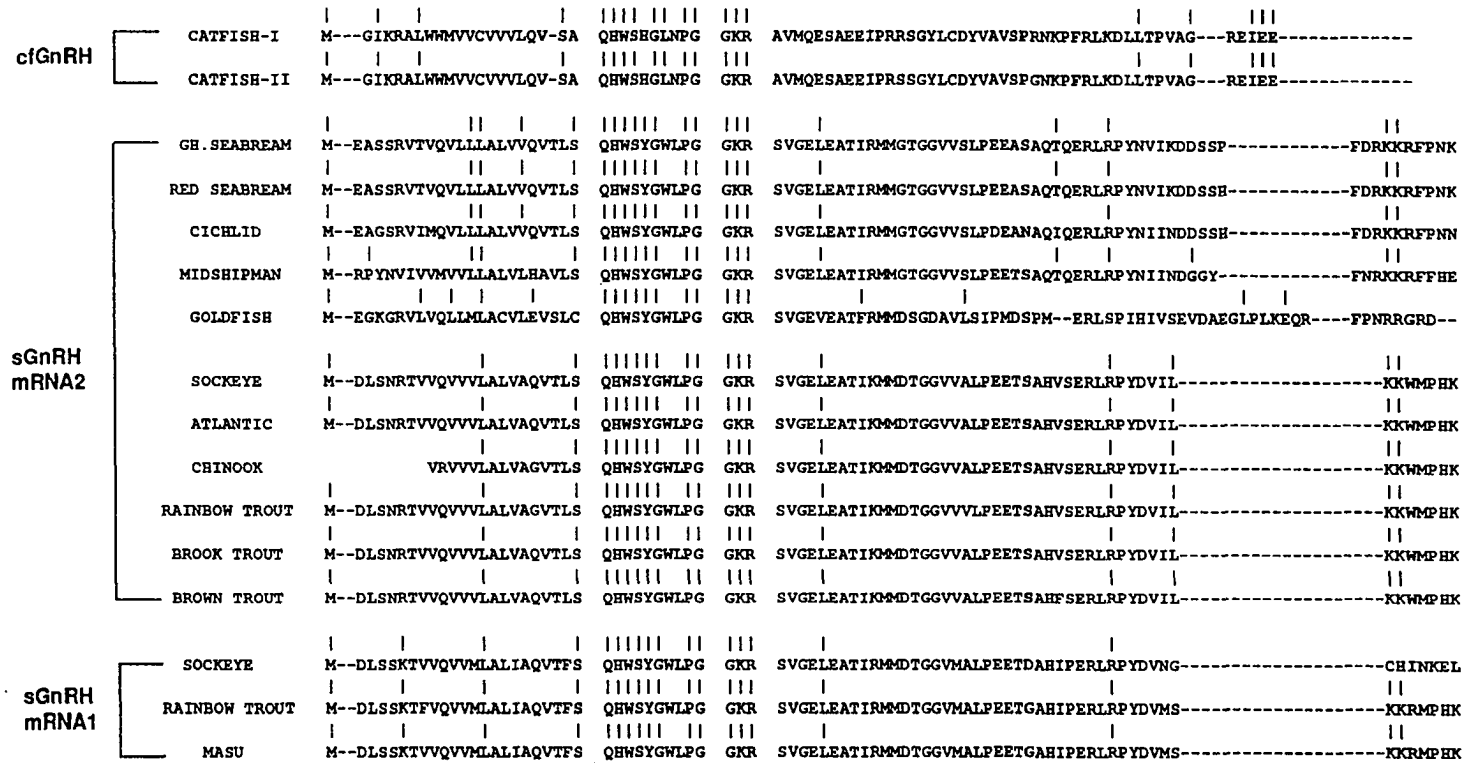
III. GnRH cDNA transcripts.

Each GnRH mRNA encodes a preproprotein with four regions: 1)- a signal region, 2)- the GnRH decapeptide, 3)- a glycine-lysine-arginine processing site and 4)- a GnRH-associated peptide (GAP). The mRNAs encoding the distinct members of the GnRH family have the same four regions, but are obviously different in the region that encodes the hormone and even more variable in the region encoding the GAP (Fig. 1-4). The GAP moiety is thought to hold the preproprotein in the correct conformation for cleavage of the mature hormone.

The cDNA encoding the mGnRH precursor has been characterized for human (Seeburg and Adelman, 1984 and Adelman *et al.*, 1986) , rat (Adelman *et al.*, 1986,

Fig. 1-4. A comparison of known preproGnRHs at the amino acid level. A line (|) indicates that the amino acid below matches the amino acid in human mGnRH. The single letter code is used to designate amino acids. The preproGnRH is shown for seven types of GnRH: mammalian GnRH (human, rat, mouse, frog); guinea pig GnRH (guinea pig); chicken GnRH-I (chicken); seabream GnRH (gilthead seabream, cichlid (*H. burtoni*) and striped bass); chicken GnRH-II (tree shrew to goldfish); catfish GnRH-I (catfish) and salmon GnRH (gilthead seabream to masu).


SIGNAL
GnRH
GAP
23
10
3
56
mGnRH
HUMAN M--KPIQKLLAGLILLTWCVEGCSS QHWSYGLRPG GKR DAENLIDSFQEIVKEVGQLAETQRFECTTHQPRSPLRDLKGALESLIEEETGQ------KKI
RAT M--ETIPKLMAAVVLLTVCLEGCSS QHWSYGLRPG GKR NTEHLVDSFQEMGKEEDQMAEPQNFECTVHWPRSPLRDLRGALERLIEEEAGQ------KKM
MOUSE M----ILKLMAGILLLTVCLEGCSS QHWSYGLRPG GKR NTEHLVESFQEMGKEVDQMAEPQHFECTVHWPRSPLRDLRGALESLIEEEARQ------KKM
TREE SHREW M--ELVPKFLAGLILLTLCVGGCYA QHWSYGLRPG GKR NAENLIDSFQEIAKEADQLAEPQHFECTISQPRSPLRALKGALESLIEEETGQ------KKI
FROG M-KAFPTFALLFLVLLF-SAHVSDA QHWSYGLRPG GKR DTESLQDMYHETPNEVALFPELERLECSVPQSR--LNVLRGAIMNWLEGENR-------KKI
gpGnRH
GUINEA PIG M--GLIPKLLAGLVLLTLCVENGSG QYWSYGVRPG GKR NIEPLVDSFQEMAKEIDQLAEPQHFECTLHQPRSPLRDLKGALESLMEEETGQ------KKI
cGnRH-I
CHICKEN M--EKSRKILVGVLLFTASAAICLA QHWSYCLQPG GKR NAENLVESFQEIANEMESLGEGQKAECPGSYQHPRLSDLKETMASLIEGEARR------KEI
sbGnRH
GH.SEABREAM MAPQTSNLWILLLLVVVMMMSQGCC QHWSYGLSPG GKR DLDSLSDTLGNIIERFPHVDS----PCSVLGCVEEPHVPRMYRMKGFIGSERDIGHRMYKK-
CICHLID MAAKILALWLLLAGTVF---PQGCC QHWSYGLSPG GKR DLDNFSDTLGNMVEEFPRVEA----PCSVFGCAEESPFAKMYRVKGLLASVAERKMDTGHSRNERFL
STRIPED BASS MAPQTFALWLLLVGTLL---GQGCC QHWSYGLSPG GKR ELDGLSETLG-IVGGFPHVET----PCRVLGCAVESPFPKIYRMKGDAVTDRENGPRTYKK-
cGnRH-II
TREE SHREW MASSMLGFLLLLLLLMAAHPGPSEA QHWSHGWYPG GKR ASNSPQDPQSALRPPAPSAAQTAHSFRSAALASPEDSVPWEGRTTAGWSLRRKQHLMRTLLSAAGAPR..
CATFISH M-VSVCRLLLVAALLLCLQAQLSFS QHWSHGWYPG GKR EIDSYSSPEISGEIKL-CEAG----ECSYL---RPLRTNILK-SILIDTLARKFQ----KRK
GH.SEABREAM M--CVSRLVLLLGLLLCVGAQLSNG QHWSHGWYPG GKR ELDSFGTSEISEEIKL-CEAG----ECSYL---TPQRRSVLR-NILLDALARELQ----KRK
CICHLID M--CVSRLALLLGLLLCVGAQLSFA QHWSHGWYPG GKR ELDSFGTSEISEEIKL-CEAG----ECSYL---RPQRRSILR-NILLDALARELQ----KRK
STRIPED BASS M--CVSRLVLLFGLLLCVGAQLSNA QHWSHGWYPG GKR ELDSFGTSEISEEIKL-CEAG----ECSYL---RPQRRNVLR-NIILDALARELQ----KRK
GOLDFISH-I M-VHICRLFVVMGMLLCLSAQFASS QHWSHGWYPG GKR EIDVYDSSEVSGEIKL-CEAG----KCSYL---RPQGRNILK-TILLDAIIRDSQ----KRK
GOLDFISH-II M-VHICRLFVVMGMIMFLSVQFASS QHWSHGWYPG GKR EIDVYDPSEVSEEIKL-CNAG----KCSFL---IPQGRNILK-TILLDALTRDFQ----KRK


cfGnRH
CATFISH-I
CATFISH-II
sGnRH mRNA2
GH.SEABREAM
RED SEABREAM
CICHLID
MIDSHIPMAN
GOLDFISH
SOCKEYE
ATLANTIC
CHINOOK
RAINBOW TROUT
BROOK TROUT
BROWN TROUT
sGnRH mRNA1
SOCKEYE
RAINBOW TROUT
MASU

Azad *et al.*, 1991 and Oikawa *et al.*, 1990), mouse (Mason *et al.*, 1986), tree shrew (*Tupaia glis belangeri*) (Kasten *et al.*, 1996) and frog (Hayes *et al.*, 1994) (Fig. 1-4). An *in situ* hybridization study using a probe to the mGnRH cDNA confirmed that the mRNA in a mammal (mouse) is present in the preoptic area of the brain (see Seeburg *et al.*, 1987).

The cDNA encoding a novel form of GnRH has been isolated from the hypothalamus of guinea pig (*Cavia cobaya*) (Jimenez-Linan *et al.*, 1997) and has not been reported in any other species to date (Fig. 1-4). The GnRH decapeptide differs from its mammalian counterparts by two amino acid substitutions in positions 2 and 7. These two amino acid substitutions are unique in comparison to all other known forms of GnRH.

Only one cDNA for cGnRH-I has been identified. The cGnRH-I cDNA was isolated from chicken brains (Dunn *et al.*, 1993), but *in situ* hybridization has not been reported.

Seabream GnRH cDNA has been isolated from gilthead sea bream, cichlid and striped bass (Gothilf *et al.*, 1995, White, S.A. *et al.*, 1995 and Chow *et al.*, 1998). This form of GnRH represents the third form of GnRH in several species of fish and hence the use of a specific cDNA probe to localize the neurons that express sbGnRH was important. In two cichlids (*H. burtoni* and tilipia), the sbGnRH was expressed in neurons in the preoptic area, whereas sGnRH was in the rostral forebrain and cGnRH-II was in the midbrain (White, S.A. *et al.*, 1995 and Parhar, 1997) (Fig. 1-4).

cDNAs for cGnRH-II have been isolated from monkey (Terasawa, personal communication), tree shrew (Kasten *et al.*, 1996), catfish (*Clarias gariepinus*) (Bogerd *et al.*, 1993), gilthead seabream (Gothilf *et al.*, 1996), cichlid (White *et al.*, 1994) and striped bass (Chow *et al.*, 1998). An interesting aspect of molecular phylogeny is that goldfish are tetraploid and hence two cDNAs encoding the cGnRH-II precursor were isolated (Lin and Peter, 1997). Specific probes for cGnRH-II have been used to definitively identify this form of GnRH in the midbrain of tree shrew (Kasten *et al.*,

1996), catfish (Zandbergen *et al.*, 1995), two cichlids (White, S.A. *et al.*, 1995 and Parhar, 1997) and the gilthead seabream (Gothilf *et al.*, 1996).

Two cDNAs encoding catfish GnRH (cfGnRH) were isolated from African catfish (*C. gariepinus*) (Bogerd *et al.*, 1993). The two cDNAs encode the identical hormone, but differ by four nucleotide substitutions that lead to the change of two amino acids in the GAP region. *In situ* hybridization has localized the cfGnRH form to the rostroventral forebrain and preoptic region (Zandbergen *et al.*, 1995).

The cDNA encoding sGnRH mRNA2 has been sequenced for six salmonids (Klungland *et al.*, 1992 a and b, Ashihara *et al.*, 1995, Coe *et al.*, 1995), three perciforms (a cichlid and both red and gilthead sea bream) (Bond *et al.*, 1991, Okuzawa *et al.*, 1995 and Gothilf *et al.*, 1996), one batrachoidiform (midshipman) (Grober *et al.*, 1995) and one cyprinid species (goldfish) (Lin and Peter, 1996) (Fig. 1-4). *In situ* hybridization studies done on the brains of different salmonids detected cell bodies with sGnRH from the olfactory bulb to the preoptic area of the brain (Suzuki *et al.*, 1992, Bailhache *et al.*, 1994).

Due to the tetraploidization of the salmonids, additional cDNAs that encode sGnRH mRNA1 have been characterized for sockeye salmon (Ashihara *et al.*, 1995), rainbow trout (Chapter 3) and masou salmon (Suzuki *et al.*, 1992). The portion of the mRNA1 encoding the hormone has two nucleotide substitutions in comparison to the sGnRH mRNA2 transcripts, but because these changes occur in the wobble position of the codons for amino acids 2 and 6, the sGnRH decapeptides translated from these two different sGnRH-encoding mRNAs are the same. Differences among the signal and GAP regions of the sGnRH mRNA1-encoded products put them in their own separate class from their sGnRH mRNA2 counterparts (Fig. 1-4).

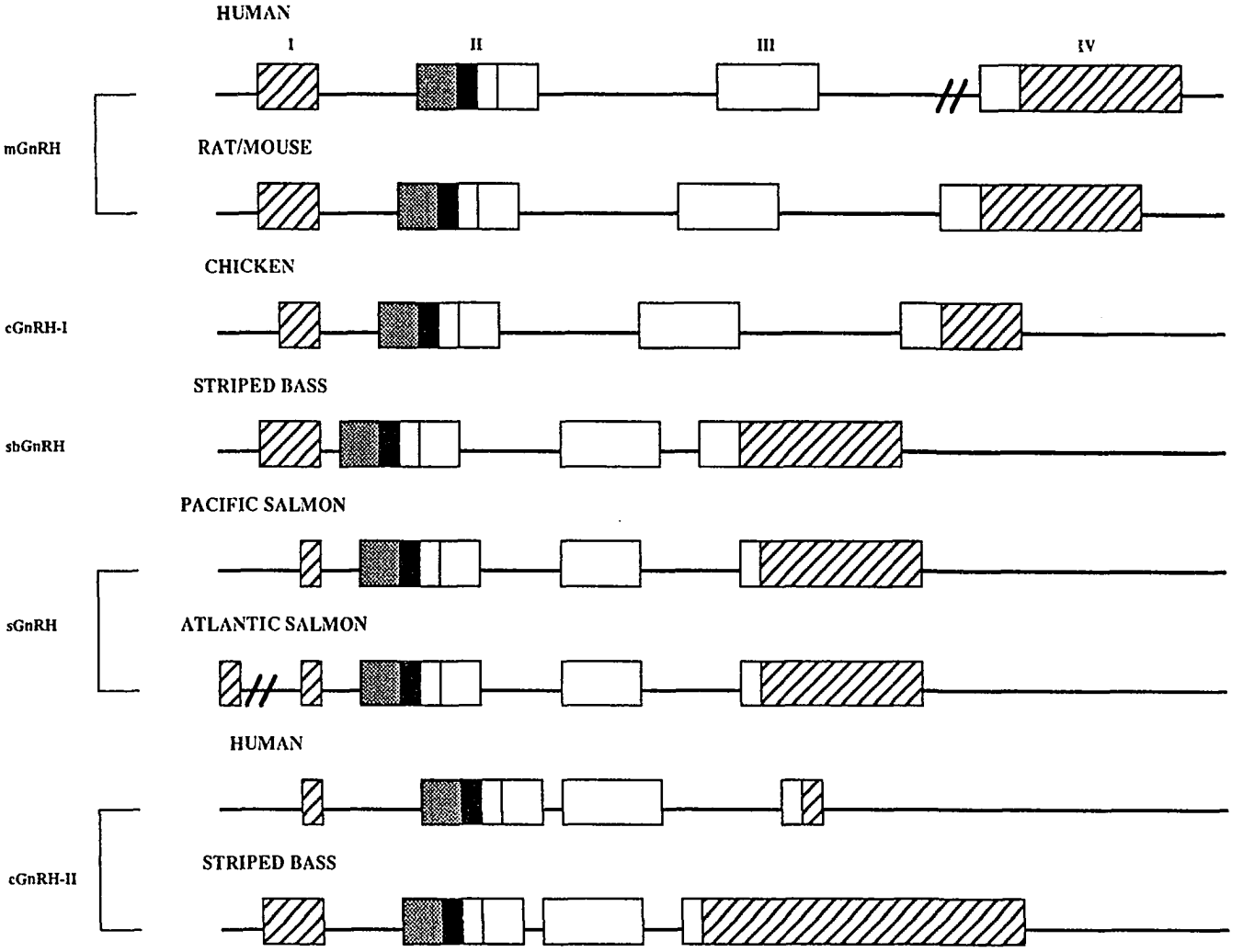
To trace the evolution of GnRH in detail requires more information than provided by the ten amino acid GnRH peptide. The precursor molecule of each peptide as deduced from the cDNAs is useful in preparing an evolutionary map. Very little conservation of

the GAP region of the precursors is observed (Fig. 1-4), but this region may be crucial for determining the diversification of the GnRH family in finer detail. For example, in the mGnRH, gpGnRH and cGnRH-I precursors, the GAP amino acids are closer in identity than between mGnRH and any other GnRH precursor (Fig. 1-4), confirming the idea that cGnRH-I was derived from mGnRH relatively recently. In the salmonid and human GAPs, however, only 4-9% identity is found, but the secondary structural level has some conservation. A central cysteine can be aligned in each GAP for mGnRH, gpGnRH, cGnRH-I, cGnRH-II (except tree shrew) and sbGnRH, but not for sGnRH (Fig. 1-4). Also, cGnRH-II has a second cysteine that can be aligned with cfGnRH's cysteine. Although speculative, I suggest cfGnRH may be derived from a duplication of the cGnRH-II gene. Amino acid identity is not helpful here as cfGnRH is 2 amino acids different from either mGnRH or cGnRH-II. Furthermore, a leucine in mGnRH (position 5 of GAP) is repeated in cGnRH-I, sbGnRH (except cichlid) and sGnRH (except goldfish), but not in cGnRH-II or cfGnRH. The sum of evidence supports the idea that mGnRH was ancestral to salmon GnRH, seabream GnRH and chicken GnRH-I. The accuracy of determining the lineage of GnRH forms will increase as more cDNAs are sequenced and the GAP regions can be compared for shorter evolutionary distances.

IV. GnRH genes in vertebrates.

Five different genes that encode GnRH in vertebrates have been identified to date; each of these genes encodes only one form of GnRH: mGnRH, sbGnRH, cGnRH-I, cGnRH-II or sGnRH (Fig. 1-5). The gene from which mGnRH is transcribed has been identified in human (Adelman *et al.*, 1986, Hayflick *et al.*, 1989 and Radovick *et al.*, 1990), rat (Adelman *et al.*, 1986, Bond *et al.*, 1989 and Kepa *et al.*, 1992) and mouse (Mason *et al.*, 1986), whereas the cGnRH-I gene was identified in chicken (Dunn *et al.*,

Fig. 1-5. Comparison of the GnRH genes for five distinct forms of GnRH: mammalian GnRH (mGnRH), chicken GnRH-I (cGnRH-I), seabream GnRH (sbGnRH), salmon GnRH (sGnRH) and chicken GnRH-II (cGnRH-II). The sGnRH gene has been isolated and sequenced from an Atlantic salmon (*Salmo salar*) and from a Pacific salmon (*Oncorhynchus nerka*, sockeye salmon). The cGnRH-I and sbGnRH gene has been isolated only from chicken and striped bass, respectively, but the mGnRH gene has been characterized from mouse, rat and human. The cGnRH-II gene has been isolated in both striped bass and human. Exons I, II, III and IV are labeled. Introns are shown as lines. The coding for the signal peptide is stippled, the hormone is black, the processing site and GnRH-associated peptide (GAP) are white, and the 5'- and 3'-untranslated regions are shown by diagonal lines.


HUMAN
I
II
III
IV
mGnRH
RAT/MOUSE
CHICKEN
cGnRH-I
STRIPED BASS
sbGnRH
PACIFIC SALMON
sGnRH
ATLANTIC SALMON
HUMAN
cGnRH-II
STRIPED BASS

1993) and the salmon GnRH gene from two species of salmon: an Atlantic salmon (*Salmo salar*) (Klungland *et al.*, 1992b) and a Pacific salmon (*Oncorhynchus nerka*) (Coe *et al.*, 1995). The genes encoding sbGnRH and cGnRH-II were recently characterized in striped bass (Chow *et al.*, 1998). The cGnRH-II-encoding gene has been isolated in monkey and human, but the sequences have not yet been published (Terasawa, personal communication and White *et al.*, 1998). The architecture of the genes encoding the five different GnRH forms is the same with a common organization of four exons separated by three introns (Fig. 1-5). In each gene, exon 1 encodes the 5'-untranslated region (5'UTR); exon 2 has the signal peptide, GnRH, a proteolytic cleavage site and the amino terminus of a GnRH-associated peptide (GAP); exon 3 has the central GAP moiety; and exon 4 has the carboxy terminus of GAP and the 3'-untranslated region (3'UTR).

Several interesting patterns are apparent in the regulatory 5'-flanking regions of GnRH. The 5'-regulatory regions of the genes encoding mGnRH (Radovick *et al.*, 1990, Kepa *et al.*, 1992) and cGnRH-I (Dunn *et al.*, 1993) differ from the fish genes encoding sGnRH (Klungland *et al.*, 1993, Coe *et al.*, 1995) because the former contain obvious regions of alternating purines (adenine or guanine) and pyrimidines (thymine or cytosine) or blocks of polydeoxyadenosine residues. Many of the AT-rich regions in each gene may serve as targets for members of the Pit-1/Oct-1/Unc-1 (POU) family of transcription factors (Bendall *et al.*, 1993) for modulation of GnRH expression. Interestingly, one footprint in the proximal promoter region contains an AT-rich element that has been implicated as a target for protein kinase C (PKC)-mediated repressor activity resulting in downregulation of the rat GnRH promoter (Eraly and Mellon, 1995). Furthermore, a distal enhancer region within the rat GnRH promoter also contains AT-rich elements that exert stimulatory (Clark and Mellon, 1995) and inhibitory (Belsham *et al.*, 1996) effects on transcription.

Although much is known about the presence and distribution of multiple forms of GnRH within the brain, relatively little is known about their regulation. Two or more GnRH genes within a single species indicate that each gene probably serves a distinct task. The high degree of sequence variability and lack of obvious regions of sequence conservation in the promoters of GnRH-encoding genes between species points to differences in the regulation of each gene. A comparison of the promoters from known GnRH-expressing genes and the factors that control their transcription will be discussed in an upcoming chapter of this thesis.

V. GnRH function.

A number of studies using immunocytochemical methods and characterization of amino acids and cDNAs all document the existence of multiple forms of GnRH within the brain. However, very little is known about the distinct functions for individual GnRHs. Location of each peptide may give a clue about function. For example, close proximity of GnRH-containing neurons to the pituitary implies a primary role as a gonadotropin releaser; detection in the forebrain, near olfactory nerve and/or retinal projections, implies a role in integrating olfactory and visual cues involved in reproductive behaviour; discovery in the midbrain of GnRH neurons with axons that terminate within the brain or spinal cord implies neuromodulatory roles possibly modifying motor-associated reproductive behaviours.

The difference in distribution of the vertebrate GnRHs is attributable to at least two distinct embryonic origins: one in the olfactory placode, which gives rise to the terminal nerve-septo-preoptic system, and one in the midbrain, which gives rise to the posterior cGnRH-II system (Muske, 1993). The embryonic origin of a third form of GnRH in the brain, at least for sbGnRH, is also the olfactory placode. The distribution gives a clue as to the functions of the forms of GnRH.

A. Release of gonadotropin. One clear function of GnRH is to activate the synthesis and release of gonadotropins from the pituitary. Neurons that contain immunoreactive mGnRH, cGnRH-I, sGnRH, cfGnRH or sbGnRH have been localized in the basal forebrain-septo-preoptic system and their axons shown to terminate on the hypothalamo-hypophysial portal vessels in tetrapods or in the pituitary in teleost fish. This location suggests that these GnRHs are predominantly involved in reproductive control as gonadotropin-releasing factors. Teleost bony fish offer a unique insight into which form of GnRH is the gonadotropin releaser because the neurites containing GnRH end directly in the pituitary. The number of forms of GnRH found in terminals in the pituitary, however, varies with the species. In fish with three forms of GnRH, the pituitary of the cichlid contained only sbGnRH (Powell *et al.*, 1995), but the pituitary of the sea bream fish (Powell *et al.*, 1994) and tilapia (Parhar, 1997) contained both sbGnRH and cGnRH-II. In fish with two forms of GnRH, the pattern also varies: goldfish (Kim *et al.*, 1995 and Yu *et al.*, 1988) have two forms of GnRH (sGnRH and cGnRH-II) in the pituitary; eels and catfish have predominantly one form of GnRH (mGnRH or cfGnRH, respectively) in the pituitary, but very small amounts of cGnRH-II also have been detected (Montero *et al.*, 1995 and Zandbergen *et al.*, 1995); salmon have only sGnRH (Amano *et al.*, 1991). Therefore, more than one form of GnRH may act on the pituitary. Physiologically, all three forms of GnRH, if given exogenously, release gonadotropin in sea bream (Zohar *et al.*, 1995) and both forms release gonadotropin or induce ovulation in the goldfish (Habibi *et al.*, 1992).

The (Hyp^9)mGnRH form, which is found in mammals and amphibians, binds to mGnRH-like pituitary receptors to stimulate the release of LH and FSH *in vitro* and *in vivo* in a similar dose-dependent but less potent manner than mGnRH (Gautron *et al.*,1992). In mammals and amphibians, post-translational enzymatic modification of existing GnRH molecules, rather than gene duplication and subsequent emergence of

novel GnRH forms, may be an alternate strategy to achieve molecular heterogeneity and diversity of function.

B. Release of growth hormone and prolactin. GnRH in vertebrates releases gonadotropins from the pituitary, but in some species of fish, GnRH also releases growth hormone (Marchant *et al.*, 1989 and Melamed *et al.*, 1995) and prolactin from the pituitary (Weber *et al.*, 1997). The three native forms of GnRH in tilapia brain stimulate the *in vitro* release of prolactin from the pituitary with the following order of potency: cGnRH-II>sGnRH>sbGnRH. This effect was shown to be a direct effect on the prolactin cells in the pituitary. It is not surprising that GnRH affects both growth hormone and prolactin as these two hormones are members of the same protein family and are thought to have resulted from an earlier gene duplication. Comparable doses of GnRH were effective in stimulating the release of gonadotropin, growth hormone and prolactin from pituitary fragments of fish (Weber *et al.*, 1997). In addition, the order of potency for the three types of GnRH for release of gonadotropin in sea bream (Zohar *et al.*, 1995) was the same as for release of prolactin in tilapia (Weber *et al.*, 1997).

C. Putative effects on behaviour. The localization of cGnRH-II in midbrain neurons in all vertebrates including primates (Lescheid *et al.*, 1997) indicates this GnRH form may serve a neuromodulatory role in reproduction and behaviour. Axons containing cGnRH-II extend to many regions of the brain (Lepretre *et al.*, 1993 and Muske, 1993) and are known to descend in the spinal cord (Millar and Kriebel, 1986, Wright and Demski, 1991). Indirect evidence shows that cGnRH-II is the form that can elicit the late, slow excitatory-postsynaptic potential in frog sympathetic ganglia (Jones, 1987). In addition, the application of GnRH to the midbrain resulted in changes in reproductive behaviour (Pfaff, 1973). The early expression of cGnRH-II in fish

embryos (White, R.B. *et al.*, 1995) and fetal monkeys (Quanbeck *et al.*, 1997) suggests that additional roles are important.

D. Local action in reproductive organs. GnRH is important in the development and maturation of reproductive tissues of vertebrates. In mammals GnRH has been utilized to serve new roles as demonstrated by its postulated involvement in the development of the placenta (Kelly *et al.*, 1991) and in embryo implantation and growth (Li *et al.*, 1993). A common theme exists for GnRH transcripts expressed in human placenta, mammary gland, ovary and testis whereby the 5'-untranslated region (UTR) is longer than in brain transcripts (Dong *et al.*, 1993). The elongated 5'-UTRs can result from use of a different upstream start site compared to brain transcription (Dong *et al.*, 1993) or to the retention of intron 1 in the mRNA (Seeburg and Adelman, 1984, Radovick *et al.*, 1990, Dong *et al.*, 1993). The long 5'-UTRs in placental, breast and gonadal tissue compared with brain indicates differential regulatory mechanisms. To date, only preliminary data exists on GnRH expressed in the gonads of lower vertebrates, but GnRH-like activity and/or binding sites are present in the ovary of goldfish, carp and African catfish (see Habibi *et al.*, 1994). sGnRH mRNA has also been detected in midshipman ovary and testis by Northern analysis (Grober *et al.*, 1995) and both sGnRH and cGnRH-II cDNAs have been isolated from the goldfish ovary (Lin and Peter, 1996). Finally, the vestiges of the early role of GnRH in gonadal development are still seen in the tunicate where gonadogenesis fails to occur in the absence of the nerve plexus containing GnRH (see Irons, 1986).

E. Functions in ancestral animals. In a protochordate, the injection of tunicate(t) GnRH-I or tGnRH-II monomers into the visceral blood sinus of adult tunicates results in a measurable increase in estradiol content of the gonads within 6 hours (Craig *et al.*, 1997). This observation as well as immunocytochemical studies showing numerous ir-GnRH neurons surrounding adult and juvenile gonads and gonoducts, suggest tGnRH

peptides are released into the bloodstream to act directly on the gonads. Current evidence suggests that it is unlikely tunicates contain an organ homologous to the pituitary. Therefore, their neurohormones may act directly on the gonads or other tissues. The observation that tunicate GnRHs affect the estradiol content of the gonad provides evidence for the early existence of GnRH receptors.

The structure of a molluscan GnRH-like substance has not been reported, but an extract from ganglia of *H. trivolvis* released gonadotropins from dispersed goldfish pituitary cells (Goldberg *et al.*, 1993). This suggests that the GnRH family may be widely distributed.

VI. Purpose of thesis.

Pacific salmon are an important biological resource. They provide food for over 22. species of mammals and birds and act as a huge nitrogen and carbon sink for coastal watersheds. Their dramatic life cycle and incompletely understood migratory habits provide a mystique that still permeates the culture of coastal aboriginal peoples.

I chose both sockeye salmon and rainbow trout to study because of their differences in habitat and reproductive patterns. Sockeye salmon spend the bulk of their adult lives in the ocean and then return to spawn in natal freshwater streams (anadromous) and die (semelparous). In contrast, rainbow trout spend their entire lives in freshwater lakes and streams and can spawn more than once (iteroparous). Some rainbow trout spawn in spring, whereas others spawn in the fall. Atlantic salmon, which are anadromous like the sockeye salmon, can spawn, return to sea and spawn again, sometimes as many as four times in a lifetime.

Reproduction is dependent on external cues such as water temperature, light and the behaviour of conspecifics. Age, fat content and the physiological milieu also play an important role in the readiness of a fish to mate and reproduce. Much, if not all, of this information ultimately converges on the brain or gonads to signal transcription,

synthesis and secretion of GnRH, which is central to reproduction. This thesis examines the control and location of expression of GnRH in these two contrasting fish species to improve our understanding of the mechanisms involved that integrate the events governing reproductive processes.

The main purpose of this thesis is to study the regulation and expression of GnRH in the brain and reproductive tissues of sockeye salmon and rainbow trout. The first objective was to isolate and characterize the gene and cDNA that encodes sGnRH in a salmonid. I isolated the first gene encoding sGnRH from the tetraploid sockeye salmon. This particular gene is referred to as the sGnRH gene2. At the time this was achieved only the gene encoding mGnRH and cGnRH-I had been established and characterization of the gene encoding sGnRH mRNA2 in Atlantic salmon had just been completed. Characterization of the sGnRH gene provides the basis to determine which regions of this particular gene are conserved after the duplication of the entire genome as occurred in salmon approximately 27 million years ago.

For my second objective the start site used to generate the sGnRH mRNA2 transcripts in the brain was determined unequivocally by primer extension analysis. Also a Southern analysis was done on sockeye salmon genomic DNA using a portion of the sGnRH gene2 as a probe to provide evidence that other genes encoding GnRH were also present in the salmonid genome.

My third objective was to sequence the 5'-flanking region of the gene encoding sGnRH mRNA1 from rainbow trout so I could compare the regulatory regions for GnRH from rainbow trout, Pacific salmon (sockeye salmon) and Atlantic salmon. For example, a large segment of 1152 nucleotides in the promoter region of the sockeye salmon sGnRH gene2 is missing in comparison to the Atlantic salmon gene. Furthermore, all three genes share strong conservation in the most proximal 200 bps of their regulatory 5'-flanking DNA. However, except for 100 bps in the upstream 5'-flanking region, the sGnRH gene1 bears no resemblance to either of the two genes that

encode sGnRH mRNA2. This indicates that all the sGnRH-encoding genes characterized to date are governed by different upstream regulatory factors or that the response elements are organized in a different pattern so that the sGnRH genes share some common control elements.

Because GnRH has been shown to be present in mammalian placenta, ovary and testis, salmonid retina and in amphibian sympathetic ganglia, my fourth objective was to examine various salmonid tissues at different life stages to determine the expression pattern of GnRH in tissues outside of the salmonid brain. My early work provided evidence that GnRH was indeed expressed as mRNA in the gonadal tissue of 1.5-year-old sockeye salmon. This work was followed by the monthly examination of ovarian and testicular tissue from both sockeye salmon and rainbow trout during the first two years of their lives.

These tissue expression studies revealed that the regulation of GnRH in the brain and gonad of salmonids differs. I found that sGnRH mRNA2 transcripts were generated for much shorter durations in the ovaries and testes of sockeye salmon than in rainbow trout. For each of the two years that the reproductive tissues were extensively examined, sockeye salmon expressed GnRH mRNA for no more than one month (October) of the first year and not at all in the second year, whereas rainbow trout expressed GnRH from May through October of the first year and in December of the second year. Fish that mature precociously in year 2 in both species express GnRH in May and June (sockeye salmon) or from June to October (rainbow trout), suggesting that reproductive maturity is coordinated with an increase in GnRH expression in the gonads.

My fifth research objective was to study the regulation of expression of the sGnRH mRNA2 transcripts in the salmonid ovaries and testes. I found that the transcripts were generated from an alternative upstream promoter that differed from that used in the brain. I determined from primer extension analysis that an additional 322 nucleotides of 5'-untranslated region is included in the gonadal transcript, but not the brain transcript.

Also, intron 1 may be retained and when coupled to the alternative upstream start site results in sGnRH cDNA2s in the gonads with much longer 5'-UTRs than their brain counterparts.

A study of the regulation and expression of sGnRH mRNA2 in both sockeye salmon and rainbow trout embryonic tissue was my sixth objective. Tissues collected every other day for rainbow trout 1 to 42 days after fertilization (DAF) and sockeye salmon 1 to 68 DAF were examined by reverse transcriptase-polymerase chain reactions. sGnRH mRNA2 was expressed in rainbow trout and sockeye salmon tissue beginning from 10 and 30 DAF, respectively. Use of the upstream alternative promoter for each species was temporal, but was used consistently just prior to and following hatching. Detection of GnRH expression was not evident in gonadal tissue following this developmental period until four months later and only in rainbow trout.

As described above, transcripts encoding mGnRH, sGnRH and cGnRH-II have been isolated and characterized from the ovaries of various species. However, to date, translation of these messages into protein has not been shown unequivocally. My seventh objective was to determine if GnRH expressed in the salmonid ovary and testis was translated into protein. I examined rainbow trout ovaries and testes by HPLC and competitive RIA. RIA of HPLC fractions eluted from extracts of immature or precocious (jill or jack) 17-month-old ovaries and testes showed weak cGnRH-II immunoreactivity for only the jill ovaries. Unexpectedly, RIA detected strong immunoreactivity for four different forms of GnRH in both the ovaries and testes of 18- to 20-month-old rainbow trout that matured precociously. HPLC elution and cross-reactivity with specific antiserum indicated the presence of mGnRH, cGnRH-II, sGnRH and an unknown GnRH in these tissues. Subsequent studies on normal developing tissues gathered from 21-month-old fish did not detect GnRH immunoreactivity. This indicates that detectable GnRH peptide is present only for a short period during the mid-summer in precociously mature rainbow trout.

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Chapter 2

Characterization of the Pacific salmon gonadotropin-releasing hormone gene, copy number and transcription start site

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*The contribution of the first two authors was equal.

Summary

To study the origin and regulation of the GnRH gene, I isolated and sequenced the salmon form of GnRH from a Pacific salmon (*Oncorhynchus nerka*). A Southern blot showed a single band that strongly hybridized to a probe for the gene reported here and weaker bands that may represent genes for related forms of GnRH. There is strong conservation of sequence in the hormone coding region and of the gene organization between fish and mammals. However, the GnRH-associated peptide (GAP) shows very little sequence identity with the mammalian GAPs, questioning its physiological role. I also show for the first time the transcriptional start site for a GnRH gene in a non-mammalian species. Interestingly, a large segment of 1152 basepairs in the promoter region of the Pacific salmon GnRH gene is missing compared with the Atlantic salmon (*Salmo salar*) gene. These gene rearrangements suggest that these two salmonid species, which have been geographically separated for 8 to 15 million years, have evolved promoters with different mechanisms for control and transcription of GnRH.

Introduction

The overall objective in this initial project was to study the evolution and regulation of the GnRH gene. I selected a salmon (sockeye salmon, *Oncorhynchus nerka*) because this teleost fish is separated in evolution by about 400 million years from humans and hence offers a good comparison for determining the regions of the gene that have been conserved. Salmon are also interesting in that they are tetraploid. The duplication of the genome means that two copies of each gene may exist which allows one to study the changes that occur in duplicate genes.

My first goal was to determine the sequence and organization of the gene that encodes salmon GnRH (sGnRH). The question was whether there is conservation of sequence and gene organization between fish and mammals. Also, I was interested in conservation of the GnRH-associated peptide (GAP) sequence compared with the mammalian GAPs, as the physiological role of GAP is controversial. If the GAP structure is tightly conserved, the function might also be conserved. My Southern analysis of salmon genomic DNA was carried out to determine if other genes encode forms of GnRH in addition to the gene that encodes the specific form of GnRH reported here. This analysis was designed to show whether other genes encode related forms of GnRH due to both the tetraploid nature of salmon and the presence of a second form of GnRH in sockeye salmon. I also examined for the first time the transcriptional start site for a GnRH gene in a nonmammalian species. Changes in promoter regions may also be important in evolution. Therefore I compared the promoter region of the sockeye salmon sGnRH gene with the mammalian and Atlantic salmon (*Salmo salar*) genes to determine if any gene rearrangements had occurred.

Materials and Methods

Genomic library screening

An amplified sockeye salmon liver genomic library in Lambda FIX II (Stratagene, La Jolla, CA), obtained from Dr. Robert Devlin at West Vancouver Laboratories, Dept. of Fisheries and Oceans, was screened using a 435 basepair (bp) ^{32}P-radiolabelled (Dupont/New England Nuclear, Boston, MA) cDNA encoding salmon GnRH from a cichlid fish (*Haplochromis burtoni*) (Bond *et al.*, 1991). Approximately 700,000 phage were screened using duplicate filter lifts. Screening took place in plaque screening buffer containing the radiolabeled probe (5 x 10^8 cpm/mg), 6X standard saline citrate (SSC), 0.1% sodium dodecyl sulfate (SDS) and 5X Denhardt`s solution at 42° C for 24 h. Filters were rinsed three times at room temperature in 2X SSC/0.1% SDS, then twice in 2X SSC/0.1% SDS at 37° C for 1 h and finally in 1X SSC/0.1% SDS. Positives were rescreened five times to ensure purity and bacteriophage DNA was prepared by standard methods (Ausubel *et al.*, 1989). Four positives were identified and the DNA further characterized.

Identification of a GnRH-containing clone by asymmetrical PCR

A sense primer against the conserved sequences encoding the GnRH decapeptide (5'-CAGCACTGGTCGTATGGCTGGCTACCTGG-3') was used without an antisense primer to amplify single-stranded polymerase chain reaction (PCR) products from each of the four positive clones. PCR samples were prepared in a 50.0 µL volume containing 0.5 µL lysate DNA, 1x Promega buffer (50.0 mM KCl, 10.0 mM Tris-HCl, 1.5 mM $MgCl_2$ and 0.1 % Triton X-100), 200 mM dNTPs, 2.5 units of Taq DNA polymerase (Promega, Madison, WI) and 30 pmol GnRH primer. The first step cycle of PCR was programmed for 3 min of denaturing at 94° C, one min of annealing at 55° C and 2 min of primer extension at 72° C. The profile of the remaining 39 cycles was one min at 94° C, one minute at 55° C and 2 min at 72° C, with a 7 min extension at 72° C in the last cycle.

The retrieved single stranded PCR products were incubated at room temperature for 3 h in a 1x oligolabeling buffer containing 0.5 M Tris-HCl, 0.1 M $MgSO_4$, 1.0 mM dithiothreitol (DTT), 0.6 mM of each deoxynucleoside triphosphate (dNTP), 100 ng hexameric random-sequence oligodeoxynucleotides and Klenow polymerase (Pharmacia, Dorval, Quebec, Canada) to make them double-stranded. They were then ligated into pBluescript II KS^+ vector and cloned in XL-1 Blue host cells (Stratagene). Recombinant plasmids were sequenced by the chain termination method (Sanger *et al.*, 1977) using the Sequenase Version 2 kit (United States Biochemicals, Cleveland, OH). Sequence analysis revealed that a PCR product from one clone contained pro-GnRH sequences from the start of GnRH through the stop codon of the GAP.

Isolation of the sockeye salmon prepro-GnRH gene

The 15000 bp genomic clone encoding GnRH was XbaI digested yielding three fragments of approximately 6000, 5400 and 3400 bp. The 5400 bp fragment was determined to contain the sequences encoding GnRH by asymmetrical PCR and further digested with KpnI to yield three fragments of 2833, 1520 and 1050 bp. Each KpnI-cut fragment was ligated into Bluescript plasmid, subcloned and sequenced by using Circumvent (New England Biolabs, Beverly, MA) or Sequenase Version 2. The 2833 bp fragment was determined to contain 2666 bp of salmon GnRH from position -791 to 1875 (Fig. 2-1). The orientation of the 1520 and 1050 bp subclones was determined by asymmetrical PCR on the GnRH genomic clone. Each of these subclones was partially sequenced with the result that the 1050 bp KpnI-cut subclone provided downstream sequence information for the 3'-flanking region as shown from position 1876 to 2240 (Fig. 2-1). The 1520 bp subclone was downstream from the 1050 bp subclone.

cDNA synthesis and analysis

Brain poly(A^+)-rich RNA extracted as described above was incubated for 1 h at 37° C with 10 U RNase-free DNase followed by 10 min at 90° C. First-strand cDNA was synthesized using Superscript RT RNase H^- reverse transcriptase by methods recommended by the manufacturer (Life Technologies, Inc., Gaithersburg, MD). Amplification of cDNA was achieved with 35 cycles of 1 min at 94° C, 1 min at 55° C and 1 min at 72° C using primers corresponding to positions 13 to 39 and 1031 to 1048 of exon 1 and exon 4, respectively (Fig. 2-1). The integrity of the cDNA was also confirmed by a separate PCR using primers which correspond to bases 523 to 545 and 719 to 740 specific for a salmon tubulin cDNA clone (Coe *et al.*, 1992).

The PCR products were separated on a 1.5 % agarose gel (Seakem FMC Bioproducts, Rockland, ME) and the GnRH DNA band retrieved by electroelution in dialysis tubing. The 286 bp fragment was subcloned into pBluescript II KS^+ vector and sequenced (Fig. 2-2).

RNA extraction and primer extension

Sockeye salmon were anesthetized by bubbling carbon dioxide; brains were immediately excised and frozen in liquid nitrogen. Poly(A^+) mRNA was isolated using a FastTrack mRNA isolation kit (Invitrogen Corp., San Diego, CA).

The transcription initiation site of the sockeye salmon prepro-GnRH gene was determined by extension of an antisense oligonucleotide primer 5'-ATACTTTCTCCGTTCTGTG-3' complementary to nucleotides 30 to 44 of exon 1. The primer (500 ng) was 5'-end labeled with [gamma-^{32}P]-ATP (Dupont/NEN) by T4 polynucleotide kinase and purified to a specific activity of 3 x 10^8 cpm/μg. A mixture of 2.4 ng labeled primer and 20 μg of sockeye salmon brain poly(A^+) mRNA was combined

in 1x first strand buffer and heated to 85° C for 5 min. After primer hybridization at 50° C for 4.5 h in the presence of 64.8 U RNAguard ribonuclease inhibitor (Pharmacia), the annealed primer was extended upon addition of 10 mM DTT, 0.5 mM of each dNTP and 4 U of Superscript reverse transcriptase (Bethesda Research Laboratories, Bethesda, MD) when incubated at 42° C for 3 h. The reaction was stopped by the addition of 1.0 µL 0.5 M EDTA (pH 8.0) and 1.0 µL DNase-free RNase (10 mg/mL) and the mixture extracted once with phenol/chloroform and ethanol precipitated. The purified extended products were dissolved in 4 µL TE (pH 8.0) and 4 µL sequencing buffer, then electrophoresed on a 6% polyacrylamide/8M urea gel in parallel with sequencing products of the 2833 bp KpnI subclone extended from the 5'-end labeled primer by the Sequenase Version 2 method. The gel was dried and exposed to Kodak XAR5 film (Rochester, NY) at -80° C for 24 h.

Southern blotting

Genomic DNA was extracted from a single sockeye salmon brain (Ausubel *et al.*, 1989) and digested with either HpaI, BspHI, PvuII, HindIII or HincII in duplicate. It was then electrophoresed through a 0.9% agarose gel and capillary transferred to nitrocellulose membranes (Hybond-N^+; Amersham, Oakville, Ontario, Canada). The membranes were prehybridized at 45° C for 4 h in 0.5 M NaH_2PO_4 (pH7.2), 1% bovine serum albumin, 1 mM EDTA, 7% SDS (Church and Gilbert, 1984) plus 100 µg/mL denatured sea urchin (*Strongylocentrotus purpuratus*) sperm DNA. Membrane-bound DNA was then hybridized with a 921 bp HpaI-cut fragment of the sockeye salmon prepro-GnRH gene radiolabeled with [^{32}P]dCTP (Dupont/NEN) by random priming to a specific activity of 1 x 10^9 cpm/µg. This probe did not overlap any of the five restriction sites used to digest the DNA. The membranes were washed with one final wash in 0.1X SSC/0.1% SDS for 15 min at 50° C for low stringency or 60° C for high stringency, then exposed to Kodak XAR5 film at -80° C for 5 days or 3 days, respectively.

Results

Isolation and characterization of the GnRH gene

Four positive clones were isolated from the sockeye salmon genomic library using the cichlid GnRH probe and screening at high stringency. A synthetic oligonucleotide primer encoding salmon GnRH was used in asymmetrical PCR for each of the four genomic clones. The 15000 basepair (bp) clone yielded a PCR product that was subcloned, sequenced and found to encode GnRH.

A 5400 bp XbaI subclone of the 15,000 bp clone was shown to contain the sockeye salmon GnRH gene (sGnRH gene2). This subclone was further digested with KpnI to yield a 2833 bp clone containing most of the gene from position -791 to 1875 and a 1050 bp subclone containing additional downstream information from position 1876 to 2240. The third subclone of 1520 bp contained information that was further downstream from the second subclone and was not recognized by comparative sequence analysis using BLASTN (Altschul *et al.*, 1990).

The structure of the complete sGnRH gene2 is shown in Fig. 2-1. Exon/intron boundaries were determined by sequence analysis of the PCR product prepared from brain cDNA (Fig. 2-2). The primary structure of the predicted prepro-GnRH precursor includes a signal peptide of 23 amino acids, a GnRH decapeptide followed by a glycine-lysine-arginine processing site and a 46 residue GAP (Fig. 2-1). The deduced GnRH is identical with the decapeptide isolated from chum salmon (*Oncorhynchus keta*) brains and designated as salmon GnRH (Sherwood *et al.*, 1983).

Introns 1, 2 and 3 are 189, 254 and 307 bp in length, respectively. Each intron presents the typical GT (donor) and AG (acceptor) splice sites (Shapiro and Senapathy, 1987) except where the consensus GT donor splice site of exon 2 is changed to GC at position 377. This nucleotide substitution is within a typical consensus splice site of 5'-GTAAGT-3'. A polyadenylation signal sequence begins at position 1228 (Fig. 2-1).

Figure 2-1. The complete nucleotide sequence of the sGnRH gene2 isolated from sockeye salmon. CAAT boxes, TATA box and polyadenylation signal sequence are in bold and underlined. Exon 1 (1 to 44), exon 2 (234 to 376), exon 3 (631 to 711) and exon 4 (1019 to 1243) are in capital letters. The prepro-GnRH precursor is comprised of a 23-amino-acid signal peptide, a GnRH decapeptide (boxed), a 3-residue glycine-lysine-arginine processing site followed by the 46-amino-acid GAP. Putative GHF-2 and AP-1 binding sites are underlined and labelled above. Putative EREs are labelled above. Possible ER binding sites are indicated by dotted overline. Positions are given for nucleotides on the left and amino acids on the right.

```
          GHF-2 GHF-2       GHF-2
-791  gatcataaataaatagaatttaaattcaaatgacctggttacccagttattaagtgttaaggcactccacagggctatcc
-711  ttcatgccaaaggtacatttatggattcatagcctgttaggtaaagatacaagtgtaaggattatgtcttcttacagaac
-631  atttagaatcagtcttgtgtacagagtatgaaattgagccataatgtagcgctcaagcatcttgttcctgtatatctctt
                                                          GHF-2
-551  gtcataagtctgtatgaagttaaaatttcatttgtaaaatctgtccgcaataaatgcattgaaaataaacaaatttgcca
                                                  .....  ERE   .....
-471  ttaatttctgaatatttgtaaaagatacaaaacatgggtatcaaagtcacttttttgttgaccagcagatattgtttcat
-391  ttacaaacatgacatattttagaatatgtacactacttgccgtttccagctacaatagtaatttacaacattaacaatgt
        ... ERE  .. AP-1                                                      .....
-311  ctacactgtatttctgatccaatttgatgttattttaatggacaaaaatgttgcttttctttcaaaaacaaggacatttc
      ERE .....                                          GHF-2
-231  taagtgacctcaaactgttgaacggtagtgctcactgacttcacctcttaacacattataaatatgtttgtttgcatcaa
                                                          ..  ERE ... AP-1
-151  atgcagtttgaagcttatgcactaagcaggtgccattagtgacatttagtgtgcattaggcacttagtgtgtcacacctg
 -71  tggagaagggattctaatcctgatgacacagactggtgcatgtctaacgacccctataaaagggactcatgATATTCCCA
                                                  AP-1
  10  CCACAGTGTAGGAAGGAATACACAGAACGGAGAAAgtatgtgattcatataagtatatttaaaattgttaactaatgtgc
  90  atttgtgggtagttcatatatactgtacaatgtgcatattcaataggtaatcattgcaagatgatcgcaaacttctgctt
 170  gatgtaaaaatacattatttttgacgatcacatttagctggtatttgactagctttctttccagCTCCC ATG GAT
                                                                        M   D       2

 245  CTT AGC AAC AGA ACG GTC GTG CAG GTT GTG GTG TTG GCG TTG GTA GCG CAG GTC ACG CTC
      L   S   N   R   T   V   V   Q   V   V   V   L   A   L   V   A   Q   V   T   L    22

 305  TCT CAG CAC TGG TCG TAT GGC TGG CTA CCT GGA GGG AAG AGA AGT GTT GGG GAG CTG GAG
      S   Q   H   W   S   Y   G   W   L   P   G   G   K   R   S   V   G   E   L   E    42

 365  GCC ACC ATC AAG gcaagtactatttacctctacctgtaactactgttacagctatgtctacatgtgcatactgtac
      A   T   I   K                                                                    46

 441  atgtaattgtcctttaaggaaattactgtgtcttgaaaacctgtgaacatttaaatgtggcatttatagggatagtttgt
 521  gtgtgaatattagttcaagaggccttgtatattaattcaactctgtaagggtgttgcataaagggtatatgggtaatagc
 601  attacaaatttcctacatattgctatccag ATG ATG GAC ACA GGA GGT GTA GTG GCT CTT CCT GAG
                                     M   M   D   T   G   G   V   V   A   L   P   E    58

 667  GAG ACA AGT GCA CAT GTC TCA GAG AGA CTG AGA CCA TAT GAT GTA gtaagtagtcatattaattt
      E   T   S   A   H   V   S   E   R   L   R   P   Y   D   V                    73

 732  attaggttagataatgtattcattgtattcctaatggccaattatttatgtttcatctgaagatgctacatttttctcctg
 812  gtaaaatatgttgcaaacaatattccaaagtatctaaaataaatgtttagataagagtaatggctgacaaatggaaatct
 892  tccctgaatttagcgaagtgatacctacaaatgtagggagacaatgttgctcatgtctgacacatcagtataatatagat
 972  atacaacataaagtagacttctgaagttaacgctttgatttccctag ATA TTG AAG AAA TGG ATG CCC CAT
                                                      I   L   K   K   W   M   P   H    81

1043  AAA TAA ACAACTGAGACCATTATTCACAAAAGAAGCGAGAAGACAACATCAAGCAGACATACAGCATCACTATCAACA
      K   *                                                                           82

1121  TCAATGATGGAGCATTCAACTACAGTTCTGTCCATATCCAACAAACATCACATTTAGATTGATGTTATTTACTTGAAGTA
1201  TAACACTTTAACCTTCTGTAAAATTGTAATAAAGAAGTGATTCaaaaggagaagtgtctttgtatgtctattgaacgtcc

1281  ttacaatacaggagcttaagtgtcctatcccaaattttaattgatcaattcgtgatccatccaaatctttagggtggagtt
1361  gacagaccattaattgattattaacatacatttgcttgctatgaccgacaagcatgatcaacttgtgatacaacaatcgt
1441  agcatgacaacagctggtaattaagtcaatttataaaacatctatggtgtcattagattgaaacaggggtttatgtccct
1521  tttgtaattagattatagattattgcttgtcggtcatgcataatttcaaagggaaaatccacactaccaggacatttgtt
1601  tcacctcaaatttctgtgagagtatgtcacttgttgaataattgaatttgcatatttaaaaataattctgaaatgtcatg
1681  agggttgacatgtttctcatagttttgccaggaccataggcatcacaaaaagaaaatgtgttcaaactttcatgtcgttc
1761  aataaattgctgttaattataaaaattggaattgcatatcaggcaaggaaaggaattagcagggggcatatgttttaaaga
1841  gaactcatttctgcaaagccccacagactgggtaccgtactaatcgcgtagtgtattaggggcccatgattcatgaactg
1921  tactgacacgagggacaccctacactggtagcgtgtggttgtgatgcacaccaatgaacccagaccactggtacttttgg
2001  gaaaaggtgaaccgtaaaagcatgtagaattggaaagaaataaaaccaatttccattgcactttcagaacaatggttatt
2081  catttgtgatacctcatattgttagtttaattttcatggttcaatttgcagtgaatgcaatacttttctttgatggggaa
2161  agacatgtattttactttaaaagcgtacttactcatttttgttgctggagaacatgctccaactattgcaactaaacac
```

Figure 2-2. Nucleotide sequences of sGnRH-encoding cDNA isolated from sockeye salmon brain. For completeness, also shown are sequences deduced from the gene sequence that correspond to regions beyond DNA amplified with primer set B/C. Numbers on left indicate position of amino acid residue or nucleotide in comparison to the sequences that encode the GnRH hormone (boxed). The polyadenylation signal sequence is underlined. An asterisk marks the stop codon.

-118
ATATTCCCACCACAGTGTAGGAAGGAATACACAGAACGGAGAAACTCCC

-23/-69
ATG GAT CTT AGC AAC AGA ACG GTC GTG CAG GTT GTG GTG TTG GCG TTG GTA
Met Asp Leu Gly Tyr Arg Met Val Val Gln Val Val Val Leu Ala Leu Val

-6/-18 1/1 GnRH
GCG CAG GTC ACG CTC TCT CAG CAC TGG TCG TAT GGC TGG CTA CCT GGA GGG
Ala Gln Val Thr Leu Ser Gln His Trp Ser Tyr Gly Trp Leu Pro Gly Gly

12/34
AAG AGA AGT GTT GGG GAG CTG GAG GCC ACC ATC AAG ATG ATG GAC ACA GGA
Lys Arg Ser Val Gly Glu Leu Glu Ala Thr Ile Lys Met Met Asp Thr Gly

29/85
GGT GTA GTG GCT CTT CCT GAG GAG ACA AGT GCA CAT GTC TCA GAG AGA CTG
Gly Val Val Ala Leu Pro Glu Glu Thr Ser Ala His Val Ser Glu Arg Leu

46/136
AGA CCA TAT GAT GTA ATA TTG AAG AAA TGG ATG CCC CAT AAA TAA ACAACTGA
Arg Pro Tyr Asp Val Ile Leu Lys Lys Trp Met Pro His Lys *

189
GACCATTATTCACAAAAGAAGCGAGAAGACAACATCAAGCAGACATACAGCATCACTATCAACATCAA

257
TGATGGAGCATTCAACTACAGTTCTGTCCATATCCAACAAACATCACATTTAGATTGATGTTATTTAC

325
TTGAAGTATAACACTTTAACCTTCTGTAAAATTGTAATAAAGAAGTGATTCA-poly(A)

Identification of the transcription start site

A typical TATA box is located at position -17 and is preceded by four putative CAAT boxes at -34, -99, -119 and -158 (Fig. 2-1). The transcription initiation site of the sockeye salmon prepro-GnRH gene was determined by primer extension analysis (Fig. 2-3). The cDNA transcript has a transcription start site that begins at an adenine 10 bp downstream from the TATA box. This is different by 10 nts from the transcription start site proposed, but not determined experimentally, for the GnRH transcript expressed in Atlantic salmon brain (Klungland *et al.*, 1992).

Organization and nucleotide sequence of the promoter region

A large section of 1152 nucleotides of the promoter region of the Atlantic salmon gene is not present in the sockeye salmon gene (Fig. 2-4). Binding sites for several transcription factors, as well as a second region of potential promoter sequences, are present in this region of the Atlantic salmon GnRH gene (Klungland *et al.*, 1992), but are absent in the sockeye salmon gene.

Analysis of the 5'-flanking region of the sockeye salmon prepro-GnRH gene also revealed several consensus elements (MacVector; Kodak) that may be involved in regulation of the expression of GnRH. Two palindromic and two nonpalindromic sequences which conform to putative estrogen response elements (EREs) demonstrated to bind human estrogen receptor (hER) (Radovick *et al.*, 1991; Klungland *et al.*, 1993) are found at positions -427 to -409/ -240 to -223 and -308 to -298/ -93 to -81, respectively (Fig. 2-1). Consensus sites reported to bind Pit-1 (GHF-2) (Castrillo *et al.*, 1989; Rhodes *et al.*, 1993) are found at positions -789, -785, -774, -504, -176; another six in the complementary strand are in positions -767, -688, -624, -386, -324 and -101 (Fig. 2-1). Additionally, binding elements for the factor GH-CSE2 (Ye *et al.*, 1988), overlap with several GHF-2 consensus sequences at -784 and -503. Three putative AP-1 binding sites

Figure 2-3. Determination of the transcription start site of the sGnRH gene2 by primer extension in sockeye salmon brain. A 5'-end labelled, 19-nucleotide antisense strand primer, complementary to the downstream portion of exon 1, was annealed with sockeye salmon brain poly(A^+) RNA and GnRH DNA. The arrow marks the transcriptional start site of the cDNA transcript. The bracket outlines antisense sequences which comprise the TATAAAA box. The extended products from the 2833 basepair KpnI-cut subclone were electrophoresed in parallel with the primed cDNA products.


G A T C

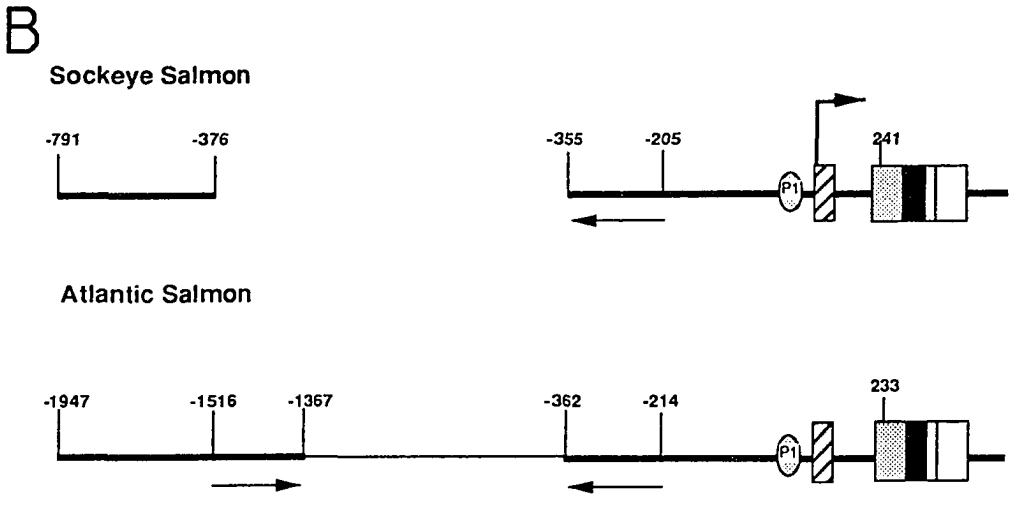
Figure 2-4A and 2-4B. A nucleotide sequence comparison of the 5'-flanking regions of Pacific (P) (sockeye) and Atlantic (A) salmon sGnRH gene2. Putative CAAT boxes, the TATA box and the translational start site of the sockeye salmon GnRH gene are in bold. Exon 1 is underlined. Blank spaces denote identical nucleotide residues. An open triangle represents a deletion of 154 basepairs and a filled triangle represents a deletion of 981 basepairs in the sockeye salmon GnRH gene.

B. A schematic showing the best alignment of the 5'-flanking regions of the two salmon GnRH genes. A large block of 1152 nucleotides present in the Atlantic salmon 5'-flanking region is not present in the sockeye salmon GnRH gene. A small region between -375 to -356 (not shown) may align with the Atlantic salmon GnRH gene. A region between -1516 to -1367 in the Atlantic salmon gene is also found in the opposite orientation with high sequence identity in both salmon genes in positions -355 to -205 in Pacific salmon and -362 to -214 in Atlantic salmon. P1 indicates the proximal promoter region. Positions 241 and 233 indicate translation start sites for the GnRH prepropeptides.

A

```
          -791
(P)   GATCATAA ATAAATAGAA TTTAAATTCA AATGACCTGG TTACCCAGTT ATTAAGTGTT AAGGCACTCC  -724
(A)                                      T

(P)   ACAGGGCTAT CC--TTCATG CCAAAGGTAC ATTTATGGAT TCATAGCCTG TTAGGTAAAG ATACAAGTGT  -656
(A)    T   A       TT

(P)   AAGGATTATG T--------- -----CTTCT TACAGAACAT TTAGAATCAG TCTTGTGTAC AGAGTATGAA  -600
(A)              AATCACATA AGGGT    A                    AT

(P)   ATTGAGCCAT AATGTAGCGC TCAAGCATCT TGTTCCTGTA TATCTCTTGT CATAAGTCTG TATGAAGTTA  -530
(A)        GT           A                                                      T

(P)   AAATTTCATT TGTAAAATCT GTCCGCAATA AATGCATTGA AAATAAACAA ATTTGCCATT AATTTCTGAA  -460
(A)                              T                                      C

(P)   TATTTGTAAA AGATACAAAA CATGGGTATC AAAGTCACTT TTTTGTTGAC CAGCAGATAT TGTTTCATTT  -390
(A)                G G

(P)   ACAAACATGA CATA △  TTTTAGAATA TGTACACTAC ▲TTGCCG TTTCCAGCTA CAATAGTAAT  -330
(A)          T            AA          C   -  G     G   A              G     C

(P)   TTACAACATT AACAATGTCT ACACTGTATT TCTGATCCAA TTTGATGTTA TTTTAATGGA CAAAAATGTT  -260
(A)   G                                        -                              -

(P)   GCTTTTCTTT CAAAAACAAG GACATTTCTA AGTGACCTCA AACTGTTGAA CGGTAGTGCT CACTGACTTC  -190
(A)                    T                     C                     T

(P)   ACCTCTTAAC ACATTATAAA TATGTTTGTT TCCATCAAAT GCAGTTTGAA GCTTATGCAC TAAGCAGGTG  -120
(A)                  C

(P)   CCATTAGTGA CATTTAGTGT CCATTAGGCA CTTAGTGTGT CACACCTGTG GAGAAGGGAT TCTAATCCTG   -50
(A)    G

(P)   ATGACACAGA CTGGTCCATG TCTAACGACC CCTATAAAAG GGACTCATGA T-ATTCCCAC CACAGTGTAG    20
(A)                T                                          T                T

(P)   GAAGGAATAC ACAGAACGGA GAAAGTATGT GATTCATATA AGTATATTTA AAATTGTTAA CTAATGTGCA    90
(A)           T                                  C

(P)   TTTGTGGGTA GTTCATATAT ACTGTACAAT GTGCATATTC AATAGGTAAT CATTGCAAGA TGATCGCAAA   160
(A)                                  C                                        C

(P)   CTTCTGCTTG ATGTAAAAAT ACATTATTTT TGACGATCAC ATTTAGCTGG TATTTGACTA GCTTTCTTTC   230
(A)     A CT              A TA A           T        G     T        C

(P)   CAGCTCCCATG 241
```

B



exist at positions -297 (on the opposite strand), -84 and 50 in the sockeye salmon GnRH gene promoter (Fig. 2-1).

Analysis of Southern blot

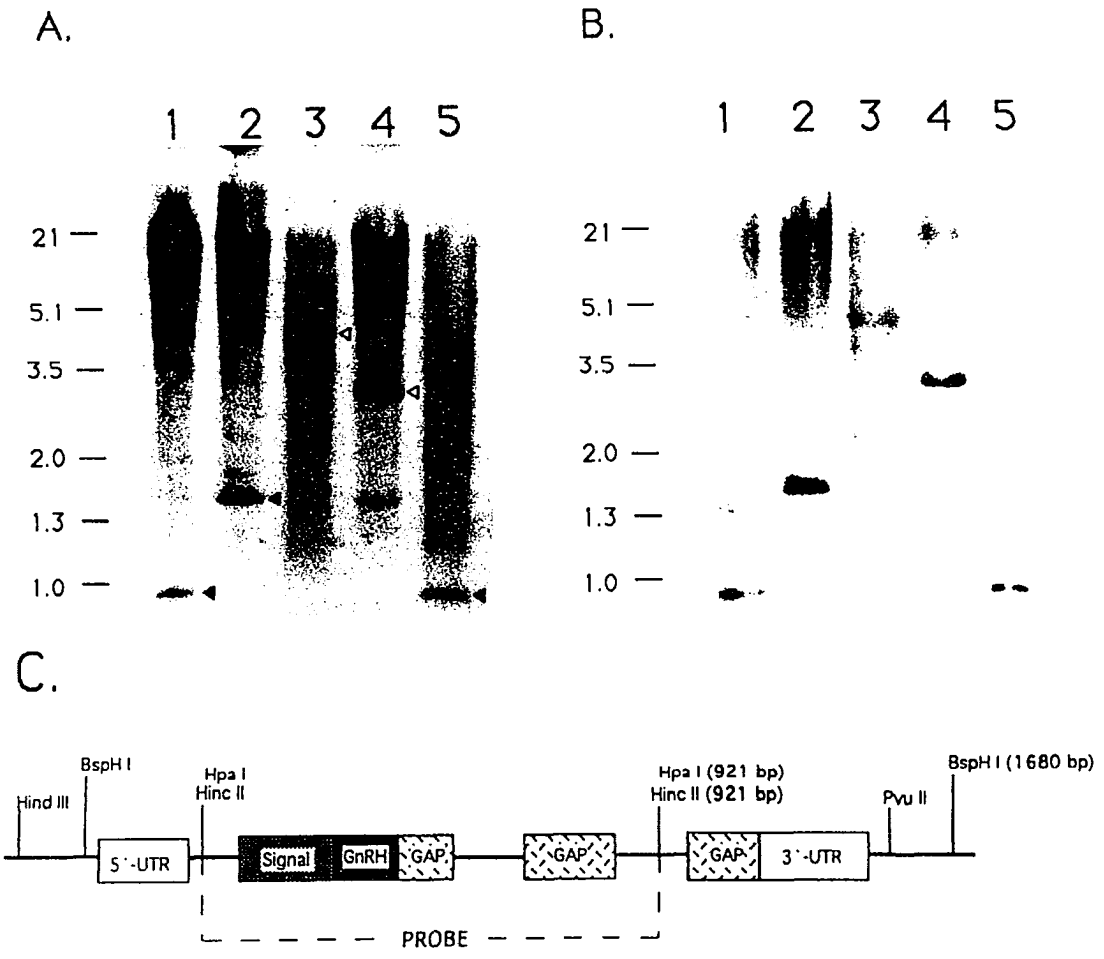
Southern blots of genomic DNA extracted from a single sockeye salmon brain were prepared at low and high stringency as shown in Figs. 2-5A and 2-5B. Digestion of the genomic DNA with various restriction enzymes followed by hybridization with a HpaI-cut fragment spanning exons 2 and 3 from position 79 to 999 revealed under high stringency conditions one major band of strong intensity in each lane (Fig. 2-5B). In Fig. 2-5A, under low stringency conditions, the same bands are marked by filled arrows corresponding to fragments of the expected sizes based on restriction site analysis of the gene characterized in this report (Fig. 2-5C). The bands found at approximately 4800 and 3200 bp in lanes 3 and 4 (open arrows) probably also represent fragments derived from the sockeye salmon GnRH gene, but one restriction site is outside of the area sequenced, which prevents identification by fragment length (Fig. 2-5A). Three other bands, shown in lanes 2, 3 and 5, show weak hybridization to the specific probe used here.

Discussion

In this study we report the sequence, organization and copy number of a sockeye salmon GnRH gene. These data also show for the first time the transcriptional start site for a GnRH gene in a nonmammalian species. The transcriptional start site of the GnRH gene has been determined from hypothalamic tissue of human (Radovick *et al.*, 1990), rat and mouse (Kepa *et al.*, 1992). Only one GnRH transcript was detected in the brain of salmon or hypothalamic tissue of the human and mouse (Radovick *et al.*, 1990; Kepa *et al.*, 1992) in primer extension studies. However, alternative transcription start sites of GnRH in human reproductive tissues (Radovick *et al.*, 1990; Dong *et al.*, 1993) suggest

Figure 2-5A and 2-5B. Southern blot analysis to determine GnRH gene copy number in the sockeye salmon genome. Genomic DNA was blotted and hybridized with a 921 bp Hpa I-digested fragment of the sockeye salmon GnRH gene at low (A) and high (B) stringency. The genomic DNA was cut with the following enzymes: lane 1, Hpa I; lane 2, BspH I; lane 3, Pvu II; lane 4, Hind III, lane 5, Hinc II. Filled arrows on the right in lanes 1, 2 and 5 of Fig. 2-5A denote fragments of expected sizes from restriction site analysis of the sockeye salmon GnRH gene as shown below in Figure 2-5C. Open arrows on the right of lanes 3 and 4 indicate fragments which are also probably derived from the sockeye salmon GnRH gene. Size markers are shown at the left (Kb).

Figure 2-5C. A schematic representing a partial restriction map of the sockeye salmon GnRH gene. The Hpa I-cut probe spans exons 2 and 3. Sockeye salmon genomic DNA digested with Hpa I, BspH I and Hinc II generates hybridizing fragments of 921, 1680 and 921 bp, respectively. Pvu II and Hind III cut only once in the characterized gene at the positions shown.


A.
1 2 3 4 5
21
5.1
3.5
2.0
1.3
1.0
B.
1 2 3 4 5
21
5.1
3.5
2.0
1.3
1.0
C.
Hind III
BspH I
Hpa I
Hinc II
Hpa I (921 bp)
Hinc II (921 bp)
Pvu II
BspH I (1680 bp)
5'-UTR
Signal
GnRH
GAP
GAP
GAP
3'-UTR
PROBE

differences in the regulation of GnRH. These may be important for tissue- or species-specific control of GnRH expression.

The overall similarity in gene organization between salmon and mammalian GnRH genes suggests that they are both derived from a common ancestral gene (see Sherwood *et al.*, 1997). The sockeye salmon gene shows a similar exon/intron organization compared with the human (Adelman *et al.*, 1986), rat (Adelman *et al.*, 1986), mouse (Mason *et al.*, 1986), Atlantic salmon (Klungland *et al.*, 1992) and other GnRH genes that have been reported after the completion of this study (Fig. 1-5). For each gene, the mRNA is generated from the splicing of four exons, each of which has a distinct role. Exon 1 encodes the 5'-untranslated region; exon 2 has the signal peptide, GnRH, a proteolytic cleavage site and the N-terminus of GAP; exon 3 has the central GAP moiety; and exon 4 has the C-terminus of GAP and the 3'-untranslated region.

Particularly striking are the differences observed between the 5'-flanking region of the sockeye salmon and Atlantic salmon genes. With the possible exception of a small region between -1360 and -1342, a 5'-regulatory region between -1516 and -362 of the Atlantic salmon gene is not present in the sockeye salmon gene (Fig. 2-4A and 2-4B).

If the sockeye salmon and Atlantic salmon GnRH genes are aligned with the best fit for 1937 nucleotides, beginning at their transcription start sites and ending about 700 nucleotides beyond the polyadenylation signal, there is a 6% difference in nucleotide identity including substitutions, deletions and insertions. Seven nucleotide changes exist in the prepro-GnRH coding region of sockeye salmon compared to Atlantic salmon, but these changes remain silent since they are all in wobble positions of their respective codons. Furthermore, if the region between -1516 to -1376 in Atlantic salmon is completely inverted, the Atlantic salmon nonsense strand is 93% identical to the coding sense strand between positions -362 to -214 in the Atlantic and the corresponding region (-355 to -205) in the sockeye prepro-GnRH genes (Fig. 2-4A and 2-4B). Interestingly, this region immediately follows the largest deletion site observed between these two genes

(Fig. 2-4B). These gene rearrangements must have become fixed after the separation of the two species in the last 8 to 15 million years (Stearley, 1992).

The sockeye salmon GnRH gene has four putative estrogen response elements (EREs) in the 5'-flanking region: two are palindromic at -240 to -223 and -427 to -409 and two are nonpalindromic at -93 to -81 and -308 to -298. The most upstream ERE is only 316 basepairs (bp) away from the most downstream ERE. In Atlantic salmon six EREs are present, although Klungland and coworkers (1993) only discuss four. A comparison of sockeye and Atlantic salmon promoters shows that three possible EREs are in similar positions close to the start site. Thus, the loss of over 1000 nucleotides in the sockeye salmon promoter did not interfere with the position of these putative estrogen receptor (ER) binding sites. There is controversy about whether estrogen acts directly on GnRH neurons or indirectly through estrogen-responsive neurons with afferents that synapse onto the GnRH cells. Radovick *et al.* (1991) and Klungland *et al.* (1993) have demonstrated binding of human ER (hER) to EREs in the promoters of human and Atlantic salmon GnRH genes. However, binding of hER occurred on the two palindromic, but not on the two nonpalindromic, EREs in the Atlantic salmon GnRH gene (Klungland *et al.*, 1993).

Examination of the 5'-flanking region of the sockeye salmon prepro-GnRH gene reveals possible sites that may bind other regulatory proteins. Several of these sites resemble elements that bind factors known to engage and transactivate the mammalian growth hormone and Pit-1 promoters. At least 5 consensus sites (Castrillo *et al.*, 1989; Rhodes *et al.*, 1993) for an alternatively spliced variant of rat Pit-1 (GHF-2) (Theill *et al.*, 1992) are present in the 5'-end of the sockeye salmon GnRH gene, in addition to 6 more in the complementary strand. Also, a number of Pit-1-Oct-1-Unc-1 (POU) domain octamer-binding factors with relaxed binding specificities for AT-rich elements (Bendall *et al.*, 1993) could be candidates for modulating salmon GnRH expression. Indeed, one footprint region containing such an AT-rich element has been implicated in targeting

protein kinase C (PKC)-mediated repressor activity resulting in downregulation of the rat GnRH promoter (Eraly and Mellon, 1995). Two other consensus elements that may bind a cell-specific factor (GH-CSE2) important to the regulated expression of the growth hormone gene (Ye *et al.*, 1988) are located in the salmon GnRH gene at positions -784 and -503. Numerous GHF-2 and GH-CSE2 consensus sequences are also found within the intronic regions of the salmon gene.

The primary response genes, *fos*, *jun* and *junB*, have been reported to be mobilized during activation of GnRH-secreting neurons through stimulation of PKC (Stojilkovic *et al.*, 1994). It is possible that these transcription factors form heterodimers at AP-1 sites to regulate transcription of the GnRH gene. Indeed, putative AP-1 binding sites exist in the promoter regions of both the rat and human GnRH genes (Bruder and Wierman, 1994), indicating that proto-oncogene activity may modulate the expression of GnRH. Three elements in sockeye salmon at positions -297 (on the opposite strand), -84 and 50 diverge by only one nucleotide from consensus AP-1 sites (5'-TGAGTCA-3' or 5'-TGGGTCA-3'). These putative AP-1 binding sites occur in similar positions in the Atlantic salmon prepro-GnRH gene. Furthermore, the portion of the Atlantic salmon prepro-GnRH gene not present in sockeye salmon contains another possible AP-1 site at -1194, a putative cAMP response element at -833 and a canonical Sp1 site at -793. Together with the arrangement of the EREs discussed above, these differences suggest that the two salmon prepro-GnRH genes may be regulated differently.

The main sequence identity between the mammalian genes and teleost genes is within the small region that encodes the GnRH decapeptide and its processing site. It is possible that the regulatory processes governing GnRH expression and hence the neural control of reproduction has changed considerably since the genes encoding salmon GnRH and mammalian GnRH diverged. The GnRH peptide expressed in mammals is identical to one of the two GnRH peptides in the sturgeon, a phylogenetically ancient bony fish (*Acipenser spp.*) (Sherwood *et al.*, 1991; Lescheid *et al.*, 1995). The divergence between the

mammalian and salmon GnRH peptides appears to have occurred in early teleosts in which the mammalian form of GnRH disappeared and the salmon form appeared (Sherwood *et al.*, 1997). It is possible that the 5'-flanking region of the mammalian prepro-GnRH gene expressed in fish such as sturgeon resembles more closely the gene characterized in mammals than in salmon.

Southern analysis under high stringency conditions shows there is one band in each lane that strongly hybridizes to our specific probe (Fig. 2-5). These digested fragments are the expected sizes for restriction site analysis of the sockeye salmon GnRH gene reported here. In addition, there are bands that hybridize less strongly suggesting there could be related GnRH genes in sockeye salmon. Salmonid fish are thought to be tetraploid and this would explain a doubling of the genes, although some of the duplicated chromosomes are thought to have been lost since the tetraploid event (Allendorf and Thorgaard, 1984). In addition, the primary structure for two distinct forms of GnRH has been determined for salmon brains. The forms are salmon GnRH (Sherwood *et al.*, 1983) and chicken GnRH-II (Powell *et al.*, personal communication). One explanation for the multiple bands in each lane of the Southern blot under low stringency conditions is that the strongest bands represent the salmon GnRH gene presented here and the weaker bands are variants of chicken GnRH-II or variants of the duplicated genes. Indeed, as shown here and in the next chapter, the gene presented here is the sGnRH gene2. The structure of the sGnRH gene1 is presented in Chapter 3.

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Chapter 3

Differences in the regulation and expression of the gonadotropin-releasing hormone gene in the brain and gonads in rainbow trout

Summary

The gonadotropin-releasing hormone (GnRH) gene is transcribed in both the brain and gonads, but it is not clear if the regulation is distinct in these tissues. Although the GnRH peptide in the brain is critical for reproduction, the function and importance of GnRH in the ovary and testis is an open question. I characterized the GnRH transcripts and their expression pattern during development in undifferentiated and juvenile gonads over a two year period. Rainbow trout was chosen because unlike other salmonids in the same genus (*Oncorhynchus*), rainbow trout mature early at three years, they are repeat spawners that do not all die after spawning and they are available because they remain in fresh water throughout their lives. As salmonids, these fish also are tetraploid with two copies of the GnRH gene so that additional information is available about the regulation of the duplicated genes.

I isolated and sequenced two different genes and their cDNAs that encode the same GnRH known as salmon GnRH (sGnRH). Each cDNA (cDNA1 and cDNA2) was isolated from the ovary and from the testis. In the gonads sGnRH cDNA1 uses the same promoter that is used in the brain and does not use an upstream promoter. However, sGnRH cDNA2 uses the same promoter in the gonads as in the brain, as well as a more upstream promoter. The cDNA2 transcript is generated in both gonads from an alternative start site that is 322 bp upstream from that utilized in the brain. Of the two long transcripts that use the upstream promoter, one retains intron 1 completely in the mRNA, whereas the other does not. The function of the long 5'-untranslated region may be for distinct regulation of the mRNA in the gonads compared to the brain. The promoters in the two GnRH genes are highly conserved only in the 215 basepairs proximal to the start site, but diverge completely in the upstream 5'-flanking region, especially in regard to estrogen response elements.

Introduction

Gonadotropin-releasing hormone (GnRH) is the key regulatory molecule involved in the control of reproduction in vertebrates. The central role of this decapeptide is to modulate the synthesis and release of the gonadotropins from the pituitary. However, differences in the ontogeny and location of different types of GnRH found within the brains of single species indicate GnRH serves roles in addition to that of a hypophysiotropic factor (Sherwood *et al.*, 1997).

Also, recent studies show that GnRH is not made exclusively in neurons. The isolation and localization of GnRH and its receptor in various reproductive tissues supports an extrapituitary autocrine/paracrine role for GnRH in reproduction. For example, mammalian GnRH (mGnRH) cDNA has been isolated or detected in the primate ovary and testis (Dong *et al.*, 1993, Dong *et al.*, 1996), as well as placenta (Radovick *et al.*, 1990, Dong *et al.*, 1996). In situ hybridization studies in females localize mGnRH and its receptor primarily in granulosa cells (Clayton *et al.*, 1992, Whitelaw *et al.*, 1995). In males, GnRH is present in Sertoli cells and its receptor in Leydig cells (Bahk *et al.*, 1995).

GnRH is first expressed in the developing mouse brain at approximately 10 days after fertilization (Pfaff *et al.*, 1994) and in the salmonid brain at 17 days after fertilization (Chiba *et al.*, 1994, Parhar *et al.*, 1995). GnRH is then continuously expressed in the brain throughout the life of the animal (Chiappa and Fink, 1977, Okuzawa *et al.*, 1990). The pattern of expression of GnRH in reproductive tissues is less clear. In the rat ovary, GnRH is first observed in primary follicles (Clayton *et al.*, 1992) and the GnRH receptor was detected at five days after birth (Whitelaw *et al.*, 1995). Both the receptor and GnRH messages are expressed in primary, secondary and tertiary follicles in the ovary (Clayton *et al.*, 1992, Whitelaw *et al.*, 1995). In the gonad, studies have not been conducted to detect GnRH in mammalian fetal or early postnatal testicular cells. The GnRH receptor

was not detected in the rat testis at 20.5 DAF (Dufau *et al.*, 1984), but was detected at five days after birth (see Gnessi *et al.*, 1997).

Data is not available currently on the transcription factors involved in activation of the GnRH gene in the gonads. However, one area of regulatory control at the post-transcriptional level could be the extended 5'-UTRs present in the GnRH mRNAs isolated from these tissues. GnRH mRNAs transcribed in the ovary and testis of human (Dong *et al.*, 1993) and monkey (Dong *et al.*, 1996), but not in rodents (see Dong *et al.*, 1996), use an upstream promoter.

Although GnRH has been demonstrated to have inhibitory and stimulatory influences on the development and maturation of ovary (see Richards, 1994) and testis (see Gnessi *et al.*, 1997), the precise role of GnRH in the gonads is still not clear. To determine if the processes governing GnRH regulation and expression are conserved in vertebrates, I have taken an evolutionary approach by studying GnRH in rainbow trout. Rainbow trout are an unusual example of the salmonid group because they mature early at three years of age, spawn every year for a number of years and spend their lives exclusively in fresh water. However, like other salmonids, rainbow trout are tetraploid (Allendorf and Thorgaard, 1984); this means that comparison and analysis of the 5'-flanking regions of the two genes that encode sGnRH (gene1 and gene2) can be used to understand the changes that have occurred since duplication in the coding and regulatory regions of the two genes. In addition, this study investigates whether different splicing strategies are used by the two genes in the ovary and testis compared to the brain.

Materials and Methods

Raising of rainbow trout

Eggs from rainbow trout were obtained in December from Frazer Valley Trout Hatchery, Abbottsford, B.C. The eggs were fertilized by gently mixing the eggs and milt by hand. The eggs were then washed with partial exchanges of water. Approximately 200 fertilized eggs were placed in each nest, which is an open container made from a PVC tube of 12 cm diameter and 10 cm in height with a piece of fine mesh glued on the bottom. The nests were placed in Heath trays at the University of Victoria. The eggs and prehatch larvae were raised in fresh water at a temperature of 14°C and a flow rate of 720 L/h. At hatching, which occurred 22 days after fertilization, the alevin were transferred from the Heath trays to 30 L holding tanks. The fry were raised for 7 months and then transferred to 500 L rearing tanks for the remainder of the study. The holding and rearing tanks were exposed to natural light conditions throughout the study. In addition, rainbow trout (1.5 years and older) were obtained from Mountain Trout Sales (Sooke, B.C.).

Isolation of RNA from ovary and testis

Beginning at 5 months of age, several fish were quickly killed by decapitation. Ovarian or testicular tissue was removed, placed on dry ice and stored at -80° C. Total RNA was extracted in Trizol as recommended by the manufacturer (Bethesda Research Laboratories, Bethesda, MD).

cDNA synthesis and RT-PCR

First-strand cDNA was synthesized from 2 to 5 μg of total RNA from extracted tissues using Superscript RT RNase H^- reverse transcriptase by methods recommended by the manufacturer (Life Technologies Inc., Gaithersburg, MD). The reverse transcription reaction product (0.5 to 1.0 μL) was diluted to a final 50 μL volume containing 1x Promega buffer (50.0 mM KCl, 10.0 mM Tris-HCl, 1.5 mM $MgCl_2$ and

0.1 % Triton X-100), 200 mM dNTPs, 2.5 units of Taq DNA polymerase (Promega, Madison, WI) and 30 pmol of sense or antisense strand primers. Amplification of cDNA was achieved with 40 cycles of 1 min at 94° C, 1 min at 50° C and 1 min at 72° C using the sense strand primers A (5'-GAAGCTTATGCACTAAGCAGG-3'), B (5'-TAGGAAGGAATACAGAACGG-3'), F (5'-AGGACATTTCTAAGTGACC-3') or G (5'-CTACACTGTATTTCTGATC-3') in combinations with antisense strand primers C (5'-TTATTTATGGGGCATCCATTTC-3') or D (5'-CAGCCATACGACCAGTGCTG-3') (as shown in Fig. 3-2A). The design of these primers was based on the sockeye salmon sGnRH gene2 (Chapter 2).

The sense strand primers A, F and G are respectively located in positions -149 to -129, -248 to -230 and -318 to -300 upstream from the transcription start site used for brain GnRH. Sense strand primer B corresponds to positions 10 to 29 of exon 1. Antisense strand primers were located as follows: C (exon 4, positions 1019 to 1040), D (exon 2, against the sequence that encodes GnRH) and E (exon 1, antisense to bases 16 to 34).

The synthesis and amplification of sGnRH-I cDNA were achieved using RNA derived from 1.5-year-old rainbow trout ovary by the same methods as described above. The sense strand primer S (5'-AGGAATAGACCGAACGGAC-3') was complementary to exon 1 (position 12 to 30) in the rainbow trout sGnRH gene1 described below (Fig. 3-2A). The antisense strand primer T (5'-TTGAATGCTCCATCATCGC-3') was designed against a consensus region for the 3'-UTR of both sockeye salmon (Ashihara *et al.*, 1995) and masou salmon (Suzuki *et al.*, 1992) sGnRH cDNA1. The 3'-UTR region was used as it is distinct from its counterpart in the sGnRH gene2. The integrity of each cDNA for each examined tissue was confirmed by separate PCRs using primers that were specific for a salmon tubulin cDNA clone (bases 523 to 545 and 719 to 740) (Coe *et al.*, 1992).

The PCR products were separated on a 1.5% agarose gel containing ethidium bromide and retrieved by electroelution in dialysis tubing. The retrieved DNA was subcloned into

pGEM-T vector (Promega) and recombinant plasmids were sequenced on both strands by the chain termination method using the Sequenase Version 2 kit (United States Biochemicals, Cleveland, OH) or Circumvent Thermal Cycle sequencing kit (New England Biolabs, Beverly, MA).

Primer extension

Rainbow trout ovaries, testes and brains were excised and quickly frozen. Total RNA was isolated as discussed above. An oligonucleotide, primer E (5'-TCTCCGTTCTGTGTATTCC-3'), was used in each reverse transcription reaction for each tissue (Fig. 3-2A). The primer (500 ng) was 5'-end labeled with [gamma-^{32}P]-ATP (Dupont/NEN, Boston, MA) by T4 polynucleotide kinase and purified to a specific activity of 3 x 10^8 cpm/μg. A mixture of 3.0 ng labeled primer and 20 μg of ovarian, testicular or brain total RNA from rainbow trout were separately combined in 1 x first strand buffer and heated to 90° C for 5 min. After primer hybridization at 50° C for 4.5 h in the presence of 50 U of RNAguard ribonuclease inhibitor (Pharmacia, Dorval, Quebec, Canada), the annealed primer was extended upon addition of 10 mM DTT, 0.5 mM of each dNTP and 4 U of Superscript II reverse transcriptase (Bethesda Research Laboratories, Bethesda, MD) when incubated at 45° C for 2 h. The reaction was stopped by the addition of 1.0 μL 0.5 M EDTA (pH 8.0) and 1.0 μL DNase-free RNase (10 mg/mL). The mixture was extracted once with phenol/chloroform and precipitated with 2.5 mM ammonium acetate and 2.5 volumes of ethanol. The purified extended products were dissolved in 4 μL TE (pH 8.0) and 4 μL sequencing buffer, then electrophoresed on a 6% polyacrylamide/8 M urea gel in parallel with sequencing products generated from extension of primer E from a complementary site within a subclone containing a 2833 bp fragment of the sGnRH gene2 (Chapter 2). The gel was dried and exposed to Kodak Biomax film (Rochester, NY) at -80° C for 24 h.

Characterization of the promoter region for the trout sGnRH gene 1

Genomic DNA isolated from rainbow trout ovary was amplified by PCR using a sense strand primer complementary to a 5'-flanking region of sockeye salmon sGnRH gene2 (position -583 to -565) and primer D. Gel fractionation yielded two amplification products of 1257 bps and 920 bps in length. Sequence analysis revealed that the largest fragment represented the promoter for the rainbow trout sGnRH gene1.

Results

I isolated and characterized two different cDNAs that encode salmon (s)GnRH; both transcripts are present in the ovary and testis of rainbow trout. In addition, I characterized a partial gene for each cDNA. The cDNAs encode an identical GnRH hormone (deduced amino acids), but differ in the signal peptide, associated peptide, promoter, alternative splice sites and expression during development.

Comparison of coding regions of cDNA1 and cDNA2

Amplification of sGnRH cDNA1 was achieved using primers designed specifically to the 5'-untranslated region (UTR) and 3'-UTR to differentiate the sGnRH cDNA1 from its sGnRH cDNA2 counterpart in both gonads (Fig. 3-1). It can be deduced that the two transcripts are each translated into preproprotein, which includes the signal peptide, GnRH hormone and GnRH-associated peptide (exons 2 to 4). The preproprotein translated from the sGnRH mRNA2 differs by 15 amino acids (aa) in comparison to the sGnRH mRNA1 (Fig. 3-1). Although two nucleotide changes occur in the region that encodes GnRH (aa codons 2 and 6) the processed hormone is identical when translated from each template. Thus, the 15 aa changes are restricted to the signal peptides (6 aa changes) and the GnRH-associated peptide (GAP; 9 aa changes).

Figure 3-1. Nucleotide and amino acid sequence comparison of sGnRH cDNA1 and cDNA2 isolated from rainbow trout ovary and testis (from exon 2 to the stop codon of exon 4). Changes in nucleotides or amino acids are shown in bold for the sGnRH cDNA2 transcript in comparison to the sGnRH cDNA1. The portion of the preproprotein that is processed to become the mature hormone is boxed.

```
                          M   D   L   S   S   K   T   F   V   Q   V   V   M
sGnRH mRNA1        cttcc  ATG GAT CTT AGC AGC AAA ACG TTT GTG CAG GTG GTG ATG

sGnRH mRNA2        ctccc  ATG GAT CTT AGC AAC AGA ACG GTC GTG CAG GTG GTG GTG
                                          N   R       V                   V

                                                        GnRH
     L   A   L   I   A   Q   V   T   F   S  | Q   H   W   S   Y   G   W   L   P   G  |
I    TTG GCG TTG ATA GCT CAG GTC ACT TTC TCT| CAG CAT TGG TCG TAT GGG TGG CTA CCT GGA|
                                            |                                        |
II   TTG GCG TTG GTA GCG CAG GTC ACG CTC TCT| CAG CAC TGG TCG TAT GGC TGG CTA CCT GGA|
                 V                   L

     G   K   R   S   V   G   E   L   E   A   T   I   R   M   M   D   T   G   G   V
I    GGA AAG AGA AGT GTT GGG GAG CTG GAG GCC ACC ATC AGG ATG ATG GAC ACA GGT GGT GTA

II   GGG AAG AGA AGT GTT GGG GAG CTG GAG GCC ACC ATC AAG ATG ATG GAC ACA GGA GGT GTA
                                                     K

     M   A   L   P   E   E   T   G   A   H   I   P   E   R   L   R   P   Y   D   V
I    ATG GCT CTT CCT GAG GAG ACA GGT GCC CAT ATC CCA GAG AGA CTG AGA CCA TAT GAT GTA

II   GTG GTT CTT CCT GAG GAG ACA AGT GCA CAT GTC TCA GAG AGA CTG AGA CCA TAT GAT GTA
     V   V                       S           V   S

     M   S   K   K   R   M   P   H   K   *
I    ATG TCG AAG AAA CGA ATG CCA CAT AAA TAA

II   ATA TTG AAG AAA TGG ATG CCC CAT AAA TAA
     I   L           W
```

Characterization and sequence analysis of extended cDNA2 for trout sGnRH

To determine if the salmonid GnRH mRNAs in the reproductive tissues were transcribed from an alternative start site, I designed primers A and F that are complementary to a region upstream from the start site in the brain for rainbow trout (Fig. 3-2A). In PCR analysis of first-strand cDNA made from ovarian or testicular total RNA using primers A/C, I observed PCR products that were 629, 467 and 440 bps in length (Fig. 3-3A) following gel fractionation. PCRs with primers F/D resulted in products of 567, 405 or 378 bps in length (Fig. 3-3B). Subsequent cloning and sequencing of these PCR products revealed that each amplification product contained sequences upstream from exon 1 and that the larger transcripts retained intron 1 (but not introns 2 or 3) (Fig. 3-2B; Fig. 3-3A and 3-3B). Primers B/C were used to identify the brain transcripts (Fig. 3-3C). That the use of the upstream start site is specific for the ovary and testis is shown in Figs. 3-3A and 3-3B by the lack of observable bands for the brain with the A/C and F/D primer sets, but amplification of a PCR product in Fig. 3-3C with primer set B/C at 286 bps.

The RT-PCR results presented here represent mRNA and not genomic contamination or RNA intermediates for several reasons. First, none of the amplification products isolated and sequenced contained introns 2 or 3. Second, the shorter testis GnRH transcript (467 bps) contained a unique intron-exon splice site that is 27 nucleotides (nts) upstream from the site more commonly used at the beginning of exon 2 (Fig. 3-2B). Third, the smaller 440 bp product from the ovary (Fig. 3-3A) was isolated, cloned and sequenced to confirm that introns 1 to 3 were not retained and that the start site originated upstream from that shown for the brain. Modifying the PCR parameters failed to yield the larger intron 1-containing transcript observed for the rainbow trout testis (Fig. 3-3A). However, using the sense primer F in conjunction with antisense primer D in PCRs of the same gonadal cDNA, I found amplification of two bands: one (567 bp) retained intron 1, but the other (378 bp) did not (Fig. 3-3B). These results were obtained even following

Figure 3-2. Schematic showing positions of primers used in RT-PCR and four different sGnRH cDNA2 transcripts isolated from gonads. (A) Schematic presents position of primers (arrows) used in RT-PCR of sGnRH cDNA1 or cDNA2. Each filled box represents one of four exons that comprise brain-type GnRH mRNA. (B) Summary of different sGnRH cDNA2 transcripts found in the salmonid ovary and testis. The transcript with the longest 5'-untranslated region is shown by the thick line and black box (exon 1) to represent sGnRH mRNA2 transcribed from the upstream start site with unspliced intron 1. The shorter transcript found in the rainbow trout testis contained 27 nucleotides of the 3-end of intron 1. In rainbow trout ovary, the shorter transcript generated from the upstream start site did not contain intron 1. Also, primer extension analysis indicates that some transcripts in the reproductive tissue are generated from the same start site as that utilized in the brain.

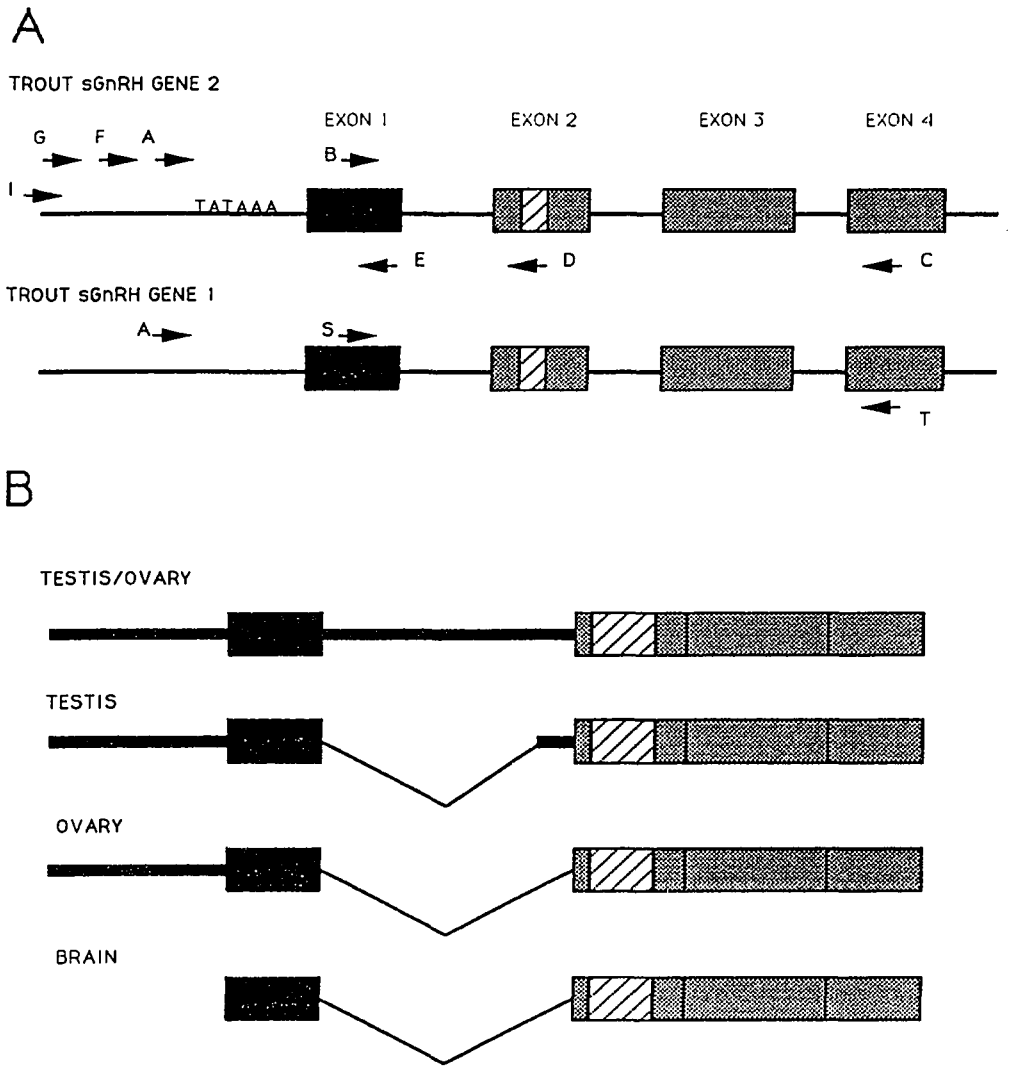
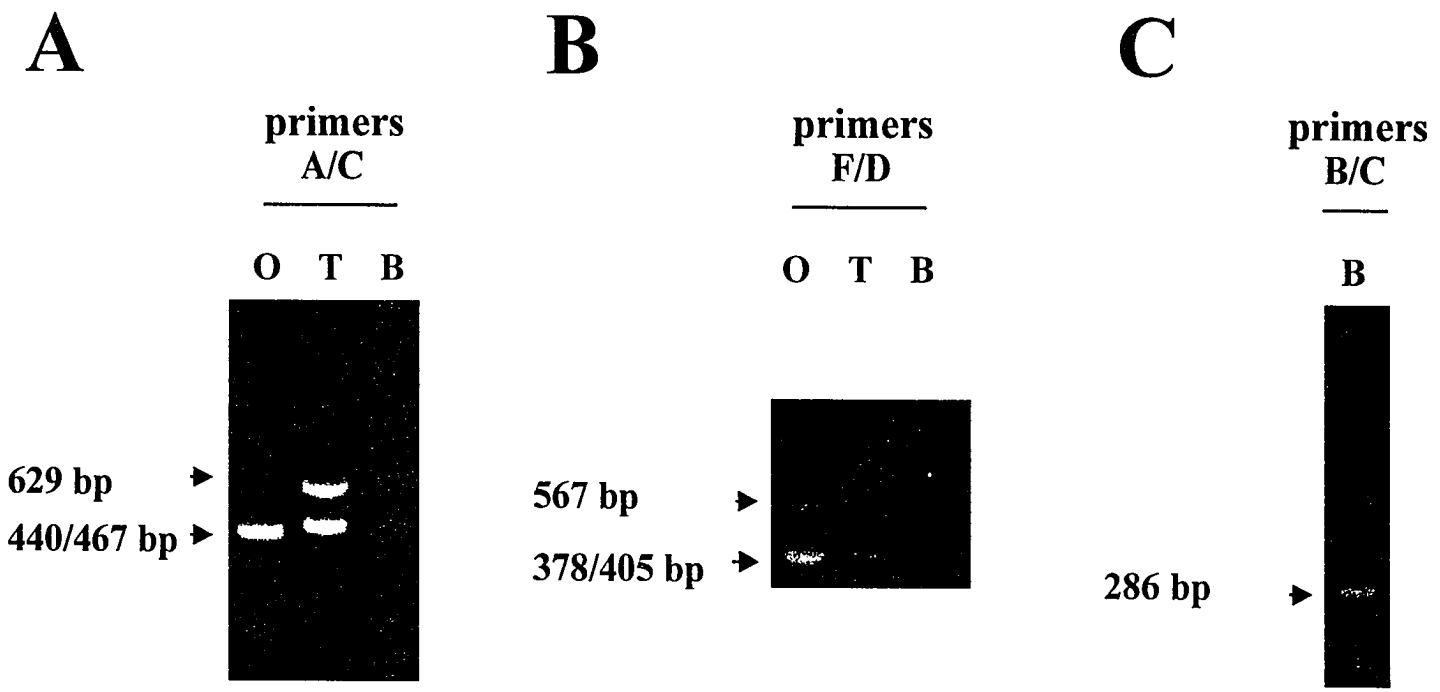

A
TROUT sGnRH GENE 2
EXON 1
EXON 2
EXON 3
EXON 4
G
F
A
B
I
TATAAA
E
D
C
TROUT sGnRH GENE 1
A
S
T
B
TESTIS/OVARY
TESTIS
OVARY
BRAIN

Figure 3-3. RT-PCR analysis of RNA extracted from two-year-old rainbow trout ovary, testis and brain. Examination of GnRH expression and use of the upstream promoter in rainbow trout ovary (O), testis (T) and brain (B) with primer sets A/C (Fig. 3-3A) and F/D (Fig. 3-3B) and for brain with primer set B/C (Fig. 3-3C). Proof that both transcripts can be amplified in the rainbow trout ovary is demonstrated using primer set F/D.


A
primers
A/C
O T B
629 bp
440/467 bp
B
primers
F/D
O T B
567 bp
378/405 bp
C
primers
B/C
B
286 bp

DNase I treatment of total RNA derived from rainbow trout ovary or testis before the RT-PCRs were initiated. However, it should be noted that the ovary did not consistently provide both products, but rather the larger or smaller fragment alone when primers F/D were used for PCR.

Determination of the start site in the rainbow trout ovary and testis by primer extension analysis (see below) permitted the design of primer G. PCRs done with primers G/D produced bands of the expected sizes that were cloned and sequenced to confirm that each transcript was generated from the upstream start site (Fig. 3-2B and 3-4). The full-length sGnRH cDNA2 and the various ways it is spliced is detailed in Fig. 3-4. PCR products were not produced with a sense primer (I) complementary to a region upstream of the alternative start site (data not shown).

Identification of the transcription start site in ovary and testes

The transcription intiation site of sGnRH gene2 in rainbow trout ovaries and testes was determined by primer extension analysis. GnRH transcripts in the salmonid ovaries and testes are synthesized from the same start site that is utilized in the brain, as well as two additional tissue-specific start sites further upstream (Fig. 3-5C). The largest extension was confirmed by PCR and represents a transcript that contains 322 nts of 5'-UTR upstream from the start site found in the brain transcript (Fig. 3-5A). It is not clear whether the primer extension product that migrated to position -81 indicates a site of termination of an extended sGnRH cDNA because I did not isolate a cDNA of the appropriate size (Fig. 3-5B).

The sGnRH gene1 does not appear to use an upstream promoter in reproductive tissues to produce sGnRH mRNA1. Both sense primer A and antisense primer T are complementary to regions in the sGnRH gene1 (Fig. 3-2A). Amplification products were not generated using these two primers together in RT-PCRs of gonadal tissues of different developmental stages (data not shown).

Figure 3-4. Nucleotide sequence of the full-length sGnRH cDNA2 transcript isolated from rainbow trout ovary and testis. For completeness, also shown are sequences that correspond to region beyond DNA amplification deduced from primer extension analysis. Note that the transcription start site for the cDNA begins 322 nucleotides upstream from the start site used in the brain. The TATA box and putative CAAT boxes used by the transcription apparatus to stimulate GnRH expression in the brain are underlined. Note also that this transcript includes all of intron 1 which is spliced out of the smaller sGnRH cDNA2 expressed in rainbow trout ovary (shown in bold). In rainbow trout testis, the smaller message contains 27 nucleotides of the 3'-end of intron 1 due to processing at an alternative acceptor, as shown by the bold underlined ag and 27 overhead asterisks. Positions are given for nucleotides on the left and amino acids on the right. Arrows indicate positions of introns 2 and 3, respectively.

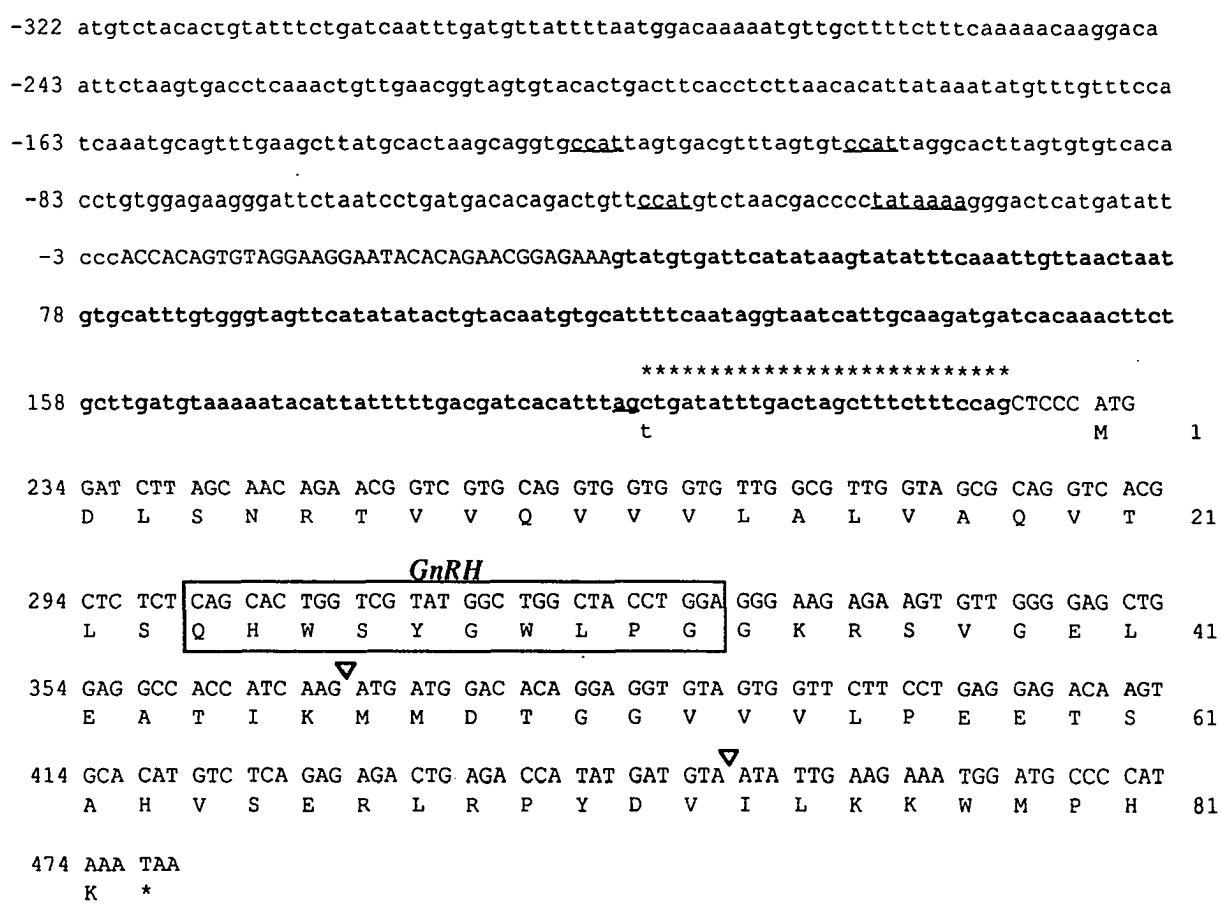
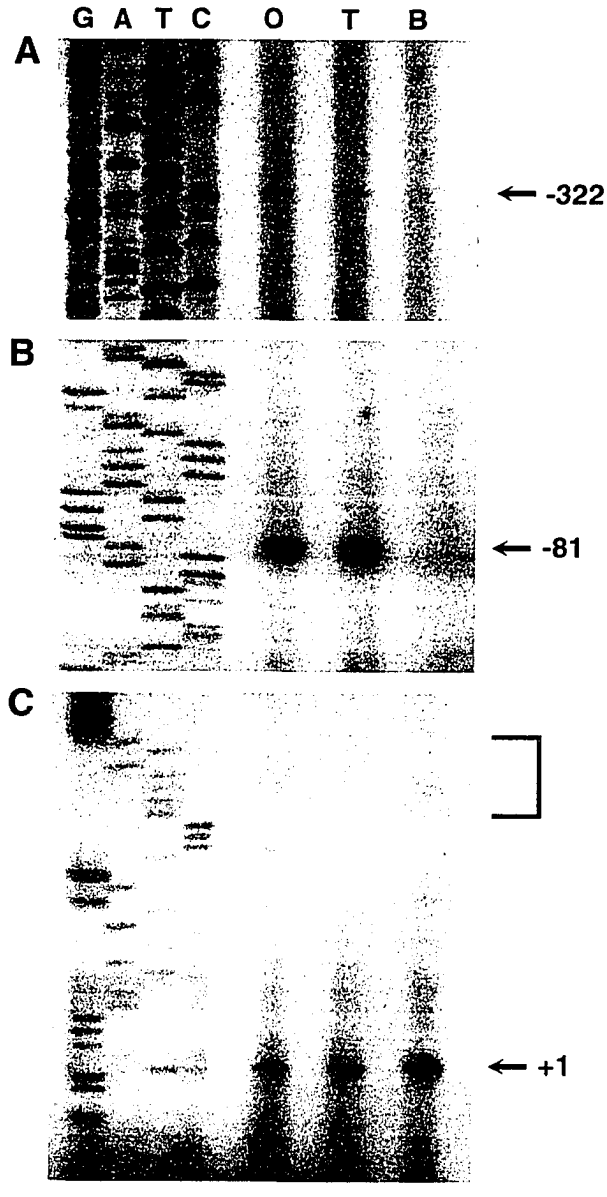

-322 atgtctacactgtatttctgatcaatttgatgttattttaatggacaaaaatgttgcttttctttcaaaaacaaggaca
-243 attctaagtgacctcaaactgttgaacggtagtgtacactgacttcacctcttaacacattataaatatgtttgtttcca
-163 tcaaatgcagtttgaagcttatgcactaagcaggtgccattagtgacgtttagtgtccattaggcacttagtgtgtcaca
-83 cctgtggagaagggattctaatcctgatgacacagactgttccatgtctaacgaccccctataaaagggactcatgatatt
-3 cccACCACAGTGTAGGAAGGAATACACAGAACGGAGAAAgtatgtgattcatataagtatatttcaaattgttaactaat
78 gtgcatttgtgggtagttcatatatactgtacaatgtgcattttcaataggtaatcattgcaagatgatcacaaacttct
158 gcttgatgtaaaaatacattatttttgacgatcacatttagctgatatttgactagctttctttccagCTCCC ATG
t M 1
234 GAT CTT AGC AAC AGA ACG GTC GTG CAG GTG GTG GTG TTG GCG TTG GTA GCG CAG GTC ACG
D L S N R T V V Q V V V L A L V A Q V T 21
GnRH
294 CTC TCT CAG CAC TGG TCG TAT GGC TGG CTA CCT GGA GGG AAG AGA AGT GTT GGG GAG CTG
L S Q H W S Y G W L P G G K R S V G E L 41
354 GAG GCC ACC ATC AAG ATG ATG GAC ACA GGA GGT GTA GTG GTT CTT CCT GAG GAG ACA AGT
E A T I K M M D T G G V V V L P E E T S 61
414 GCA CAT GTC TCA GAG AGA CTG AGA CCA TAT GAT GTA ATA TTG AAG AAA TGG ATG CCC CAT
A H V S E R L R P Y D V I L K K W M P H 81
474 AAA TAA
K *

Figure 3-5. Determination of the upstream transcription start site used by sGnRH cDNA2 transcripts in trout ovary and testis. RNA extracted from rainbow trout ovary (O), testis (T) or brain (B) was hybridized with a labelled primer made antisense to exon 1 and extended with reverse transcriptase. sGnRH gene2 messages in the salmonid ovary and testis are initiated from the same start site that is utilized in the brain (3-5C), as well as two additional tissue-specific start sites further upstream (3-5A and 3-5B). The largest extension represents a transcript that contains 322 nucleotides of 5'-UTR upstream in contrast to that found in the brain transcript (3-5C). The bracket indicates the antisense sequence that comprises the TATAAAA box.

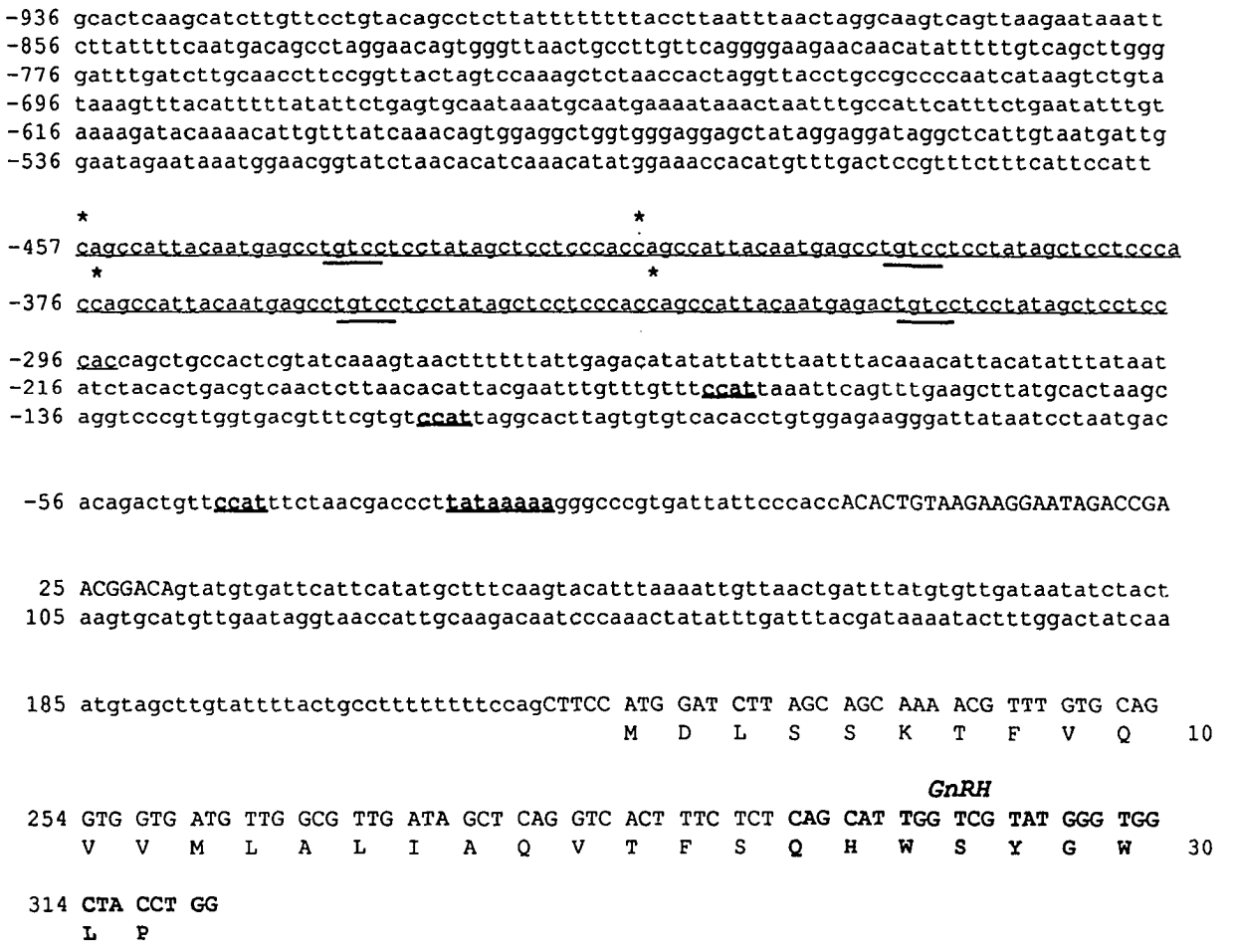

G A T C
O
T
B
A
-322
B
-81
C
+1

Organization and sequence of the promoter region for trout sGnRH gene1

The 5'-flanking region of the rainbow trout sGnRH gene1 is shown in Fig. 3-6. The transcriptional start site for this gene was deduced from the start site for sockeye (Ashihara *et al.*, 1995) and masou (Suzuki *et al.*, 1992) salmon sGnRH cDNA1. The promoter of the trout sGnRH gene1 is highly conserved in the 215 bps proximal to the transcription start site compared to the sGnRH gene2 found in Atlantic salmon (Klungland *et al.*, 1992), sockeye salmon (Chapter 2) and rainbow trout (Fig. 3-7). However, the 400 bps of DNA beyond -215 (-589 to -215) in the rainbow trout sGnRH gene1 diverge completely from similar positions in the two other salmonid sGnRH gene2 promoters that are known (Fig. 3-6 and 3-7). Thereafter, about 100 bps of flanking region in the rainbow trout sGnRH gene1 (-708 to -589) has high sequence identity to the sGnRH gene2 in both sockeye salmon (-551 to -426) and Atlantic salmon (-1689 to -1565). This region of identity is found about 60 bps upstream from a site in the sockeye salmon gene that appears to have lost 1152 bps of DNA in comparison with the Atlantic salmon gene (Chapter 2).

In the rainbow trout between the upstream and downstream start sites are two nonpalindromic estrogen response elements (EREs) and one palindromic ERE. This is similar to the sockeye salmon and Atlantic salmon proximal promoter regions. In the sockeye salmon the upstream start site is centred between two palindromic EREs, that are within 100 bps on each side (Chapter 4). These two palindromic EREs have 100% identity to EREs shown to bind hER in the Atlantic salmon GnRH gene (Klungland *et al.*, 1993), although they lie in a downstream section of DNA in the opposite orientation in the region examined here (Chapter 2; Fig. 6-1). Furthermore, sequences in position 6 to 18 closely resemble a putative ERE found near to the start site for GnRH expression in human and monkey reproductive tissues (Dong *et al.*, 1993 and 1996). The position of

Figure 3-6. Nucleotide sequence of the 5'-flanking region of the rainbow trout sGnRH gene1. CAAT boxes and the TATAAAA box are bold and underlined. The sequence encoding GnRH is in bold. The underlined region between position -457 to -294 contains four repeating blocks of DNA (each block begins with a C *). Each repeating block of DNA contains a putative estrogen receptor-binding TGTCC half-site (double-underlined). Positions are given for nucleotides on the left and amino acids on the right.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-616 aaaagatacaaaacattgtttatcaaacagtggaggctggtgggaggagctataggaggataggctcattgtaatgattg
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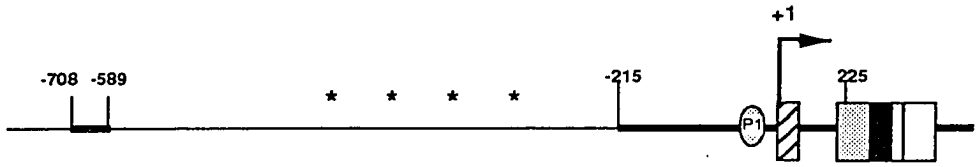
-376 ccagccattacaatgagcctgtcctcctatagctcctcccaccagccattacaatgagactgtcctcctatagctcctcc
-296 caccagctgccactcgtatcaaagtaacttttttattgagacatatattatttaatttacaaacattacatatttataat
-216 atctacactgacgtcaactcttaacacattacgaatttgtttgtttccattaaattcagtttgaagcttatgcactaagc
-136 aggtcccgttggtgacgtttcgtgtccattaggcacttagtgtgtcacacctgtggagaagggattataatcctaatgac
-56 acagactgttccatttctaacgaccctataaaaagggcccgtgattattcccaccACACTGTAAGAAGGAATAGACCGA
25 ACGGACAgtatgtgattcattcatatgctttcaagtacatttaaaattgttaactgatttatgtgttgataatatctact
105 aagtgcatgttgaataggtaaccattgcaagacaatcccaaactatatttgatttacgataaaatactttggactatcaa
185 atgtagcttgtattttactgccttttttttccagCTTCC ATG GAT CTT AGC AGC AAA ACG TTT GTG CAG
M D L S S K T F V Q 10
GnRH
254 GTG GTG ATG TTG GCG TTG ATA GCT CAG GTC ACT TTC TCT CAG CAT TGG TCG TAT GGG TGG
V V M L A L I A Q V T F S Q H W S Y G W 30
314 CTA CCT GG
L P

Figure 3-7. A schematic comparing the 5'-flanking regions of the known salmonid sGnRH-encoding genes. The thick lines represent regions of DNA that are highly conserved for each gene. The asterisks above the sGnRH gene1 represent the four blocks of DNA that are exact repeats of one another. The bent arrow denoted by +1 indicates the brain transcription start site from exon 1. No similarity exists between -589 to -215 in the sGnRH gene1 in comparison to the sGnRH genes2. A large block of 1152 bps present in Atlantic salmon gene2 is not present in the sockeye salmon sGnRH gene2. P1 and P2 indicate downstream and upstream promoter regions, respectively. Positions 225, 233 and 241 indicate translational start sites for each of the GnRH preproproteins.

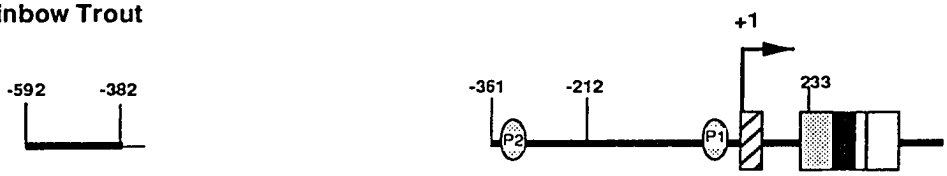
SALMONID sGnRH GENE1:

Rainbow Trout

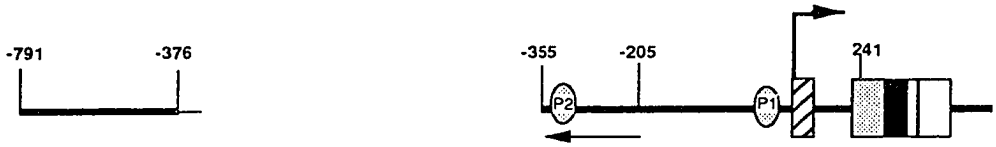


SALMONID sGnRH GENE2:

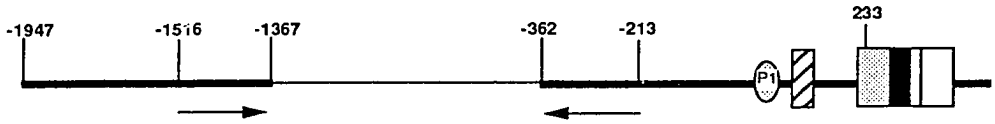
Rainbow Trout



Sockeye Salmon



Atlantic Salmon



the start site in relation to these EREs in all three salmonids could point to the involvement of estrogen receptor in the responsiveness of the upstream promoter in gene2.

Developmental expression of GnRH in the ovary and testis

My original intent was to determine whether alternative promoters are used in the ovary and testis during early development. In studying the expression pattern, I observed an unusual pattern of expression during development in the gonads compared to the brain.

At five to eight months after fertilization (May to August) the gonads were undifferentiated and yet they expressed GnRH (Fig. 3-8A). Once the gonads became differentiated in the first year, expression of GnRH was limited to a short period: September and October in the ovary and only October in the testis. This coincided with the time of year in which they would eventually mature and spawn in their third year and thereafter. GnRH was not expressed in rainbow trout ovary and testis from November of the first year through May of the second year (Fig. 3-8A and 3-8B). Expression of GnRH was not detected in juvenile tissue at any stage in the second year except in December (Fig. 3-8B).

In the second year of life, beginning in June, about 20 % of the fish showed precocious sexual development in which there was advanced ovarian ("jills") and testicular ("jacks") maturation. The expression of GnRH was tested separately in these fish (Fig. 3-8C). GnRH was expressed in each precocious tissue examined in June through October except in ripe ovaries (August) and in "jack" testes (October) (Fig. 3-8C). The testes examined from June through October were very large compared to their immature counterparts. The ovaries examined were considered to be "jills" because they had well-defined features indicating they were maturing (visible eggs, orange coloration, larger size).

The undifferentiated gonadal tissue did not use the upstream promoter in June (year 1), but did use it in August to generate GnRH mRNA2 (data not shown) (Fig. 3-8A).

Figure 3-8A to 3-8D: GnRH expression in ovary and testis of rainbow trout in first and second year of their lives using primer sets B/C and/or A/C with RT-PCR.

A: RT-PCR of RNA from undifferentiated gonads of fish that were 5 months (May) to 8 months (August) of age. Only in September (ovary) or October (testis) of first year did differentiated organs express GnRH.

B: RT-PCR of juvenile ovary and testis showed GnRH expression only in December.

C: RT-PCR of gonads from fish that matured precociously in year 2 beginning in June. Maturing ovary expressed GnRH from June to October, except for ripe ovary examined in August. Large testes taken from precocious males expressed GnRH in June, July and August, but not October.

D: Life history of rainbow trout showing development from egg to death. Larvae from fall-spawning rainbow trout overwinter in gravel spawning beds and hatch approximately three months following fertilization, depending on water temperature, flow rate and other factors. The alevin live on their yolk sac until roughly mid-March when they begin to feed as fry. The fry may undergo smoltification by one year of age and ultimately develop into mature, reproductively competent adult fish by three years of age. In the second year, about 20% of the population may have well-developed reproductive tissue. The males in this precocious group are called "jacks" and the females are called "jills". In the present experiment the eggs were fertilized in December and hatching occurred in January. Differentiation of the gonads was observed in September when the fry were 10 months old.

A Year 1- Normal

	ovary	testis
May	YES	
June	YES	
July	YES	
Aug	YES	
Sept	YES	NO
Oct	YES	YES
Nov	NO	NO
Dec	NO	NO

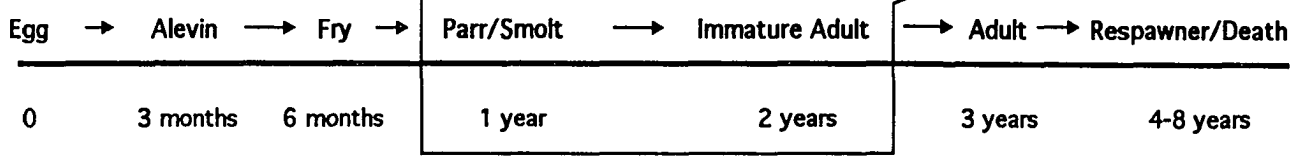
B Year 2-Normal

	ovary	testis
Jan	NO	NO
March	NO	NO
April	NO	NO
May	NO	NO
July		NO
Aug		NO
Oct		NO
Dec	YES	YES

C Year 2-Precocious

	ovary	testis
June	YES	YES
July	YES	YES
Aug	YES/NO	YES
Oct	YES	NO
Dec	N/A	N/A

D Life History



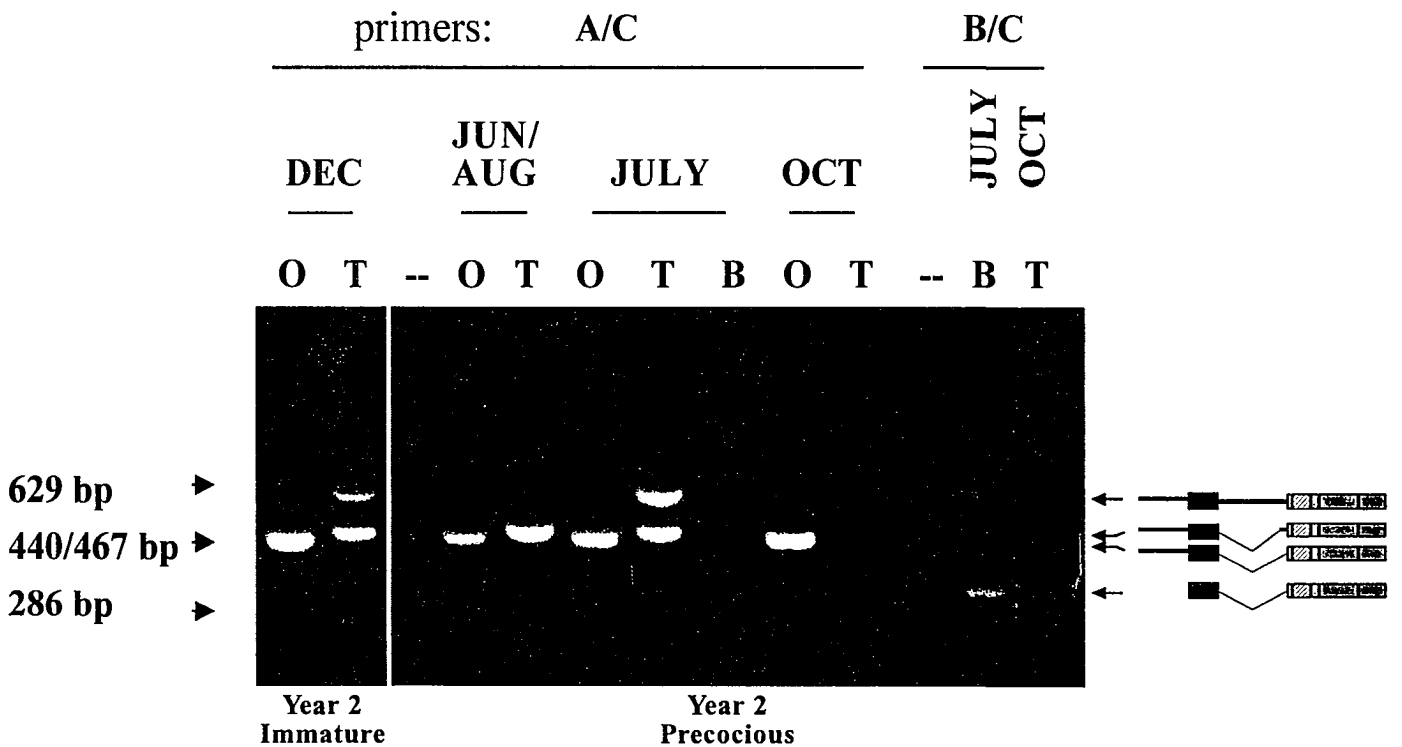
Both May and July gonadal tissue were examined for expression of GnRH only with primer set B/C, which detects use of the downstream promoter. The upstream promoter was used in all juvenile fish in September to October (year 1) and again in December (year 2) (Fig. 3-8A and 3-8B) (Fig. 3-9). The same was true for the precocious fish examined in year 2 (Fig. 3-8C and Fig. 3-9).

Discussion

My data show that ovary and testis use an alternative upstream promoter and retain intron 1 to generate GnRH mRNAs bearing 5'-UTRs longer than their brain counterparts in rainbow trout. I found that the ovarian and testicular transcripts have two 5'-UTRs that differ in length from the brain 5'-UTRs. In salmonids, such as rainbow trout, which have two genes encoding identical GnRH peptides, the organization of the 5'-flanking regions is distinct beyond the 215 basepairs (bps) proximal to the start site. During development, GnRH mRNA is expressed even in undifferentiated gonads during the first year and then in both the ovary and testis in an intermittent pattern during the juvenile stage. This is unlike the brain where GnRH is continuously expressed from before hatching through adult life.

In developing gonads, upstream promoters were used in late stages of undifferentiated gonads and throughout the juvenile and precocious stages. In the ovary and testis, the same promoter that is used in the brain is utilized, as well as the more upstream alternative promoter, for transcription of the sGnRH mRNA2 transcripts. This generates at least three sGnRH mRNA2s; two transcribed from the upstream promoter (with or without intron 1) and at least one from the more downstream promoter. Testes transcripts also have a variation due to an extra 27 nucleotides (nts) from the alternative splice site between intron 1 and exon 2. A similar requirement for long 5'-UTRs has been demonstrated for mammalian (m)GnRH mRNA in the reproductive tissue of monkey (Dong *et al.*, 1996) and human (Dong *et al.*, 1993). Although the rat ovary has been shown to express

Figure 3-9. Examination of GnRH expression and use of the upstream promoter in two-year-old rainbow trout ovary (O), testis (T) and brain (B) with primer sets A/C and B/C. sGnRH mRNA2 is expressed in precocious ovary and testis from June to October of the second year. All results from rainbow trout testis for June to August are from RT-PCR analysis of RNA extracted from large testes taken from jack males. In October, larger testes from jack males no longer expressed sGnRH cDNA2. GnRH expression observed in juvenile rainbow trout ovary and testis was only in December as shown here. No amplification products were observed for the brain with primer set A/C, but amplification of a PCR product is detected using primer set B/C at 286 bp.


primers: A/C
B/C
DEC
JUN/ AUG
JULY
OCT
JULY
OCT
O T -- O T O T B O T -- B T
629 bp
440/467 bp
286 bp
Year 2 Immature
Year 2 Precocious

GnRH, neither rat nor mouse appear to utilize upstream promoters for transcription of GnRH in reproductive tissues (Dong *et al.*, 1993 and 1996). Also, a comparison of transcript sizes from Northern analysis indicates that the GnRH mRNA expressed in the gonads of goldfish (Lin and Peter, 1996) and midshipman (Grober *et al.*, 1995) are not generated from upstream promoters. It therefore appears that the mechanism for control of GnRH expression in the ovary and testis of rodents, as well as for goldfish and midshipman, differs from that of the primates and salmonids.

It has been demonstrated in the mammalian brain that various neurotransmitters and modulators regulate GnRH levels by modulating mRNA stability (Gore and Roberts, 1997). Perhaps the extended 5'-UTRs of sGnRH and mGnRH transcripts found in the salmonid and mammalian gonads are required by nonneuronal tissue to similarly modulate GnRH levels. Specific sequences contained within a variety of mRNAs have been demonstrated to influence the half-life of the mRNA (for a review see Ross, 1988). The extended 5'-UTRs characterized here may contain regulatory sequences that are recognized by stabilization or destabilization factors that may be associated with RNases in the ovary and testis. Alternatively, the 5'-UTR may inhibit or enhance GnRH translation (through facilitatory binding proteins) at important periods during development and maturation. These functional questions need to be addressed to complete our understanding of the dynamic processes that govern GnRH in the gonad.

To my knowledge only mGnRH and sGnRH transcripts bearing these unusually long 5'-UTRs have been isolated from reproductive tissue. That both mGnRH and sGnRH genes utilize two different promoters and generate alternative transcripts provides further support for the hypothesis that the gene encoding sGnRH arose from the ancient mammalian GnRH gene in bony fish (Sherwood *et al.*, 1997). We have shown previously that mGnRH of identical structure is present in humans and an ancient bony fish, the sturgeon (Sherwood *et al.*, 1991, Lescheid *et al.*, 1995). The salmon form of GnRH (sGnRH) arose as mGnRH disappears in fish that evolved later than the sturgeon.

A series of potential AUG translational start sites are present in the extended 5'-UTRs and in intron 1. However, these potential start sites neither resemble the functional translational start sites (CT^{C}/TCCAUGG) nor the Kozak consensus sequence rule (CC^{A}/GCCAUGG) (Kozak, 1991). Furthermore, when read in frame the potential start sequences are always followed by termination codons. It is doubtful that translation begins at any other site in the extended transcripts except the same site that is used in the brain.

In human placental or breast tumor cell lines, the major promoter for mGnRH was shown to be the more upstream one (Dong *et al.*, 1993). The sequences permitting tissue-specific retention of intron 1 may be present in the 5'-end of intron 1 where the 4th nucleotide of the salmonid or the 5th nucleotide of the human (Radovick *et al.*, 1990) GnRH gene is changed from the intron consensus 5'-GTAAGT-3' donor site. These changes may provide some flexibility or recognition by the spliceosome for intron retention or splicing. In rainbow trout testis, the mechanism for selection of the more upstream AG acceptor site (27 nts upstream from the acceptor used in the brain) is clear because both acceptor sites for intron splicing closely match the splice site consensus sequences found in vertebrates ($(T/C)_{11}$NCAG) (Padgett *et al.*, 1986). Use of this alternative splice site in the 3'-end of intron 1 was observed in preliminary studies on 1.5 year-old sockeye salmon (Chapter 4) and therefore may occur more frequently amongst salmonids than reported here.

The promoters of the two genes characterized here have 94 % sequence identity within the region proximal to the start site. Whatever alterations have occurred to the promoters after duplication of the ancestral sGnRH gene at the transition from a diploid to tetraploid fish, the proximal 215 bps have been conserved. This core promoter region is presumably important for binding of the basic transcription factors near to the start site.

Remarkably, there are no large regions of similar sequences beyond the 215 nt at the start site that are shared between the salmonid GnRH promoters and any of the known

mammalian GnRH promoters. However, small consensus sequences that resemble binding motifs for mammalian transcription regulators are present in the sGnRH gene2 (Chapter 2 and 6). Also, the A/T-rich block of DNA that is conserved among the salmonid sGnRH-encoding genes (-708 to -589 in the trout sGnRH gene1; -551 to -426 in the sockeye salmon sGnRH gene2 and -1689 to -1565 in the Atlantic salmon sGnRH gene2) do possess recognition sequences that may be important for GnRH regulation in both the brain and the gonads. For example, consensus elements are present in each promoter that potentially could bind members of the POU homeodomain family of transcription regulators that are involved in morphogenesis and neurogenesis (He *et al.*, 1989). Each promoter in this conserved region contains two elements that strongly resemble recognition sequences for mammalian Brn-2 (CATnTAAT) and at least one centrally-positioned element that could be engaged by Oct-type factors (ATGCAAAT) (Li *et al.*, 1993). Importantly, members of this family of regulators have been isolated in both the mammalian brain and gonad (He *et al.*, 1989).

The block of repeating DNA between position -457 to -294 in the sGnRH gene1 contains no apparent consensus sites for factors that direct GnRH transcription in mammals. However, this GC-rich region does contain four repeating blocks of DNA (each 41 bps in length), that each hold a potential estrogen response element (ERE) half-site. The Atlantic salmon sGnRH gene2 promoter contains six EREs, three that are palindromic and three that are non-palindromic (Klungland *et al.*, 1993; Chapter 2). These TGTCC half-sites were shown to bind human estrogen receptor only if they were part of a complete palindromic element in footprinting assays and gel retardation experiments studies on the Atlantic salmon sGnRH gene2 (Klungland *et al.*, 1993). However, unlike the sGnRH gene2 of Atlantic and sockeye salmon, complete palindromic EREs are not present in the sGnRH gene1 promoter, indicating that if estrogen is involved in activation of sGnRH-I expression in the gonads, it may not be through ER binding to the half-site.

Also, the more proximal portion of the Atlantic salmon promoter is highly homologous to both the sockeye salmon and rainbow trout sGnRH gene2 promoters (Klungland *et al.*, 1993). This is of some interest considering that the alternative upstream promoter of GnRH in salmonids, as well as in both human (Dong *et al.*, 1993) and monkey (Dong *et al.*, 1996), contains non-palindromic EREs which were demonstrated by Radovick to stimulate GnRH expression (Radovick *et al.*, 1991).

Little is currently known about the regulation of GnRH transcription in the gonad. I speculate that potential EREs in the salmonid and primate GnRH promoters means that ER or other steroid receptors play a role in the transcription of GnRH in the ovary and testis. Future analysis using promoter deletion and electrophoretic mobility shift assays need to be conducted to define the regulatory factors and the specific recognition motifs that the promoters bind.

The intermittent expression of GnRH in juvenile gonads during the first two years of life suggests a very different function compared to that in the brain where GnRH is continuously expressed (Chiappa and Fink, 1977, Okuzawa *et al.*, 1990, Parhar *et al.*, 1995). GnRH is expressed in juvenile gonads only in September and October of the first year and in December of the second year (see Fig. 3-8). Longer periods of expression are observed in precocious gonads in the second year. There are strong indications from several physiological studies that GnRH acts as a meiosis stimulating factor in the oocytes of rat (Hillensjo and LeMaire, 1980) and fish (goldfish and seabream; see Nabissi *et al.*, 1997). As well, GnRH has been implicated in lymphocyte blastogenesis (Wilson *et al.*, 1995), gonadotrope differentiation (Aubert *et al.*, 1985) and in tunicate gonadogenesis (Irons, 1986). In this light, the expression of GnRH in rainbow trout might be needed as part of a program for growth and differentiation of a new wave of germ cells required by the iteroparous rainbow trout. This regeneration requirement is also displayed in mammals (human and rat) and a fish (goldfish) where GnRH mRNA is present at all stages of follicular development (except very early in development and following

ovulation). The expression of GnRH in undifferentiated gonads suggests the role of GnRH is considerably broader than presently understood.

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Chapter 4

Regulation and expression of gonadotropin-releasing hormone in embryonic and gonadal tissue of sockeye salmon

Summary

Gonadotropin-releasing hormone (GnRH), in addition to its classical role of releasing pituitary hormones, also exerts its effects at sites other than the pituitary. The isolation and localization of GnRH and its receptor in mammalian and fish reproductive tissues supports an extrapituitary role for GnRH in reproduction. However, little is known about the regulation and expression of GnRH during early development in the gonads.

I studied the expression of GnRH in embryonic tissues of both sockeye salmon and rainbow trout and in several extraneural tissues in sockeye salmon. In whole embryos, an upstream promoter was transiently used for expression of GnRH beginning as early as 14 days after fertilization in rainbow trout and 30 days after fertilization in sockeye salmon. Both sGnRH cDNA1 and cDNA2 were expressed in embryonic tissue in both species. The expression in whole embryos is most likely to be in the brain and possibly in gonadal tissue because analysis of individual tissues in juvenile fish showed expression of GnRH mRNA in brain, ovary and testis, but not in heart, liver, gut, adrenal, spleen or retina. In sockeye salmon, when undifferentiated gonads (at 5 to 8 months) or individual ovary and testis (at 9 months) could be dissected, GnRH was expressed in the first year only at 10 months (October), but not during year 2. However, in precociously mature fish, GnRH was expressed in both ovary and testis in May and June.

I also isolated and sequenced both forms of sGnRH-encoding cDNAs from the ovary and testis of sockeye salmon. sGnRH mRNA2 was expressed as transcripts of two sizes in both of these tissues from an alternative start site 315 bp upstream from that utilized in the sockeye salmon brain. One of these transcripts retained intron 1 completely whereas the other message does not. Although sockeye salmon and rainbow trout are in the same genus (*Oncorhynchus*), there are distinct differences in the cDNA sequence and in GnRH expression pattern in the gonads of juvenile or precociously mature fish.

Introduction

Considerable information has been reported for the brain in regard to GnRH. Two forms of the GnRH peptide, sGnRH and cGnRH-II, have been isolated and sequenced from the brains of chum salmon (Sherwood *et al.*, 1983; Powell, personal communication). These two forms of GnRH have been identified also from the brains of both masou salmon and rainbow trout by radioimmunoassays (Okuzawa *et al.*, 1990, Amano *et al.*, 1992). Differences in the location and levels of synthesis have been measured for these two GnRHs (Okuzawa *et al.*, 1990). Furthermore, two different cDNAs (sGnRH cDNA1 and sGnRH cDNA2) that each encode sGnRH have been isolated from the brain of sockeye salmon (Ashihara *et al.*, 1995). At the nucleotide level these transcripts have 80% identity, but they both encode identical sGnRH decapeptides.

A more recent emphasis is on the nonneuronal roles of GnRH. In fish, Northern analysis was used to detect a transcript encoding sGnRH mRNA2 in midshipman ovary and testis (Grober *et al.*, 1995). Both sGnRH and cGnRH-II cDNAs have been isolated from the goldfish ovary (Lin and Peter, 1996). GnRH-like activity and binding sites in goldfish, carp, African catfish and gilthead seabream ovary have also been described (see Nabissi *et al.*, 1997).

In this study I use embryos to examine the stage-specific pattern of sGnRH expression in the brain and gonads. I also examine the gonads in sockeye salmon to determine whether expression of GnRH is distinct from that in the brain. The types of transcripts and the use of an alternative promoter or retention of intron 1 are determined for embryos, juveniles and adults. Other tissues are studied to see whether sGnRH is expressed in nonneural sites in addition to the gonads.

Materials and Methods

Raising of salmon and tissue collection

Eggs from sockeye salmon were obtained from Pitt River, B.C. in November. Rainbow trout eggs were obtained from the Frazer Valley Trout Hatchery in Abbottsford, B.C. in December. The eggs were fertilized by gently mixing them with the milt by hand. The eggs were then washed with partial exchanges of water. Approximately 200 fertilized eggs/nest were placed in a Heath tray system (see Chapter 3). The sockeye salmon eggs were raised in fresh water at 13° C and at a flow rate of 30 L/h. Rainbow trout eggs were raised at 14° C and at a flow rate of 720 L/h. Every other day for 68 days after fertilization (sockeye salmon) or 42 days after fertilization (rainbow trout), whole embryos (n=20) were collected for each species and directly placed into liquid nitrogen and stored at -80° C. Following hatching, alevin (under 2 cm in length) were collected every other day for several weeks and their heads and trunks were separated and placed onto dry ice.

Sockeye salmon alevin from the group described above were transferred from the Heath trays to 30 L holding tanks and raised for 7 months, then transferred to 500 L rearing tanks for the remainder of the study. The holding and rearing tanks were exposed to natural light conditions throughout the study. Sockeye salmon at age 1.5 years and older were obtained from Rosewell Creek Hatchery (Rosewell, B.C.). Beginning at 5 months of age (dated from fertilization), several fish were quickly killed by decapitation and ovarian or testicular tissue removed and placed on dry ice.

Isolation of RNA from embryos and adult ovaries and testes

Total RNA was extracted from eggs, embryos and posthatch fish or juvenile and adult ovaries and testes. Trizol was used as recommended by the manufacturer (Bethesda Research Laboratories, Bethesda, MD).

cDNA synthesis and RT-PCR

First-strand cDNA was synthesized from 2 to 5 ug of total RNA from extracted tissue using Superscript RT RNase H^- reverse transcriptase by methods recommended by the manufacturer (Life Technologies Inc., Gaithersburg, MD). 0.5 to 1.0 µL of the reverse transcription reaction product was diluted to a final 50 µL volume containing 1x Promega buffer (50.0 mM KCl, 10.0 mM Tris-HCl, 1.5 mM $MgCl_2$ and 0.1 % Triton X-100), 200 µM dNTPs, 2.5 units of Taq DNA polymerase (Promega, Madison, WI) and 30 pmol of sense or antisense strand primers. Amplification of sGnRH-II cDNA was achieved with 40 cycles of 1 min at 94° C, 1 min at 50° C and 1 min at 72° C using the sense strand primers A (5'-GAAGCTTATGCACTAAGCAGG-3'),
B (5'-TAGGAAGGAATACAGAACGG-3'),
H (5'-CAGCACTGGTCGTATGGCTGGCTACCTGG-3'),
F (5'-AGGACATTTCTAAGTGACC-3') or G (5'-CTACACTGTATTTCTGATC-3') in combination with antisense strand primers C (5'-TTATTTATGGGGCATCCATTTC-3') or D (5'-CAGCCATACGACCAGTGCTG-3') (as shown in Fig. 4-2A). The sense strand primers A, F and G are located in positions -141 to -121, -240 to -222 and -310 to -292 upstream from the sockeye salmon brain GnRH transcription start site, respectively (Fig. 2-1). In rainbow trout, the corresponding positions for primers A, F and G are -149 to -129, -248 to -230 and -318 to -300, respectively (Fig. 3-4). Sense strand primers B and H correspond in the sockeye salmon gene2 to the respective positions 18 to 37 of exon 1 and positions 308 to 336 of exon 2 (complementary to GnRH). Antisense strand primers were located as follows: C (exon 4, positions 1031 to 1048) and D (exon 2, against the sequences that encode GnRH).

The PCR products were separated on a 1.5% agarose gel containing ethidium bromide and retrieved by electroelution in dialysis tubing. The retrieved DNA was subcloned into pGEM-T vector (Promega, Madison, WI) and recombinant plasmids were sequenced on both strands by the chain termination method (Sanger) using the Sequenase Version 2 kit

(USB) or Circumvent Thermal Cycle sequencing kit (New England Biolabs, Beverly, MA).

RT-PCR of sGnRH cDNA1 from embryo and ovary

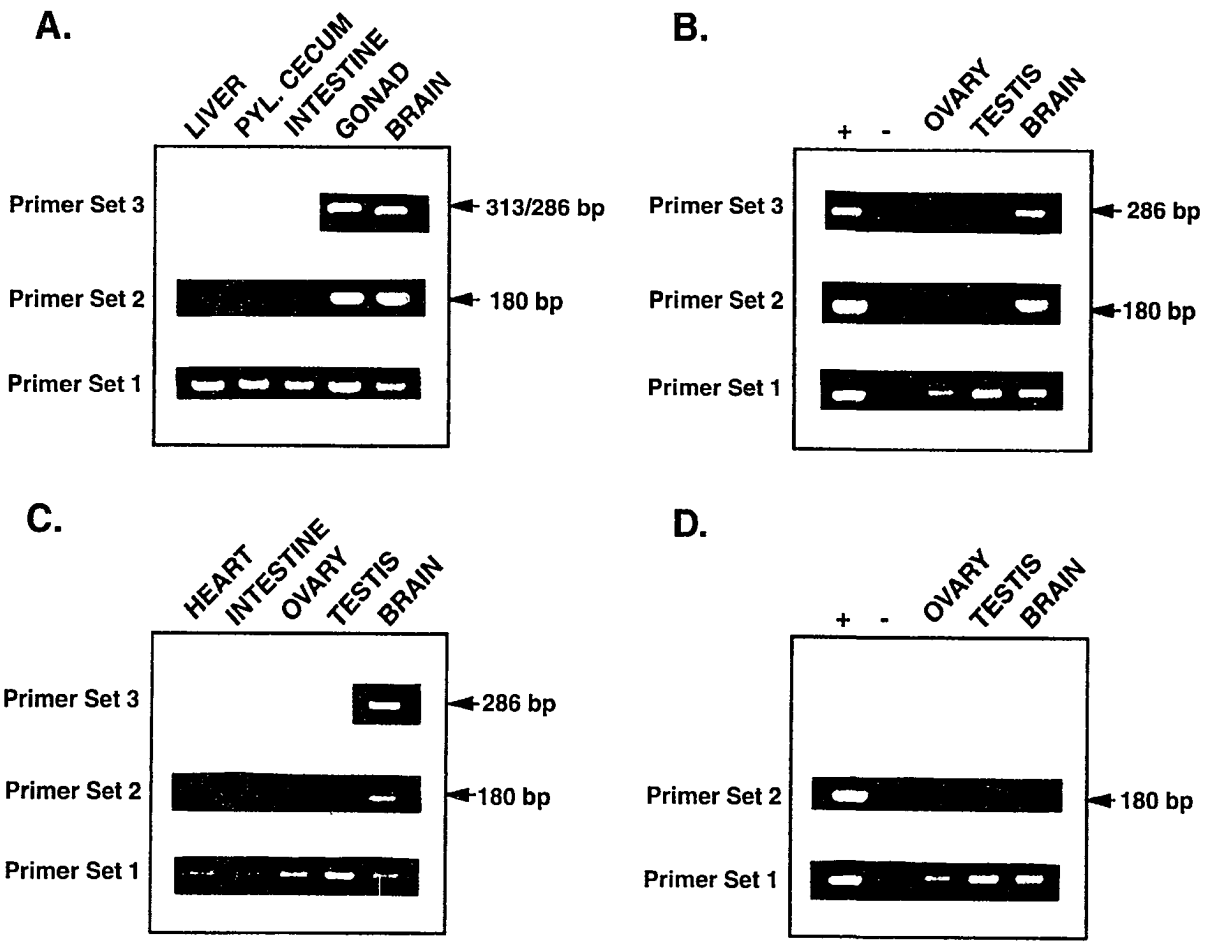
The synthesis and amplification of sGnRH cDNA1 was achieved using RNA derived from both 18 day-old embryos and ovary of 1.5 year-old rainbow trout by the same methods as described above. The sense strand primer S (5'-AGGAATAGACCGAACGGAC-3') was complementary to the 5'-UTR (position 10 to 28 of exon 1) for the sockeye salmon brain sGnRH cDNA1 (Ashihara *et al.*, 1995) (Fig. 4-2A). The antisense strand primer T (5'-TTGAATGCTCCATCATCGC-3') was designed against a consensus region for the 3'-UTR of both sockeye salmon (Ashihara *et al.*, 1995) and masou salmon (Suzuki *et al.*, 1992) sGnRH mRNA1. The 3'-UTR region was used as it is distinct from its sGnRH mRNA2 counterparts. The integrity of each cDNA for each examined tissue was confirmed by separate PCRs using primers that correspond to bases 523 to 545 and 719 to 740 specific for a salmon tubulin cDNA clone (Coe *et al.*, 1992).

Results

Expression of GnRH in extraneural tissue

To determine if GnRH mRNAs were expressed in tissue other than the brain, RNA derived from liver, pyloric cecum, intestine, gonad, heart, ovary and testes of sockeye salmon was examined by RT-PCR (Fig. 4-1). Panels A and B present RT-PCR results for tissues from 1.5-year-old (collected in early May) and nearly two-year-old (collected in August) fish, respectively. Panels C and D present RT-PCR results for RNA derived from tissues of four year-old fish (approximately one month before spawning when entering the Frazer River in late July) and from tissues of fish that are12 hours from

Figure 4-1. Tissue- and stage-specific study of various sockeye salmon tissues examined by RT-PCR. The expression of GnRH was analysed using primer sets H/C (Primer Set 2) and B/C (Primer Set 3). Amplification of PCR products with primers specific for salmon tubulin (Primer Set 1) confirmed the integrity of the cDNA used in this study. RT-PCR analysis of tissues from (A) 1.5-year-old fish (B) two-year-old fish (C) fish collected approximately one month before spawning and (D) fish collected approximately 12 hours before spawning. The PCR results presented in Panels A and B or C and D were conducted simultaneously with a positive (brain cDNA) and negative control (no cDNA).


A.
LIVER
PYL. CECUM
INTESTINE
GONAD
BRAIN
Primer Set 3
Primer Set 2
Primer Set 1
313/286 bp
180 bp
B.
+
-
OVARY
TESTIS
BRAIN
Primer Set 3
Primer Set 2
Primer Set 1
286 bp
180 bp
C.
HEART
INTESTINE
OVARY
TESTIS
BRAIN
Primer Set 3
Primer Set 2
Primer Set 1
286 bp
180 bp
D.
+
-
OVARY
TESTIS
BRAIN
Primer Set 2
Primer Set 1
180 bp

spawning (early November), respectively. The PCR results presented in Panels A and B or C and D were conducted simultaneously with a positive and negative control.

The use of primers H/C (Primer Set 2) and B/C (Primer Set 3) (Fig. 4-2A) shows expression of GnRH in both the gonad and brain of 1.5-year-old fish, but in only the brains of 2- and 4-year-old sockeye salmon (Fig. 4-1 A-C). All other tissues examined appeared to be devoid of GnRH expression, at least at the times that tissue collection took place. We also examined spleen, adrenal and retina of 1.5-year-old sockeye salmon and found no GnRH expression (data not shown). Sequence analysis of the cloned PCR product (313 bps) obtained from the 1.5-year-old gonad (Fig. 4-1A) revealed that 27 nucleotides (nts) of the most 3'-end of intron 1 was retained in the cDNA compared to the brain transcript of 286 bps. The integrity of each cDNA for each examined tissue was confirmed by separate PCRs using primers that correspond to bases 523 to 545 and 719 to 740 specific for a salmon tubulin cDNA clone (Primer Set 1) (Coe *et al.*, 1992).

Subsequent sampling and analysis of ovaries and testes monthly during the first two years of life of sockeye salmon by RT-PCR revealed significant differences in the expression of GnRH (Fig. 4-3). To determine if the salmonid GnRH transcripts found in the reproductive tissues were transcribed from an alternative start site, I designed primers A and F (Fig. 4-2A) that are complementary to a region upstream from the transcription start site in the brain of sockeye salmon. In PCR analysis of first-strand cDNA made from ovarian and testicular total RNA using primers A/C, I observed PCR products 629 and 440 bps in length (Fig. 4-4) following gel fractionation. Subsequent cloning and sequencing of these PCR products revealed that each amplification product contained sequences upstream from exon 1 and that the larger transcripts retained intron 1 (but not introns 2 or 3) (Fig. 4-2B; Fig. 4-5).

In the first year, sockeye salmon expressed GnRH in both ovaries and testes in October and at no other time during the year (Fig. 4-3A). GnRH was not expressed in sockeye salmon ovaries and testes from November of the first year through April of the

Figure 4-2. Analysis of the upstream transcription start site of the salmonid GnRH gene. (A) Schematic presents position of primers (arrows) used in RT-PCR. The four boxes represent the exons that comprise brain-type GnRH mRNA. (B) Summary of different sGnRH cDNA2 transcripts found in the sockeye ovary and testis and immature gonad. The transcript with the longest 5'-untranslated region is shown by the thick line and black box (exon 1) to represent sGnRH mRNA2 transcribed from the upstream start site with unspliced intron 1. In sockeye salmon ovary and testis, the shorter transcripts generated from the upstream start site do not contain intron 1. The undifferentiated gonad generated a sGnRH cDNA2 transcript containing 27 nucleotides of the most 3'-end of intron 1.

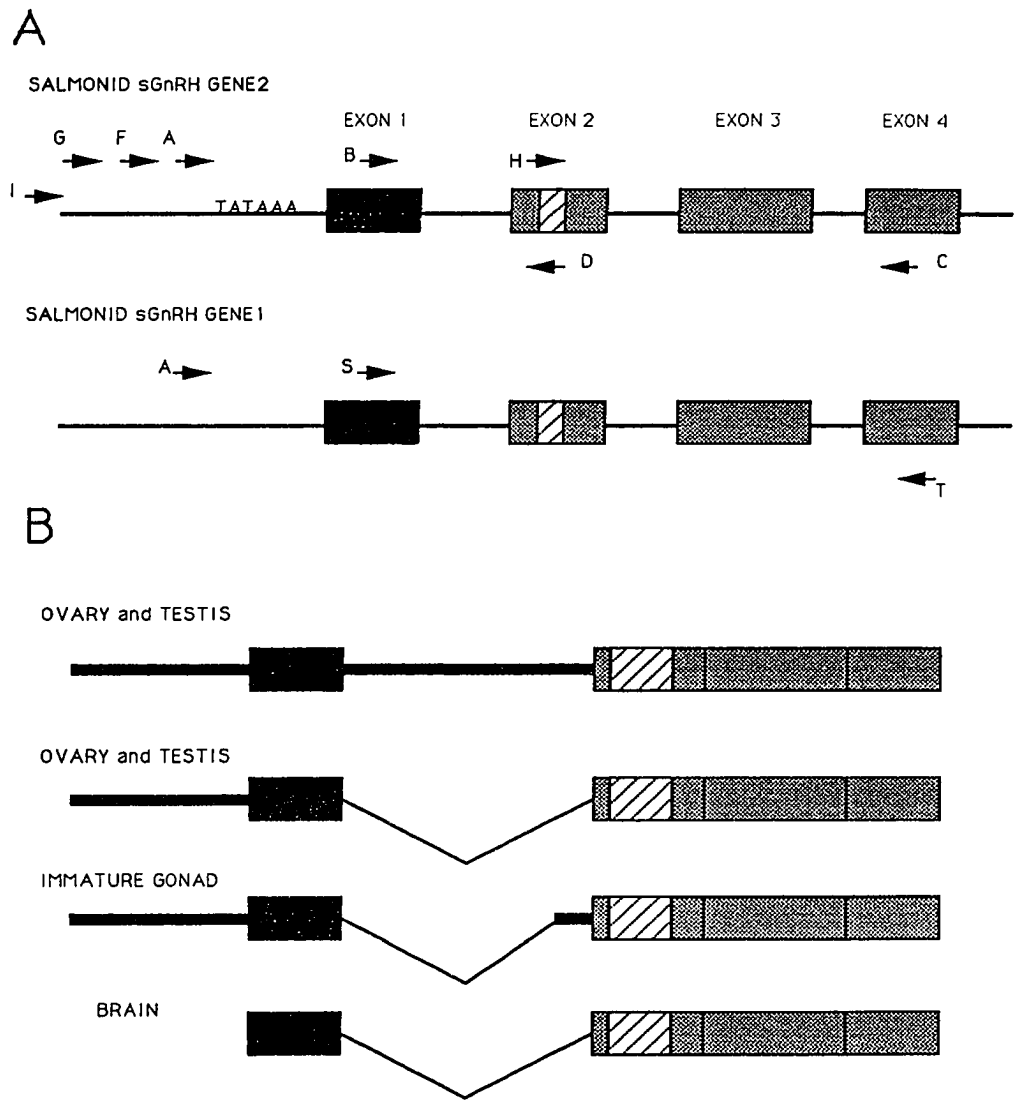

A
SALMONID sGnRH GENE2
EXON 1
EXON 2
EXON 3
EXON 4
G
F
A
B
H
I
TATAAA
D
C
SALMONID sGnRH GENE1
A
S
T
B
OVARY and TESTIS
OVARY and TESTIS
IMMATURE GONAD
BRAIN

Figure 4-3: GnRH expression using RT-PCR in reproductive tissues of sockeye salmon in the first and second years of their lives.

A: RNA from pooled undifferentiated gonads was examined by RT-PCR from May to August. Only in October of the first year did differentiated ovaries and testes express GnRH.

B: At no time during the second year did juvenile ovary or testis express GnRH. Gonadal tissue from 1.5-year-old fish (May) did express GnRH (data not shown) (see Fig. 4-1).

C: In May to October, all positive results were from RT-PCR analysis of RNA extracted from precocious animals.

D: General life history of sockeye salmon showing development from egg to spawning and death. Sockeye salmon larvae overwinter in gravel spawning beds and hatch approximately three months following fertilization, depending on the water temperature, flow rate and other factors. The alevin live on their yolk sac until roughly mid-March when they begin to feed as fry. The fry usually undergo smoltification by one year of age. Following smoltification, the majority of the young sockeye salmon migrate to the ocean where they will spend the rest of their lives until returning to their natal stream or river at 4 to 6 years of age where they will spawn in the fall and die. In the second year, a percentage of the population may have well-developed reproductive tissue. The fish in this precocious group may be comparable to most fish at 3 years of age.

A Year 1-Normal

	ovary	testis
May	NO	
June	NO	
July	NO	
Aug	NO	
Sept	N/A	
Oct	YES	YES
Nov	N/A	
Dec	N/A	

B Year 2-Normal

	ovary	testis
Jan	NO	NO
March	NO	NO
April	NO	NO
June	NO	NO
July	NO	NO
Oct	NO	NO

C Year 2-Precocious

	ovary	testis
May	YES	YES
June	YES	YES
July	NO	NO
Aug	NO	NO
Oct	NO	NO
Dec	N/A	N/A

D Life History

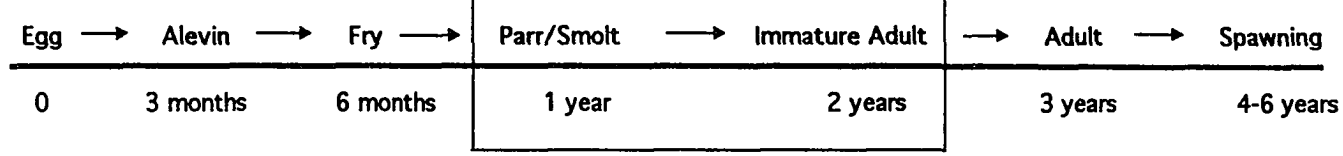


Figure 4-4. RT-PCR analysis of RNA extracted from two-year-old salmonid ovary (O), testis (T) and brain (B). Examination of GnRH expression and use of the upstream promoter in sockeye salmon ovary, testis and brain with primer sets A/C and B/C. GnRH was expressed from the upstream promoter in both the ovary and testis in June and from the ovary in May. In the second year, GnRH was not expressed, at least not in July or October. In May, the downstream start site was used by testis. The larger band at 629 bps represents a transcript in which intron 1 has not been spliced, whereas the smaller transcript at 440 bps is devoid of introns. I demonstrate that the use of the upstream start site is specific for the ovary and testis since no amplification products are observed for the brain with primer set A/C, but amplification of a PCR product is detected using primer set B/C at 286 bp.

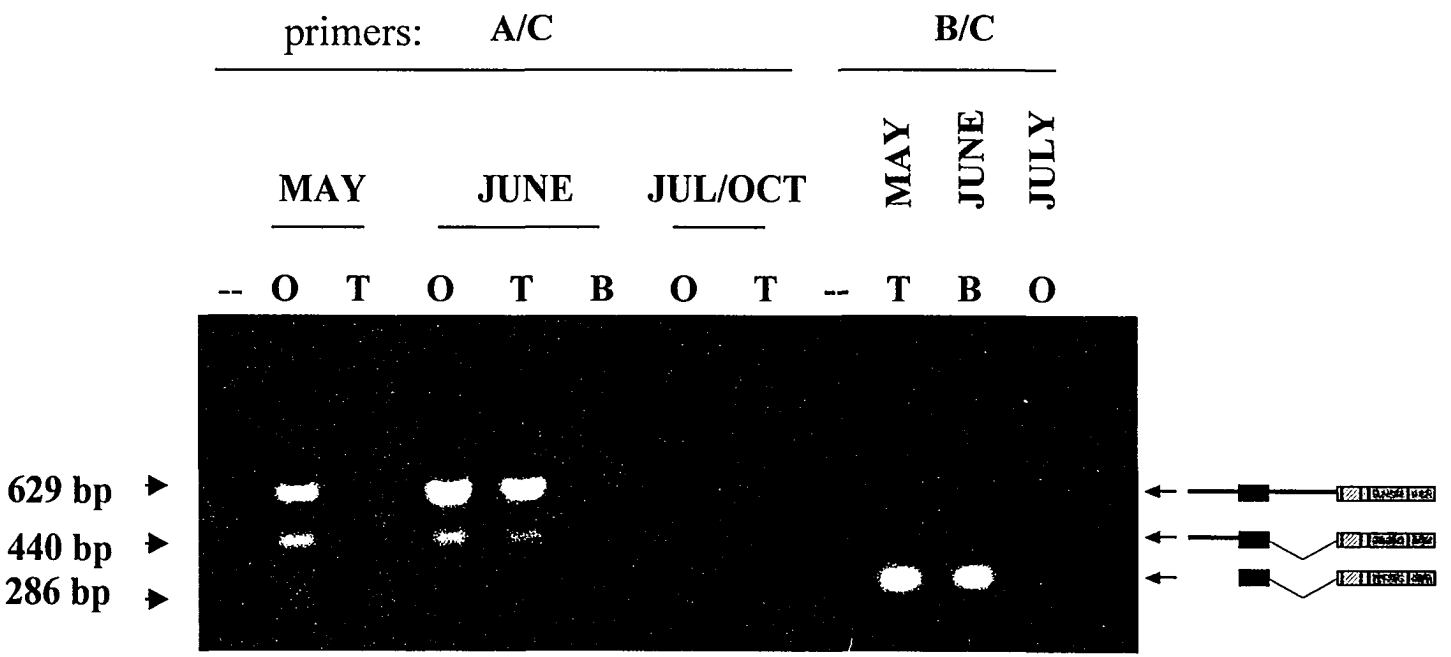
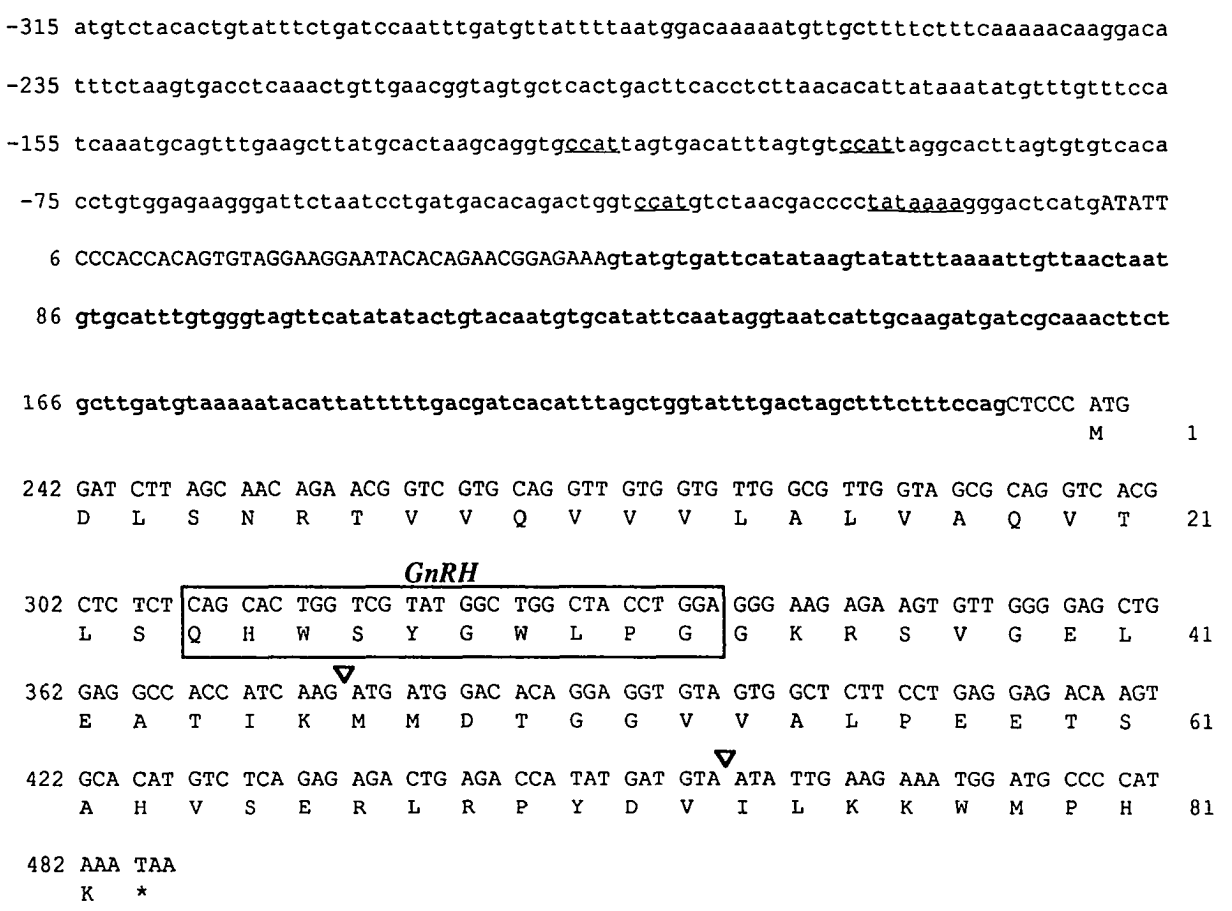

primers: A/C
B/C
MAY
JUNE
JUL/OCT
MAY
JUNE
JULY
-- O T O T B O T -- T B O
629 bp
440 bp
286 bp

Figure 4-5. Nucleotide sequences of the full-length sGnRH cDNA2 isolated from ovary and testis of sockeye salmon. Note that the start site for the cDNA begins 315 nucleotides upstream from the start site used in the brain. The start site for GnRH expressed in the brain was determined for sockeye salmon by primer extension analysis (Chapter 2). The TATA box and putative CAAT boxes used by the transcription apparatus to stimulate GnRH expression in the brain are underlined. Note also that this transcript includes all of intron 1 (shown in bold) which is spliced out of the smaller sGnRH cDNA2 messages expressed in the ovary and testis of sockeye salmon. Positions are given for nucleotides on the left and amino acids on the right. Arrows indicate positions of introns 2 and 3, respectively.


-315 atgtctacactgtatttctgatccaatttgatgttattttaatggacaaaaatgttgcttttctttcaaaaacaaggaca
-235 tttctaagtgacctcaaactgttgaacggtagtgctcactgacttcacctcttaacacattataaatatgtttgtttcca
-155 tcaaatgcagtttgaagcttatgcactaagcaggtgccattagtgacatttagtgtccattaggcacttagtgtgtcaca
-75 cctgtggagaagggattctaatcctgatgacacagactggtccatgtctaacgaccccctataaaagggactcatgATATT
6 CCCACCACAGTGTAGGAAGGAATACACAGAACGGAGAAAgtatgtgattcatataagtatatttaaaattgttaactaat
86 gtgcatttgtgggtagttcatatatactgtacaatgtgcatattcaataggtaatcattgcaagatgatcgcaaacttct
166 gcttgatgtaaaaatacattatttttgacgatcacatttagctggtatttgactagctttctttccagCTCCC ATG
M 1
242 GAT CTT AGC AAC AGA ACG GTC GTG CAG GTT GTG GTG TTG GCG TTG GTA GCG CAG GTC ACG
D L S N R T V V Q V V V L A L V A Q V T 21
GnRH
302 CTC TCT CAG CAC TGG TCG TAT GGC TGG CTA CCT GGA GGG AAG AGA AGT GTT GGG GAG CTG
L S Q H W S Y G W L P G G K R S V G E L 41
362 GAG GCC ACC ATC AAG ATG ATG GAC ACA GGA GGT GTA GTG GCT CTT CCT GAG GAG ACA AGT
E A T I K M M D T G G V V A L P E E T S 61
422 GCA CAT GTC TCA GAG AGA CTG AGA CCA TAT GAT GTA ATA TTG AAG AAA TGG ATG CCC CAT
A H V S E R L R P Y D V I L K K W M P H 81
482 AAA TAA
K *

second year (Fig. 4-3A and 4-3B). In the second year, GnRH was expressed only in precocious sockeye salmon ovaries and testes in May and June (Fig. 4-4; Fig. 4-3C). GnRH expressed in the testes in May appeared to be generated only from the more downstream start site since PCRs with primers A/C produced no detectable products. GnRH mRNA was not present in any reproductive tissue examined in July through October. No immature reproductive tissue from two-year-old sockeye salmon expressed GnRH, other than the 17-month-old undifferentiated gonadal tissue examined in May. In July, neither immature gonadal tissue or sperm duct nor jack testes expressed GnRH.

Identification of the transcription start site in ovary and testis

Determination of the start site in the rainbow trout ovaries and testes by primer extension analysis (Chapter 3) permitted the design of primer G. RT-PCRs from both ovary and testis conducted with primers G/D produced bands of the expected sizes that were cloned and sequenced to confirm each sockeye salmon transcript was generated from the same upstream start site (Fig. 4-5). PCRs conducted using a sense primer (primer I) complementary to a region upstream of the alternative start site did not produce an observable amplification product (data not shown).

Also, RT-PCRs of whole embryos or adult reproductive tissues from either rainbow trout or sockeye salmon failed to yield sGnRH cDNA1 amplification products when using primer set A/T.

Sequence analysis of the rainbow trout sGnRH cDNA1

To confirm that the sGnRH sequence in embryonic tissue was identical to that in juveniles, I amplified sGnRH cDNA1 from rainbow trout that were 18 DAF and compared the sequence to that from the ovary of 1.5-year-old fish. The primers were designed specifically to differentiate sGnRH cDNA1 from its cDNA2 counterpart. This sequence is compared to the rainbow trout sGnRH cDNA2 and the sockeye salmon cDNA1 and

cDNA2 transcripts (Fig. 4-6). The isolated 332 bp transcript was cloned, sequenced and confirmed to be the sGnRH cDNA1 transcript by sequence comparison to other known salmonid sGnRH cDNA1 (Suzuki *et al.*, 1992; Ashihara *et al.*, 1995). sGnRH cDNA1 PCR products of 332 bps were observed in rainbow trout at various stages of embryogenesis (14, 34, 46 and 55 DAF) and in 17-month-old precocious ovary and 20-month-old precocious testis, but not immature 24-month-old ovary or testis (data not shown). Expression of sGnRH cDNA1 was observed at 51 DAF and in precocious testis, but not immature ovary, of 17-month-old sockeye salmon (data not shown).

Expression of GnRH in rainbow trout and sockeye salmon embryonic tissue

The expression of GnRH during the early development of salmonid embryos and hatchlings was followed by RT-PCR analysis (Fig. 4-7 and 4-8). We examined sockeye salmon tissue from embryos at 30 to 68 days after fertilization (DAF) and rainbow trout tissue from 4 to 42 DAF. Primers B/C produced a band of 286 bps which is evidence that GnRH is expressed from at least 30 to 68 DAF in sockeye salmon (except 40 DAF) (Fig. 4-7). Using primers F/D, I also showed the transient use of the upstream start site in the amplification of PCR products of 567 and 378 bps in length. Expression of GnRH in rainbow trout embryonic tissue was first observed at 10 DAF and continued uninterrupted for the rest of the study (Fig. 4-8). The earliest day in which the upstream promoter is used in rainbow trout embryos is 14 DAF. Each species of salmonid began to use the alternative promoter for GnRH expression roughly 10 days pre-hatch and throughout examined hatchling stages. Examination of RNA from gonadal tissue for 3 and 4-month-old rainbow trout (data not shown) and up to 8 month-old sockeye salmon (Fig. 4-3A) failed to yield any GnRH RT-PCR products.

The RNA used in the developmental RT-PCR study was extracted from whole embryonic tissues and the head from the hatchlings at the times presented in Fig. 4-7 and

Figure 4-6. Nucleotide and amino acid sequence comparison of the sGnRH cDNA1 and cDNA2 transcripts isolated from sockeye salmon (ss) and rainbow trout (Rt). Nucleotides that indicate where changes exist in comparison to the sGnRH cDNA1 transcripts are shown in bold. Amino acid identity among the different cDNAs is shown by the consensus amino acid sequences presented by the letter code in bold. The position at which a deletion is present in the sockeye salmon sGnRH cDNA1 is aligned with the other transcripts between codons 74 to 77. The sGnRH decapeptide is boxed. The rainbow trout sGnRH mRNA sequences are from Chapter 3; the sockeye salmon sGnRH mRNA1 sequence is from Ashihara *et al.* (1995) and the sockeye salmon sGnRH mRNA2 is from Chapter 2.

```
ss.sGnRH mRNA1          cttcc  ATG GAT CTT AGC AGC AAA ACG GTT GTG CAG GTG GTG ATG
Rt.sGnRH mRNA1          cttcc  ATG GAT CTT AGC AGC AAA ACG TTT GTG CAG GTG GTG ATG
ss.sGnRH mRNA2          cgcc-  ATG GAT CTT AGC AAC AGA ACG GTC GTG CAG GTT GTG GTG
Rt.sGnRH mRNA2          ctccc  ATG GAT CTT AGC AAC AGA ACG GTC GTG CAG GTG GTG GTG
consensus                      M   D   L   S           T       V   Q   V   V
```

GnRH

```
 I   TTG GCG TTG ATA GCT CAG GTC ACT TTC TCT CAG CAT TGG TCG TAT GGG TGG CTA CCT GGA
 I   TTG GCG TTG ATA GCT CAG GTC ACT TTC TCT CAG CAT TGG TCG TAT GGG TGG CTA CCT GGA
II   TTG GCG TTG GTA GCG CAG GTC ACG CTC TCT CAG CAC TGG TCG TAT GGC TGG CTA CCT GGA
II   TTG GCG TTG GTA GCG CAG GTC ACG CTC TCT CAG CAC TGG TCG TAT GGC TGG CTA CCT GGA
     L   A   L       A   Q   V   T       S   Q   H   W   S   Y   G   W   L   P   G

 I   GGA AAG AGA AGT GTT GGG GAG CTG GAG GCC ACC ATC AGG ATG ATG GAC ACA GGT GGT GTA
 I   GGA AAG AGA AGT GTT GGG GAG CTG GAG GCC ACC ATC AGG ATG ATG GAC ACA GGT GGT GTA
II   GGG AAG AGA AGT GTT GGG GAG CTG GAG GCC ACC ATC AAG ATG ATG GAC ACA GGA GGT GTA
II   GGG AAG AGA AGT GTT GGG GAG CTG GAG GCC ACC ATC AAG ATG ATG GAC ACA GGA GGT GTA
     G   K   R   S   V   G   E   L   E   A   T   I       M   M   D   T   G   G   V

 I   ATG GCT CTT CCT GAG GAG ACA GAT GCC CAT ATC CCA GAG AGA CTG AGA CCA TAT GAT GTA
 I   ATG GCT CTT CCT GAG GAG ACA GGT GCC CAT ATC CCA GAG AGA CTG AGA CCA TAT GAT GTA
II   GTG GCT CTT CCT GAG GAG ACA AGT GCA CAT GTC TCA GAG AGA CTG AGA CCA TAT GAT GTA
II   GTG GTT CTT CCT GAG GAG ACA AGT GCA CAT GTC TCA GAG AGA CTG AGA CCA TAT GAT GTA
             L   P   E   E   T       A   H           E   R   L   R   P   Y   D   V

 I   A-- --- --- --A CGG ATG CCA CAT AAA TAAAGAACTG TGA
 I   ATG TCG AAG AAA CGA ATG CCA CAT AAA TAA         *
II   ATA TTG AAG AAA TGG ATG CCC CAG AAA TAA
II   ATA TTG AAG AAA TGG ATG CCC CAT AAA TAA
             K   K       M   P   H   K   *
```

Figure 4-7. RT-PCR analysis of RNA extracted from sockeye salmon embryos during early stages of development. GnRH expression in sockeye salmon tissue from 30 to 68 days after fertilization (DAF) using primer sets B/C and F/D. Expression of GnRH by use of downstream start site is observed for each DAF examined (except E 40) by amplification of a 286 bp product. Temporal use of the upstream promoter is observed by RT-PCR products of 567 and 378 bps in size.

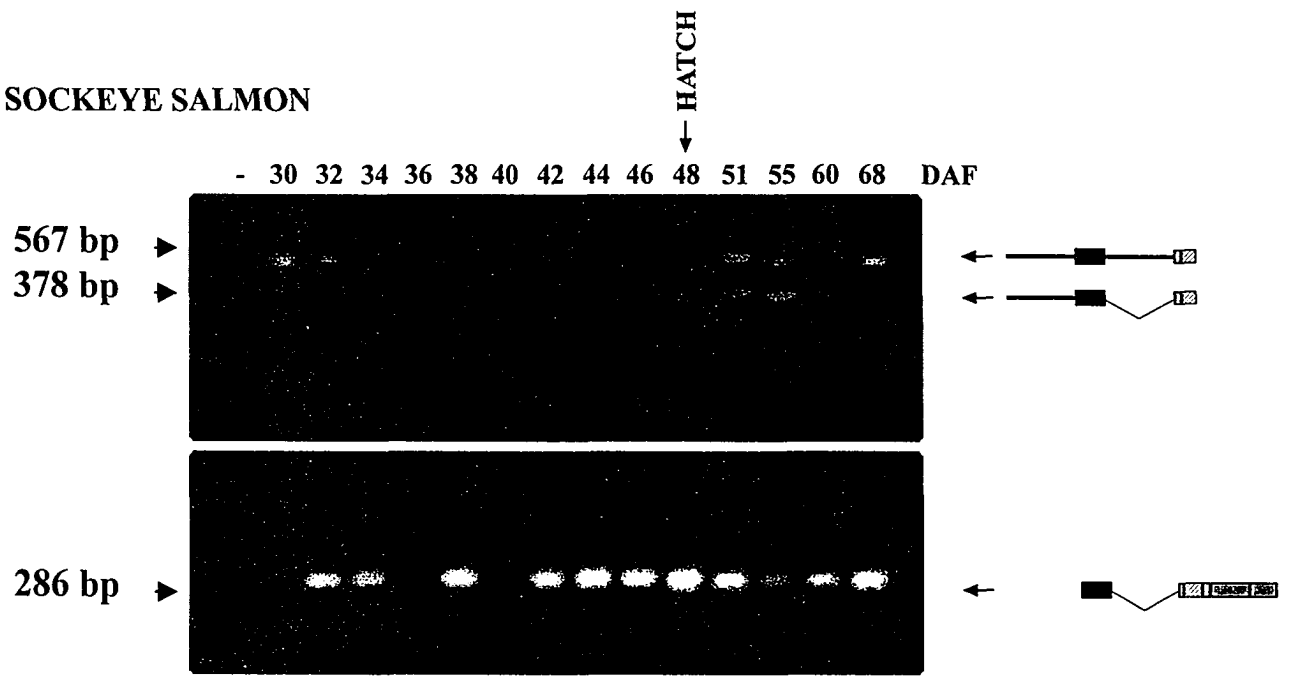
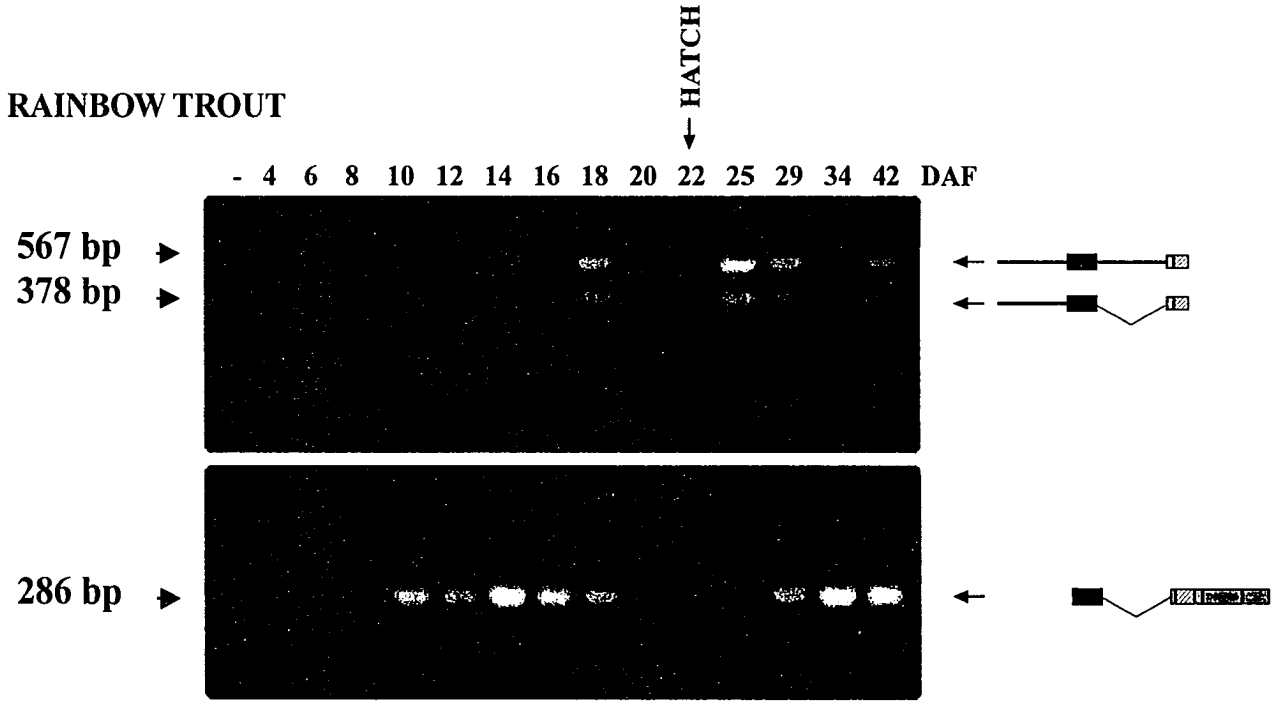

SOCKEYE SALMON
HATCH
- 30 32 34 36 38 40 42 44 46 48 51 55 60 68 DAF
567 bp
378 bp
286 bp

Figure 4-8. RT-PCR analysis of RNA extracted from rainbow trout embryos during early stages of development. Examination of GnRH expression in rainbow trout tissue from 4 to 42 DAF using primer sets B/C and F/D. Expression of GnRH by use of downstream start site is observed for each DAF examined by amplification of a 286 bp product. Temporal use of the upstream promoter is observed by RT-PCR products of 567 and 378 bps in size.


RAINBOW TROUT
HATCH
- 4 6 8 10 12 14 16 18 20 22 25 29 34 42 DAF
567 bp
378 bp
286 bp

4-8. This approach was designed to differentiate brain- from gonad-specific expression of GnRH in sockeye salmon 68 DAF and rainbow trout 42 DAF. Milt, eggs (data not shown) and embryos at 1 to 9 DAF from each species were analyzed, but did not express GnRH.

Discussion

I have examined the tissue-specific expression of GnRH in sockeye salmon. Expression of GnRH was not detected in any tissue other than the brain or gonad. Both sGnRH mRNA1 and mRNA2 were expressed in an intermittent pattern in whole embryos and in the ovary and testis of juvenile and adult sockeye salmon.

sGnRH cDNA2 expression in the present study was observed in whole embryos as early as 10 DAF in rainbow trout and by 30 DAF in sockeye salmon. The trout results at 10 DAF are the earliest expression of GnRH in a salmonid embryo and may represent the rapid development of rainbow trout, at least in my holding conditions. Chiba *et al.* (1994) used immunocytochemical studies on brain sections taken from chum salmon to show GnRH-immunoreactive cells in the olfactory placode at 16 DAF. Parhar *et al.* (1995) used *in situ* hybridization studies to detect GnRH mRNA in sockeye salmon brain at 19 DAF which is earlier than 30 DAF. Neither author reported on the gonads. Expression and biosynthesis of GnRH is detectable at all times once initiated in the brain of fish (Okuzawa *et al.*, 1990, Amano *et al.*, 1992, Parhar *et al.*, 1995) or mammals (Chiappa and Fink, 1977).

The difference in the time it took for the sockeye and rainbow trout to hatch may reflect the differences in the flow rate of the water in which they were raised (See Materials and Methods). In the wild, it would take three to four months for these eggs to hatch, whereas under our experimental conditions it took roughly 48 DAF for the sockeye and 22 DAF for the rainbow trout eggs to hatch. The PCR method is very sensitive for detection of GnRH mRNA, but the location within the embryo is not revealed. However, it appears that once

GnRH mRNA is synthesized, it is continuously generated (except at 40 DAF in sockeye salmon), at least from the downstream promoter. It therefore appears that expression of GnRH in the developing gonad is intermittent whereas expression of GnRH in the early brain is constant.

I cannot completely discount the possibility that the GnRH-expressing neurons in the early brain are transiently using the upstream promoter to generate GnRH mRNA. I do know, however, that at no time in RT-PCRs conducted on RNA derived from 5 month or older salmonid brains (with primers A/C or F/D) did I observe upstream amplification products. *Primer extension analysis of rainbow trout brain RNA to determine the GnRH* start site also confirms that GnRH transcripts are not generated from any other site in the brain than 18 nucleotides (nts) downstream from the TATAAA box (Chapter 3). Clearly, microscopic excision of the developing brain from surrounding tissues and subsequent RT-PCR analysis is needed to answer this question unequivocally.

In the present study, rainbow trout expressed GnRH in the gonads longer than sockeye salmon in the first two years of life in normal and precocious fish. In the first year of life, sockeye salmon expressed sGnRH mRNA2 in immature ovary and testis in October only, whereas rainbow trout expressed GnRH in the gonads in May through October (Fig. 3-8A). Sockeye salmon do not express GnRH in the second year, but rainbow trout express GnRH in December of the second year (see Fig. 4-3B and Fig. 3-8B). In precocious ovary and testis of sockeye salmon, GnRH mRNA2 was present in May and June of the second year, whereas rainbow trout express GnRH throughout the latter half of the second year (June through October) (Fig. 3-8C). The difference in pattern of expression between species may be that rainbow trout are reproductively competent in year 3 and continue each year as repeat spawners whereas sockeye salmon do not spawn until 4 to 6 years of age and then only spawn once before death. Also rainbow trout remains in fresh water only, whereas sockeye salmon move out to sea at about one to two years of age.

Older adult sockeye salmon did not appear to express GnRH in tissue other than the brain (at least at the times examined) and at 12 hours before spawning this tissue no longer synthesized sGnRH mRNA. Although not examined as extensively as cDNA2, sGnRH cDNA1 was expressed in precocious ovary and testis of both sockeye salmon and rainbow trout, but not in normal juvenile tissue.

It is clear that there is an increase in the expression of GnRH in maturing or precocious reproductive tissues in comparison to immature tissue. The precocious reproductive tissues examined from the second year are probably comparable to tissue of three-year-old fish. The role in the reproductive competence of the salmonids by locally produced GnRH remains to be elucidated.

Each of the four sGnRH-encoding mRNAs described here (cDNA1 and cDNA2 for two species) generates the identical sGnRH (Fig. 4-6). In the translated region of the message that encodes sGnRH preproprotein, the precursor1 molecule differ from their precursor2 counterpart by only 15 amino acid residues in both species. The amino acid changes are restricted to the signal peptides (6 changes) and the GnRH-associated-peptides (GAP; 9 changes). The 3'-UTR region of the sockeye salmon sGnRH cDNA1 has 10 nts that are missing between amino acid residues 74 and 77 in comparison to the rainbow trout sGnRH cDNA1 and the sGnRH cDNA2 transcripts in both species. This deletion occurs exactly at the boundary of intron3/exon4 and reflects a change in or near the splice site of the sockeye salmon sGnRH gene1. However, the rainbow trout sGnRH cDNA1 and cDNA2 resemble the sockeye salmon sGnRH cDNA2 in this region of the 3'-UTR whereby the deletion has not taken place. Thus the 9 final amino acid residues of the GAP region for both rainbow trout preproproteins and sockeye salmon preproprotein2 have no identity with the sockeye salmon sGnRH preproprotein1.

The reproductive tissue of both sockeye salmon and rainbow trout (Chapter 3) use an alternative promoter and retain intron 1 to generate sGnRH cDNA2 bearing 5'-UTRs that are longer than their brain counterparts. In sockeye salmon, these long GnRH trancripts

are generated from a TATA-less promoter region that is highly conserved in the two genes that encode sGnRH mRNA2 in Atlantic (Klungland *et al.*, 1992) and sockeye salmon (Chapter 2). At least two sGnRH mRNA2 transcripts are generated from the upstream promoter in both the ovary and testis of sockeye salmon: one which retains intron 1 and at least one without intron 1. Also, a cDNA2 transcript isolated from undifferentiated gonad of 17-month-old sockeye salmon retained 27 nts at the most 3'-end of intron 1, similar to the sGnRH cDNA2 transcript expressed in rainbow trout testis (Chapter 3). The sGnRH mRNA1 is not generated from an alternative promoter like its mRNA2 counterpart. However, I did observe PCR products (amplified with primer set S/T) of sizes potentially representing retention of intron 1 in precocious ovary and testis of both sockeye salmon and rainbow trout (data not shown).

The alternative upstream promoter of the sockeye salmon sGnRH gene2 lacks TATA or CAAT boxes, which is in contrast to the downstream promoter that has TATA and CAAT boxes. However, the upstream promoter contains sequences that loosely match initiator (Inr) and downstream promoter element (DPE) motifs required to facilitate binding of the basal transcriptional apparatus in the promoters of other genes that lack a TATA-box (Purnell *et al.*, 1994; Burke and Kadonaga, 1996). The Inr $CAA_{+1}TGT$ at -2 to 4 resembles the consensus sequences of Py-Py-A_{+1}-N-$^{T}/_{A}$-Py-Py or T-C-A_{+1}-$^{G}/_{T}$-T-$^{T}/_{C}$ for mammalian (Javahery *et al.*, 1994) or *Drosophila* (Purnell *et al.*, 1994) genes. Three candidate DPEs are found in positions 44 to 50 (GGACAAA), 76 to 82 (GGACATT) and 108 to 114 (GGTAGTG) that are homologous to the consensus $^{A}/_{GG}{}^{A}/_{TCGTG}$ established for the *Drosophila* DPE (Burke and Kadonaga, 1996).

The upstream start site is centred between two palindromic estrogen response elements (EREs), that are within 100 basepairs (bps) on each side in the sockeye salmon gene2 (Fig. 2-1). Although these EREs are oriented differently in the context presented here (see Fig. 6-1), they both were shown to bind human estrogen receptor in the Atlantic salmon sGnRH gene2 (Klungland *et al.*, 1993). Furthermore, sequences are present in the

opposite orientation at position 6 to 18 that closely resemble a putative ERE that is found also in close proximity to the start site for GnRH expression in primate reproductive tissues (Dong *et al.*, 1993, Dong *et al.*, 1996). The position of the start site in relation to these EREs could point to the involvement of estrogen receptor in the responsiveness of the upstream promoter. Finally, the alternative transcription initiation site begins approximately 60 bps downstream from a site at which a block of 1152 bps are missing in the sockeye salmon sGnRH gene2 in comparison to the Atlantic salmon gene2 (Fig. 2-4).

I can only speculate on the need for GnRH expression in these developing tissues, but it could be important for cell-cell communication, movement or mitogenesis. If, as I suggest, the TATAAA-box deficient alternative promoter requires estradiol (or another steroid) for activation of the GnRH gene in the developing gonad, it is important to note that estradiol and other steroids are present in fertilized eggs of chum salmon (de Jesus and Hirano, 1992).

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Chapter 5

Characterization of immunoreactive GnRH peptide in the brain and gonads of rainbow trout

Summary

There is growing evidence for the local synthesis of GnRH in reproductive tissues from nucleic acid research. Ample physiological evidence exists that shows GnRH can exert changes in the development and maturation of the gonads. However, the unequivocal demonstration of endogenous GnRH peptide synthesis in the ovary or testis has not been conclusive.

To determine whether GnRH peptide is present in the developing ovary and testis, high pressure liquid chromatography and radioimmunoassays were used for peptide detection. I examined extracts of immature and precocious ovarian and testicular tissue taken from 17- to 21-month-old rainbow trout. The presence of at least three forms of GnRH in the precociously mature ovaries and testes from 18 to 20 month-old fish is demonstrated.

Introduction

The salmon form of GnRH (sGnRH) was first isolated and the primary structure determined from the brain of chum salmon by high pressure liquid chromatography (HPLC) and radioimmunoassay (RIA) in 1983 (Sherwood *et al.*, 1983). Subsequent work isolated and sequenced a second peptide named chicken GnRH-II (cGnRH-II) from the same species (Powell, personal communication). The same two forms of GnRH were detected by HPLC/RIA in the brain of both masou salmon (Amano *et al.*, 1991) and rainbow trout (Okuzawa *et al.*, 1990). Based on the anatomical distribution of these two GnRHs from immunocytochemical studies (Okuzawa *et al.*, 1990, Amano *et al.*, 1991, Bailhache *et al.*, 1994) it was deduced that sGnRH is the gonadotropin-releaser and that cGnRH-II serves a neuromodulatory function in the salmonid brain. Synthetic sGnRH injected into reproductively mature salmon advanced the date of ovulation (see Sherwood *et al.*, 1983).

Local expression of GnRH has also been demonstrated in various reproductive tissues. For example, expression of GnRH has been shown in the ovary and testis in human (Dong *et al.*, 1993) and in two monkey species (Dong *et al.*, 1996). I have also demonstrated the expression of sGnRH mRNA in the ovary and testis of two salmonids, sockeye salmon and rainbow trout (Chapters 3 and 4). However, to date, translation of these GnRH messages into a functional GnRH peptide has not been clearly demonstrated (see Hseuh *et al.*, 1994, Richards, 1994).

I therefore investigated the presence of GnRH peptides in the ovary and testis of rainbow trout by HPLC and RIA. The ovary and testis were examined at various stages of development in rainbow trout at 17- to 21-months of age. I use immunological and chromatographical methods to determine whether multiple forms of GnRH are present and are developmentally regulated in the salmonid ovary and testis.

Materials and Methods

Tissues

The two-year-old male and female rainbow trout used in this study were raised in an open lake fed by a natural stream in Sooke, B.C. (Mountain Trout Sales). The fish were exsanguinated and the ovaries, testes and brains removed, immediately frozen on dry ice and subsequently stored at -80° C. Ovaries, testes and brains were taken from a total of 109 fish that were either 17-, 19- or 21-months of age (see Table 1 for details).

Tissue Extraction

The age, organ number and total weight of the tissue examined is listed in Table 1.

The frozen tissue was crushed to a powder using a cold mortar and pestle. For larger extractions a Waring blender was used to powder the material. Peptides were extracted with an acetone-HCl mixture and soluble lipids removed as previously described (Lovejoy et al., 1992). The final acetone-water soluble mixture was reduced in a vacuum centrifuge to approximately 2 mL and filtered through a 45-µm filter.

High Pressure Liquid Chromatography

The filtered extracts (2 mL) were loaded in repeated injections of 600 µL each at 2-min intervals onto a 1-mL loop. The filtrate was loaded onto a Supelco Supelcosil LC-18 column (25.0 cm x 4.6 mm; 5-µm particle size) (Supelco Canada, Oakville, Ontario) with a guard column of the same material attached. The material was eluted using an isocratic program of 83 % 0.25 M triethylammonium formate (pH 6.5) and 17 % acetonitrile over a 10-min period at a flow rate of 1 mL/min. After 10 min, the percentage of acetonitrile was elevated to 24 % over 7 min and maintained there for an additional 43 min. Sixty fractions of 1 mL each were collected in polyallomer tubes; 100 µL were removed from each fraction,vacuum dried, reconstituted in PBS with 0.1 % gelatin and assayed for irGnRH.

Table 5-1. Details of fish and amount of GnRH peptide per organ. The age, sex and number of fish collected for study, the reproductive status (development stage of ovaries and testes) and weight of average organ are shown. The amount of each GnRH peptide detected per organ is shown in last two columns (total GnRH detected in pg per gonad). m, mGnRH; c-II, cGnRH-II; u, unknown GnRH; s, sGnRH.

Table 1. REPRODUCTIVE DATA FROM RAINBOW TROUT

AGE	SEX	REPROD. STATUS	NUMBER OF FISH	WEIGHT/ORGAN (mg.)			GnRH pg/Gonad				GnRH pg/Brain	
				ovary	testis	brain	m	c-II	u	s	c-II	s
17 Months	Female	immature	21	143		230 *	–	–	–	–	918 *	945
	Male	immature	19		192		–	–	–	–		
	Female	precocious	4	312			–	35	–	–		
	Male	precocious	6		725		–	–	–	–		
18-20 Months	Female	precocious	8	244			232	106	106	19		
	Male	precocious	1		5600		167	174	100	–		
21 Months	Female	maturing	37	195		166 *	–	–	–	–	436 *	1088
	Female	ripe	2	8625			–	–	–	–		
	Male	ripe	11		5782		–	–	–	–		

- not detectable

* 50 brains pooled for age mates

Injections for each set of extracts were preceded by an overnight wash and a blank run in which a 600 µL volume of Milli-Q (Millipore, Bedford, MA) water was injected onto the column. The blank run was assayed between each application of extract to ensure that the column was free of any contaminating residual GnRH from previous HPLC analyses. Synthetic standards were applied to the column after each set of extracts were assayed. Four GnRH forms: mGnRH, cGnRH-II, dfGnRH and sGnRH were combined at 200 ng each and applied to the HPLC system as described for the above extracts. The elution positions of the standards on the chromatograph were confirmed by absorbance peaks (A = 280 nm) and GnRH-specific radioimmunoassay (RIA).

Radioimmunoassay

Aliquots of 100 µL from each fraction collected for each HPLC run were dried and assayed for irGnRH by methods previously described (Sherwood *et al.*, 1986). The reconstituted extracts were assayed using various antisera and ^{125}I-labeled synthetic GnRH tracers in a competitive RIA. The assay systems are as follows: mGnRH standard, GF-6 antibody at a final dilution of 1:25,000 and mGnRH ^{125}I trace; cGnRH-II standard, 7CR-10 antibody at 1:37,500 and cGnRH-II ^{125}I trace; and mGnRH standard, B-7 antibody at 1:10,000 and mGnRH ^{125}I trace. Serial dilutions were done on brain fractions collected in May or September if values of irGnRH was less than $B/B_0 = 20\%$ and the value closest to 50% B/B_0 was used to estimate the quantity of irGnRH present.

Antiserum GF-6, 7CR-10 and B-7 were raised in rabbits in our laboratory against sGnRH, mGnRH and dfGnRH, respectively. GF-6 cross-reacts with a number of forms of GnRH. It detects mGnRH (100 % cross reactivity with mGnRH trace), sbGnRH (94.1 %), sGnRH (23.7 %) and cGnRH-II (10.5 %) (Quanbeck *et al.*, 1997). Antibody 7CR-10 detects cGnRH-II (100 % cross reactivity with a cGnRH-II trace), sGnRH (84.8 %), dogfish GnRH (25.0 %) and lamprey GnRH-III (12.6%) (Lescheid *et al.*, 1997). B-7 detects mGnRH (100 % cross reactivity with mGnRH trace) (Lescheid *et al.*, 1997).

Results

Ovaries and testes from seventeen-month-old rainbow trout

The GF-6 antibody that detects many forms of GnRH did not detect any peaks indicative of immunoreactive GnRH-like material in the extracts of immature ovaries or testes or in the precociously mature testes (Fig. 5-1). However, GF-6 detected a small amount of immunoreactive material (0.075 ng) in fractions 24 to 25 of the extract from the precocious ovaries. Synthetic cGnRH-II standard eluted in position 24 under the same HPLC conditions. With an antiserum that detects cGnRH-II (7CR-10), 0.282 ng (total in two fractions) of immunoreactive GnRH was found in fractions 24 to 25 (Fig. 5-1). The mGnRH-specific antibody, B-7, did not detect mGnRH immunoreactive material in the extract from the precocious ovaries.

Ovaries and testes from eighteen/nineteen-month-old rainbow trout

In precociously mature ovaries the GF-6 antibody that cross-reacts with several forms of GnRH detected four peaks indicative of GnRH-like immunoreactivity: mGnRH (fractions 18 to 20), cGnRH-II (fractions 24 to 26), an unknown GnRH (fractions 30 to 32) and sGnRH (fractions 39 to 41) (Fig. 5-2). The dfGnRH and sGnRH standards consistently eluted at positions 31/32 and 39/40, respectively, throughout the study under the same HPLC conditions. However, it is not clear whether the GnRH-like immunoreactivity that eluted in fractions 30 to 32 corresponds to dfGnRH or if it is a novel form. There was 3.7 ng (total in three fractions) of immunoreactive mGnRH compared to only 308 pg immunoreactive sGnRH in these fractions. The amount of immunoreactive cGnRH-II and of the unknown GnRH form were roughly the same (1.7 ng) (total in three fractions). Synthetic mGnRH, cGnRH-II and sGnRH eluted in positions corresponding to the native material under the same HPLC conditions.

Figure 5-1. Immunoreactive GnRH in 17-month-old immature and precocious ovary and testis extracts. HPLC elution position (min) and the amount of immunoreactivity (nanograms per fraction) detected by antisera GF-6 or 7CR-10 are shown. The arrow above HPLC elution profiles shown for the precocious ovaries represent the detection of cGnRH-II by antiserum 7CR-10 as well as where the synthetic form elutes under the same HPLC conditions.

17 Months

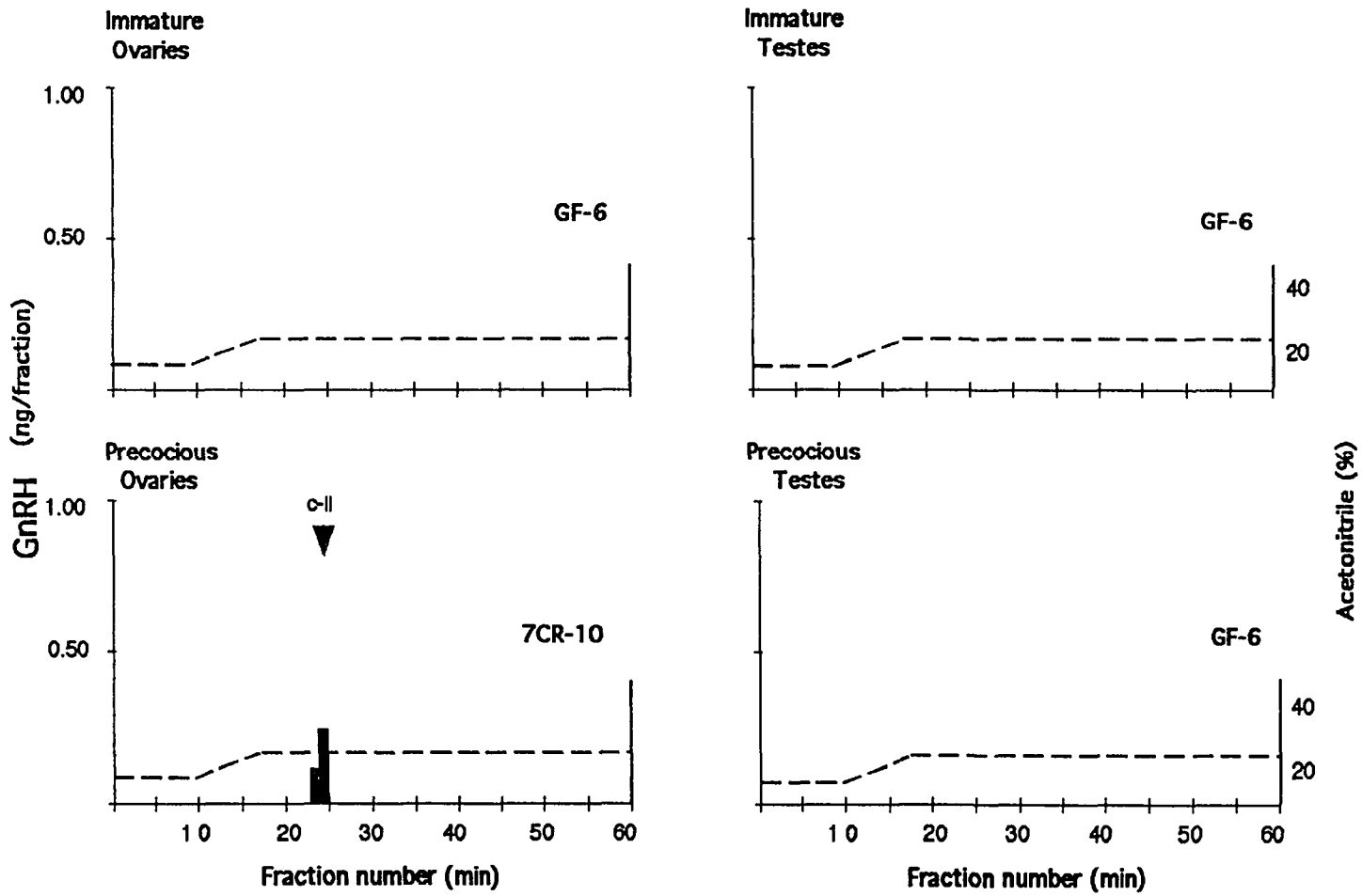
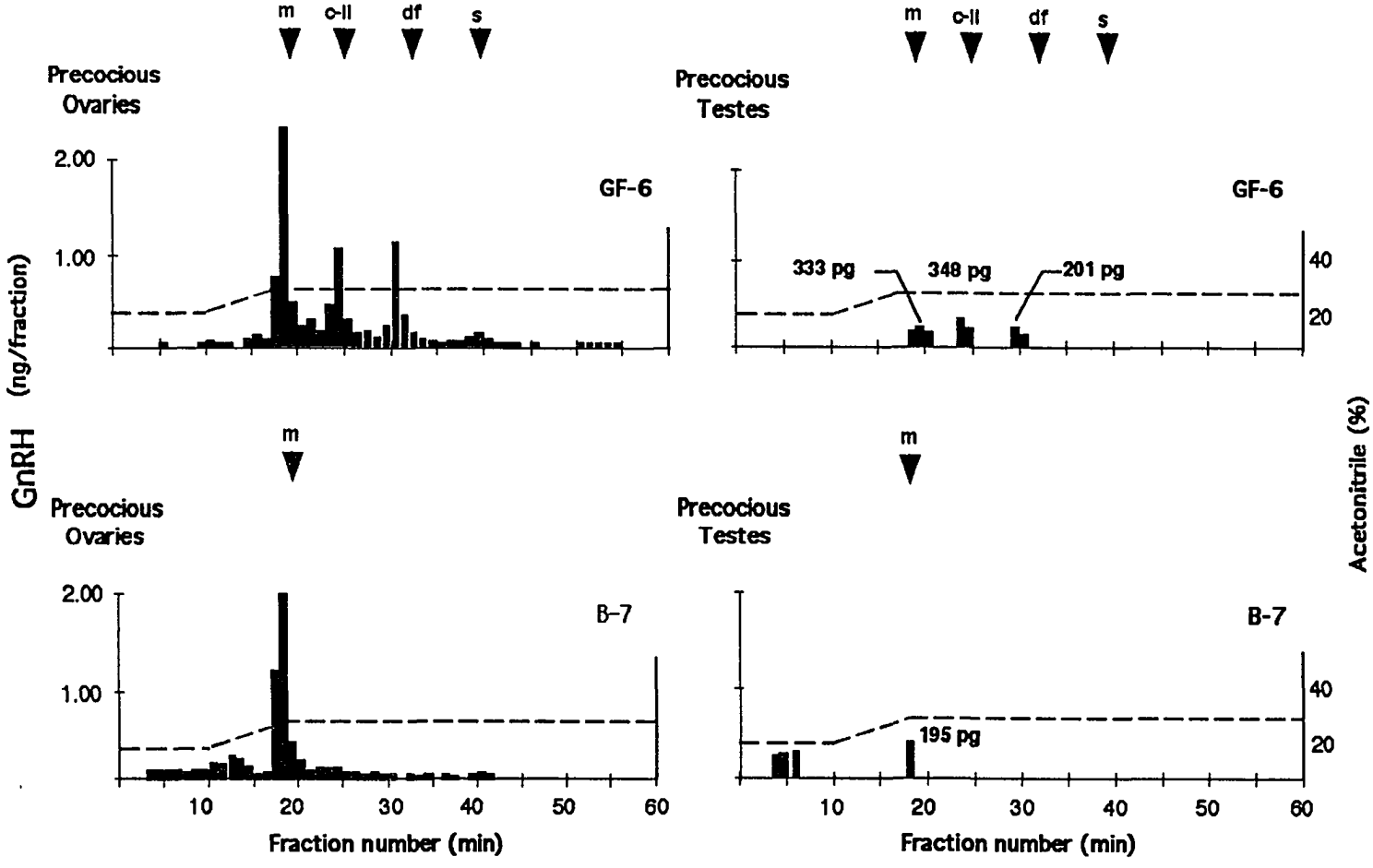

Immature
Ovaries
1.00
0.50
GF-6
Immature
Testes
GF-6
40
20
GnRH (ng/fraction)
Precocious
Ovaries
c-II
1.00
0.50
7CR-10
Precocious
Testes
GF-6
40
20
Acetonitrile (%)
10
20
30
40
50
60
Fraction number (min)
10
20
30
40
50
60
Fraction number (min)

Figure 5-2. Immunoreactive GnRH in 18-to 20-month-old precocious ovary and testis extracts. HPLC elution position (min) and the amount of immunoreactivity (nanograms per fraction) detected by antisera GF-6 and B-7 are shown. In the upper figure, arrows above HPLC elution profiles represent the different forms of GnRH detected by antiserum GF-6 as well as where the synthetic forms elute under the same HPLC conditions. m, mGnRH; c-II, cGnRH-II; df, dfGnRH; s, sGnRH. In the lower figure, the arrow above HPLC elution profiles represent the detection of mGnRH-II by antiserum B-7 as well as where the synthetic form elutes under the same HPLC conditions.

18 - 20 Months


m
c-II
df
s
Precocious
Ovaries
Precocious
Testes
GF-6
B-7
2.00
1.00
333 pg
348 pg
201 pg
195 pg
40
20
GnRH (ng/fraction)
Acetonitrile (%)
10
20
30
40
50
60
Fraction number (min)

Analysis of the precocious testis extract showed two small immunoreactive peaks corresponding to elution positions of synthetic mGnRH and cGnRH-II (Fig. 5-2). The third peak in fractions 30 and 31 could represent a novel GnRH form. Immunoreactive mGnRH-like (333.2 pg) and cGnRH-II (348.0 pg) material was detected with GF-6 in fractions 19 to 21 and 24 to 25, respectively (Fig. 5-2). sGnRH-like immunoreactivity was not detected in the precocious testis extract.

Also, all the HPLC values have been converted to pg/organ for comparison purposes (Table 1). The total amounts of immunoreactive mGnRH, cGnRH-II, unknown GnRH and sGnRH detected in each precocious ovary was 232.5, 106.2, 106.2 and 19.2 pg, respectively. The total amounts of immunoreactive mGnRH, cGnRH-II and unknown GnRH detected in each precocious testis was 166.6, 174.0 and 100.5 pg, respectively.

Confirmation of the presence of mGnRH-like immunoreactivity in both the precocious ovaries (3.5 ng) and testes (195.0 pg) is shown by the immunoreactive peaks, primarily in fractions 18 and 19, using the mGnRH-specific antiserum, B-7 (Fig. 5-2).

Ovaries and testes from twenty-one-month-old rainbow trout

GF-6 antiserum did not detect any peaks of immunoreactive GnRH-like material in the extracts of ripe ovaries or in the maturing ovaries or testes (normal development) in 21-month-old fish (Fig. 5-3). Also, each fraction of the extract from the ripe ovaries was reassayed with 7CR-10, but immunoreactive GnRH material was not detected.

Brains from seventeen- or twenty-one-month-old rainbow trout

Both cGnRH-II-like (fractions 24 to 28) and sGnRH-like (fractions 38 to 40) immunoreactivity are detected in brain extracts from 17- and 21-month-old fish (Figs. 5-4 and 5-5). Peaks corresponding to immunoreactive mGnRH were not detected with GF-6 in brain extracts (see fractions 18 to 20) (Figs. 5-4 and 5-5). However, a small amount of immunoreactive GnRH that could correspond to the unknown form observed in the

Figure 5-3. Immunoreactive GnRH in 21-month-old maturing ovary and testis extracts after HPLC elution. HPLC elution position (min) and the amount of immunoreactivity (nanograms per fraction) detected by antisera GF-6 or 7CR-10 are shown. Detection of GnRH was not made with either antiserum in any tissue.

21 Months

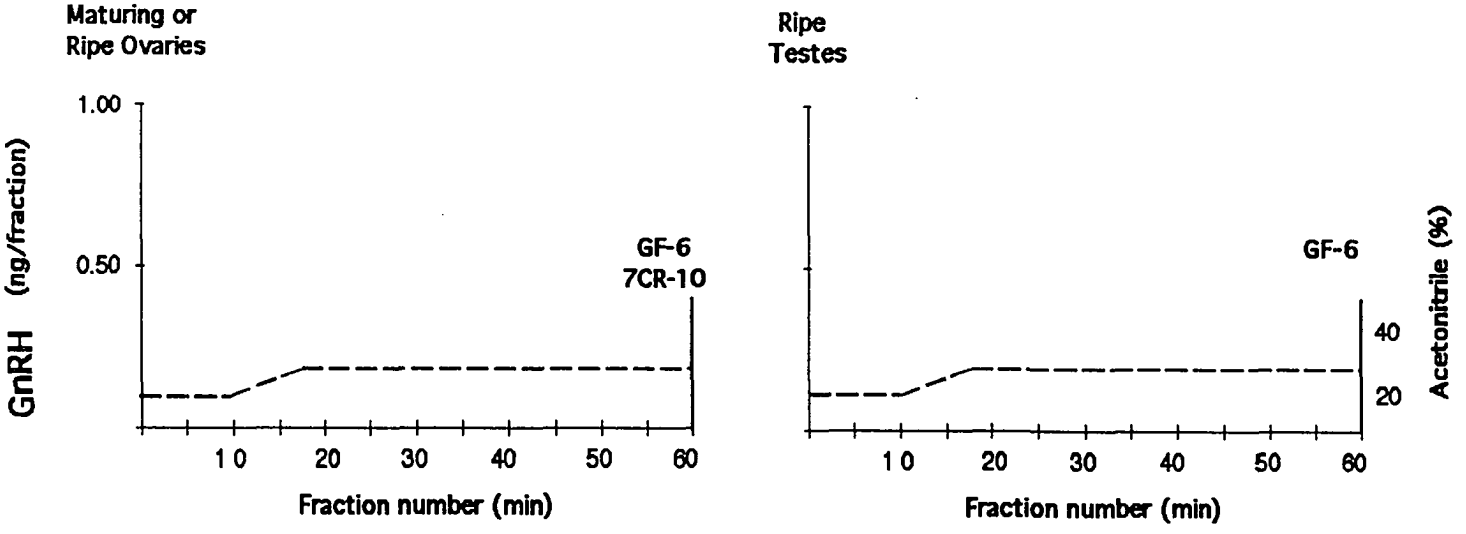
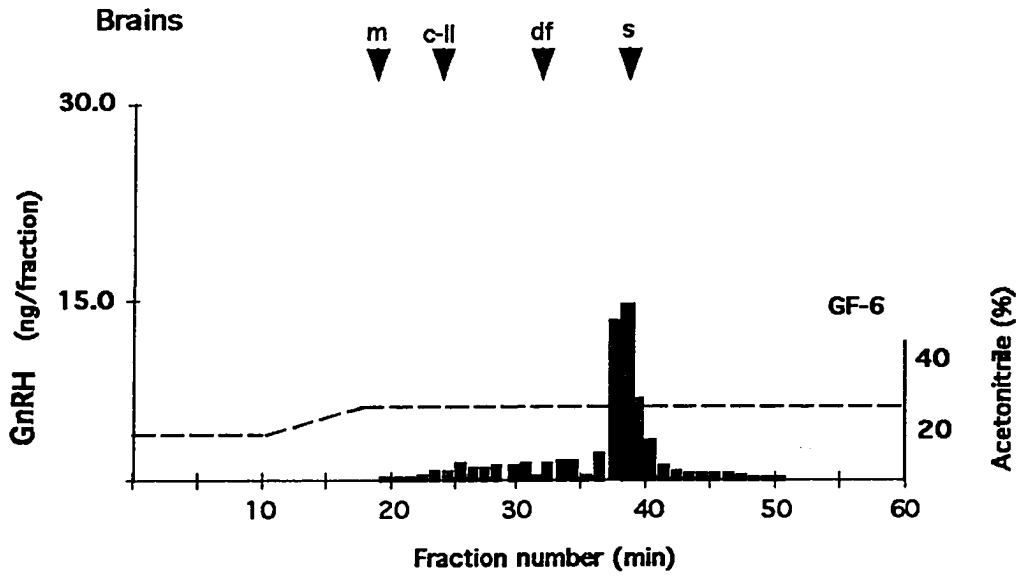

Maturing or
Ripe Ovaries
1.00
0.50
GnRH (ng/fraction)
GF-6
7CR-10
1 0
20
30
40
50
60
Fraction number (min)
Ripe
Testes
GF-6
40
20
Acetonitrile (%)
1 0
20
30
40
50
60
Fraction number (min)

Figure 5-4. Immunoreactive GnRH in 17-month-old brain extracts showing HPLC positions and amounts of immunoreactivity. HPLC elution position (min) and the amount of immunoreactivity (nanograms per fraction) detected by antisera GF-6 or 7CR-10 are shown. Arrows above HPLC elution profiles represent the different forms of GnRH detected by either antiserum GF-6 or 7CR-10 as well as where the synthetic forms elute under the same HPLC conditions. m, mGnRH; c-II, cGnRH-II; df, dfGnRH; s, sGnRH.

17 Months


Brains
m
c-II
df
s
30.0
15.0
GnRH (ng/fraction)
GF-6
40
20
Acetonitrile (%)
10
20
30
40
50
60
Fraction number (min)

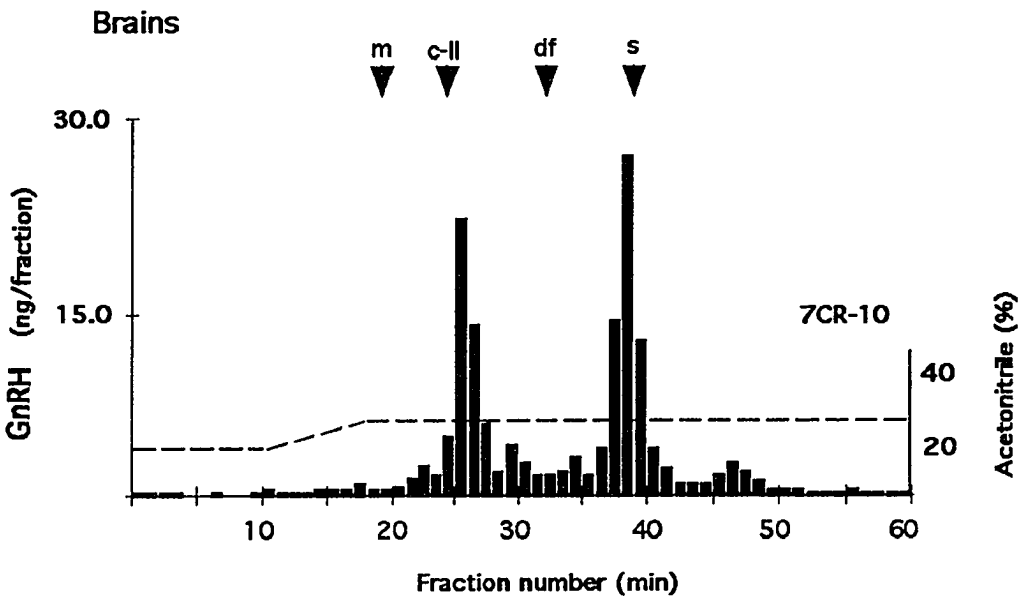
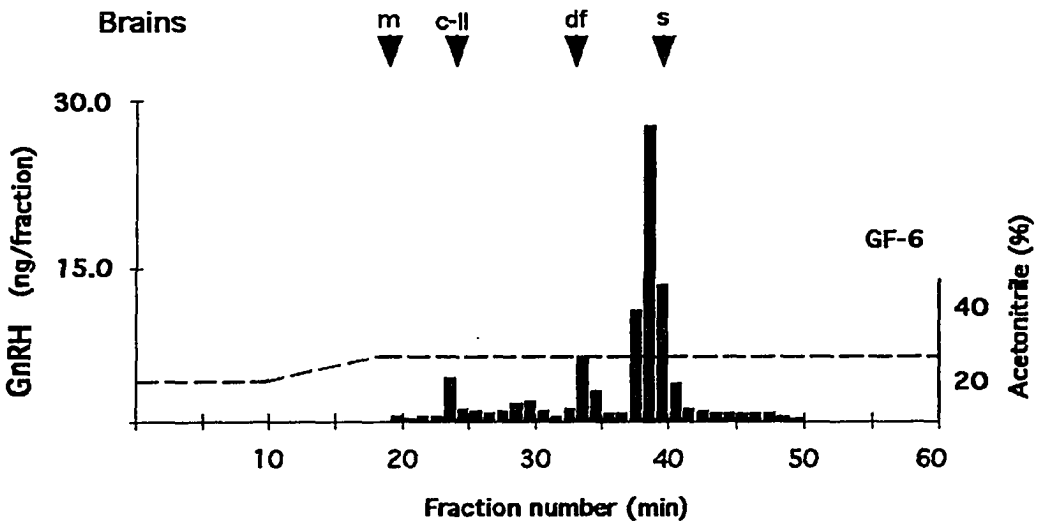
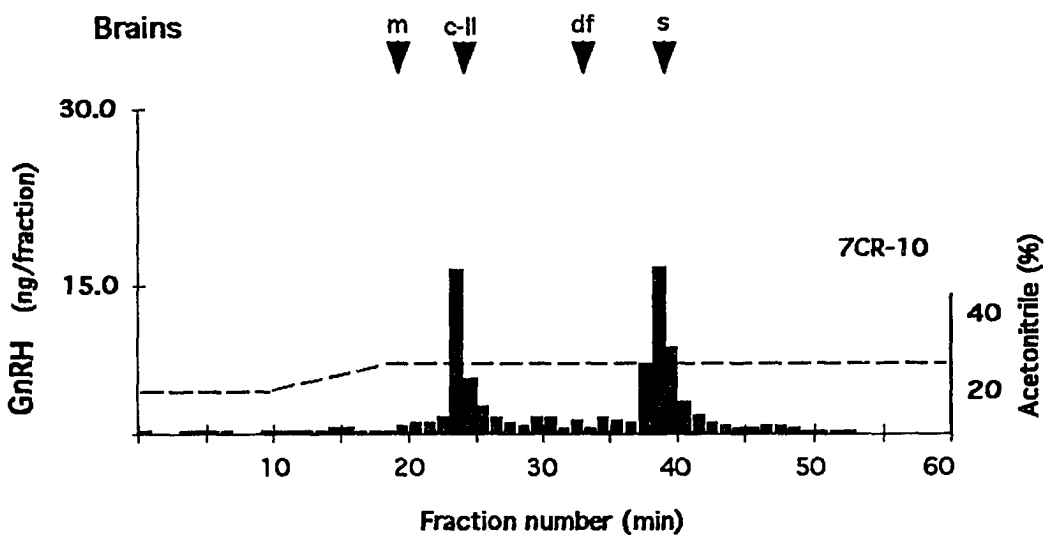

Brains
m
c-II
df
s
30.0
15.0
GnRH (ng/fraction)
7CR-10
40
20
Acetonitrile (%)
10
20
30
40
50
60
Fraction number (min)

Figure 5-5. Immunoreactive GnRH in 21-month-old brain extracts showing HPLC positions and amounts of immunoreactivity. HPLC elution position (min) and the amount of immunoreactivity (nanograms per fraction) detected by antisera GF-6 or 7CR-10 are shown. Arrows above HPLC elution profiles represent the different forms of GnRH detected by either antiserum GF-6 or 7CR-10 as well as where the synthetic forms elute under the same HPLC conditions. m, mGnRH; c-II, cGnRH-II; df, dfGnRH; s, sGnRH.

21Months


Brains
m
c-II
df
s
30.0
15.0
GnRH (ng/fraction)
GF-6
40
20
Acetonitrile (%)
10
20
30
40
50
60
Fraction number (min)


Brains
m
c-II
df
s
30.0
15.0
GnRH (ng/fraction)
7CR-10
40
20
Acetonitrile (%)
10
20
30
40
50
60
Fraction number (min)

ovaries and testes is present in position 30 to 31 (Figs. 5-4 and 5-5). The irGnRH detected in position 33-35 has previously been analysed and determined to be a breakdown product of sGnRH (Powell, personal communication).

Discussion

Strong evidence is provided here for the first time for the local presence of GnRH peptide in both the ovary and testis. My earlier work using PCR amplification showed that sGnRH mRNA transcripts are present in the ovary and testis (Chapters 3 and 4). These transcripts were identical to those in the brain except that extended 5'-UTRs were present in the gonadal transcripts. In the present study, GnRH protein is shown to be present in large amounts in the brain (ng) and in precociously mature ovaries in fish that are 18 to 20 months old. In contrast, GnRH peptide is shown to be present in small (pg) amounts at one stage in immature ovaries and in precociously mature testes.

My HPLC/RIA work on extracts from immature (normal) and precocious ovary and testis of 17-month-old fish showed the presence of small amounts (0.282 ng) of cGnRH-II in only the precocious ovary. Large amounts of total mGnRH (3.7 ng), cGnRH-II (1.7 ng) and unknown GnRH (1.7 ng) were present in pooled extracts from 18- to 20-month-old precocious ovaries. Only 308 pg of sGnRH was detected in the 18- to 20-month-old pooled ovaries. The extract from the 19-month-old testes showed the presence of small amounts of mGnRH (333.2 pg), cGnRH-II (348.0 pg) and the unknown GnRH (201.0 pg), but not sGnRH. However, the amounts of mGnRH, cGnRH-II and the unknown GnRH present in each precocious ovary and testis was similar (see Table 1). GnRH was not detected in extracts from ovary or testis at various stages of development in 21-month-old fish.

What is extraordinary about the work presented here is the finding that two forms of GnRH are present in the rainbow trout ovary and testis in addition to the forms of GnRH found in the brain (sGnRH and cGnRH-II). One corresponds to mGnRH and the other is

an unknown form eluting at position 31. An examination of an earlier HPLC/RIA report indicates these two forms of GnRH may also be present in very low quantities in rainbow trout brain extracts (Sherwood *et al.*, 1984). The present work indicates that mGnRH is not expressed in the rainbow trout brain, at least not in May or September of the second year of their lives (Figs. 5-4 and 5-5). However, I do detect small amounts of irGnRH-like material in positions 30 to 32 that could represent the expression of an unknown form of GnRH in the brain. Clearly, cGnRH-II and sGnRH are the dominant forms of GnRH in the salmonid brain, expressed in quantities that far exceed amounts of any GnRH form detected in the reproductive tissues. Nevertheless, I have found that four different forms of GnRH are synthesized at specific stages during ovarian and testicular cell development.

In the previous two chapters of this thesis I have shown the expression of two different sGnRH-encoding mRNAs in both the ovary and testis. Isolation and characterization of neither mGnRH- nor cGnRH-II-encoding transcripts have been reported. Of interest is the demonstrated expression of sGnRH cDNA2 in precociously mature testes from June to August (Chapter 3), but this HPLC/RIA work indicates that sGnRH is not detectable, at least not at the time the tissue was collected (July 20). Whether this is due to the sampling of only one precociously mature fish or that the levels and/or forms of GnRH fluctuate, remains to be clarified. Furthermore, although sGnRH was expressed at the cDNA level in the precocious ovary throughout the same period as shown for the precociously mature testis (Chapter 3), the amounts of sGnRH detected were low in comparison to the other GnRH forms present.

Synthesis of the messages that encode both GnRH and its receptor in ovarian and testicular cells points to a direct physiological role for this factor. Any idea that secreted hypothalamic GnRH could act peripherally on the gonads is unlikely because of its low concentration and short half-life in the blood (see Bahk *et al.*, 1995). However, the unequivocal demonstration of the local biosynthesis of GnRH decapeptide in these tissues had not been reported. The first report of a GnRH-like factor extracted from rat follicular

fluid capable of releasing FSH and LH from rat pituitary cells *in vitro* was made in 1981, but was later retracted (Ying *et al.*, 1981). Other receptor-binding or HPLC studies have detected GnRH-like activity in ovarian extracts from various species, but the protein products were shown not to be GnRH (Aten *et al.*, 1986; Aten *et al.*, 1987). Similar reports of GnRH-like immuno- and/or bioactivity in testicular tissue have been inconclusive (Gnessi *et al.*, 1997).

The role of these peptides in these tissues is less clear. There is a large body of work that demonstrates that GnRH both stimulates and inhibits normal regulatory mechanisms depending on the maturational state of the tissue. In the immature gonad, the inhibitory effects of GnRH are due primarily to the disruption of gonadotropin effects. In immature granulosa cells, for example, follicle stimulating hormone (FSH)-stimulated steroidogenesis and FSH/luteinizing hormone (LH) receptor production is inhibited by exogenous GnRH (Hsueh and Schaeffer, 1985, Richards, 1994). This FSH-suppressing action has been demonstrated to profoundly disrupt granulosa cell maturation *in vitro* (Knecht *et al.*, 1985). The GnRH inhibition of gonadotropin-mediated progesterone biosynthesis in the ovary and androgen biosynthesis in the testis is due to GnRH-suppressing action on the synthesis of LH/chorionic gonadotropin (CG) receptors and steroidogenic enzymes (Hsueh and Schaeffer, 1985), as well as enzymes involved in cAMP production and signal transduction (Knecht *et al.*, 1985, Richards, 1994, Stojilkovic *et al.*, 1994).

In contrast, GnRH is reported to have stimulatory effects in mature granulosa cells. Activation of Ca^{2+} and phospholipid fluxes by GnRH are associated with stimulatory effects on meiotic maturation, oocyte cleavage, ovulation and luteinization (Hillensjo and LeMaire, 1980, Knecht *et al.*, 1985, Richards, 1994). Stimulation of steroidogenesis is also evident in cultured adult rat Leydig cells after short-term exposure (4h) to GnRH (Sharpe and Cooper, 1982). In the ovary, GnRH may modulate follicular development by

selection of particular follicles for ovulation, whilst promoting atresia in follicles more sensitive to early GnRH inhibition (Knecht *et al.*, 1985, Hseuh *et al.*, 1994).

Therefore, depending on the differentiation state of the tissue, GnRH can act as an atretogenic factor or as an inducer of ovulation/luteinization. The mechanisms determining follicular fate are not completely understood, but it appears that the dominant follicles arise from exposure to a milieu of both atretogenic and survival factors throughout the course of their development (Hseuh *et al.*, 1994). At the penultimate stage of follicle development, FSH is apparently required for selection and escape of the dominant follicles from atresia (Hseuh *et al.*, 1994). This also may be the case for the salmonids as I observed GnRH expression at a time that roughly coincides with increasing GTH-I plasma levels (Swanson, 1991). The difference between fish and mammals, however, is that it is not known if GTH-I has the same ability as FSH to spare follicles from atresia. However, treatment of incubated preovulatory rainbow trout follicles with a partially purified salmon gonadotropin containing primarily GTH-II suppressed apoptosis by 31% in comparison to untreated follicles (Janz and Van Der Kraak, 1997). GTH-I and GTH-II are generally conceived as sharing similar characteristics as their mammalian counterparts, FSH and LH, respectively.

A similar mechanism for selection of viable gametes through atresia appears to exist in both mammals and teleosts (Van Der Kraak *et al.*, 1998). This is contrasted, however, by the fact that although the majority of mammalian oocytes and/or follicles undergo degenerative changes before ever reaching ovulation (Hseuh *et al.*, 1994), a large population of thousands of follicles develop to full maturation synchronously in the salmonids (Janz and Van Der Kraak, 1997). Investigation in this area of reproductive development clearly needs to be addressed.

If there is a requirement for GnRH at later stages for ovulation of the salmonid follicles, these tissues need to be analysed later in the year (October or November), because GnRH was not present in any tissues examined in September. However,

examination of extracts from ovarian fluid collected in the fall from three-year-old Atlantic salmon did not provide positive GnRH results (data not shown).

I propose that endogenous GnRH expression is independent of GTH-I exposure because of the small amount of cGnRH-II observed in the May precocious ovarian extracts. I did not conduct parallel studies to measure GTH-I levels, but this time in the season does coincide with the absence or only small amounts of GTH-I in the plasma (Swanson, 1991). Some support for this also comes from the report that the appearance of GnRH receptor mRNA in rat immature follicles is observed before the onset of gonadotropic stimulation (Whitelaw *et al.*, 1995).

Finally, the focus of attention should now be directed to localize the expression and to determine the function of each GnRH in these tissues. Isolation and characterization of the cDNAs that encode both mGnRH and cGnRH-II needs to be completed. Primers specifically designed for each cDNA would permit RT-PCR stage-specific analysis of each mRNA in the rainbow trout ovary and testis. Coinciding with the RT-PCR analysis, monthly HPLC/RIA examination of the same tissue would provide insight into the expression of these different forms of GnRH and the processes governing their synthesis.

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Chapter 6

General Conclusion

I. Conclusions.

This thesis focuses on the expression of GnRH in two completely different kinds of tissue----the brain and the gonad. We don't know why GnRH is expressed in two such different tissues. In this thesis it is clear that regulation of the GnRH gene is distinct in the brain and gonad. In the gonad, unlike the brain, the gene is transcribed intermittently and transcription is associated with the stage of reproduction. One clue to why GnRH is expressed in these diverse tissues might be in the development of the tissue.

A. Development of the brain and gonad. The neural tube generates the spinal cord and brain. The position and function of each neuron in the brain can be directly corelated with the time and place of their generation during brain development (Alberts *et al.*, 1994). Once established, neurons send out their axons, which are guided by specific adhesion and chemotactic molecules to set up the organized synaptic connections that compose the neural networks (Alberts *et al.*, 1994).

The differentiation and development of the cells within the ovary and testis are controlled by the orchestrated actions of regulators that are within the gonads. FSH and LH act only as initiators of stimulatory or inhibitory actions carried out by a multitude of growth factors, cytokines and steroids that modify their effects (Adashi and Rohan, 1992, Richards, 1994, Van Der Kraak *et al.*, 1998). Many of these growth factors, cytokines and steroids are associated with both the brain and the gonads. Spared from atresia by critical hormonal signals, a few follicles will survive and develop to the ovulatory stage (Hsueh *et al.*, 1994). This is of interest because a similar mechanism is featured early in the development of the brain for the survival of innervating neurons by their dependence on neurotrophic factors secreted by their target cells. These various growth factors and steroids may be important in differentiating transcription of the GnRH in the brain and the gonads.

B. Effect of GnRH on gonad physiology. Now that I have demonstrated that GnRH is transcribed and the GnRH peptide is synthesized in both the ovary and testis, it is important to review the physiological effects of GnRH in the gonads. This thesis is not a physiological study, but an understanding of the role of GnRH in the gonads may be the basis for determining the factors that regulate GnRH. An interesting aspect is that GnRH can exert opposite effects in the gonads depending on its concentration and the maturational state of the gonads. GnRH has antigonadotropic effects in immature follicles and can induce atresia in developing follicles (Hseuh *et al.*, 1994). But GnRH can also induce ovulation and luteinization in preovulatory follicles (Richards, 1994). As well, GnRH has demonstrated meiosis-inducing action in models for both mammals (Hillensjo and LeMaire, 1980) and fish (Habibi *et al.*, 1988).

Many of the opposing effects demonstrated by GnRH in the gonads may be attributable to differences between chronic versus acute exposure to GnRH. It is known that chronic exposure of the pituitary to GnRH causes the gonadotropes to become refractory to GnRH. In contrast, exposure to GnRH for a short term or in low doses promotes expression of its own receptor on gonadotropes (Katt *et al.*, 1985).

Although the GnRH receptor found in the ovary is the same as that expressed in the gonadotrope, at least for mammals (Moumni *et al.*, 1994), some of the signalling pathways activated by GnRH appear to be different in the gonads. For example, granulosa cells, in comparison to the gonadotropes, are less sensitive to GnRH, use a different Ca^{2+} channel for Ca^{2+} influx and stimulate dose-independent elevations in $[Ca^{2+}]_i$ (Stojilkovic *et al.*, 1994). Nevertheless, the stimulatory and inhibitory effects of GnRH in the gonads are probably also mediated by modulation of the receptor levels.

GnRH is known to mimic the ability of LH to induce granulosa cells of preovulatory follicles to assume the luteal phenotype (with the required up-regulation of progesterone, progesterone receptor, prostaglandin synthesis, etc.) (Richards, 1994). The

demonstration that GnRH induces meiotic maturation of oocytes was similarly indicated for LH (Hillensjo and LeMaire, 1980). Thus, the loss of follicle integrity, due in part to down-regulation of LH receptor in granulosa cells (Piquette *et al.*, 1991), could be an indirect effect of GnRH by a pathway connected to GnRH receptor modulation. Some support for this comes from the *in vitro* demonstration that exposure of granulosa-luteal cells to low doses of GnRH (1 nM) increased the messages for both GnRH and its receptor, whereas high doses of GnRH decreased both mRNAs (Peng *et al.*, 1994). This is somewhat analogous to the findings that GnRH can autoregulate its own secretion from GnRH-producing neurons through a feedback mechanism (Krsmanovic *et al.*, 1993). Also of interest is that Peng *et al.* have shown that the low doses (1 nM) of GnRH suppress progesterone production at a time that GnRH receptor levels increase (Peng *et al.*, 1994). Higher doses of GnRH had no effect on the accumulation of progesterone (Peng *et al.*, 1994). Furthermore, during late antral development, the persistent expression of GnRH receptor in the absence of LH receptor and GnRH (Whitelaw *et al.*, 1995) appears to be characteristic of follicles undergoing atresia.

Similar paradoxical GnRH effects have been demonstrated with the inhibition of FSH-dependent steroid and LH receptor production in cultured rat testicular cells (Clayton *et al.*, 1980; Hseuh *et al.*, 1983). In contrast, short-term incubations of adult rat Leydig cells with GnRH provides evidence for stimulation of steroidogenesis (Gnessi *et al.*, 1997; Sharpe and Cooper, 1982). The subtle effect of GnRH in testicular physiology is shown in an experiment whereby blockage of 90% of the receptors with a GnRH antagonist led to a decrease of 16 to 32% of the cellular content of testosterone and FSH/LH receptors (Huhtaniemi *et al.*, 1987).

At this stage of research it is clear that GnRH has specific effects in the gonads, but the overall purpose of GnRH in gonadal physiology is not clear. A coordinated approach of understanding the factors regulating the GnRH gene and the role of GnRH once synthesized in the gonadal cell is required. By understanding the function of the long 5'-

UTRs in GnRH transcripts in the gonads, we may improve our insight into the processes that GnRH controls in reproduction.

C. Differences in GnRH promoters. I have identified four different types of promoters that drive GnRH expression in the gonads of salmonids: one is TATAAA box-dependent; another is TATAAA box-independent; one has only half-site estrogen response elements (EREs) and is thought not to bind estrogen receptor (ER) (sGnRH gene1) and one has complete EREs and should bind ER (sGnRH gene2). These differences in the promoters of the genes that encode sGnRH demonstrate how promoter regions can undergo rapid change while the structural component of the gene is conserved. If the salmonid genes encoding sGnRH are an example for the other three GnRH peptides that I have identified by HPLC from rainbow trout gonads, then this indicates that there could be as many as eight GnRH-encoding genes, each with distinct promoters. Whether each of these genes is devoted to regulating reproductive processes remains to be seen.

A number of common characteristics of GnRH expression are shared across the species. However, the ploidy of an animal does not seem to have any bearing on the number of GnRH forms that are expressed. HPLC/RIA studies of brain extracts reveal that a polyploid fish like sturgeon has only two GnRHs (mGnRH and cGnRH-II), whereas the lizard *Anolis carolinensis* (Lescheid *et al.*, 1997b) and snake *Thamnophilis sirtalis* (Smith *et al.*, 1997) each have only one GnRH, cGnRH-II and cGnRH-I, respectively. Both protein and nucleic acid analysis provide evidence for three distinct GnRH genes in several perciform fish----the diploid cichlid, striped bass and the gilthead seabream (Powell *et al.*, 1995, White *et al.*, 1995, Gothilf *et al.*, 1996, Chow *et al.*, 1998). Of course, the possibility exists that other GnRH forms are expressed in *A. carolinensis* and *T. sirtalis*, but only during discrete periods. Similar difficulties in detecting a second GnRH form in primates have been reported until recently (Lescheid *et al.*, 1997a). Alternatively, expression of some forms may evade detection because their expression is only in specific

tissues. For example, it may turn out that examination of the *Anolis* gonad will reveal other forms of GnRH. Expression of GnRH in specific tissues is supported by my HPLC/RIA work on rainbow trout where it is demonstrated that four forms of GnRH are expressed in the ovary, whereas only two of these forms appear to be required in the brain (cGnRH-II and sGnRH).

Considering how important reproduction is to the continuation of a species, I find the most striking feature is that large blocks of sequence identity do not exist in the promoters of any of the GnRH-encoding genes examined. For multiple GnRH genes in the same animal this makes sense because the expression of each gene would be controlled by different developmental and physiological cues if each GnRH serves a unique function. However, in an examination of all the genes across the species that are known to encode the hypophysiotropic form of GnRH, none possess blocks of sequence identity within their promoter regions.

An alignment of the mGnRH genes (human, rat and mouse) and salmonid sGnRH genes (rainbow trout sGnRH gene1 and 2 and Atlantic salmon sGnRH gene2) reveals the strong conservation of the proximal promoter regions, but only within the same class of animals (Fig. 6-1). Only limited sequence similarity exists between the mammalian and salmon consensus sequences determined from Fig. 6-1 (Fig. 6-2). The mammalian promoter-proximal consensus sequence shows no identity to the salmonid consensus in a sequence comparison by the BLAST program. The mammalian genes diverge in sequence similarity further upstream, but large blocks of DNA are not missing and complete divergence does not occur as shown for the salmonid genes (Fig. 6-3).

The possibility does exist that common binding motifs exist for specific transcription factors that control expression of each gonadotropin-releaser (Fig. 6-3). The development of a mouse hypothalamic tumor cell line (GT-1 cells) has been indispensible in the study of the regulation of the mammalian (m)GnRH gene. Studies using the GT-1 cell-line in footprinting or deletion analysis, coupled with gel mobility shift assays, demonstrate

Fig. 6-1. Nucleotide sequence comparison of the mammalian and salmonid GnRH proximal promoters. (A) The mammalian sequences include human, rat, and mouse. (B) The salmonid sequences include rainbow trout and Atlantic salmon sGnRH gene2 and the rainbow trout sGnRH gene1. The first 240 basepairs for the respective mammalian or salmonid GnRH-encoding genes are aligned in relation to the brain transcription start sites based on human (Dong *et al.*, 1996) or the rainbow trout sGnRH gene2. These sequences are aligned to maximize homology. TATA boxes are underlined. Nucleotides conseved in two or more species are shown in bold.

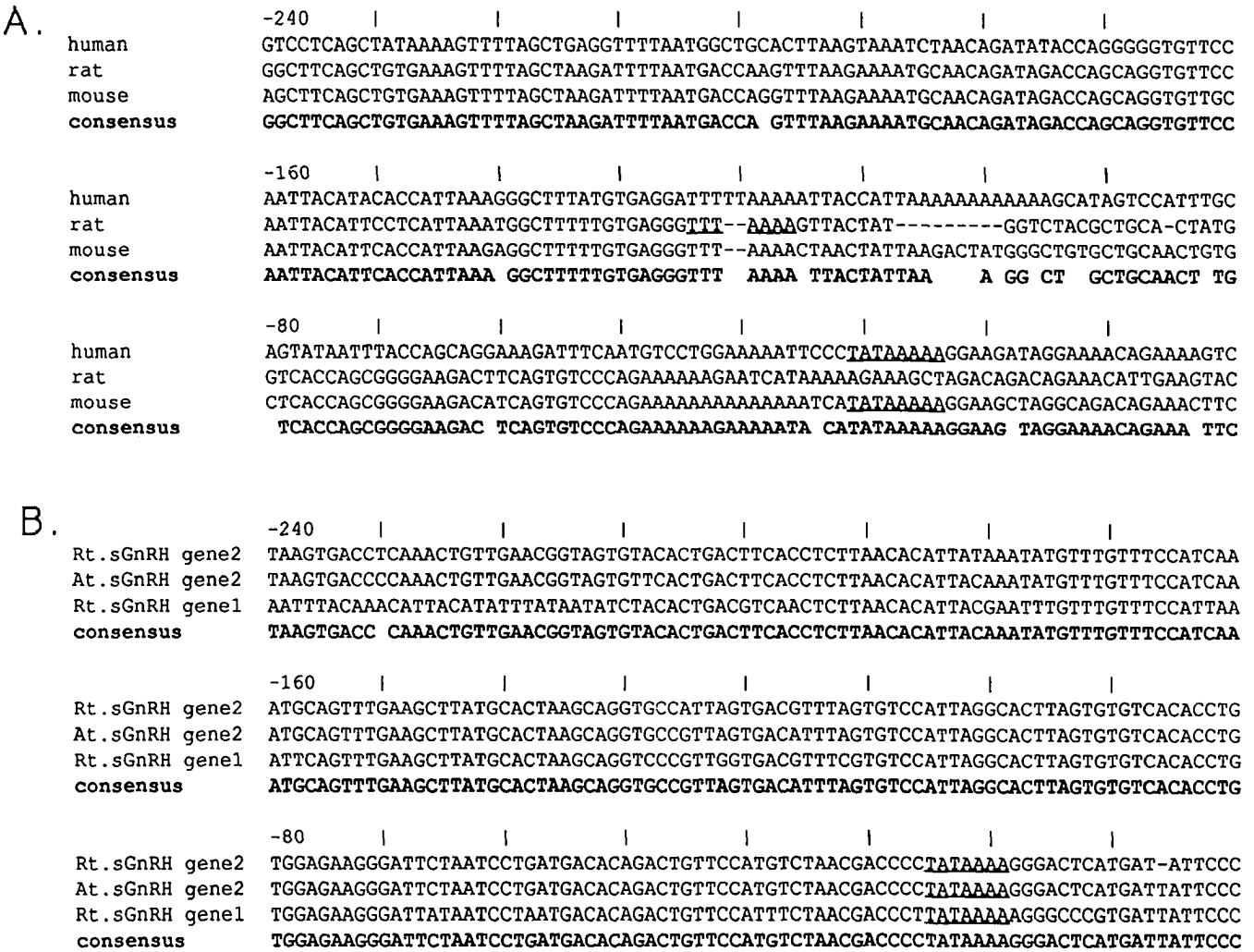

A.
-240
human GTCCTCAGCTATAAAAGTTTTAGCTGAGGTTTTAATGGCTGCACTTAAGTAAATCTAACAGATATACCAGGGGGTGTTCC
rat GGCTTCAGCTGTGAAAGTTTTAGCTAAGATTTTAATGACCAAGTTTAAGAAAATGCAACAGATAGACCAGCAGGTGTTCC
mouse AGCTTCAGCTGTGAAAGTTTTAGCTAAGATTTTAATGACCAGGTTTAAGAAAATGCAACAGATAGACCAGCAGGTGTTGC
consensus GGCTTCAGCTGTGAAAGTTTTAGCTAAGATTTTAATGACCA GTTTAAGAAAATGCAACAGATAGACCAGCAGGTGTTCC
-160
human AATTACATACACCATTAAAGGGCTTTATGTGAGGATTTTTAAAAATTACCATTAAAAAAAAAAAAGCATAGTCCATTTGC
rat AATTACATTCCTCATTAAATGGCTTTTTGTGAGGGTTT--AAAAGTTACTAT---------GGTCTACGCTGCA-CTATG
mouse AATTACATTCACCATTAAGAGGCTTTTTGTGAGGGTTT--AAAACTAACTATTAAGACTATGGGCTGTGCTGCAACTGTG
consensus AATTACATTCACCATTAAA GGCTTTTTGTGAGGGTTT AAAA TTACTATTAA A GG CT GCTGCAACT TG
-80
human AGTATAATTTACCAGCAGGAAAGATTTCAATGTCCTGGAAAAATTCCCTATAAAAAGGAAGATAGGAAAACAGAAAAGTC
rat GTCACCAGCGGGGAAGACTTCAGTGTCCCAGAAAAAAGAATCATAAAAAGAAAGCTAGACAGACAGAAACATTGAAGTAC
mouse CTCACCAGCGGGGAAGACATCAGTGTCCCAGAAAAAAAAAAAAAAATCATATAAAAAGGAAGCTAGGCAGACAGAAACTTC
consensus TCACCAGCGGGGAAGAC TCAGTGTCCCAGAAAAAAGAAAAATA CATATAAAAAGGAAG TAGGAAAACAGAAA TTC
B.
-240
Rt.sGnRH gene2 TAAGTGACCTCAAACTGTTGAACGGTAGTGTACACTGACTTCACCTCTTAACACATTATAAATATGTTTGTTTCCATCAA
At.sGnRH gene2 TAAGTGACCCCAAACTGTTGAACGGTAGTGTTCACTGACTTCACCTCTTAACACATTACAAATATGTTTGTTTCCATCAA
Rt.sGnRH gene1 AATTTACAAACATTACATATTTATAATATCTACACTGACGTCAACTCTTAACACATTACGAATTTGTTTGTTTCCATTAA
consensus TAAGTGACC CAAACTGTTGAACGGTAGTGTACACTGACTTCACCTCTTAACACATTACAAATATGTTTGTTTCCATCAA
-160
Rt.sGnRH gene2 ATGCAGTTTGAAGCTTATGCACTAAGCAGGTGCCATTAGTGACGTTTAGTGTCCATTAGGCACTTAGTGTGTCACACCTG
At.sGnRH gene2 ATGCAGTTTGAAGCTTATGCACTAAGCAGGTGCCGTTAGTGACATTTAGTGTCCATTAGGCACTTAGTGTGTCACACCTG
Rt.sGnRH gene1 ATTCAGTTTGAAGCTTATGCACTAAGCAGGTCCCGTTGGTGACGTTTCGTGTCCATTAGGCACTTAGTGTGTCACACCTG
consensus ATGCAGTTTGAAGCTTATGCACTAAGCAGGTGCCGTTAGTGACATTTAGTGTCCATTAGGCACTTAGTGTGTCACACCTG
-80
Rt.sGnRH gene2 TGGAGAAGGGATTCTAATCCTGATGACACAGACTGTTCCATGTCTAACGACCCCTATAAAAGGGACTCATGAT-ATTCCC
At.sGnRH gene2 TGGAGAAGGGATTCTAATCCTGATGACACAGACTGTTCCATGTCTAACGACCCCTATAAAAGGGACTCATGATTATTCCC
Rt.sGnRH gene1 TGGAGAAGGGATTATAATCCTAATGACACAGACTGTTCCATTTCTAACGACCCTTATAAAAAGGGCCCGTGATTATTCCC
consensus TGGAGAAGGGATTCTAATCCTGATGACACAGACTGTTCCATGTCTAACGACCCCTATAAAAGGGACTCATGATTATTCCC

Fig. 6-2. Comparison of the mammalian and salmonid GnRH consensus sequences for the proximal promoter in Fig. 6.1. Nucleotides conserved in both the mammalian and salmonid genes are shown in bold.

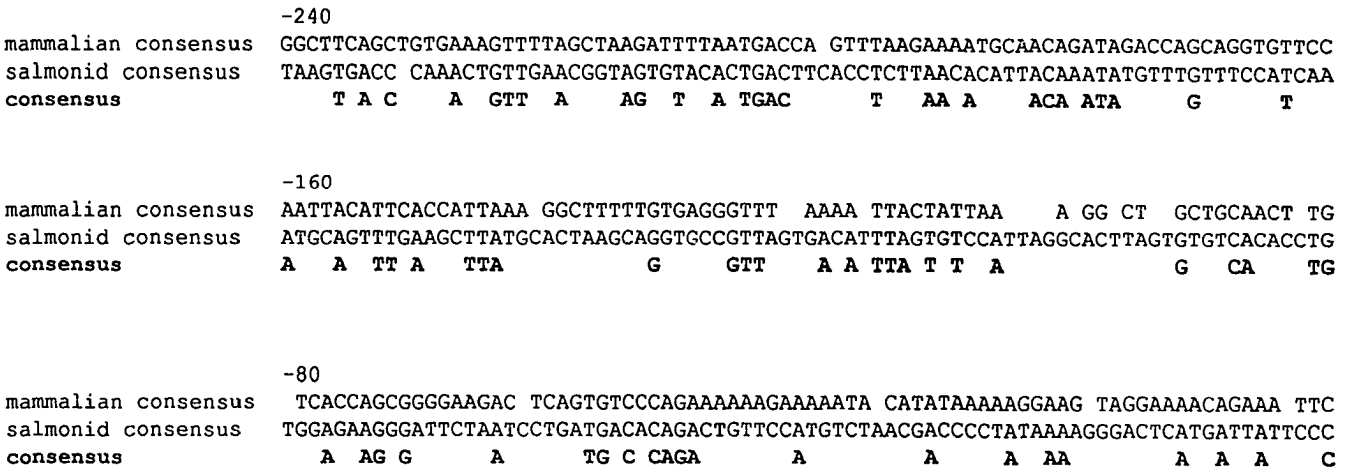
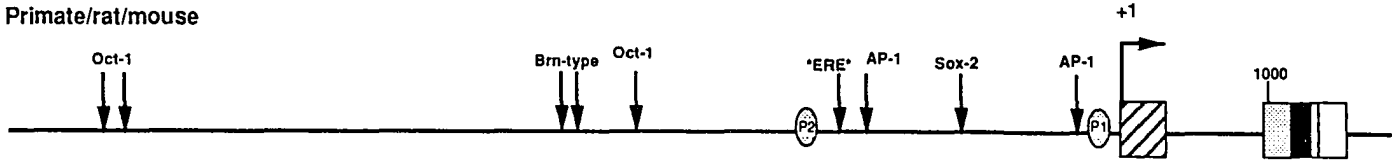
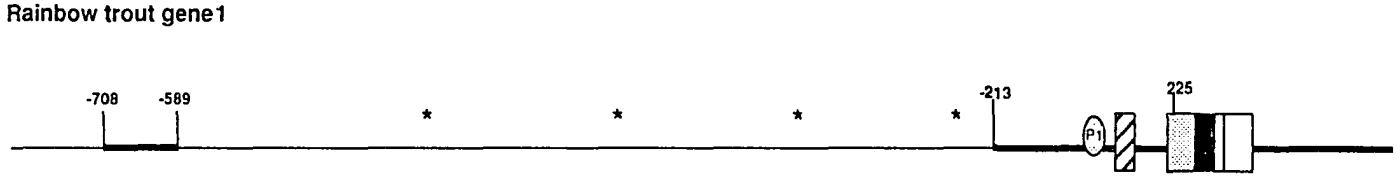

-240
mammalian consensus GGCTTCAGCTGTGAAAGTTTTAGCTAAGATTTTAATGACCA GTTTAAGAAAATGCAACAGATAGACCAGCAGGTGTTCC
salmonid consensus TAAGTGACC CAAACTGTTGAACGGTAGTGTACACTGACTTCACCTCTTAACACATTACAAATATGTTTGTTTCCATCAA
consensus T A C A GTT A AG T A TGAC T AA A ACA ATA G T
-160
mammalian consensus AATTACATTCACCATTAAA GGCTTTTTGTGAGGGTTT AAAA TTACTATTAA A GG CT GCTGCAACT TG
salmonid consensus ATGCAGTTTGAAGCTTATGCACTAAGCAGGTGCCGTTAGTGACATTTAGTGTCCATTAGGCACTTAGTGTGTCACACCTG
consensus A A TT A TTA G GTT A A TTA T T A G CA TG
-80
mammalian consensus TCACCAGCGGGGAAGAC TCAGTGTCCCAGAAAAAAGAAAAATA CATATAAAAAGGAAG TAGGAAAACAGAAA TTC
salmonid consensus TGGAGAAGGGATTCTAATCCTGATGACACAGACTGTTCCATGTCTAACGACCCCTATAAAAGGGACTCATGATTATTCCC
consensus A AG G A TG C CAGA A A A AA A A A C

Fig. 6-3. A schematic comparing positions of potential recognition motifs in the 5'-flanking regions of mammalian and salmonid GnRH-encoding genes. The bent arrows denoted by +1 indicate the brain transcription start site from exon 1 as presented by primer extension evidence. The thick lines represent regions of DNA that are highly conserved for each salmonid gene. Each salmonid gene contains the response elements presented in the sockeye salmon gene2 within the conserved regions (thick lines). Additional putative recognition motifs are presented in the Atlantic salmon gene2. Palindromic sites are shown by ERE. Sequences closely matching non-palindromic EREs shown by Radovick *et al* (1991) to bind human ER are indicated by *ERE*. Putative ERE half-sites are shown for sGnRH gene1 by asterisks. P1 indicates the position of the downstream promoter region and P2 represents the upstream promoter.

MAMMALIAN mGnRH GENES:



SALMONID sGnRH GENEs:



binding activity of POU-homeodomain regulators (Oct-1; Brn-type) in upstream (-1870 to -1840) and downstream (-959 to -831) regions of the rat (Clark and Mellon, 1995) or human (Wolfe and Radovick, 1997) GnRH promoter. An imperfect activator protein-1 complex (AP-1) has been mapped (-111 to -73) as a site at which repression of rat GnRH promoter activity occurs (Eraly and Mellon, 1995, Bruder *et al.*, 1996). An AP-1 consensus is also located at -402 to -396 in the human GnRH promoter, but its effect on transcriptional activity has not been investigated.

An interesting target element has also been identified in the proximal mGnRH promoter that directs GnRH expression. Competition for a consensus site for a SRY-like box (Sox-2) protein with Brn-4 and SCIP (Tst-1/Oct-6) at -342 to -314 results in GnRH transcription repression or stimulation (Xiong *et al.*, 1997). Sox-2 and SCIP alone or coexpressed repress GnRH promoter activity. Brn-4 alone stimulates GnRH transcription, but coexpression with Sox-2 inhibited the ability of Brn-4 to up-regulate GnRH promoter activity.

The only work on the salmonid genes that studies the binding of transcription factors has been done on the Atlantic salmon sGnRH gene2. This work demonstrated the binding of human estrogen receptor to two palindromic, but not to two nonpalindromic EREs in the 5'-flanking region of the Atlantic salmon GnRH gene2 (Klungland *et al.*, 1993). The sockeye salmon sGnRH gene2 contains these four EREs, although one palindromic and one nonpalindromic ERE are oriented in the opposite direction (between postions -355 to -205) in comparison to the Atlantic salmon gene2 (between positions -1516 to -1367) (Fig. 6-3). The rainbow trout sGnRH gene1 contains only the most proximal nonpalindromic ERE, a site that is shared by the salmonid gene2 promoters (Fig. 6-3).

When examined in this light, some common element(s) in similar contextual positions of the mammalian and salmonid GnRH promoters are revealed (Fig. 6-3). The upstream regions of both the sGnRH and mGnRH genes appear to have consensus sequences that share the potential to bind POU homeodomain regulators (Oct-1; Brn-2). Also of interest

is the position of both AP-1 and estrogen regulatory elements (Radovick *et al.*, 1991) in close proximity to the alternative upstream start site in both the mammalian (primate) and salmonid (sGnRH gene2) promoters. Finally, the strong conservation of the salmonid GnRH promoters in the proximal 200 basepairs might indicate that an element critical to GnRH regulation, similar to the the Sox recognition motif, is shared amongst these genes.

Some overlap in the types of regulators that could potentially influence GnRH expression in both the brain and gonad do exist (see Chapter 3). However, transcription of GnRH in reproductive tissues uses an upstream promoter that does not appear to be used in the brain. Hence, regulators (estrogen receptor or possibly other steroid receptors) that are not expressed in GnRH-producing neurons may be required for GnRH transcription in the reproductive tissues. Moreover, the mechanisms modulating post-transcriptional events for GnRH mRNA (turnover, storage, secretion) in the gonad are also probably different than those employed in the brain.

D. Purpose of an alternative promoter in transcription of GnRH in gonads.

I have shown that use of an alternative promoter in both the ovary and testis to regulate and express GnRH is not solely a primate attribute. I also found that alternative intron splicing mechanisms are employed in the reproductive tissues in contrast to the brain. Intron 1 is retained (or not) and in some circumstances an alternative intron acceptor site is used permitting retention of the most 3'-27 nucleotides of intron 1 (Chapter 3). The purpose of the resulting long 5'-UTRs in the sGnRH mRNA2 transcripts is not clear.

I do know that regulation of GnRH expression in the gonad has not been transferred completely to the upstream promoter. Primer extension analysis demonstrates the use also of the downstream promoter, at least at the time of testing for the particular ovary and testis in July of second year. Whether the putative EREs flanking the upstream start site provide a steroid scaffold for nucleation of the transcriptional machinery remains to be shown experimentally.

In the gonads it does not appear that the transcription of the sGnRH gene1 requires any other promoter than the same one that is used in the brain for GnRH biosynthesis. Thus, even without the generation of the sGnRH mRNA2 transcripts from the upstream start site, both types of the shorter sGnRH-encoding messages could be synthesized. Are only those GnRH-encoding mRNAs with long 5'-UTRs spared from rapid turnover and translated? Or is the differential use of the upstream and downstream promoters a mechanism to generate large numbers of GnRH molecules in a short time? This is a reasonable idea where a short window of GnRH expression occurs in the gonads; this might include juvenile gonads in salmonids and short cycling (days) gonads in mammals. In support of this, regulation of neuronal GnRH mRNA turnover appears to be controlled mostly at the post-transcriptional level rather than at the transcriptional level (Gore and Roberts, 1997).

II. Future Studies.

A. Localization of distinct sGnRH transcripts in specific cells. I have shown that both sGnRH mRNA1 and mRNA2 transcripts are expressed in the salmonid gonad. It also has been shown here (Chapters 3 and 4) and by others (Ashihara *et al.*, 1995) that both transcripts are expressed in the salmonid brain. In future studies the discovery that sGnRH mRNA2 transcripts contain long 5'-UTRs could be exploited to do *in situ* hybridization studies. This could be used to determine if these sGnRH-encoding transcripts are localized to the same or different cells in reproductive tissues. These studies would complement *in situ* hybridization studies simultaneously attempting to determine where the GnRH receptor is localized in these tissues (or if distinct types of receptors are in specific types of cells).

Similarly, differences found in the 3'-UTRs between these two sGnRH-encoding transcripts could be used to make probes to determine if these messages are transcribed in distinct subgroups of GnRH-positive neurons. An answer to this kind of question would

lead to two different experimental approaches. If both transcripts are generated in one neuron then questions on how these two genes are regulated would need to be addressed. The upstream sequences of the genes that encode these two mRNAs are very different and so are probably regulated by different transcription factors. However, if expression of each transcript is compartmentalized to distinct neurons, a different set of questions about brain development and organization would result. For example, it is possible that expression of one of the sGnRH-encoding transcripts is localized only to the neurons within the terminal nerve in the forebrain, whereas the other sGnRH message is expressed only in the preoptic area of the brain in salmonids. This would be analogous to the expression of sGnRH only in the terminal nerve and sbGnRH only in the preoptic area of the brain in the perciform fishes. If this is the case, it would be interesting to determine if these putative neurons arise early as distinct cell-types or if the hypothetical localization of the expression of each GnRH is regulated by "silencer" factors expressed in these cells following establishment of the neural network. Another possibility is that the regulation of these transcripts is governed by the type of neurotransmitters that are released onto the GnRH-positive neurons.

B. Interaction of GnRH(s) with its receptor in reproductive tissue.

I have shown by HPLC/RIA that four different GnRHs are present in the rainbow trout ovary and testis during the mid-summer. The question arises whether or not these GnRHs interact with one receptor or with four distinct receptors. Based on binding affinity and gonadotropin release studies, it is known that mGnRH (and cGnRH-II to a lesser extent) strongly interacts with the mGnRH receptor, but that all other vertebrate GnRHs interact poorly with this receptor (Sealfon *et al.*, 1997). However, all of the other vertebrate GnRHs (except lamprey GnRHs) interact with similar binding activities with non-mammalian receptors (Sealfon *et al.*, 1997). There is the possibility that only one receptor exists in the salmonid ovary and testis for GnRH binding. There is some evidence that

different GnRHs bind a single receptor, but activate different intracellular pathways (Chang and Jobin, 1991). If, however, it is ultimately shown that the four GnRHs interact with four specific receptors, it would be interesting to determine if they exert their effects through different pathways. Also, it is clear that a second form of GnRH (cGnRH-II) does exist in the primates (Lescheid *et al.*, 1997a; White *et al.*, 1998); it would be interesting to determine if both GnRHs are expressed in the gonad and whether each ligand has a distinct receptor.

C. *Determination of function of GnRH in reproductive tissues.*

To define the role of GnRH accurately within the context of changing levels of its receptor and the maturation state of the target cells is very difficult. One approach to understanding the function of GnRH in the gonads is to determine what occurs in the absence of GnRH in an otherwise unaltered physiological state. Development of a tissue-specific gene strategy is probably the best way to study this.

It is now possible to selectively knock out a gene of interest in either the brain or gonads. The usual knock out strategy would result in lack of GnRH expression in all tissues so that it would not be possible to distinguish brain and gonadal differences. However, one method to ensure GnRH knock-out specifically in the gonad would be in the design of two vectors that would be incorporated into the host genome. One vector would carry the exon encoding GnRH flanked by specific sequences that are recognized and clipped out by Cre recombinase. The second vector would carry the sequences that encode the recombinase driven by a gonad-specific promoter. The flanked exon encoding GnRH would be integrated into the genome by homologous recombination at early stages of development. It is possible to select gonad-specific promoters for the recombinase that are only activated at specific times during development. The transcription of the recombinase gene in the gonad results in the production of the recombinase enzyme that will remove the GnRH gene locally.

The major obstacle to this strategy would be in the selection of a promoter that drives expression of a gonad-specific gene product. It has been demonstrated, however, that there are a number of genes and signalling factors specific to the developing follicles (Richards, 1994). The fact that numerous isoforms of kinases, cyclases and catabolic enzymes exist in mammalian follicles could be exploited to find a promoter used by one isoform expressed exclusively in one cell type at a specific time.

D. Connection of GnRH effects with arachidonic acid metabolism in gonad.

Another area of interest is the role that arachidonic acid (AA) plays in gonadal maturation. Work conducted with various AA metabolites or polyunsaturated fatty acids in fish show opposite effects on ovarian and testicular steroidogenesis depending on the study (Van Der Kraak *et al.*, 1998). In mammals, the synthesis of prostaglandins and other eiconsanoids is a very important step in the ovulation process (Richards, 1994). In the mammalian gonadotrope, a number of leukotrienes generated by AA metabolites have been implicated in stimulation of gonadotropin release (Dan-Cohen *et al.*, 1992). Because the receptor in both the pituitary and gonad has been shown to be the same and to activate similar pathways, it is possible that GnRH regulates the gonad through diacylglycerol lipolysis (following activation of phospholipase C) or directly through phospholipase A_2 (Naor, 1990) to generate the AA metabolites. One possible function of GnRH is to modulate the synthesis of these AA metabolites. The stimulatory and inhibitory effects attributed to GnRH (see above) may be due to the actions of these eicosanoid hormones since a growing body of evidence indicates these AA compounds operate as pairs of hormones with opposing actions (Smith and Borgeat, 1985).

E. Association of GnRH expression with growth in reproductive tissues.

It is very interesting that the cDNAs encoding GnRH were not observed in any tissue we considered immature, except in October of the first year and December of the second year. The remaining positive results were from jill ovary or jack testis indicating the possibility of some association of GnRH expression and growth. It is noteworthy that IGF-I and other growth factors have survival and growth-promoting effects when added to GnRH-expressing neuronal cell lines (Zhen *et al.*, 1997). Also, IGF-I activates a signal transduction pathway that induces GnRH transcription through an AP-1 site located proximally on the human GnRH gene (Zhen *et al.*, 1997). Similar efforts should be made to determine if IGF-I or other growth factors stimulate GnRH expression in ovarian or testicular cell lines.

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