Ruprecht - Karls - Universität Heidelberg

Diplomarbeit

Attemps to clone the rodent gene for GnRH-II

vorgelegt dem
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1 Summary

Hypothalamic gonadotropin-releasing hormone (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2, GnRH-I) is named for its role in regulating reproductive function in vertebrates by controlling release of gonadotropins from the pituitary. Another form of GnRH of unknown function (pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly; GnRH-II) is expressed in the mesencephalon of all vertebrate classes except jawless fish. GnRH-II cDNA has been receantly cloned from several mammals including the tree shrew, musk shrew, human, and rhesus monkey. Attempts to clone the mouse gene for GnRH-II were undertaken by screening a mouse genomic library with a human cDNA-derived GnRH-II probe under low stringency conditions. Several putative positive clones were obtained, however none of them contained coding sequences for GnRH-II. As an alternative approach, RT-PCR was performed on mouse and rat cDNA with specific primers deduced from the GnRH-II coding sequence. While these primers were succesful in amplifying a cDNA for GnRH-II from human brain cDNA, which served as a positive control, RT-PCR from either mouse or rat total brain and kidney RNA did not yield a positive result. Cloned mouse GnRH-II cDNA thus remains elusive.

2 Introduction.

2.1 Preface. The integrating role of hormones.

The main problem of understanding how any living multicellular entities ranging from comparably simple organisms such as cnidarians to complex mammals, generation by generation, can recreate the same plan of construction during development is to delineate all players and to comprehend their roles at a molecular level .

The simplest definition of development refers to a process of progressive change by which a single cell expands into a complex multicellular organism, made up of many tissues and organs. This transition in all multicellular species in each generation takes place in indispensable and unaltered order without or with little variations and, as far as we know, does not need a centralized control mechanism that coordinates embryonic development. Each cell in the developing embryo simply responds to signals it receives from the cells that surround it. Such cell-to-cell signals regulate gene expression in the receiving cells, and altered gene expression, in turn, sets up the conditions that cause the next round of developmental events. As a developing embryo grows, long-range communication by cell-to-cell signaling becomes increasingly inefficient, and each of its parts tends to turn largely autonomous: in each part development proceeds at its own place and in a direction dictated by local interactions among cells. Since regulatory and inductive signals can extend only into a small portion of the developing animal, the regulation of development becomes spatially restricted. The autonomy of emergent developmental fields (Gilbert, Opitz, Raff, 1996) creates a problem in development regulation - how to coordinate the development of distant

parts so that the animal as a whole remains well integrated. To orchestrate the development of distant parts, it has proven advantageous to deploy some kind of long-range signaling mechanism that can signal across the entire organism and can differentially affect the timing and the rate of development of the various parts. Hormones as well as other bioregulators, such as pheromones, paracrine substances, and growth factors which do not originate from defined glandular or nervous structures and are delivered to their sites of action in many ways are almost universally used for this purpose, and the role of hormones in coordinating development and functional regulation of the entire organism is well appreciated. One of the most prominent players in this field is gonadotropin-releasing hormone.

Gonadotropin-releasing hormone (GnRH, previously called leutinizing hormone-releasing hormone, LHRH) is the final common signaling molecule used by the brain to regulate reproduction in all vertebrates. The GnRH decapeptide is synthesized by scarce neurosecretory cells (approximately 800 per animal in mice; Wray and Hoffman, 1986; Wu *et al.*, 1997) scattered in the hypothalamus, and is secreted in a pulsatile manner into the primary portal capillaries in the median eminence, to be transported to the pituitary gland by hypophyseal portal veins, where it stimulates secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from pituitary gonadotrophs, which are responsible for gonadal development and growth. Despite of the fact that the GnRH amino acid sequences vary by 50% among vertebrates, this so-called "releasing" form during the 500 million years of vertebrate evolution remains the main primary link between the brain and the pituitary in regulating gonadal functions and thus plays a central role in the coordination of reproduction. This role is well characterized but the simple picture of this function has been perturbed with the discovery of

similar genes encoding decapeptides slightly different from the hypothalamic GnRH-I form and furthermore, by the fact that the all forms are expressed not only in the brain but also in other tissues.

2.2 Short historical survey on GnRH gene discovery.

The existence of a bioregulator with GnRH function has been predicted 50 years ago (Harris, 1950) but the real history of GnRH began in 1971 with the isolation and determination of the primary structure of hypothalamic luteinizing hormone-releasing hormone from porcine hypothalami in the laboratory headed by Schally (Matsuo *et al.*, 1971). Guillemin`s group reported the amino acid sequence of ovine GnRH (Amoss *et al.*, 1971), which proved to be the same as that of porcine GnRH. Studies accomplished during the following years showed that the structure of hypothalamic GnRH is identical in mammalian species with one known exception for guinea pig, but at least 13 additional forms of GnRH that differ structurally have been identified in birds, reptiles, amphibians, fish, and protochordata. The amino acid sequence of GnRH permitted the isolation of cloned genomic and cDNA sequences encoding the precursor form of hormone in human and rat (Seeburg, Adelman, 1984; Adelman *et al.*, 1986) using short degenerate oligodeoxynucleotide probes deduced from the primary structure of decapeptide. The first evidence of an additional gene encoding a second form of GnRH in Haplochromis burtoni and African catfish was made in 1994 (White *et al.*,1994; Bogerd *et al.*, 1994) and later in numerous fish species (Ashihara *et al.*, 1995; Lin, Peter. 1996; Gore, Roberts. 1997; Penlington *et al.*, 1997; Chow *et al.*, 1998). It was not surprising since immunocytochemical labeling and HPLC (high pressure liquid chromatography) analysis showed that in numerous vertebrate species

more than one of eight GnRH forms sequenced at the peptide level at that time can be expressed in the brain. With the identification of a third GnRH cDNA in H. Burtoni (White *et al.*, 1995) three neuronal populations have been shown to express different GnRH forms by immunocytochemistry and in situ hybridization: the terminal nerve, the hypothalamic/preoptic area, and the mesencephalon (Davis & Fernald, 1990; White, Fernald, 1993; White *et al.*, 1994). GnRH-I (the releasing form, also known as LHRH, luteinizing hormone-releasing hormone, mammalian GnRH, mGnRH; this form in *H. burtoni* differs from mGnRH at one amino acid position – Ser^8 substitutes Arg^8 and also known as seabream GnRH, sbGnRH) is expressed exclusively in the hypothalamus of this species, GnRH-II (also called chicken GnRH-II, cGnRH-II) is expressed in the midbrain mesencephalon, and GnRH-III is expressed in the terminal nerve area of the telencephalon (also called salmon GnRH or sGnRH).

Since the original discovery in fish, a cDNA encoding GnRH-II has been found in several placental mammals (tree shrew, Kasten T. L. *et al.*, 1996; humans, White R. B. *et al.*,1998; rhesus monkey, Urbanski H. F. *et al.*, 1999). To date a cDNA or gene for GnRH-III was identified only in several teleost fish but was not found in higher vertebrates although recent studies using indirect methods combining reverse-phase high-performance liquid chromatography and radioimmunoassay revealed immunoreactive sGnRH-like peak in human and bovine (Yahalom D. *et al.*, 1999) and in capybara (Montaner A. D. *et al.*,1998, 1999) acid brain extracts coeluting with sGnRH synthetic standard.

2.3 Decapeptides and genes structure.

With the recent discovery of a new GnRH peptides in guinea pig (Jimenez-Linan *et al.*, 1997) and in herring (Carolsfeld J *et. al.*, 2000) there are now 11 forms in vertebrate brains and two in a protochordate (Chelyosoma productum, Ascidiacea; Powell *et al.*, 1996).

I. GnRH-I (releasing form)

N	Name	Amino acid decapeptide sequence
1	Mammalian mGnRH	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2
2	Guinea pig	pGlu-Tyr-Trp-Ser-Tyr-Gly-Val-Arg-Pro-Gly-NH2
3	Seabream sbGnRH	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Ser-Pro-Gly-NH2
4	Herring hGnRH	pGlu-His-Trp-Ser-His-Gly-Leu-Ser-Pro-Gly- NH2
5	Dogfish dfGnRH	pGlu-His-Trp-Ser-His-Gly-Trp-Leu-Pro-Gly- NH2
6	Lamprey I	pGlu-His-Tyr-Ser-Leu-Glu-Trp-Lys-Pro-Gly- NH2
7	Lamprey III	pGlu-His-Trp-Ser-His-Asp-Trp-Lys-Pro-Gly- NH2
8	Catfish ctGnRH	pGlu-His-Trp-Ser-His-Gly-Leu-Asp-Pro-Gly-NH2
9	Tunicate I	pGlu-His-Trp-Ser-Asp-Tyr-Phe-Lys-Pro-Gly- NH2
10	Tunicate II	pGlu-His-Trp-Ser-Leu-Cys-His-Ala-Pro-Gly- NH2
11	Chicken-I cGnRH I	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Gln-Pro-Gly-NH2

II. GnRH-II

	Chicken-II cGnRH II	pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH2

III. GnRHIII

	Salmon sGnRH	pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH2

Table 1. All known GnRH forns are divided into three groups. Tunicate-I, tunicate-II, lamprey-I, lamprey-III are included into the GnRH-I group because of unclear phylogenetic relationships.

In common with neuropeptides in general, GnRHs are expressed as precursors with signal and associated (GAP) peptide. The latter is separated from the decapeptide by a Gly–Lys–Arg which is considered to be a dibasic recognition sequence for a subtilisin-type protease (Seeburg *et al.*, 1987). Within each of the variants of GnRH precursors studied (alignment of the known mammalian GnRH-II precursors is shown on Figure 1), the amino acid sequences of the GnRH and the following Gly–Lys–Arg are conserved, while their nucleic acid sequences show some degeneracy. Significant similarity between the cDNA and amino acid sequences of the signal and associated peptides occurs within but not between variants.

The amino (pGlu) and carboxy (Gly-amide) residues are unchanged, and 5 of the 10 residues are identical from lamprey to humans. Residues in positions 1, 4, 9, and 10 are fully conserved in all GnRH forms, and those in positions 2 and 3 are conserved in 11 of the 13 GnRH forms.

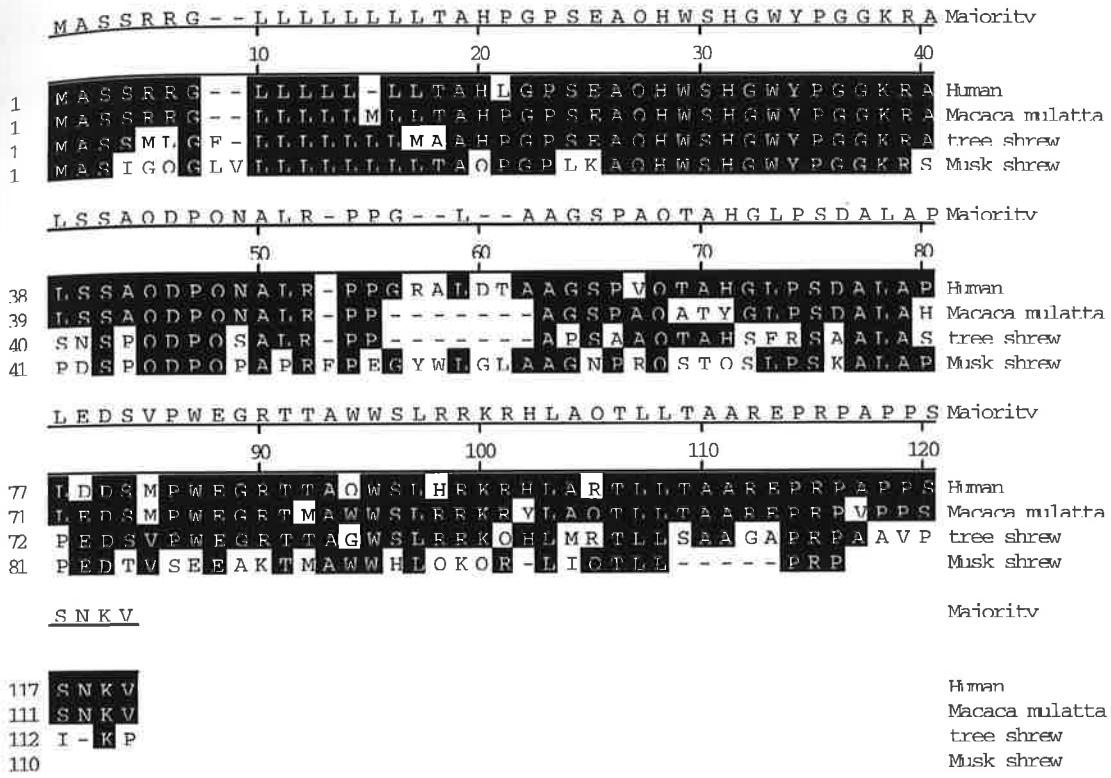


Figure 1. Alignment of the known mammalian GnRH-II precursors. The amino acid sequences of the GnRH-II and the following Gly–Lys–Arg are conserved, while their nucleic acid sequences (Figure 5) show some degeneracy.

The most stable region of the peptide, residues 1–3, is responsible for releasing gonadotropins, while the region with the most changes, residues 5–8, is thought to mediate receptor binding. Conformation–function studies of the GnRH analogs based on energy calculations (Nikiforovich G. V., 1993) suggested that the central tetrapeptide common to GnRH and its agonists possesses a β-II′ turn as the dominant conformation. It was suggested that one of the purposes of this conformation is to maintain the proper spatial arrangement of the terminal fragments, and especially of the N-terminal tripeptide, which presumably participate in direct contact with the GnRH receptor, with the His^2 ''key'' residue being held at the surface of the molecule by hydrophobic moieties.

All GnRH genes share the same basic structure. In all cases, the GnRH preprohormone mRNA encodes GnRH and the GnRH-associated peptide

(GAP), separated by a canonical cleavage site. The preprohormone mRNAs are encoded by four exons (Figure 2).

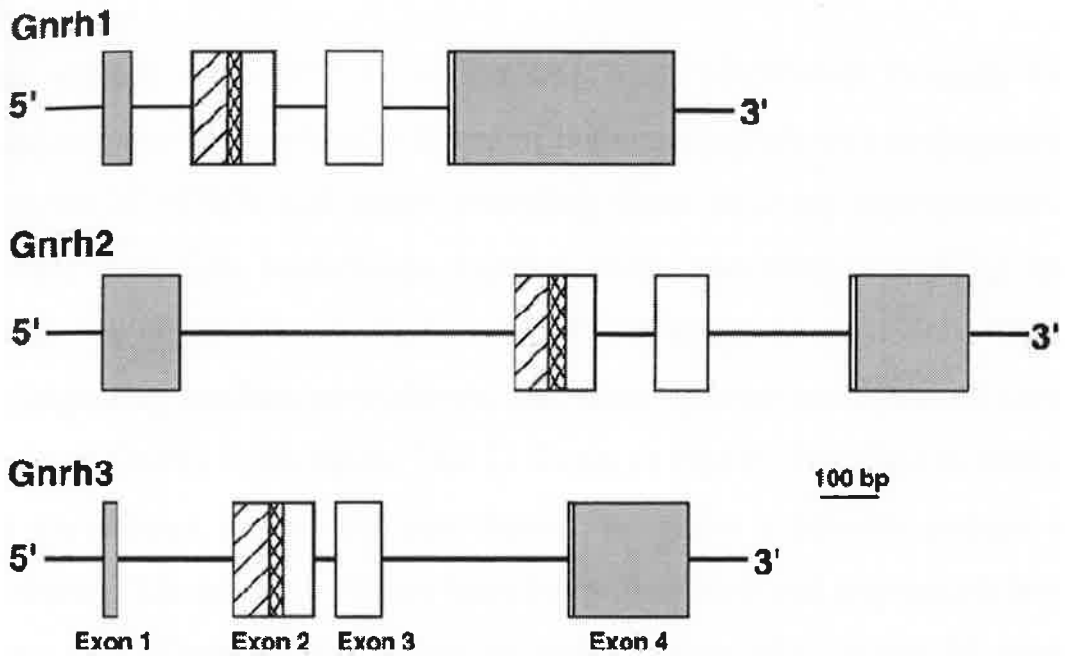


Figure 2. Schematic diagram showing organization of GnRH genes. The genes encoding GnRH in the cichlid fish, *H. burtoni*, are shown. Within each gene, a given exon encodes a corresponding preprohormone region. Single hatched bars, signal sequence coding region; cross-hatched bars, GnRH-coding region; open bars, GnRH-associated peptide (GAP) coding region. Horizontal lines connecting the exons represent introns. (Redrawn from Fernald and White, 1999).

Exon 1 encodes the 5′ untranslated region (5′-UTR) exclusively. Exon 2 encodes the signal peptide, the GnRH decapeptide, the proteolytic cleavage site, and the N-terminus of GAP. Exon 3 encodes the central portion of GAP and exon 4 encodes the C terminus of GAP along with the 3′-UTR. Among all known GnRH genes, there seem to be two generalities: (i) exons 2 and 3 are the most similar in length and (ii) the UTR sizes and intron sizes are the most different. The greatest differences within the preprohormone are within the GAP sequences. The striking contrast between the conservation of the GnRH coding sequence and lack thereof in the GAP coding sequence is evidence of differential selective pressure within the gene.

2.4 Phylogenetic relationships among GnRHs.

The pattern of GnRH evolution has been elucidated through the comparison of the molecular forms of the neuropeptide and phylogenetic analysis of cDNA and genes encoding them in many representative species from fish, amphibians, reptiles, birds, and mammals (King and Millar, 1995; Sherwood *et al.*, 1994, 1997; White *et al.*, 1998). These phylogenetic studies have shown that most species have two or three forms of GnRH in the brain. The 11 forms of GnRH identified to date in the vertebrates are widely distributed, but show a definite pattern in evolution. Six of the 11 forms have been identified and sequenced from bony fish (Osteichthyes): two of these forms also occur in other vertebrates, whereas four forms are specific to bony fish. The shared forms, which are named after the animals from which the GnRH form was identified first, are GnRH-I (mGnRH) and GnRH-II (cGnRH-II). The specific forms are salmon GnRH (sGnRH or GnRH-III), catfish GnRH (cfGnRH), seabream GnRH (sbGnRH) and herring GnRH (hrGnRH). All vertebrates tested to date excluding the jawless fish species (agnathans) have cGnRH-II present in the brain. This characteristic is shared by cartilaginous fish, bony fish, amphibians, reptiles, birds, and mammals.
Another form of GnRH in the brain of some bony fish is mGnRH. This form is expressed in lobe-finned fish (e.g., lungfish, Joss *et al.*, 1994) and the most ancient of the living ray-finned fish, (Lescheid *et al.*, 1995; Sherwood et al., 1991). The presence of GnRH-I is a characteristic shared with amphibians and mammals (Conlon *et al.*, 1993; Lescheid *et al.*, 1995). The ancient bony fish species with mGnRH include the four major groups whose ancestors evolved before those of

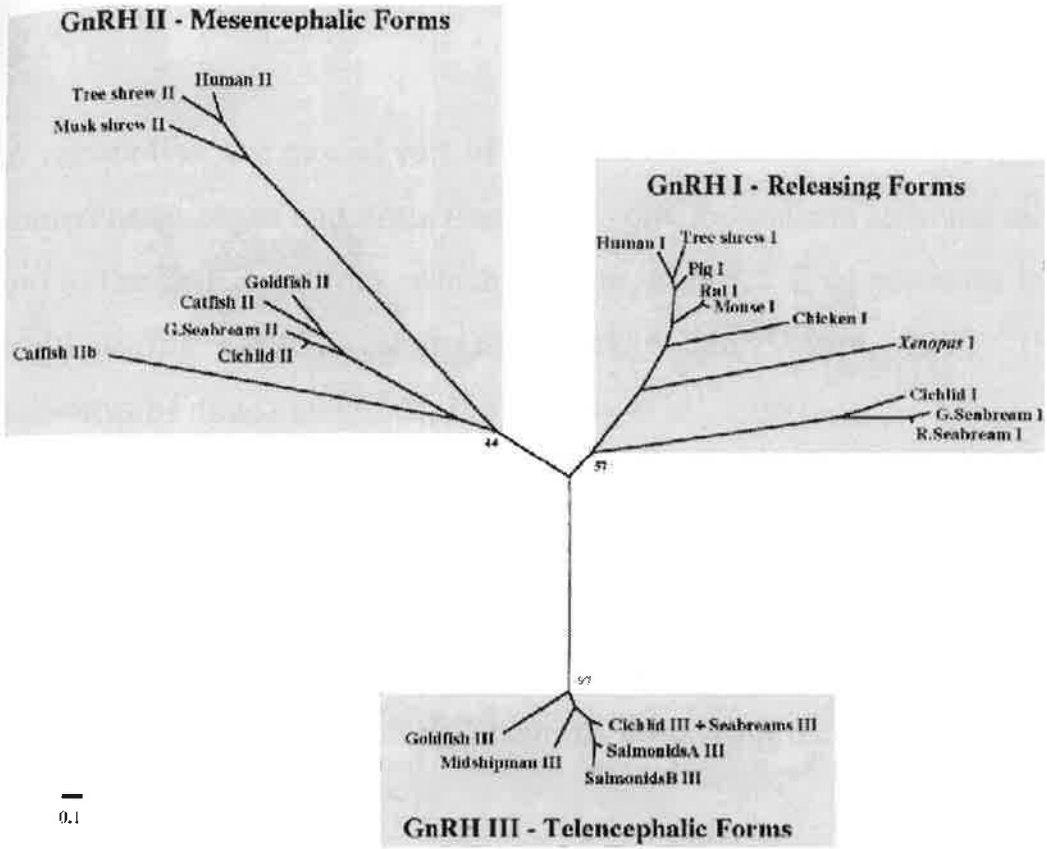


Figure 3. Unrooted neighbor-joining phylogenetic tree of cDNAs encoding GnRH. The tree was generated using the neighbor-joining algorithm and the Hall distance calculation correction procedure. Evolutionary distances are represented only by the length of the branches and not by branch angles. Bootstrap values, indicating the number of times a particular set of sequences groups together when trees are generated from resampled alignments, are indicated for some important nodes on the tree. An unrooted tree is shown because of the lack of an obvious outgroup. Some GnRH-III sequences from different species were identical and are grouped together. The scale bar corresponds to estimated evolutionary distance units as calculated by Protdist in Phylip. Redrawn from White RB et al., 1998.

the teleosts and also include one of the earliest teleosts to evolve, the eels (King et al., 1990). The other tree specialized forms of GnRH - cfGnRH, sbGnRH and hrGnRH appeared in more recent euteleosts. Thus, all GnRH forms are tightly conserved in vertebrates as might be expected for a molecule that controls reproduction. Therefore, a change in the molecular form of GnRH in vertebrates is a useful characteristic or marker in studies of evolution. Phylogenetic analysis of known cDNAs and genes encoding GnRH reveals that the GnRH gene family falls into three distinct branches (Figure 3. White RB at al., 1998)

2.5 Expression sites of different GnRH forms in vertebrates.

In vertebrates, the medial part of the olfactory placode develops into the vomeronasal organ and gives rise to the vomeronasal and terminal nerves and to the GnRH neurons, which migrate into their final positions in the brain during early development (Muske and Moore, 1987, 1990; Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989).

By using molecular probes to identify the cell types expressing distinct GnRH forms, White, R. B. and Fernald, R. D.(1995) showed the migration of both GnRH-I and GnRH-III neurons from the placode to their appropriate adult locations in the brain of *H. burtoni* and, in contrast, revealed that GnRH-II neurons arise from the germinal zone of the third ventricle.

In amphibians, both a mammalian-type GnRH (mGnRH) and cGnRH-II have been identified in the portal blood that flows from the median eminence to the pituitary gland, suggesting that both forms may mediate gonadotropin release (Licht *et al.*, 1994).

In rainbow trout, the failure to identify cGnRH-II precursor expression in other brain areas, including the pituitary gland, is in general agreement with other molecular studies (Bogerd *et al.*, 1994; Powell *et al.*, 1995; White et al., 1995), but contrary to some immunocytochemical studies (e.g. Goos *et al.*, 1995; Parhar *et al.*, 1996). Some light is shone on this discrepancy by two studies conducted on the distribution of cGnRH-II precursors in catfish. Bogerd *et al.* (1994) reported that cGnRH-II was not expressed in the pituitary gland, while a later study from the same laboratory (Goos et al., 1995) identified, with highly specific antibodies cGnRH-II in the pituitary gland of mature females of this species. Hence, cGnRH-II, is probably synthesized in the midbrain and later transported to the pituitary gland. It is therefore possible that this variant may be

involved in reproductive regulation not only in goldfish (Murthy, Peter, 1994) but also in all other fish species.

Preoptic neurons of eels were immunoreactive to chicken GnRH-II, considered the main GnRH form in the midbrain tegmentum of most vertebrates, but not seabream GnRH as in other teleosts. Furthermore, preoptic GnRH neurons are morphologically and immunologically distinct from the olfactory GnRH neurons. Preoptic GnRH neurons increase in size and number within the periventricular area. As in some teleosts but not in amphibians and mammals, preoptic GnRH neurons in the Japanese eel appear to originate from within proliferative zones of the diencephalon (Tobet *et al.*, 1996; Parhar 1997; Parhar *et al.*, 1998). In a number of teleost species, including goldfish, multiple GnRH forms are delivered to the pituitary (Yu *et al.*, 1991), where they regulate maturational gonadotropin hormone (GTH-II; luteinizing hormone-like) secretion (Chang *et al.*, 1996). Whether different endogenous GnRH neuropeptides utilize identical or distinct signal transduction mechanisms to stimulate GTH secretion is not well understood but has been most extensively studied in the goldfish (Chang *et al.*, 1996). Short (10-s) applications of the two endogenous GnRH forms (salmon (s)GnRH and chicken (c)GnRH-II) were reported to induce either rapid/transient or slow/longer-lasting elevations in $[Ca^{2+}]$ in goldfish gonadotropes (Mollard and Kah, 1996).

Although available data suggest that both GnRH peptides act on goldfish gonadotropes through a single population of receptors (Murthy *et al.*, 1993; Habibi *et al.*, 1990; Murthy and Peter, 1994; Yu *et al.*, 1998; Illing *et al.*, 1999), sGnRH and cGnRH-II signal transduction mechanisms differ in their relative dependence on intracellular and extracellular Ca^{2+} availability, protein kinase C activation, inositol phosphate production, and arachidonic acid mobilization (Jobin and Chang 1992; Chang *et al.*,

1996, 1995). These signal transduction components may be regulated by Ca^{2+} and are known to modulate Ca^{2+} fluxes in many cells, including rat gonadotropes (Stojikovic and Catt, 1995; Tse *et al.,* 1997). This raises the intriguing possibility that sGnRH- and cGnRH-II-evoked Ca^{2+} signals may have spatial and temporal differences arising from or causing differences in their signal transduction pathways.

While the cGnRH-II variant has been identified in the midbrain of fish (Bogerd *et al.*, 1994; Powell *et al.*, 1995; White et al., 1995; Gothilf *et al.*, 1996; Lin & Peter, 1996; Penlington *et al.*, 1997), relatively few studies (Rosenblum *et al.*, 1994; Goos *et al.*, 1995; Kim *et al.*, 1995) have provided evidence for its localization in the pituitary gland

To date GnRH-like factors in invertebrates have received little attention with only the snail Helisoma trivolvis (Goldberg *et al.*, 1993) having being shown to contain the peptide. However, a yeast peptide (the yeast α-mating factor, a tridecapeptide which has extensive sequence homology with GnRH (Loumaye et al., 1982), acts in the cell cycle, to arrest the G1 stage of haploid cells of opposite (a) mating types, prior to fusion with cells to produce diploid zygote.) shows structural and functional similarity with GnRH. .

The selection of the cGnRH-II decapeptide, which appears to have been conserved for 500 million years, together with its identification in birds, reptiles, amphibians, fish and recently mammals (Kasten et al., 1996) suggests it is the phylogenetically primitive form and may have a conserved and central function.

Because immunological detection techniques can produce ambiguous results in comparison with molecular hybridization, identifying precisely when and where each GnRH peptide may be produced requires genetic probes for all GnRH mRNAs within a single species in which ontogeny can be studied.

3 Materials and methods

3.1 Plating libraries and transfer to filter membranes.

The basic principle of screening recombinant DNA libraries is that bacteriophage plaques, or bacterial colonies containing plasmids or cosmids, contain relatively large amounts of insert DNA that can be detected either directly by hybridization or indirectly by the protein that may be expressed from the cloned segment. The first step in the nucleic acid hybridization screening procedure is to grow large numbers of colonies or plaques on agar plates. Replica copies of these colonies are transferred to nitrocellulose filters, where they can be screened.

The Lambda FIX® II mouse genomic library was used for screening with cDNA-derived human GnRH-II probe. Accordring to suplier`s information the Lambda FIX® II vector is digested with Xho I and a partial fill-in reaction is performed with dTTP and dCTP, resulting in 5'-TC overhangs. The vector cannot self-ligate. Insert DNA is digested with BamH I, Mbo I, Bgl II or Sau3A I and a partial fill-in reaction is performed with dGTP and dATP, resulting in 5'-GA overhangs. The insert DNA will not self-ligate, preventing multiple inserts. However, the partially filled-in vector and the partially filled-in insert will ligate. The partial fill-in technique thus eliminates the need for dephosphorylation of genomic DNA. Background can be further reduced with the Lambda FIX II vector by plating on a host strain that incorporates Spi+/P2 selection.

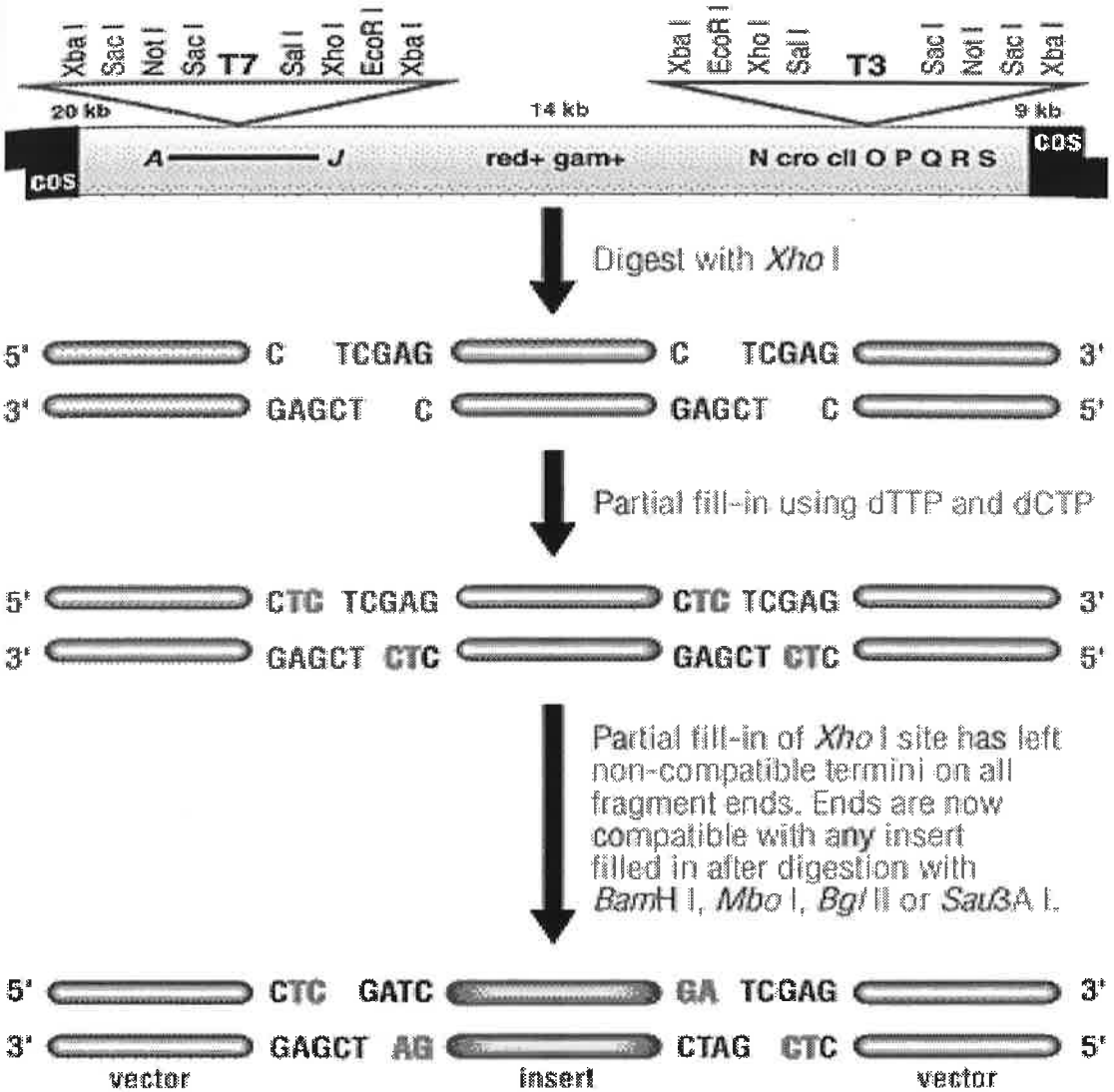


Figure 4. Scheme of the Lambda FIX® II mouse genomic library construction. (Redrawn from the Lambda FIX® II mouse genomic library. User manual, Stratagene).

The cloning region of the Lambda FIX II vector is flanked by T3 and T7 bacteriophage promoters, which can be used to generate end-specific transcripts that allow rapid chromosomal walking and restriction mapping. Not I sites flanking the T3 and T7 promoters allow isolation of a cassette containing the insert and the two promoters. High-resolution restriction maps can then be generated quickly by performing a partial digestion of the cassette and probing a Southern blot of the resulting fragments with T3 and T7 oligonucleotide probes.

Plating and Transferring Bacteriophage Libraries

Bacteriophage are plated onto agar plates at high density so that as many as 1 million different plaques can be screened. The bacteriophage plaques are then transferred to nitrocellulose filters, denatured, and baked.

Materials

Host bacteria LE 392

Recombinant phage FIXII mouse genomic library (Stratagene)

0.7% top agarose (prewarmed)

150-mm LB plates

0.2 M NaOH/1.5 M NaCl

0.4 M Tris-Cl, pH 7.6/2x SSC

2x SSC

Nitrocellulose membrane filters

20-G needle

Whatman 3MM filter paper

80^0C vacuum oven

Plating bacteriophage

1. Determine the titer of the library by serial dilution to get approximately 30000 plaques per plate (1X 10^6 plaques on 30 plates)
2. Mix recombinant phage and plating bacteria in a culture tube (12 ml Falcon tube) and incubate 20 min at 37^0C.
3. Add 0.7% top agarose (45^0 to 50^0C) to culture tube and transfer mixture to LB plates. Disperse bacteria and agarose on plates by tilting the plates back and forth.

4. Incubate plates at 37^0C until plaques cover the plate but are not confluent. Incubation time varies between 6 and 12 hr and depends on type of phage and bacteria used. Store at 4^0C.
5. Incubate plates at 4^0C for at least 1 hr before applying filters.

Transferring to nitrocellulose filters

6. Label nitrocellulose filters with a ballpoint pen and apply face down (ink side up) on cold LB plates bearing bacteriophage plaques.
7. Leave filters on plates for 1 to 10 min to allow transfer of phage particles to the filter. During this transfer period the orientation of the filter to the plate is recorded by stabbing a 20-G needle through the filter into the agar at several asymmetric points around the edge of the plate. Remove the filter slowly from the plate with blunt, flat forceps and place face up on paper towels or filter paper.
Make the second replica from each plate. Hybridization both to the DNA probe and comparison the autoradiographs of the replica filters eliminates many possible artifacts.
8. Dry the filters on the benchtop for at least 10 min.

Denaturation and baking

9. Place Whatman 3MM paper on the benchtop and saturate with 0.2 M NaOH/1.5 M NaCl. Place filters on the paper face up for 1 to 2 min.
10. Transfer filters (face up) to 3MM paper saturated with 0.4 M Tris-Cl, pH 7.6/2x SSC for 1 to 2 min and then to 3MM paper saturated with 5x SSC for 1 to 2 min.
11. Dry filters in a vacuum oven 90 to 120 min at 80^0C.

3.2 Hybridization in formamide.

Bacteriophage plaques or bacterial colonies bound to a filter membrane are detected by hybridization with a radioactive probe. Hybridization proceeds on prewet filters placed in plastic box. After hybridization the filters are removed from the plastic box, excess probe is washed off, and the filters are autoradiographed to identify the clones that have hybridized with the probe.

Materials

Nitrocellulose membrane filters bearing plaques.
Hybridization solution I
Radiolabeled probe, 1 to 15 ng/ml
High-stringency wash buffer (1XSSC)
Low-stringency wash buffer (5XSSC)
Water bath adjusted to washing temperature

Incubate filters with probe

1. Wet filters with hybridization solution I. Lay a filter membrane bearing plaques on top of 5 to 20 ml of hybridization solution I and allow solution to seep through filter. It is important to wet only one surface at a time to prevent trapping air in filter. Wet each filter in turn, producing a stack of wet filters.
2. Transfer the stack of wetted filters to an appropriately sized plastic box. Add enough hybridization solution to generously cover filters and close.

3. Prehybridize filters by placing the plastic box at RT for at least 1 hr.

4. While filters are prehybridizing, pipet the radioactive probe into a screw-cap tube, add 2 mg (1 ml) sonicated herring sperm DNA, and boil 10 min. Place boiled probes directly into ice to cool.

5. Add 2 ml hybridization solution I to the boiled probe.

6. Open the plastic box and add probe mixture.

7. Mix probe in the plastic box so that filter is evenly covered and let hybridize overnight.

Wash filters to remove nonhybridized probe

8. Warm 1 liter high-stringency wash buffer I to the "washing temperature" in a water bath.

9. Remove the plastic box containing hybridizing filters from the incubator.

10. Quickly immerse the filters in 500 ml low-stringency wash buffer I at room temperature in a glass baking dish. Separate all the filters, as they may stick together during hybridization.

11. Rinse the filters three times with 500 ml low-stringency wash buffer. Let the filters sit 10 to 15 min at room temperature in low-stringency wash buffer with each rinse.

12. Pour off the low-stringency wash buffer and pour in 500 ml high-stringency wash buffer (prewarmed to washing temperature).

13. Replace the high-stringency wash buffer with another 500 ml of high-stringency wash buffer, then place the glass dish containing the filters in incubator at wash temperature. Make sure that the temperature in the glass dish reaches the desired washing temperature by placing a thermometer directly into the bath and measuring the temperature.

Usually 15 to 20 min at the desired wash temperature is sufficient to remove most of the background radioactivity.

Autoradiographing filters

14. Remove filters and mount them either wet or dry on a plastic backing. If the filter(s) is to be exposed wet, then isolate it from the film by covering it with plastic

3.3 Purification of bacteriophage clones.

Phage plates are correctly oriented to the autoradiograph film, and a region that should contain the clone of interest can be taken from the primary plate with a Pasteur pipet, placed in SM, and this solution used to plate a small secondary library. Plaques on the secondary plates are transferred to nitrocellulose filters, hybridized to ^{32}P-labeled probe, and an isolated positive plaque is picked, diluted in SM, and regrown. This process is repeated until the desired plaque is purified.

Materials

0.7% top agarose
Host bacteria (OD_{600} 1.5 to 2 in 10 mM $MgSO_4$)
LB plates
Suspension medium
Chloroform
Pasteur pipet

Nitrocellulose membrane filters

Growth of secondary plaques

1. Insert the large end of a Pasteur pipet into the top agarose of the primary plate to cut a circular plug corresponding to the region of the autoradiogram demonstrating radioactivity. Remove this plug with the Pasteur pipet and place into 1 ml SM with one drop of chloroform. Allow to sit 1 to 2 hr and then titer. As soon as the titer is known, make 3 to 6 plates with a density of <500 phage per plate. These secondary plaques are handled as in step 4 below.

Screening secondary plaques by hybridization

2. Grow secondary plates at 37°C overnight. Transfer plaques to nitrocellulose filters, process, hybridize, wash, and expose. Mark filter orientation points on the autoradiograph and identify positive plaques on the secondary plates. Insert the small end of a Pasteur pipet into the most strongly hybridizing plaque for each clone and placed a plag into 1 ml SM for 5 min. Plate 1 µl of this phage stock and 1 and 10 µl of a 1:100 dilution onto tertiary LB plates.

3. Screen tertiary plates as above. Insert toothpick into an isolated hybridizing plaque and transfer to SM. This phage stock may be pure, but it is plated and evaluated by hybridization of these plates. If all plaques are positive, make a final SM stock from one of the plaques on these plates. Repeat these steps until the phage is pure.

3.4 PCR amplification of RNA under optimal conditions.

Materials

Total RNA

25 μg/ml cDNA primer in H_2O

3 M sodium acetate, pH 5.5

100% and 70% ethanol

400 mM Tris Cl, pH 8.3

400 mM KCl

Reverse transcriptase buffer

M-MuLV reverse transcriptase

10 mM Tris_Cl/10 mM EDTA, pH 7.5

Phenol buffered with 10 mM Tris_Cl/10 mM EDTA, pH 7.5 (store at room temperature)

24:1 chloroform/isoamyl alcohol

~150 μg/ml amplification primers in H_2O

5 mM 4dNTP mix (5 mM each dNTP in H_2O;)

10x amplification buffer

Taq DNA polymerase

Anneal the primer to the RNA

1. Coprecipitate RNA and cDNA primer by adding the following ingredients to a microcentrifuge tube:

2 μg poly(A)$^+$ RNA

25 ng (3 pmol) cDNA primer

H_2O to 90 µl.

Mix and add 10 µl of 3 M sodium acetate, pH 5.5, and 200 µl of 100% ethanol. Mix and incubate overnight at –20°C or 15 min at -70°C.
This coprecipitation step maximizes the efficiency of annealing. The template source can also be total RNA or crude RNA. Less than 2 µg can be used, depending upon the abundance of the RNA within the sample.

2. Microcentrifuge 15 min at high speed, 4°C, and discard supernatant.
3. Add 200 µl of 70% ethanol and mix gently by inversion. Microcentrifuge 5 min at high speed, room temperature, and discard supernatant. Dry pellet briefly in a Speedvac evaporator. Do not overdry the pellet or the RNA will not resuspend well.
4. Add the following to the RNA pellet:

12 µl H_2O
4 µl 400 mM Tris_Cl, pH 8.3
4 µl 400 mM KCl.

Heat to 90°C, then cool slowly to 67°C. This step removes any base-paired regions in the RNA and then allows the RNA and primer to begin to anneal. The best way to heat and cool the sample is to place the microcentrifuge tube in a 400-ml beaker of 90°C water and allow it to cool to 67°C on the benchtop.
5. Microcentrifuge the sample 1 sec to collect the condensate at the bottom of the tube. Incubate 3 hr at 52°C. Microcentrifuge 1 sec to collect condensate. Perform the centrifugation in this step quickly so the sample does not cool too much. This final annealing temperature can be adjusted

according to the base composition of the primer, and increased or decreased depending on the specificity of annealing required.

Synthesize the cDNA

6. Add 29 µl reverse transcriptase buffer and M-MuLV reverse transcriptase. Mix and incubate 1 hr at 42°C. The temperature can be adjusted from 37° to 55°C if desired. Lower temperatures may be helpful for primers with high A-T base compositions. Higher temperatures may lead to more full-length products by lessening RNA secondary structure.
7. Add 150 µl 10 mM Tris Cl/10 mM EDTA, pH 7.5, and mix. Add 200 µl buffered phenol and vortex briefly. Microcentrifuge 5 min at high speed, room temperature, and save upper aqueous phase.
8. Add 200 µl of 24:1 chloroform/isoamyl alcohol and vortex briefly. Microcentrifuge 5 min at high speed, room temperature, and save upper aqueous phase.
9. Add 20 µl 3 M sodium acetate, pH 5.5, and 500 µl of 100% ethanol. Mix and precipitate overnight at -20°C or 15 min at -70°C.
10. Microcentrifuge 15 min at high speed, 4°C, and discard supernatant.
11. Dry pellet briefly and resuspend in 40 µl water.

Amplify the cDNA by PCR

12. Mix the following:

5 µl cDNA from step 11
5 µl each amplification primer (~150 µg/ml or ~20 µM each)
4 µl 5 mM 4dNTP mix
10 µl 10x amplification buffer

70.5 µl H_2O.

Heat 2 min at 94°C. Microcentrifuge 1 sec to collect condensate.

Usually one of the amplification primers is the same as the cDNA primer. If a different amplification primer is used, the cDNA primer should be removed from the cDNA reaction.

13. Add 0.5 µl (2.5 U) *Taq* DNA polymerase, mix, and microcentrifuge 1 sec.

14. Set up the following automated amplification cycles:

39 cycles: 2 min 55°C
2 min 72°C
1 min 94°C

1 cycle: 2 min 55°C
7 min 72°C

The number of cycles can be varied depending upon the abundance of the RNA. Forty total cycles is sufficient for rare mRNAs in 2 µg poly(A)$^+$ RNA but more cycles may be necessary if smaller amounts of template are used.

15. Analyze products by electrophoresis in agarose or nondenaturing polyacrylamide gels, choosing a concentration of the gel matrix appropriate for the expected size of the amplification products.

3.5 Sequencing analysis.

All sequencing analysis was performed on ABI Prism 377 DNA sequenser. It performs electroforetic separation and spectral detection of dye-labeled DNA fragments. For sequencing applications on ABI Prism 377 DNA sequenser sequencing reactions are performed with fluorescent labels (BigDye Terminator Cycle Sequencing Ready Reaction Kit, Applied Biosystems) and all data are treated by ABI Prism Data Collection 1.1 and ABI Prism Sequencing Analysis 3.3 software.

3.6 The primers used for RT-PCR.

Degenerate primers complementary to the decapeptide coding sequence:

G1 – 5′ CAG CAC TGG TCC CAT GG(GTC) TG 3′

G2 – 5′ CTG GTC CCA TGG (GTC)TG GTA CCC 3′

G3 – 5′ CAT GG(GTC) TGG TAC CCT GG(TA) G 3

Primers for RT-PCR:

oligo(dT)-Ad primer -

5′GACCACGTCTAGATGGACTCAG(T18)(ACG) 3′

Ad primer

5′ GACCACGTCTAGATGGACTCAG 3′

4 Results

4.1 Screening of the genomic library as a method of choice.

To study whether rodents have a second GnRH form with subsequent isolation and sequencing of the complete mouse GnRH-II gene three approaches were considered: 1. Low-stringency hybridization screening of a mouse genomic library with the human cDNA probe; 2. Low-stringency hybridization screening of cDNA libraries with the same probe; 3. RT-PCR and rapid amplification of cDNA using degenerate deoxyoligonucleotide primers complementary to the sequence encoding the ten amino acids of GnRH-II in order to get partial coding sequence for further manipulations.

A genomic library in preference to cDNA libraries has been chosen to screen for GnRH-II-encoding DNA sequences in anticipation of the small number of cells expressing the hormone, a low expression level of GnRH-II, possible long 3'-end of mRNA, existing ambiguities in determining the hormone localization in the mouse brain. (Reexamined immunocytochemical data (Gestrin *et al.*, 1999) concerning the distribution of GnRH-II in the mouse brain show that GnRH-II-immunopositive cells are located in areas surrounding each of the cerebral ventricles and in areas adjacent to the hippocampus.)

I4.1.1 Screening of the mouse genomic library with the "long" human cDNA probe.

Partial human cDNA 390 bp in length encoding GnRH-II precursor cloned into the *EcoR V* site of pBSII SK (courteously donated by John P. Adelman) and spanning from exon 1 to exon 4 was sequenced in both directions and used to obtain a 329 bp PCR fragment for radiolabeling. Primers were located on the sequence for the signal peptide (5`primer) and on the boundary between exon 3 and 4 (3`primer). From the total length of the PCR product 159 bp coincided with exon 2 coding signal peptide, decapeptide sequence and 5` end of GAP (GnRH-associated peptide), 158 bp with exon 3 and 12 bp with exon 4 **(Figure 5)**.

Radiolabeling was performed using a hexanucleotide random primered labeling kit (Roche Molecular Biochemicals). The specific activity of probe was $1,5X10^9$ cpm/µg of DNA. Recombinant phage-infected *E. coli* cells (strain LE 392) were plated onto 30 plates (15-cm in diameter) to give 30,000 phage plaques of mouse Lambda FixII genomic library (Sratagene) per plate. Two replicas from each plate were made and hybridized with a ^{32}P-labeled PCR-derived probe ($1.25X10^6$ cpm of probe per filter). Probe hybridization was performed in 20% formamide/1x standard hybridization buffer at room temperature for 20 hours and filters were washed in 1X SSC at 55°C. These washing conditions eliminate the unspecific binding of probe to unrelated targets without removing putative positive signals, and so corresponded to the main prerequisite of low-stringency hybridization screening strategy – to "fish" a nucleic acid sequence similar to the probe. Out of approximately 50 hybridization signals obtained in the first round 12 were double positive and 11

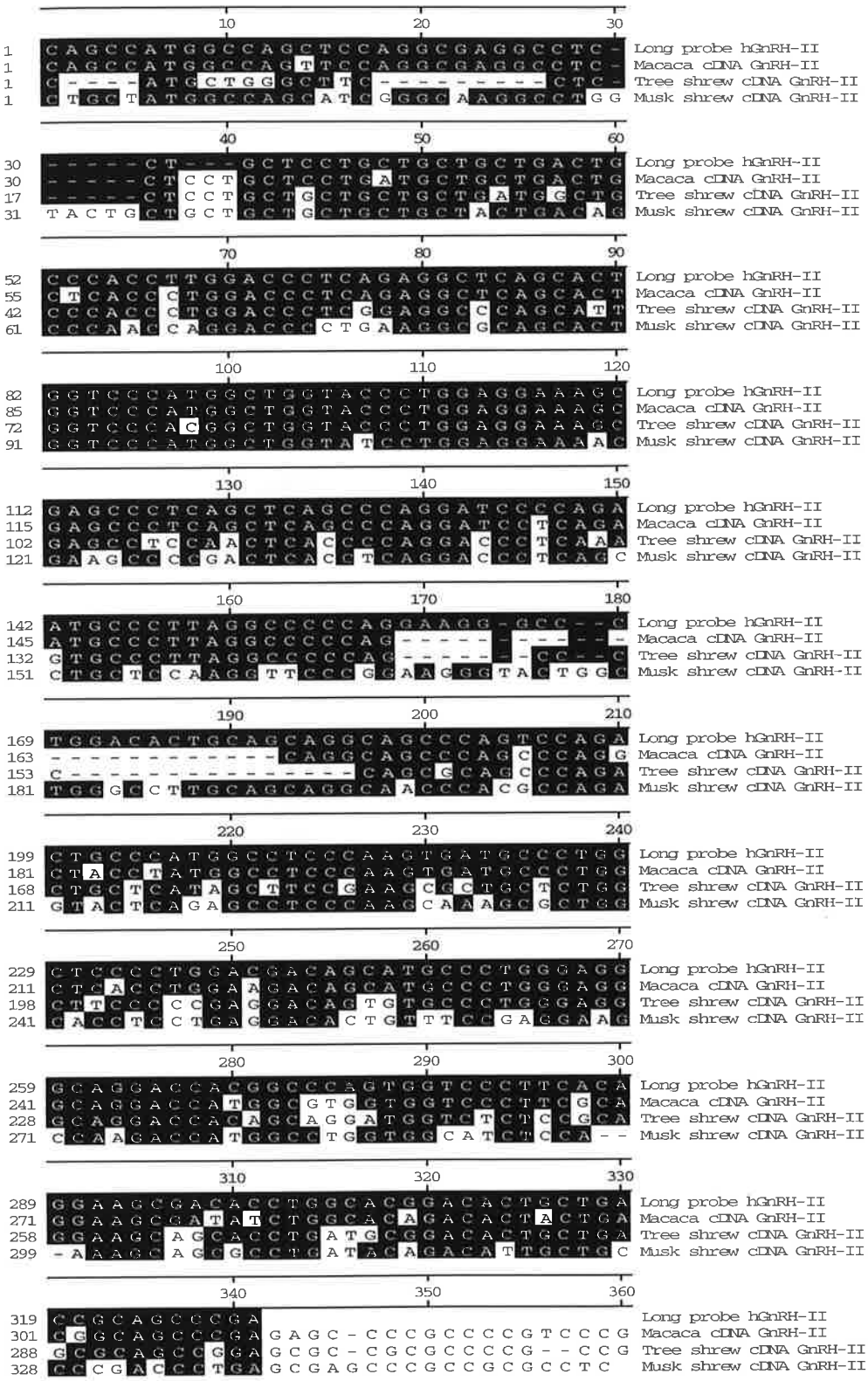


Figure 5. Alignment report of the human cDNA GnRH-II probe and known mammalian cDNA sequences for GnRH-II precursors using Clustal method with Weighted residue weight table. For the human cDNA GnRH-II: locations 1..159-exon 2, 160..317-exon 3, 318..329-exon 4, .75..104 sequence for GnRH-II, 105..113-proteolytic cleveage site, 160..181- 7 aa splice variant.

bacteriophage clones (clone No7 disappeared in the second round) corresponding to these signals were purified after three rounds of plating Genomic inserts from lambda DNA prepared by polyethylene glycol (PEG) sedimentation followed by phenol extraction were isolated with *Sal* I and subcloned into pBSII SK using a shotgun approach.

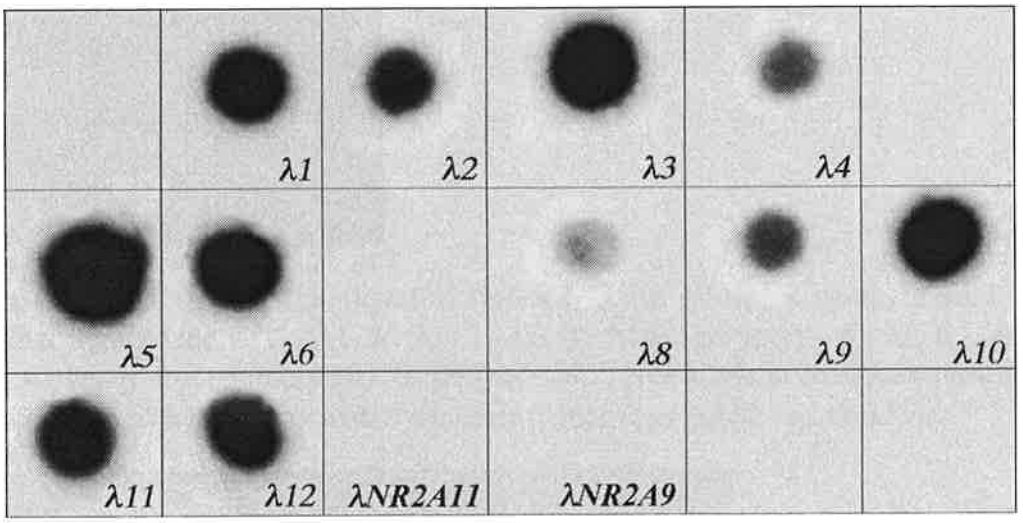


Figure 6. Dot-blot of all lambda clones obtained in the screening of the mouse genomic library with hGnRH-II cDNA probe. On each spot was loaded approximately 100-200 ηg of lambda DNA as was estimated by previous loading DNA on agarose gel. For negative control lambda DNA bearing cloned fragments of NR2A subunit was used. Washing conditions were the same as for screening of the library - 1xSSC,50°C.

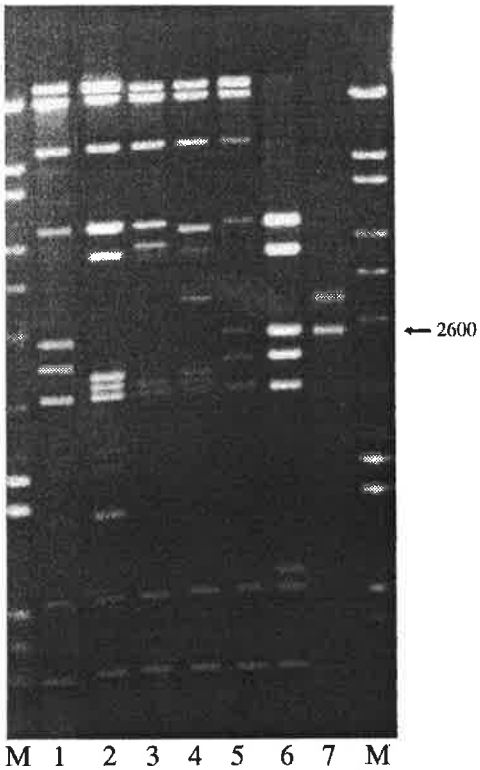


Figure 6. *SacI-XbaI* and *SacI* digest of obtained λ and pBSII SK clones group I. *SacI-XbaI* digest: lane **1** (**1**) - λ1, **2** - λ3, **3** - λ6, **4** - λ10. *SacI* digest: **5** - λ1, **6** - full-length λ1 insert cloned into pBS, **7** - pBS 1.7.26.2. Black arrow designats location 2600 bp fragment hybridizing with the human GnRH-II probe.**M** - marker lane.

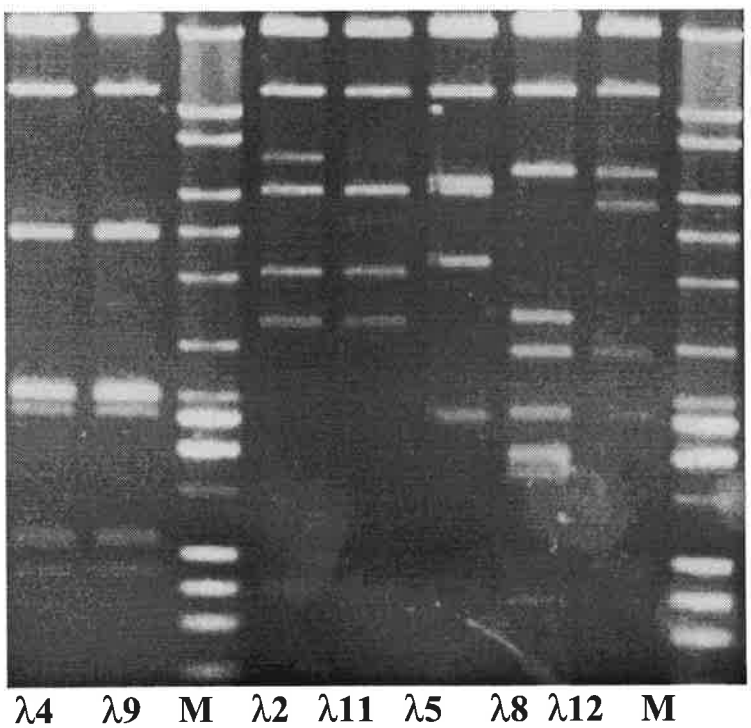


Figure 7. *SacI-XbaI* digest of obtained λ clones in the screening of the mouse genomic library with human cDNA probe.

Restriction mapping analysis showed that the clones could be divided into five distinct groups. Groups I-IV contained overlapping clones and group V included single clones **(Table 2)**.

No of group	No of clones
I	1,3,6,10
II	2,11
III	4,9
IV	8,12
V	5

Table 2. The obtained clones were divided according to restriction analysis.

A dot blot of the isolated lambda clones was used to analyze the relative hybridization to the probe **(Figure 6)** Groups No I and No V had the strongest affinity to the probe and the washing condition could be raised up to 65°C/1xSSC without noticeable weekening of the signal. This was unacceptable for group No III (N4 and N9).To get insight into the nature of nucleic acid sequences the obtained clones were digested with sets of restriction enzymes and the smallest fragments with the human GnRH-II probe were subcloned into pBSII SK vector and sequenced with T3 and T7 primers (Big Dye sequencing kit, Applied Biosystems). Unexpectedly in two of these clones (groups No I and No 5, the "strongest" among all clones) the enzimes responsible for obtaining a smoller hybridizing fragment were not found and later sequence analysis reveal the main cause of it. Four groups turned out to contain trinucleotide repeats – CTG/CTC resembling the part of the GnRH II gene that codes for the signal peptide distinguished by high contents of hydrophobic leucine residues – a trait common for signaling peptides.

Table 3 summarizes the attemps to subclone the shortest hybridizing fragments which were partially or completely sequenced.

No of group	Subclon	Subcloned fragment	Size,kB	Occurrence of CTG/CTC repeats	High G/C content
I	1.7.26.2	*SacI*	2.6	+	+
II	2.9.1200	*SacI-EcoRI*	1.2	+	+
III	4.7 st	*Xba I*	0.95	-	+
IV	12.19.6	*PvuII*	1.4	+	+
V	5s7	*BfaI-ApaI*	2.6	+	+

Table 3.

Alignment of the sequenced fragments showed a great degree of similarity among them **(Figure 8)**, and only to the part of the human cDNA for the GnRH-II coding leader peptide had such significant similarity to all of them **(Figure 9)**

Figure 8. Aligment of the sequenced hybridizing fragments subcloned from positive lambda clones obtained in the screening of the mouse genomic library with the long human GnRH-II probe

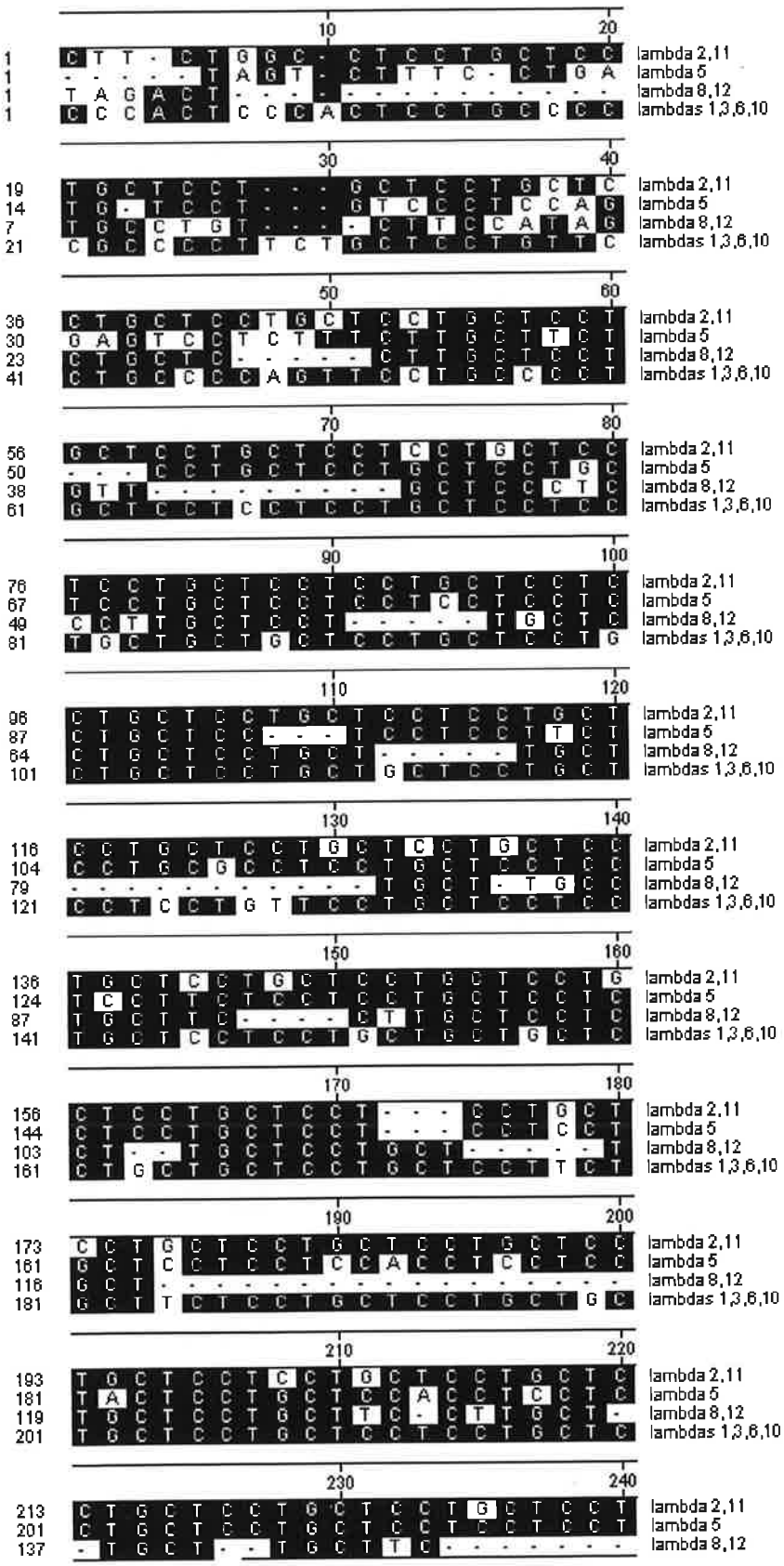
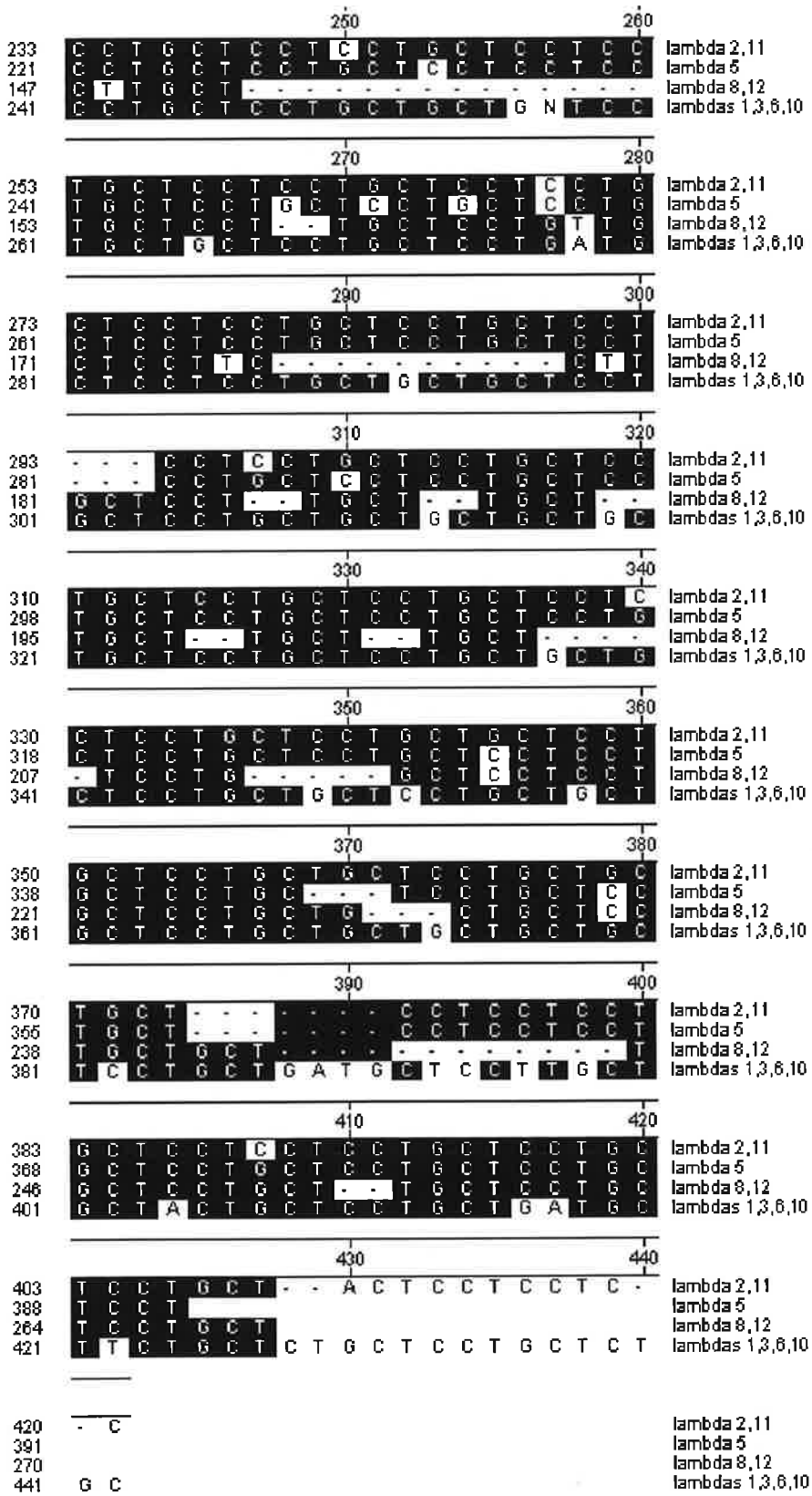


Figure 8 (continued)



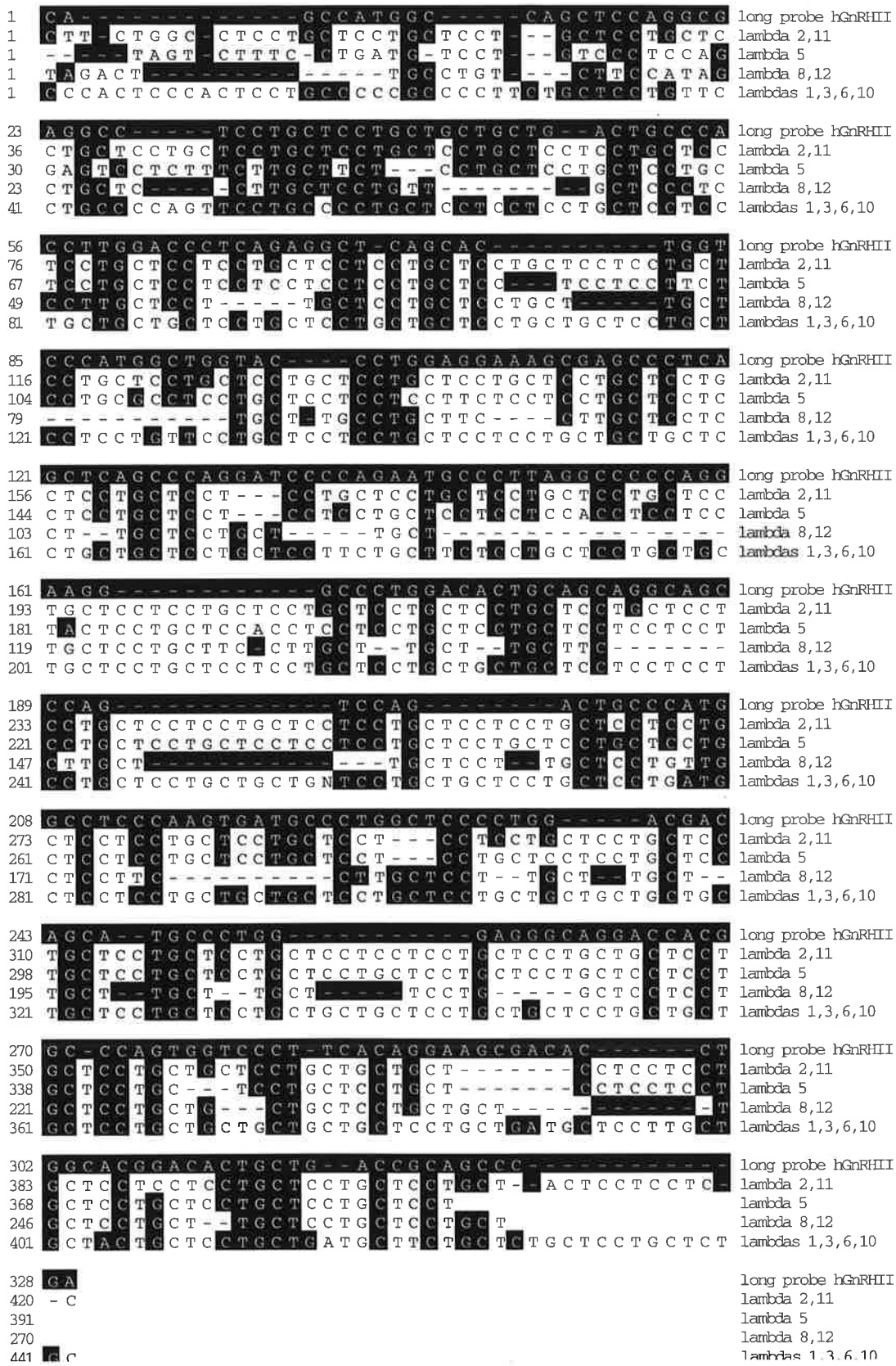
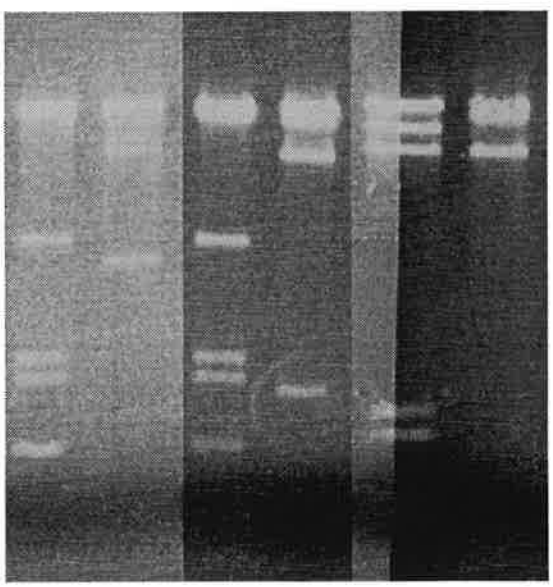


Figure 9. Alignment report of alignment human cDNA deraived GnRH-II probe with the subclones obtained from the screening of mouse genomic library. The only part of the probe which matches with the lambda subclones in noticeable degree is the sequence coding the leader peptide of GnRH-II precursor.

4.1.2 Screening of the mouse genomic library with the human cDNA probe lacking the coding sequence for signaling peptide.

To avoid interference of the codinng sequence for the signal peptide a new probe lacking this sequence was used for the second screening of the mouse genomic library (mouse Lambda FixII genomic library, Stratagene). For this purpose a PCR fragment was generated for labeling with a 5`primer matching the decapeptide sequence, and a 3'primer that was the same as for the first screening. The PCR fragment was sequenced and radiolabeled with a hexanucleotide random primered labeling kit (Roche Molecular Biochemicals). Specific activity of probe was about $2X10^9$ cpm/μg. Screening of the mouse genomic library was performed



1 2 3 4 5 6

Figure 10. Lambda clones obtained in the second screening of the mouse genomic library with the human cDNA probe lacking the coding sequence for signaling peptide. *HindIII* digest: lanes 1, 3, 5 - λ6 , λ11, λ9 accordingly; *SalI* digest:lanes 2, 4, 6 - λ6 , λ11, λ9 accordingly .

as with the previous probe and 3 clones were isolated. Restriction mapping revealed that two of them were overlapping clones that contained an internal *SalI* site **(Figure 10)**. The sequencing of the smallest hybridizing fragments cloned into pBSII SK showed no significant similarity to GnRH-II sequence. Presumably the high GC contents of the obtained clones could have evoked unspecific hybridization.

4.2 RT-PCR on total mouse and rat brain and kidney RNA.

Total RNA was extracted from fresh whole brain and kidney of mouse and rat using Tri reagent (Molecular Research Center, Inc.), based on the acid guanidinium thiocyanate–phenol–chloroform extraction method.
Total RNA was reverse transcribed with a bipartite 41mer oligo(dT)-Ad primer which consisted of 3′(TCG)-oligo$(dT)_{18}$ downstream part and 5′ upstream 22mer Ad primer (the primer sequences are listed in the "Materials and methods") using Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase and was amplified by PCR. 5′degenerate primers (G1, G2, G3) were designed according to available information on nucleotide sequences of mammalian GnRH-IIs and nested on the decapeptide coding sequence – the most conserved region among them. The common Ad reverse primer represented the 22mer 5′part of the oligo(dT)-Ad primer used for the reverse reaction. Tree primers G1 – 5′ CAG CAC TGG TCC CAT GG(GTC) TG 3′, G2 – 5′ CTG GTC CCA TGG (GTC)TG GTA CCC 3′, G3 – 5′ CAT GG(GTC) TGG TAC CCT GG(TA) G 3′ allowed to accomplish three combinations of two rounds of "seminested" PCR: (i) PCR1 - G1/Ad, PCR2 – G2/Ad; (ii) PCR1 – G1/Ad, PCR2 – G3/Ad; (iii) PCR1 – G2/Ad, PCR2 – G3/Ad. After

amplification the PCR products were electrophoretically separated on an agarose gel and stained with ethidium bromide. Nine distinct bands ranging from 180 bp to 650 bp were cut from the gel, purified with gel extraction kit (Qiagen) and cloned into the *EcoRV* site of pBSII SK(-) vector. Sequencing of obtained cDNA clones with T3 and T7 primers revealed neither an occurrence of a dibasic recognition sequence serving as a proteolytic cleavage site between decapeptide and GnRH-associated peptide (GAP) nor any significant similarity with GAP sequences, and therefore all clones were considered to be false.

4.3 RT-PCR on total human brain RNA.

In order to see if the nested RT-PCR protocol could lead to cloned cDNA for GnRH-II it was decided to undertake an attempt to amplify the human GnRH-II cDNA, preserving all conditions applied in the RT-PCR from total mouse and rat brain and kidney RNA. Human brain mRNA was considered to be an appropriate because of the known low expression level of GnRH-II in the brain compared to hGnRH-I in the brain and hGnRH-II in the kidney and because of existence two splice variants of mRNA for GnRH-II in the brain.

Total cerebral human RNA (Stratagene, age-57, male) was reverse transcribed using the bipartite 41mer oligo(dT)-Ad primer and the M-MuLV reverse transcriptase and was amplified by PCR with the same set of upstream primers used previously. In the first round of amplification with primer set G2/Ad no distinct bands were visible on agarose gel. 1µl from this reaction was dissolved in 100µl H_2O and in turn 1µl of this solution was used as template DNA in the second round of amplification

with the primer set G3/Ad. After 35 cycles of amplification (94°C - 20 sec, 54°C - 20 sec, 72°C - 40 sec) a band of aproximately 300 bp was visible on the gel **(Figure 11)**. *Sph1* digest of the PCR product generated two bands -180 bp and 120 bp as was expected from the restriction map of human GnRH-II cDNA.

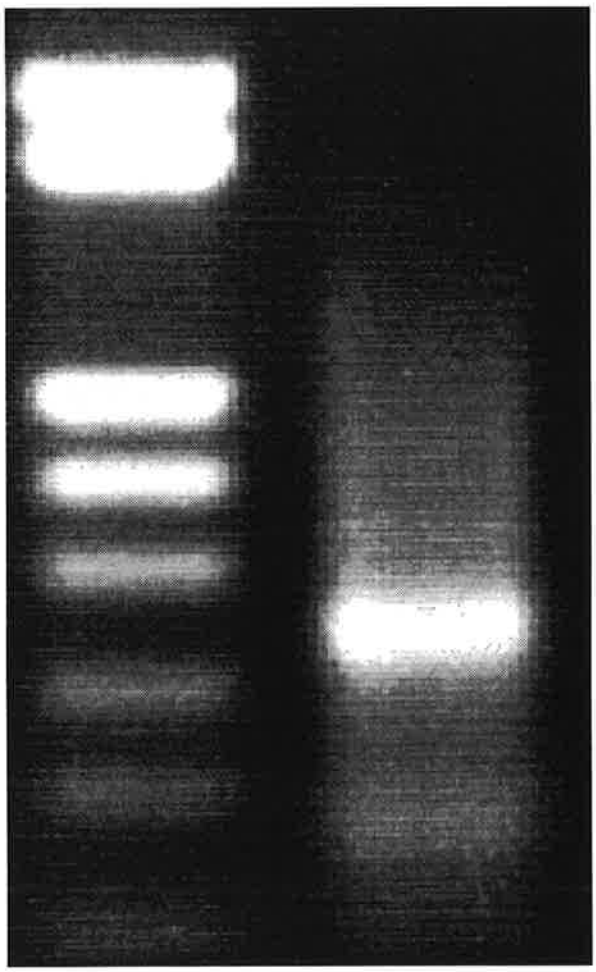


Figure 11. In the second round of PCR on human brain total cDNA with G3/Ad sets primer bands in 300 b. p. was obtained.

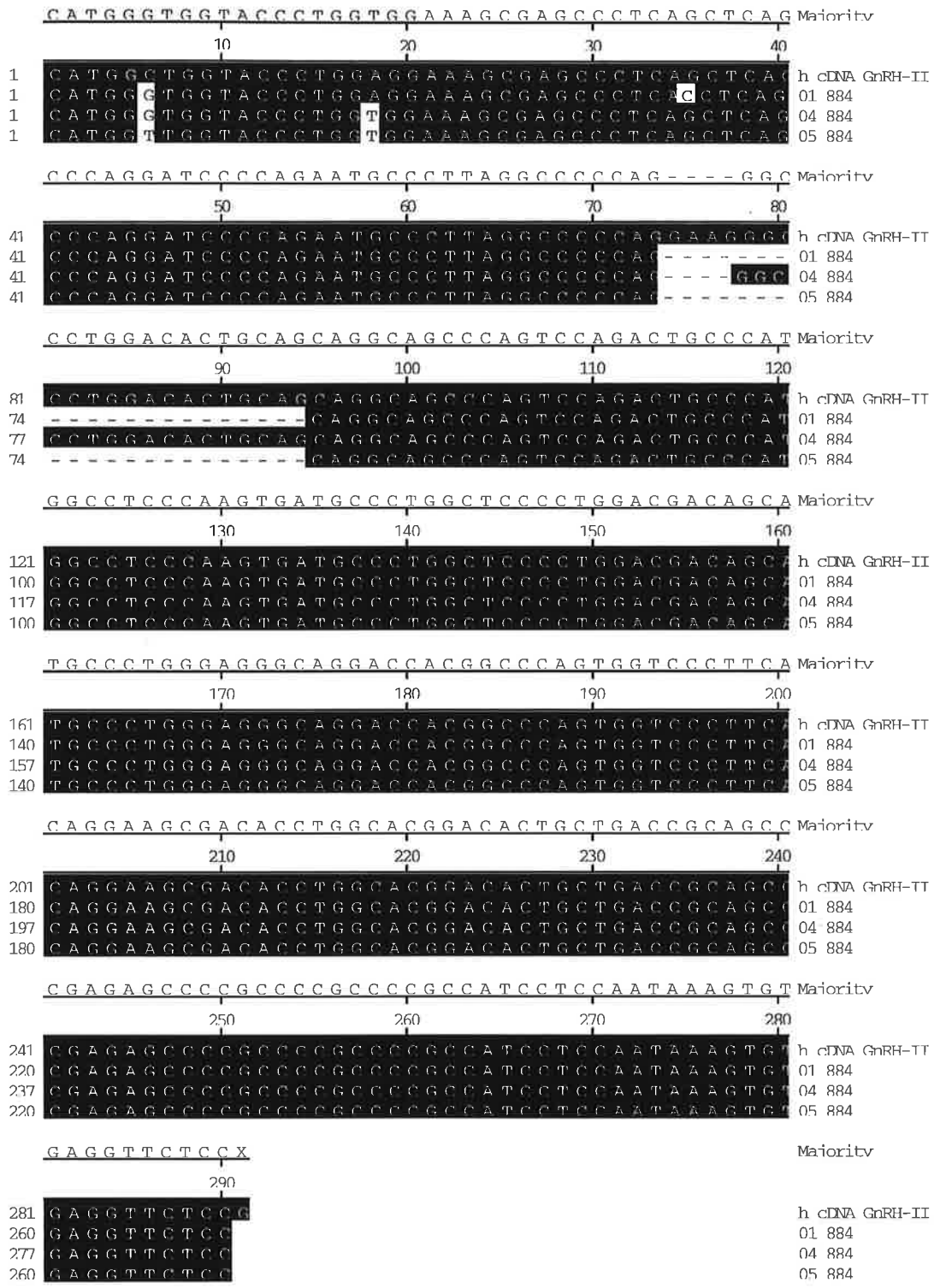


Figure 12.Alignment of the human cDNA GnRH-II with three individual clones directly sequenced after colony PCR with T3 and T7 primres. Four-nucleotide discrepancy at position 74-77 might be explained as a consequence of the recombination during 95 cycles of the PCR (35 cycles for PCRI, 35 cycles for PCRII and 25 cycles for colony PCR). 21-nucleotide gap at position 74-94 is the known splice variant for human mRNA GnRH-II (White et. al.,1998)

The PCR product was phosphorylated with T4 polynucleotide kinase, end-trimmed by T4 polymerase and cloned into the pBS EcoRV site. Three cloned fragments were directly sequenced after colony PCR with T3 and T7 and both known splice variants were obtained **(Figure12).**

This showed that (i). these degenerate decapeptide-nested primer were srecific to the human cDNA GnRH-II sequence under applied conditions; (ii) using the bipartite 41mer oligo(dT)-Ad primer in the reverse transcriptase reaction allowed to create convinient downstream site for accomplishing of the PCR with 22mer Ad primer.

5 Discussion

In the framework of this diploma thesis the rodent GnRH-II gene was not found and it's existence in rodents remains a mystery.

At this time reverse genetics, in which detailed knowledge of a gene of interest permits in vivo modification of its expression or function, becomes a powerful method for examining the physiological role of many proteins. From a historical point of view the cloning of the GnRH-I gene enabled one of the first demonstrations of the prospect for gene therapy when a GnRH transgene introduced by pronucleus injection into hypogonadal mice was shown to rescue the infertile phenotype (Seeburg PH *et al*, 1987). Probably, unravelling the genetic nature of the mysterious GnRH-II might help to unveil the evolutionary conundrum of the structural conservation of this polypeptide and shed light on the functional reasons restricting sequence diversions through the long history of the vertebrate evolution. As detailed in the introductory section, hypothalamic forms of GnRH vary among all groups of vertebrates at 5 amino acids. With striking contrast to this variation the decapeptide expressed by the mesencephalic cells is identical in all species in which the sequence has been directly determined or deduced from isolated cDNAs. Only in one vertebrate class - jawless fish - this form has not been found. It is difficult to imagine that rodents could have lost this gene during evolution. The sequence conservation of the mesencephalic form during 500 million years of vertebrate evolution could multiple, important

functions and therefore might require strict physiological constraints. Presumably, these roles might differ from the execution of gonadotropic functions, also indicated by the mesencephalic localization and the high expression level outside the brain.

In order to clone the mouse GnRH-II gene a genomic library was screened with human GnRH-II cDNA. Unfortunately all identified positive clones turned out to be false. Recent immunocytochemistry data support with a high degree of probability the occurrence of GnRH-II in mouse and rat. One of the reasons for not finding the gene could lie in the nature of this gene - only the sequences coding for the decapeptide and the cleavage site from GAP are conserved among different species. As seen from the screening of the mouse genomic library, the human cDNA probe tended to recognise the long CTG/CTC three-nucleotide genomic repeats apparently not only by virtue of occurrence of the ordered CTG/CTC repeats coding for the leucine stretch in the leader peptide but also because of the overall high G/C content of the probe. Out of 329 nucleotides of the probe 66.9% are G+C, out of 74 leader peptide nucleotides - 67.6%, out of 21bp of leucine stretch exactly two-third (66.7%) and out of GAP (216 bp) - 67.6% are G+C. The "lightest" part is the 39 bp coding for GnRH-II and cleavage site with only 61.5% of G+C.

Taking into account that CTG/CTC sequences might run several thousand bp it is easy to suppose that the hybridising signal arising under low stringency conditions would be stronger than from the true counterpart sequences. Attempts to avoid interference of the coding

sequence for the leader peptide by shortening the 5` end of the human cDNA used as a probe showed that this hypothesis makes sense because all clones obtained in the second screening had a weaker signal compared to clones isolated from the first library. In spite of this, all positive clones from the second screening did not contain the target sequence for GnRH-II. Finding two overlapping clones shows that they were not isolated by chance. Presumably, GAP's CCCA and CCCCG repeats were responsible for this result.

As alternative RT-PCR approach might be useful for cloning partial GnRH-II cDNA as was shown in the case of the human GnRH-II. Presumably there are several reasons for the failure with this method. The quality of the synthesised mouse and rat cDNA was not checked for rare mRNAs (for instance, GnRH-I). Any altered structure (length of the 3`end) also can preclude finding the GnRH-II gene. It seems to be that the RT-PCR approach after optimisation of the conditions might be the easiest and most straightforward method to clone mouse GnRH-II cDNA.

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