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Authors: Okubo, Kataaki, Suetake, Hiroaki, and Aida, Katsumi

Source: Zoological Science, 16(4) : 645-651

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.16.645

ZOOLOGICAL SCIENCE **16**: 645–651 (1999)

A Splicing Variant for the Prepro-Mammalian Gonadotropin-Releasing Hormone (Prepro-mGnRH) mRNA is Present in the Brain and Various Peripheral Tissues of the Japanese Eel

Kataaki Okubo*, Hiroaki Suetake and Katsumi Aida

Department of Aquatic Bioscience, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo, Tokyo 113-8657, Japan

ABSTRACT—We have previously demonstrated that the Japanese eel, *Anguilla japonica*, has two distinct mRNAs for prepro-mammalian gonadotropin-releasing hormone (prepro-mGnRH) and prepro-chicken GnRH-II (prepro-cGnRH-II). In the present study, we found a splicing variant for the prepro-mGnRH mRNA, but not for the prepro-cGnRH-II mRNA in the eel. Genomic Southern blot analysis revealed that each of the eel prepro-GnRHs is encoded by a single gene. Isolation and sequencing of the two prepro-GnRH genes revealed that both of them contain four exons (denoted 1, 2, 3 and 4) separated by three introns (denoted A, B and C). Staining with ethidium bromide and Southern blotting following reverse transcription-polymerase chain reaction and electrophoresis revealed that in addition to a mature prepro-mGnRH mRNA, its splicing variant with retention of intron A is present in all tissues tested including the brain, pituitary, eye, olfactory epithelium, ovary, testis, liver, kidney, spleen, heart, gill, intestine, pancreas, muscle, skin, fin and peripheral blood leukocyte. This result differed to that of mammals, the splicing variant of which is present only in the peripheral tissues, but not in the brain. In contrast, only a mature mRNA for the prepro-cGnRH-II was detected in the middle part of dissected brain, pituitary, olfactory epithelium, ovary and testis, indicating that the two prepro-GnRH genes have different splicing processes. These findings suggested that the splicing pattern which produces the intron A retention variant dates from a common ancestor to teleosts and mammals, and is conserved during evolution. Therefore the splicing may be one of the mechanisms regulating prepro-mGnRH gene expression.

INTRODUCTION

Gonadotropin-releasing hormone (GnRH) was originally isolated from mammalian hypothalamus as the physiologic stimulator of gonadotropin release from the pituitary gland (Matsuo *et al*., 1971; Amoss *et al*., 1971). It is a decapeptide synthesized as part of a larger precursor protein termed prepro-GnRH which contains another peptide, GnRH-associated peptide (GAP) (Adelman *et al*., 1986). The structural organization of all prepro-GnRH genes identified so far consists of four exons (denoted 1, 2, 3 and 4) separated by three introns (denoted A, B and C) (see Sherwood *et al*., 1997; Suetake and Aida, 1997; White *et al*., 1998; White and Fernald, 1998). To date, twelve distinct molecular forms of GnRH have been identified in vertebrates and a protochordate (Powell *et al*., 1996; see Amano *et al*., 1997; Jimenez-Linan *et al*., 1997). In addition, it has been suggested that all vertebrates have multiple molecular GnRH forms, one of which is termed chicken GnRH-II (cGnRH-II). The coexistence of multiple GnRH forms in a single species raises questions concerning their respective regulation and physiological function.

Prepro-GnRH gene expression has also been found in several peripheral tissues and various parts of brain besides the hypothalamus, indicating paracrine or autocrine role(s) for GnRH. Furthermore, in mammals, a prepro-GnRH mRNA variant with retention of intron A has been described in several tissues including the placenta, mammary gland, testis and ovary (Seeburg and Adelman, 1984; Radovick *et al*., 1990; Goubau *et al*., 1992; Dong *et al*., 1993; Dong *et al*., 1996). There is, however, no evidence for the presence of the splicing variant in lower vertebrates.

We have previously demonstrated that the Japanese eel, *Anguilla japonica*, has two distinct mRNAs for the prepro-mammalian GnRH (prepro-mGnRH) and prepro-cGnRH-II, and that both genes are expressed in various tissues (Okubo *et al*., 1999). The eel is one of the most primitive living teleost species and, therefore, is a good animal model to study the functions of GnRH and the structure and expression of its gene in

* Corresponding author: Tel. +81-3-5841-7564;
FAX. +81-3-5841-8165.
E-mail: aa86110@mail.ecc.u-tokyo.ac.jp

an evolutionary point of view. In the present study, we found a splicing variant for the prepro-mGnRH mRNA, but not for the prepro-cGnRH-II mRNA, in the eel by staining with ethidium bromide and Southern blotting following reverse transcription-polymerase chain reaction (RT-PCR) and electrophoresis.

MATERIALS AND METHODS

Genomic Southern blot analysis

Genomic DNA was extracted from peripheral blood erythrocyte of an individual eel and digested with either *Apa*I, *Eco*RI or *Hin*dIII. It was then electrophoresed on a 0.9% agarose gel and transferred to nylon membranes (Hybond-N^+, Amersham, UK). The membranes were prehybridized at 42°C for 3 hr in 6×SSC containing 50% formamide, 1×Denhardt's solution, 0.5% SDS and calf thymus DNA (100 ng/ml). Two cDNA probes were generated and radiolabeled with [α-^{32}P] dCTPs (Amersham Pharmacia Biotech, USA) from the prepro-mGnRH and prepro-cGnRH-II cDNA clones by PCR amplification using a GeneAmp PCR System 2400 (Perkin Elmer, Norwalk, CT, USA). The prepro-mGnRH cDNA probe of 217 bp was generated with the sense (5'-GGCAAGAGAGAGGAGCAGATAGT-3') and antisense primers (5'-GTCATGTTTTATTGAAG-GAATGC-3'), which are located in the N-terminus of the GAP and the 3'-untranslated region (3'-UTR), respectively. The prepro-cGnRH-II cDNA probe of 225 bp was also generated with the sense (5'-AGAATGCAGCT-ACTTGAGACC-3') and antisense primers (5'-TAGAGCGAGTCATA-GGAGAC-3'), which are located in the middle portion of the GAP and the 3'-UTR, respectively. These probes did not overlap any of the three restriction sites used to digest the genomic DNA. Membrane-bound DNA digested with *Apa*I or *Eco*RI was hybridized in the prehybridization buffer at 42°C for 18 hr, with the prepro-mGnRH cDNA probe. Membrane-bound DNA digested with *Eco*RI or *Hin*dIII was also hybridized with the prepro-cGnRH-II cDNA probe. These membranes were washed in 1×SSC containing 0.1% SDS at room temperature for 10 min, and washed twice in 0.1×SSC containing 0.1% SDS at 65°C for 10 min. They were then exposed to Fuji X-ray film (Fuji Film, Tokyo, Japan) at –80°C for 2 days.

Isolation and characterization of the prepro-GnRH genes

The eel genomic DNA extracted as described above was amplified in a reaction volume of 20 µl containing 1×PCR buffer (Takara, Otsu, Shiga, Japan), 200 µM of dNTPs (Takara), 0.5 U *Taq* DNA polymerase (Takara) and 0.5 µM each of sense and antisense strand primers with a cycle protocol as follows: denaturation at 94°C for 5 min, followed by 40 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. The final elongation step was carried out at 72°C for 7 min. The sense (5'-AGACTGACAGAGAAGAGAACGC-3') and antisense primers (5'-GTCATGTTTTATTGAAGGAATGC-3') to amplify the prepro-mGnRH gene fragment are located in the 5'-UTR and 3'-UTR, respectively. The sense (5'-ACATCCACACAGCGAC-TCTG-3') and antisense primers (5'-AATATAGAGTTGCAGTGTG-TGTCAG-3') to amplify the prepro-cGnRH-II gene fragment are located in the 5'-UTR and 3'-UTR, respectively. One half of each PCR reaction mixture was analyzed on a 2% agarose gel and stained with ethidium bromide. The cDNA fragments were ligated into pBluescript II SK(–) vector (Stratagene, La Jolla, CA, USA). The plasmid DNA was purified, and both strands of it were sequenced with T3 or T7 primer, using a SQ-5500 DNA sequencer (Hitachi, Tokyo, Japan).

RT-PCR and Southern blot analysis

Four immature eels of both sexes, body weight 285–357 g and gonadosomatic index 0.16–0.32, were anesthetized in 0.06% 2-phenoxyethanol and sacrificed by decapitation. The brain was removed and divided into three parts: part A, olfactory bulb and telencephalon; part B, diencephalon, optic tectum and cerebellum; part C, medulla oblongata and spinal cord (Fig. 1). Peripheral blood leukocyte (PBL) was collected as described previously (Okubo *et al.,* 1999). Total RNA from the brain part A, B, C, pituitary, eye, olfactory epithelium, ovary, testis, liver, kidney, spleen, heart, gill, intestine, pancreas, muscle, skin, fin and PBL was isolated using the RNA extraction solution, ISOGEN (Nippongene, Toyama, Japan), according to the manufacturer's instructions. Each total RNA was treated with DNase (Promega, WI, USA). Four micrograms of total RNA was reverse transcribed using Ready-To-Go™ T-primed first-strand cDNA synthesis kit (Amersham Pharmacia Biotech, USA) according to the manufacturer's instructions. One microliter of the cDNA synthesis reaction was amplified by PCR with the same cycle protocol as described above. The sense (5'-AGACTGACAGAGAAGAGAACGC-3') and antisense primers (5'-GTCATGTTTTATTGAAGGAATGC-3') were used to amplify the prepro-mGnRH gene transcript. The sense (5'-GCTTTCAGTGCCCGACATAGG-3') and antisense primers (5'-GGATACAGTTGCTATGTGAAG-3') were used to amplify the prepro-cGnRH-II gene transcript. Both of these two primer sets are located in the exons 1 and 4. One half of each PCR reaction mixture was analyzed on 2% agarose gels and stained with ethidium bromide. For negative control, PCRs were also conducted with the absence of cDNA template to examine if PCR products result from cross-contamination of sample.

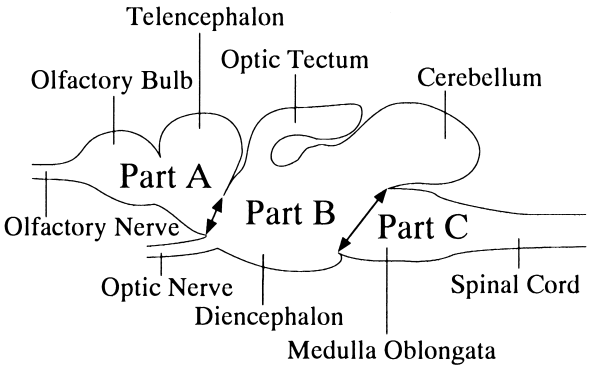

Fig. 1. Dissection diagram of the eel brain. The brain was divided into three parts: part A, olfactory bulb and telencephalon; part B, diencephalon, optic tectum and cerebellum; part C, medulla oblongata and spinal cord.

The PCR products in the gels were transferred to nylon membranes (Hybond-N^+) for Southern blot analysis. The prepro-mGnRH cDNA-specific probe of 278 bp was generated with the sense (5'-GTCCATCTGACAAGAATGGC-3') and antisense primers (5'-CGTCCAGTTTCTCTGTCAGCCAG-3'), and the prepro-cGnRH-II cDNA-specific probe of 311 bp was generated with the sense (5'-CTTTAGCTGTGATGGTGAAC-3') and antisense primers (5'-GGATACAGTTGCTATGTGAAG-3'). Labeling of the probes, prehybridization, hybridization and washing were carried out as described above. The membranes were then exposed to Fuji X-ray film (Fuji Film, Tokyo, Japan) at –80°C for 1 hr.

Two cDNA fragments of 565 and 367 bp obtained with the prepro-mGnRH cDNA-specific primer set and a 452 bp fragment obtained with the prepro-cGnRH-II cDNA-specific primer set were subcloned and sequenced as described above to confirm their authenticity as prepro-GnRH cDNA amplicons.

RESULTS

Genomic Southern blot analysis

Two probes specific to the prepro-mGnRH or prepro-cGnRH-II cDNA sequence were used for genomic Southern blot analysis in order to examine the copy number of the

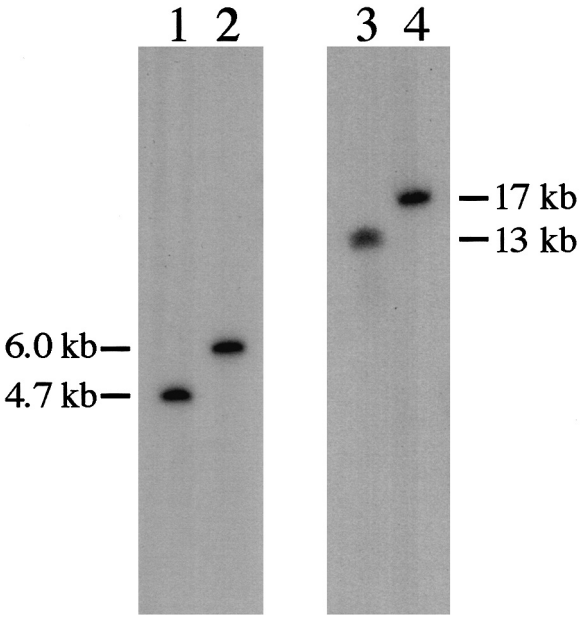

Fig. 2. Genomic Southern blot analysis of the prepro-mGnRH and prepro-cGnRH-II genes of the eel. The eel genomic DNA digested with *Apa*I (lane 1), *Eco*RI (lanes 2 and 3) or *Hin*dIII (lane 4) was electrophoresed and hybridized with the prepro-mGnRH cDNA-specific probe (lanes 1 and 2) or prepro-cGnRH-II cDNA-specific probe (lanes 3 and 4). The sizes of detected bands are indicated.

prepro-mGnRH and prepro-cGnRH-II genes. Digestion of the genomic DNA with *Apa*I and *Eco*RI was followed by hybridization with the prepro-mGnRH cDNA-specific probe. Digestion with *Eco*RI and *Hin*dIII was followed by hybridization with the prepro-cGnRH-II cDNA-specific probe. The Southern blots revealed a single band at different size in each lane (Fig. 2). Hybridization with the prepro-mGnRH cDNA-specific probe gave a single band of approximately 6.0 and 4.7 kb for *Apa*I and *Eco*RI digestions, respectively. Hybridization with the prepro-cGnRH-II cDNA-specific probe gave a single band of approximately 17 and 13 kb for *Eco*RI and *Hin*dIII digestions, respectively.

Isolation and characterization of the prepro-GnRH genes

We isolated and characterized the eel prepro-mGnRH and prepro-cGnRH-II genes in order to determine their exon/intron organizations. The nucleotide sequences of these genes are shown in Fig. 3. The exon/intron organization of these genes was defined by comparing them with their respective cDNAs reported previously (Okubo *et al*., 1999). The coding sequences of the genes are perfectly identical to the sequences of their respective cDNAs. Both the prepro-mGnRH and prepro-cGnRH-II genes have four exons separated by three introns (Fig. 4). Exon 1 of each prepro-GnRH gene contains only a 5'-UTR. Each exon 2 encodes a signal peptide followed by the GnRH decapeptide, a Gly-Lys-Arg processing site and the N-terminus of a GAP (11 and 9 amino acids for the prepro-mGnRH and prepro-cGnRH-II respectively). Each exon 3 encodes the middle portion of the GAP (32 and 28 amino acids for the prepro-mGnRH and prepro-cGnRH-II respectively). Each exon 4 contains the C-terminus of the GAP (13 amino acids for each prepro-GnRH) and a 3'-UTR. The length of introns A, B and C of the prepro-mGnRH gene are 198, 538 and 245 bp, respectively. The length of introns A, B and C of the prepro-cGnRH-II gene are 355, 321 and 189 bp, respectively.

RT-PCR and Southern blot analysis

Staining with ethidium bromide and Southern blotting following RT-PCR and electrophoresis were utilized in an attempt to characterize the expression patterns of the prepro-mGnRH and prepro-cGnRH-II genes in the brain part A, B and C, pituitary, eye, olfactory epithelium, ovary, testis, liver, kidney, spleen, heart, gill, intestine, pancreas, muscle, skin, fin and PBL. Representative results are presented in Fig. 5. A band of expected size (367 bp) was observed in all tissues examined with the prepro-mGnRH gene-specific primer set by staining with ethidium bromide. Furthermore, a longer cDNA fragment was also observed in some tissues. Subsequent Southern blot analysis revealed that this longer band was present in all tissues examined. In addition, another cDNA fragment which was slightly above the longer band was observed in some tissues. In contrast, electrophoresis and staining with ethidium bromide following RT-PCR with the prepro-cGnRH-II gene-specific primer set revealed a single band of expected size (452 bp) in the brain part B, pituitary, olfactory epithelium, ovary and testis. The same band was detected by Southern blotting with the prepro-cGnRH-II cDNA-specific probe.

These cDNA fragments were subcloned and sequenced. The 367 bp fragment amplified with the prepro-mGnRH gene-specific primer set was determined to be the expected prepro-mGnRH product. The two longer fragments amplified with the prepro-mGnRH gene-specific primer set revealed to have just identical sequence of 565 bp, which contained intron A, besides four exons of the prepro-mGnRH gene. The single cDNA fragment of 565 bp probably appeared as the two bands in some tissue samples on the agarose gel. The 452 bp fragment amplified with the prepro-cGnRH-II gene-specific primer set was determined to be the expected prepro-cGnRH-II product.

The possibility of genomic DNA contamination is ruled out, since the sizes of the PCR products differ to the sizes calculated from the genomic DNA sequences. The possibility of cross-contamination seems highly unlikely, since there were no products in negative controls.

DISCUSSION

The present study demonstrated that a splicing variant for the prepro-mGnRH mRNA is present in various tissues of the eel. Genomic Southern blot analysis showed that each of the eel prepro-GnRHs is encoded by a single gene. Also, the sequencing of the RT-PCR product of 565 bp revealed that it indeed contains the sequence of intron A. Therefore the two

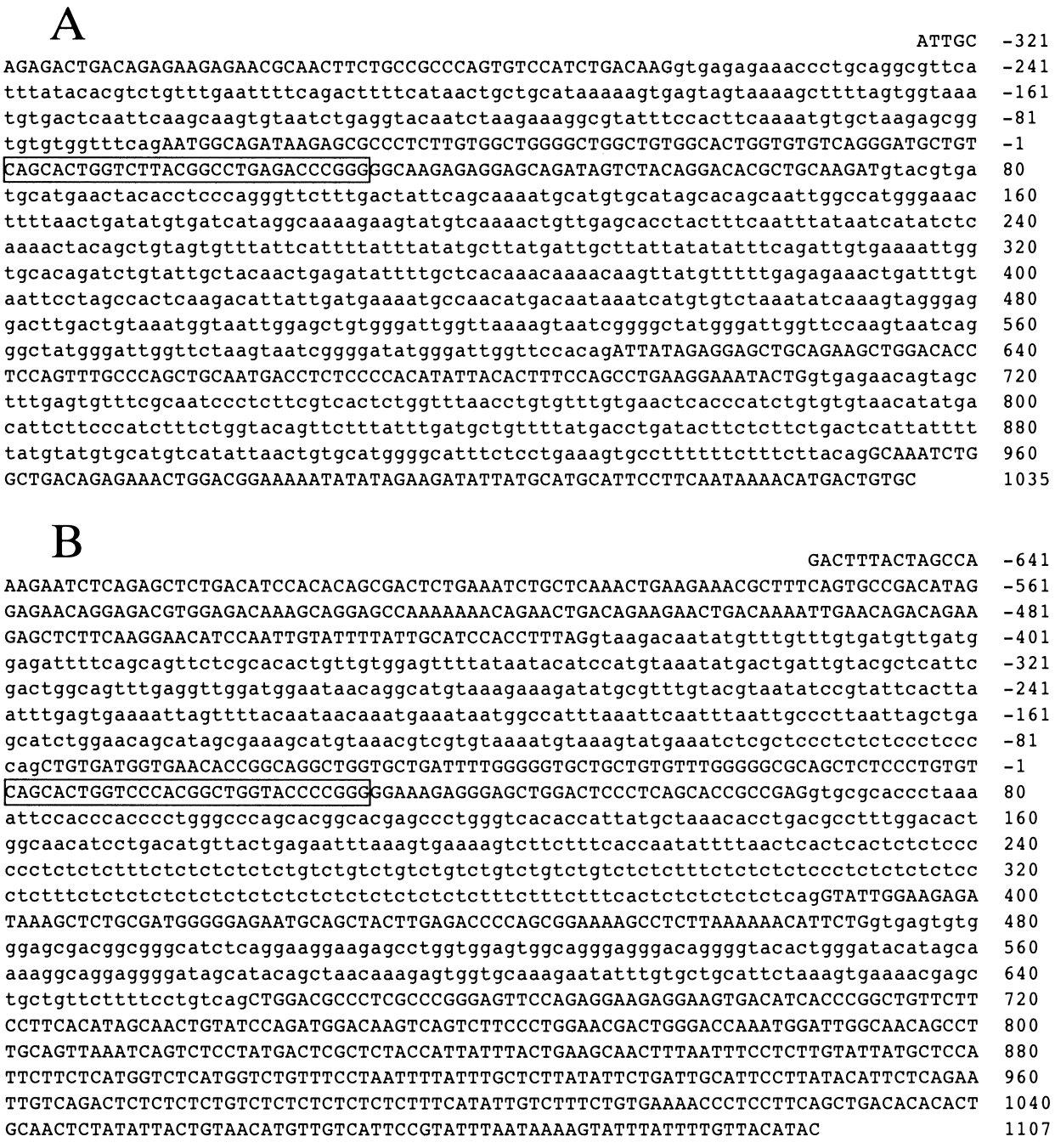

Fig. 3. Genomic nucleotide sequences of the prepro-mGnRH and prepro-cGnRH-II genes of the eel. (**A**) The prepro-mGnRH gene. Exon 1 (–325 to –266), exon 2 (–67 to 72), exon 3 (611 to 706) and exon 4 (952–1035) are in capital letters. The mGnRH decapeptide region is boxed. These sequences have been deposited in GenBank under accession number AB026991. (**B**) The prepro-cGnRH-II gene. Exon 1 (–654 to –433), exon 2 (–77 to 66), exon 3 (388 to 471) and exon 4 (661 to 1107) are in capital letters. The cGnRH-II decapeptide region is boxed. These sequences have been deposited in GenBank under accession number AB026992.

fragments obtained by the RT-PCR with the prepro-mGnRH gene-specific primer set were the products differentially spliced from the same gene but not derived from distinct genes. Although the mature prepro-mGnRH mRNA represented the abundant type in all tissues tested, the longer variant with retention of intron A was also demonstrated in these tissues. To date, studies for the splicing variant of the prepro-GnRH gene have been limited to mammals. Thus, this is the first report for the presence of a splicing variant of prepro-GnRH mRNA in vertebrates other than mammals. This finding showed that such a splicing pattern dates from a common ancestor to teleosts and mammals, and is conserved during evolution. Therefore splicing of mRNA may be one of the mechanisms regulating prepro-mGnRH gene expression. In mammals, the relative expression of the mature mRNA and its variant with retention of intron A differs between the central

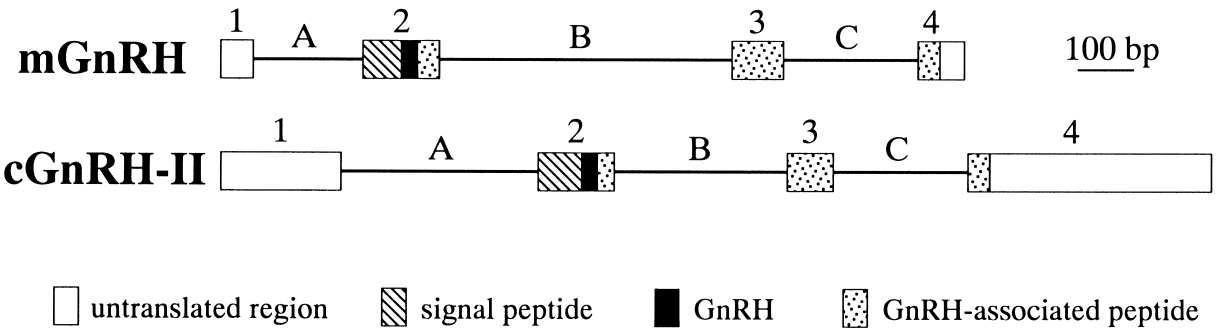

Fig. 4. Schematic diagram showing the organization of the prepro-mGnRH and prepro-cGnRH-II genes of the eel. Boxes represent exons. Horizontal lines adjacent to exons represent introns.

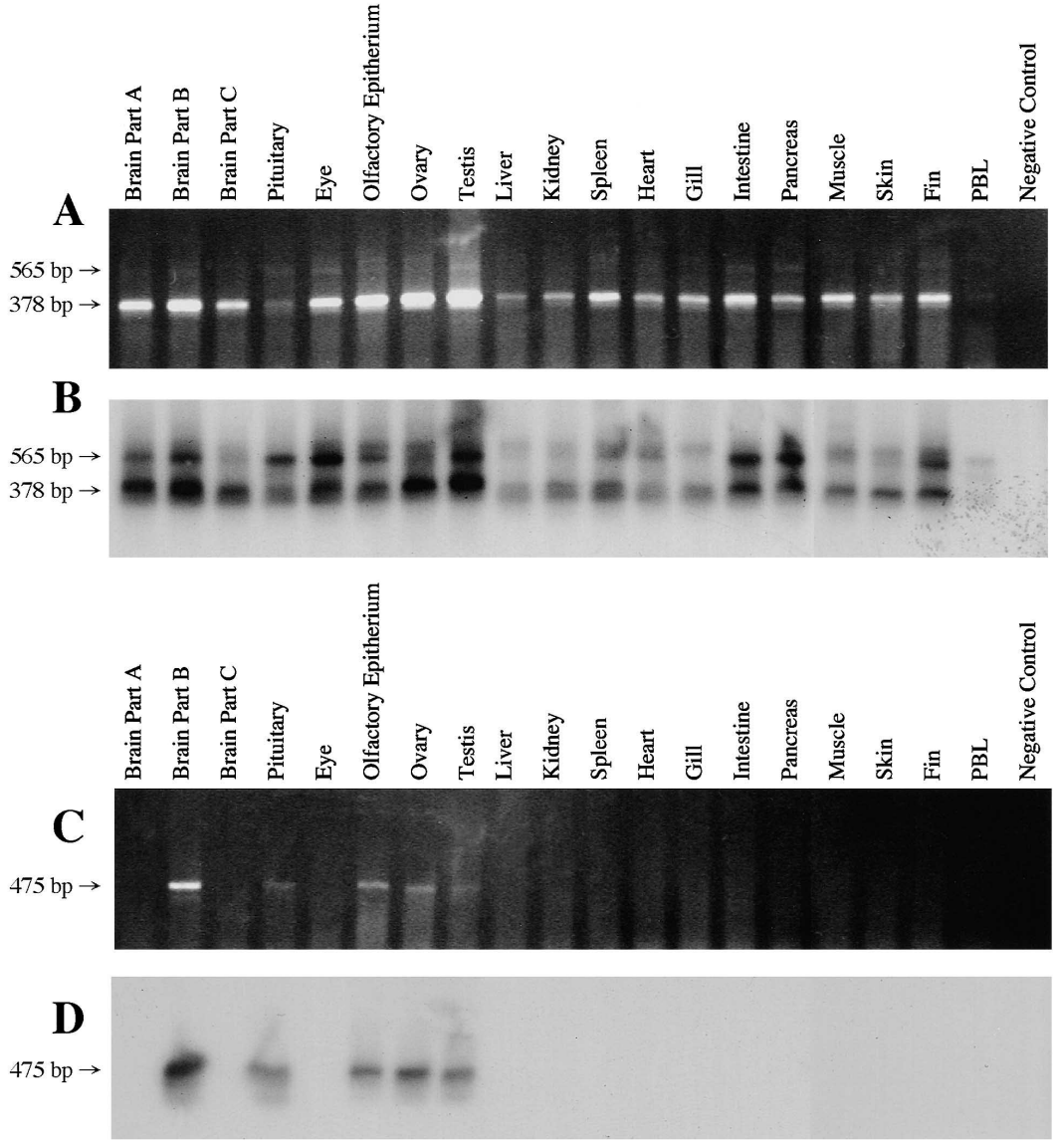

Fig. 5. Staining with ethidium bromide and Southern blotting following RT-PCR and electrophoresis to test the expression patterns of the prepro-mGnRH and cGnRH-II genes in various tissues of the eel. The PCR products were electrophoresed and stained with ethidium bromide after the amplification with the prepro-mGnRH gene-(**A**) and prepro-cGnRH-II gene-specific primer sets (**C**). The products amplified with the prepro-mGnRH and prepro-cGnRH-II gene-specific primer sets were transferred to nylon membranes and hybridized with the prepro-mGnRH (**B**) and prepro-cGnRH-II cDNA-specific probes (**D**), respectively.

nervous system and peripheral tissues: only the mature type is expressed in the former whereas a greater proportion of the transcripts in the latter contains intron A (Seeburg and Adelman 1984; Radovick *et al.*, 1990; Goubau *et al.*, 1992; Dong *et al.*, 1993; Dong *et al.*, 1996). In contrast, such a tissue-specific splicing pattern is not detected in the eel prepro-mGnRH gene. The mature mRNA for the prepro-mGnRH is more abundant than its variant with retention of intron A in all tissues examined in the present study. On the other hand, we could not obtain the evidence for the presence of splicing variant for the prepro-cGnRH-II mRNA. These results indicated that the two eel prepro-GnRH genes have different processes of RNA splicing as well as transcription.

There raises a question as to the function of the splicing variant with retention of intron A. Although this variant has extremely long 5'-UTR, it encodes a functional prepro-GnRH protein. An analysis of the sequences of this mRNA revealed two possible AUG translational start sites within its intron A. However, both of the AUG sites are followed rapidly by terminator codon (data not shown). In addition, neither of the two AUG sites have a typical Kozak consensus sequence (Kozak 1981; Kozak 1984), which is present at the functional translational start site in exon 2 for producing the prepro-GnRH protein. Therefore the intron A retention type should produce the same functional prepro-GnRH protein as mature mRNA does. Further studies are required to investigate, for example, the translational efficiency of this mRNA.

The present study confirmed our previous observation of the eel prepro-GnRH gene expressions in various tissues (Okubo *et al.*, 1999). The present study further demonstrated that the prepro-mGnRH gene is expressed in all three dissected parts of the brain whereas the prepro-cGnRH-II gene expression is detected only in a part of the brain including diencephalon, optic tectum and cerebellum. This result shows that the eel prepro-mGnRH gene has a widespread distribution of expression not only in the peripheral tissues but also in the brain.

We also demonstrated here the structures of the two distinct eel prepro-GnRH genes. Each prepro-GnRH gene contains four exons separated by three introns, and the positions of the exon/intron junctions are similar to those of other prepro-GnRH genes identified so far (see Sherwood *et al.*, 1997; Suetake and Aida 1997; White *et al.*, 1998; White and Fernald 1998). This result indicated that all prepro-GnRH genes are derived from a common ancestral gene, which has such a exon/intron organization. However, conservation of the length and sequence is not present between the introns of the two eel prepro-GnRH genes, suggesting that the separation of these two genes by gene duplication occurred long before the emergence of the primitive teleost.

Messenger RNA splicing requires the presence of conserved nucleotide sequences, the 5' splice donor site and 3' splice acceptor site, branch point sequence and polypyrimidine tract in a pre-mRNA. These sequence elements are recognized by splicing factors. All of the exon/intron junction sites of the eel prepro-GnRH genes correspond to the consensus splice sequence, which is characterized as GT (donor) and AG (acceptor). All introns of the two eel prepro-GnRH genes lack the consensus sequence of the lariat branch point (UNYURAY). Intron C of the prepro-mGnRH gene and all three introns of the prepro-cGnRH-II gene have a polypyrimidine tract near the 3'-splicing sites. In particular, a very long polypyrimidine tract over 150 bp is found in intron B in the prepro-cGnRH-II gene. Introns A and B of the prepro-mGnRH gene, however, lack such sequences. It remains to be studied how these unique features of the two prepro-GnRH genes affect the regulation of splicing. Also, it is interesting to note that the 27 bp sequence motif (AAGTAATCGGGGCTATGGGATTGGTTC) appears four times with only eight mismatches in intron B of the prepro-mGnRH gene. Such a repeat of the sequence motif has also been described in intron B of the prepro-salmon GnRH (prepro-sGnRH) genes of the masu salmon, *Oncorhynchus masou* (Higa *et al.*, 1997).

In summary, we demonstrated the presence of a splicing variant for the prepro-mGnRH mRNA, but not for the prepro-cGnRH-II mRNA, with retention of intron A in all tissues tested. This finding indicated that the eel prepro-mGnRH and prepro-cGnRH-II mRNAs have different splicing processes, and that such a splicing pattern for prepro-mGnRH mRNA is conserved during evolution. It is possible that the splicing is one of the mechanisms regulating prepro-mGnRH gene expression.

ACKNOWLEDGMENTS

We thank T Ohira and T Usami, the University of Tokyo, for technical assistance. This study was supported in part by Grants-in-Aid from the Japan Society for the Promotion of Science (Research for the Future Program) and from the Ministry of Agriculture, Forestry and Fisheries.

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(Received March 31, 1999 / Accepted April 30, 1999)