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Molecular Biological and Immunohistochemical Studies of Natriuretic Peptide Receptor Type B in the Euryhaline Eel *Anguilla japonica*

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1996

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Abbreviations

ANP	atrial natriuretic peptide or A-type natriuretic peptide
BNP	brain natriuretic peptide or B-type natriuretic peptide
CNP	C-type natriuretic peptide
DNP	natriuretic peptide of *Dendroaspis anguisticeps*
VNP	ventricle natriuretic peptide
NPR-A	natriuretic peptide receptor type A
NPR-B	natriuretic peptide receptor type B
NPR-C	natriuretic peptide receptor type C
cGMP	3', 5'-cyclic guanosine monophosphate
DMEM	Dulbecco's modified Eagle's medium
PCR	polymerase chain reaction
IBMX	3-isobutyl-1-methylxanthine
DASPMI	2-(4-dimethylaminostyryl)-1-methylpyridinium iodide
bp	base pairs
MOPS	4-morpholinepropanesulfonic acid
PIPES	1,4-piperazinediethanesulfonic acid
GC	guanylyl cyclase

SUMMARY

A comparative study of natriuretic peptide receptor NPR-B was performed by cloning and expressing, in COS-1 cells, the cDNA for NPR-B gill of the eel *Anguilla japonica.* This receptor exhibited a strong guanylyl cyclase activity in response to C-type natriuretic peptide (CNP). Immunohistochemical studies demonstrated that the CNP/NPR-B system is the one of the most important systems in "chloride cells" which are located on the gill and involved in homeostasis of water and ions. Resembling other mammalian NPR-B receptors, the eel NPR-B receptor consists of a ligand-binding extracellular domain, a hydrophobic transmembrane domain, a kinase-like domain and a guanylyl cyclase domain. Sequence comparisons among eel and mammalian receptors revealed a relatively low degree of similarity (ca. 44%) in the extracellular domain, as compared to a very high degree of similarity (ca. 84%) in the cytoplasmic regulatory and catalytic domains. The low degree of similarity allowed identification of the amino acid residues or candidate regions that might be important for ligand-binding activity.

An immunohistochemical study demonstrated that a highly specific antiserum against NPR-B stained the chloride cells in freshwater-adapted eels. Northern blot analysis and measurements of the intracellular

accumulation of cGMP also revealed that, in the chloride cells, the mRNA for NPR-B was abundant and CNP stimulated the marked elevation of levels of cGMP. After transfer of eels from freshwater to seawater, both the mRNA for NPR-B and the response of cGMP to CNP almost disappeared within several days. After complete adaptation to seawater, a marked elevation of the levels of cGMP by ventricle natriuretic peptide (VNP) instead of CNP was observed in the chloride cells. This observation strongly suggests the existence of new receptors that respond to VNP.

RNase protection analysis of the mRNA for eel NPR-B also demonstrated that the mRNA was predominantly located in the liver as well as in the gills with moderate to low levels in the brain, ventricle, esophageal sphincter, stomach, posterior intestine, and kidney. When eels were transferred from freshwater to seawater, the high levels of NPR-B mRNA in the liver and atrium decreased markedly. Similar changes are known to occur in the levels of CNP. These results suggested that CNP/NPR-B system might play an important role in the successful adaptation of the eels to changes in salinity.

INTRODUCTION

The natriuretic peptide system is one of the most important homeostatic systems that regulate natriuresis and vascular tension for maintenance of salt and water balance and blood pressure in mammals. This system consists of three structurally related ligands, atrial natriuretic peptide (ANP) (21, 33) brain natriuretic peptide (BNP) (60, 69, 73, 74), and C-type natriuretic peptide (CNP) (75), which share a common structural motif that consists of an amino acids disulfide loop of 17 (59). The physiological importance of this system is underlined by the existence of closely related peptide in the venom of green mamba *Dendroaspis angusticeps* (DNP) (68). ANP and BNP are mainly synthesized in the heart as endocrine peptides (they are found at concentration of 10^{-12}-10^{-10} M in the plasma) (1, 2, 50, 85) and act on vascularized organs, namely, blood vessels and kidney (7, 8, 13, 14, 88). These two peptides control cardiovascular function and body-water homeostasis. CNP has unique structural and physiological properties as compared to the other two peptides. The plasma concentration of CNP is undetectable even by radioimmunoassay ($<10^{-12}$ M) (51, 86). Consequently, so low as to be, CNP should be considered to be a paracrine, autocrine or neurocrine peptide. It lacks the carboxy terminal tail found in the other

two peptides and is synthesized in the central nervous system (42), vascular endothelial cells (76), tracheal epithelial cells (20), chondrocytes (30) and reproductive organs (20). Some of these tissues seemed to have no involvement in the body-water homeostasis. CNP affects neither natriuresis nor vascular relaxation at physiological concentrations and its functions are not well understood. It has, however, been shown to inhibit the growth of several types of cell (27, 30).

Recent studies of the receptors in mammals have revealed at least three receptor subtypes in the natriuretic peptide system: natriuretic peptide receptor-A (NPR-A) (17, 47, 53) and natriuretic peptide receptor-B (NPR-B), (16, 23, 67) which each has a tyrosine kinase-like domain and a guanylyl cyclase (GC) domain in the intracellular region, and natriuretic peptide receptor-C (NPR-C) (26, 46, 56, 70), which lacks tyrosine kinase-like domain and guanylyl cyclase domain. The relationship between the ligands and the various receptors is summarized in Figure 1. NPR-A responds to both ANP and BNP to a similar extent and produces intracellular cGMP as a second messenger, but it does not respond to CNP (44). NPR-A is expressed in the adrenal gland, kidney, blood vessels and some organs that are directly involved in body-water homeostasis (64, 87), and its expression has been detected in all the organs in which ANP is biologically active, as previously described. NPR-B responds very weakly to ANP and BNP but responds strongly to CNP

(44). Although NPR-B is widely expressed in various tissues (64, 87), its biological function is unclear. To date, its only action is the inhibition of growth of chondrocytes and vascular smooth muscle cells (27, 30) and this activity has been suggested to be related to cell differentiation and vascular remodeling, respectively. NPR-C binds all natriuretic peptides (11), and its expression is very sensitive to environmental changes *in vivo* (10, 28, 48, 65) and *in vitro* (15, 31, 36, 37, 39, 41) but its physiological function is unknown. It has only a short cytoplasmic domain and it is still unclear how it transduces a signal to the cytoplasm. Some investigators have suggested that NPR-C is coupled to G_i and inhibits adenylyl cyclase activity (4-6). Some other investigators have proposed that it is only a clearance receptor that regulates concentrations of natriuretic peptide in the plasma and never transduces a signal into a cell (49).

Considerable efforts have been directed toward resolving these issues. Comparative studies with animals with specialized anatomical and physiological features may be a fruitful way of addressing these problems. In fact, with respect to the ligands, there are several reports on the structure, function and metabolism of the natriuretic peptides of non-mammalian species, such as chicken (3, 9), frog (43, 45, 62, 89), eel (80, 82), killifish, dogfish (57, 66), and trout (78) (Fig. 2). It has been shown, for example, that ANP induces anti-natriuresis rather than

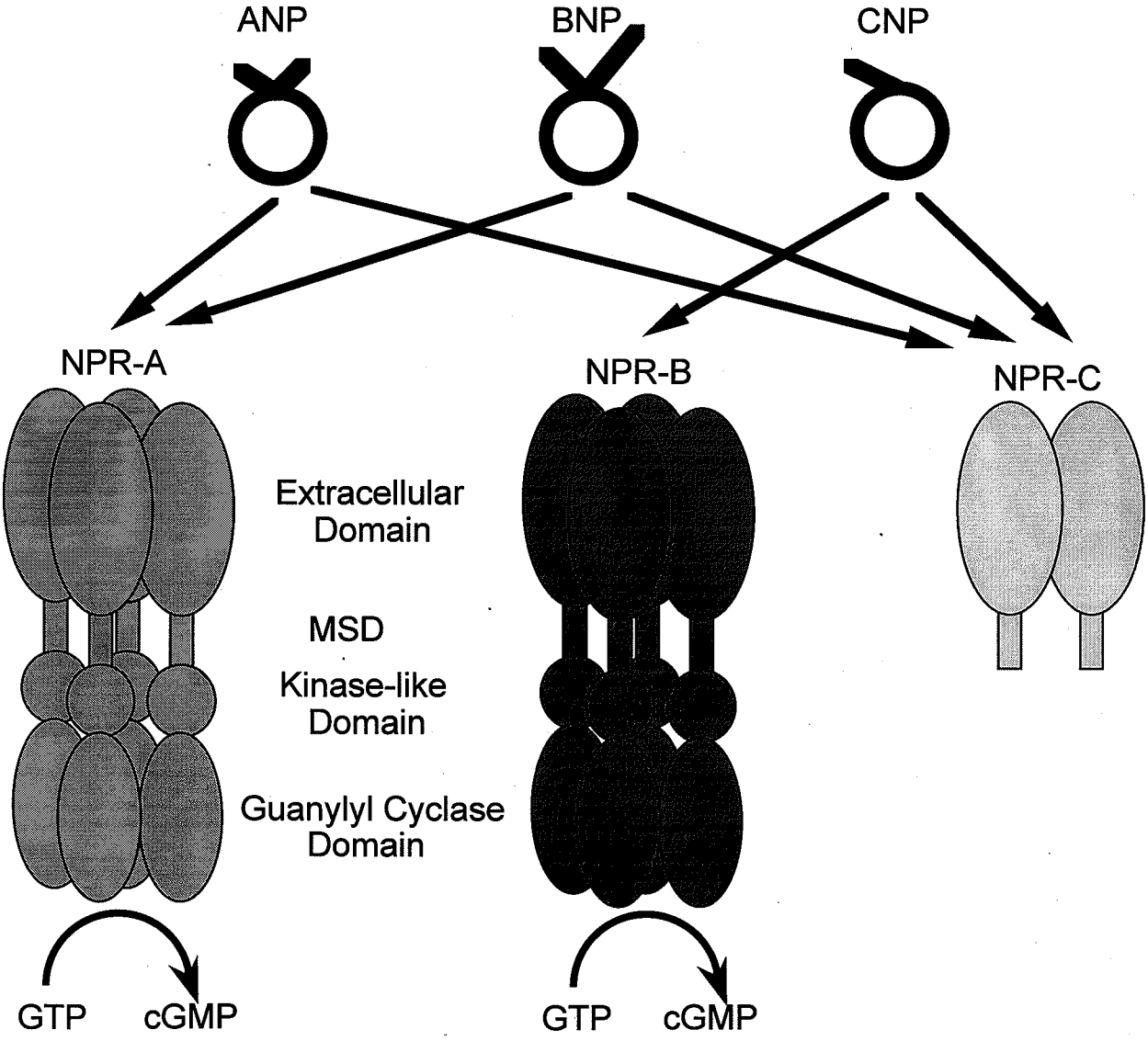

Fig. 1. **Schematic representation of natriuretic peptides and their receptors.**

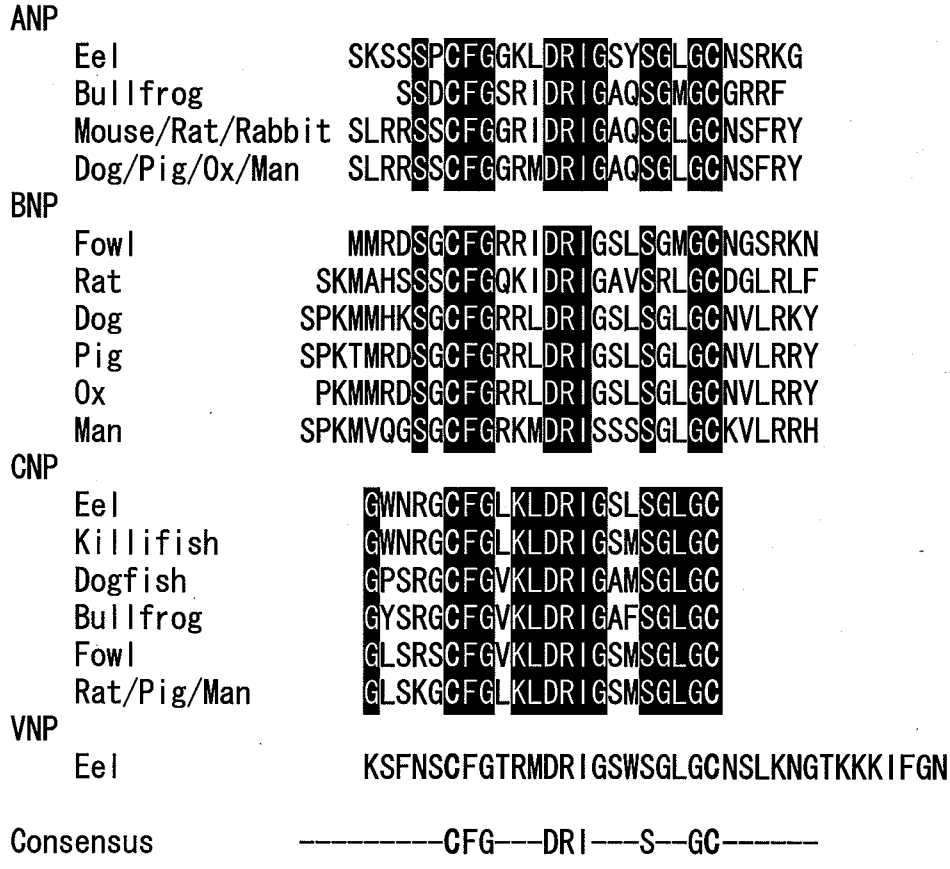

Fig. 2. **Amino acid sequences of eel and other vertebrate natriuretic peptides.** All conserved residues are shaded. Cysteine residues involved in disulfide linkages are bold.

natriuresis in eel (84), that CNP is the most highly conserved in natriuretic peptide (38), and that there exist two kinds of CNP in frog (43, 89).

In the present study, with comparative analysis as a goal, a molecular biological study of the eel NPR-B receptor system was performed. The eel *Anguilla japonica* and and its NPR-B were chosen because three natriuretic peptides, ANP, CNP and a novel peptide designated VNP (81, 83), have been isolated from the eel and well characterized (Fig. 3). Eels are euryhaline species and their efficient osmoregulatory mechanisms appear to be of interest in relation to the natriuretic peptide system. In contrast to mammals, in which CNP is a neuropeptide rather than a circulating hormone, the eel has been demonstrated to contain a relatively large amount of CNP in the plasma. Moreover, plasma levels decrease dramatically after adaptation to seawater, suggesting that studies on the tissue distribution of eel NPR-B, a receptor subtype that is highly specific for CNP, might provide clue to the physiological role of CNP.

In the present study, a comparison of amino acid sequences of eel and mammalian NPR-B receptors allowed comparative location or conserved sequences essential for receptor function. RNase protection analysis revealed the widespread distribution of NPR-B in various tissues of the eel. For example, substantial amounts of the mRNA for NPR-B were detected in the liver, heart, gill and brain, with the highest level in the

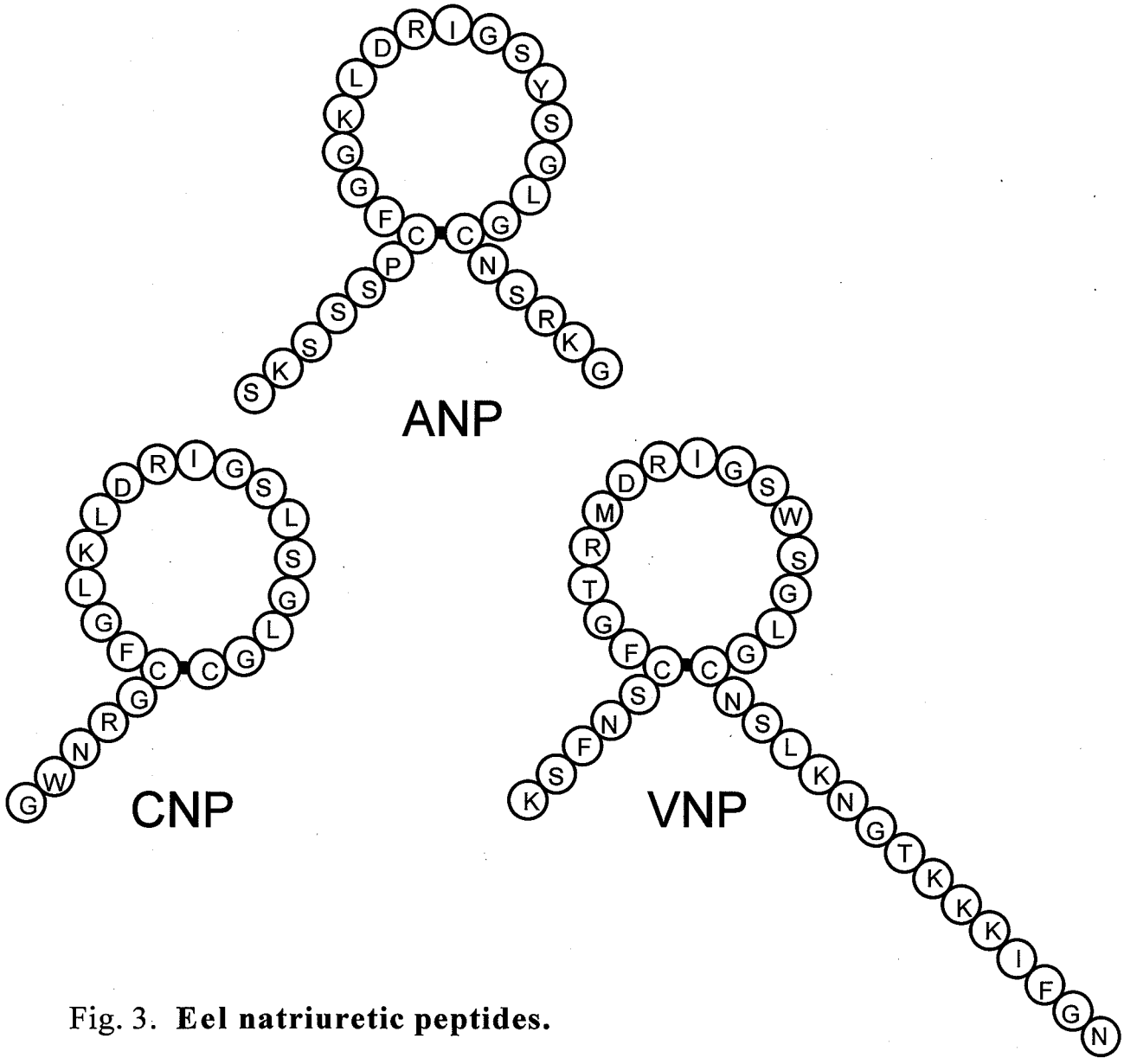

Fig. 3. **Eel natriuretic peptides.**

liver. Similar analysis using preparations of RNA from eels that had adapted to seawater or freshwater indicated that the levels of mRNA changed in response to changes in environmental osmolarity. Immunohistochemical staining revealed high-density localization of NPR-B in the chloride cells of freshwater-adapted eels, but no expression in the chloride cells of seawater-adapted eels. RNase protection analysis also demonstrated that expression of the mRNA decreased during adaptation from freshwater to seawater. CNP stimulated the NPR-B-dependent production of cGMP in epithelial cells from freshwater-adapted eels, although it did not do so in those from seawater-adapted eels.

EXPERIMENTAL PROCEDURES

Materials

Restriction enzymes were obtained from Toyobo ,Osaka, and Boehringer Mannheim, Mannheim, Germany; Time-Saver cDNA synthesis kit and *double-stranded* nested deletion kit were from Pharmacia, Uppsala, Sweden; *Purococcus furiosus* (*Pfu*) DNA polymerase, λ ZAP II, Gigapack II Gold *in vivo* packaging kit and RNA transcription kit were from Stratagene, San Diego, CA, USA; mRNA Separator poly(A)-rich RNA purification kit was from Clonetech, Palo Alto, CA, USA; Sequenase version 2.0 sequencing system was from United States Biochemical, Cleveland, OH, USA; Dulbecco's modified Eagle's medium (DMEM) and penicillin-streptomycin antibiotics mixture were from Life Technologies, Gaithersburg, MD, USA; fetal bovine serum was from Bocknek; [α-^{32}P]dCTP and [α-^{32}P]UTP were from Du Pont-New England NuclearBoston, MA, USA; cGMP assay system was from Yamasa, Chiba; and synthetic eel ANP (eANP), eel CNP, and eel ventricular natriuretic peptides (eVNP) were from Peptide Institute, Osaka; A mitochondria-specific fluorescent dye 2-(4-dimethylaminostyryl)-1-methylpyridinium iodide (DASPMI), Tricaine

(3-aminobenzoic acid ethyl ester methanesulfonate; MS-222) and phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) were obtained from Sigma, St. Louis, MO, USA. An avidin-biotin-complex staining system was from Vectastain, Vector Laboratory, Burlingame, CA, USA.

Construction of an eel gill cDNA library

Total RNA was prepared from eel gills by the guanidium thiocyanate/cesium chloride method (18) and fractionated on oligo(dT)-cellulose resin to obtain poly(A)-rich RNA. Complementary DNA was synthesized by reverse transcription of 5 μg of the eel gill poly(A)-rich RNA with a Time-Saver cDNA synthesis kit. The cDNA was ligated to the λ ZAP II phage vector and packaged with a Gigapack II Gold *in vitro* packaging kit. Approximately 3×10^6 recombinant phages were obtained.

Preparation of a cDNA probe

To obtain a cDNA probe for NPR-B, part of the sequence of bovine NPR-B cDNA covering the GC domain was first amplified by PCR.

Bovine adrenal cDNAs were synthesized as described previously and used as templates for PCR, which was performed with *Purococcus furiosus* (*Pfu*) DNA polymerase and the following oligonucleotide primers: 5'-GAGACAGTAACGGGGCATCTT-3' (antisense) and 5'-ATGGAGCAGTACGCGAACAA-3' (sense). PCR consisted of 35 cycles of denaturation at 94°C for 1 min, annealing at 45°C for 2 min, and polymerization at 75°C for 3 min. The product was size-fractionated by electrophoresis on an agarose gel, cloned into pBluescript (Stratagene) and sequenced. The PCR-amplified cDNA fragment was 525 bp in length and it encode a protein that exhibited 98.9% homology to the GC domains of human and rat NPR-B receptors.

Screening of cDNA library

The above-described cDNA probe was labeled with [α-^{32}P]dCTP using random primers. Approximately 4 x 10^5 plaques were screened by plating recombinant phages of a density of 30,000 plaques/15-cm plate together with the host, *Escherichia coli* XL-1 Blue, on plates of agar-solidified Luria-Bertani medium. Replicate filters were prehybridized at 37°C for 2 h and hybridized for 16 h in the presence of the ^{32}P-labeled

probe. Prehybridization and hybridization buffers contained 6x SSC (1x SSC contains 0.15 M NaCl, 15 mM sodium citrate, pH 7.0), 5x Denhardt's solution (Denhardt's solution contains 0.02% each of bovine serum albumin, polyvinylpyrrolidone and Ficoll) and 0.1% SDS. After hybridization for 16 h at 37°C, filters were washed twice with 2x SSC containing 0.05% SDS at room temperature for 5 min and twice with 2x SSC at 37°C for 1 h. Then they were exposed to Kodak X-Omat AR5 (Eastman Kodak Co., Rochester, NY, USA) film with an enhancer screen (Konica, Tokyo) at -80°C overnight. Double-positive clones were plaque purified and the longest cDNA insert was used as a probe for a second round of screening to isolate a full-length cDNA. Approximately 1.3 x 10^6 plaques were rescreened with the homologous probe under higher-stringency conditions. Prehybridization and hybridization buffers used for the rescreening contained 20% formamide, 6x SSC, 5x Denhardt's solution and 0.1% SDS. Hybridization was carried out at 42°C for 18 h. Washing was performed twice with 2x SSC that contained 0.05% SDS at room temperature and twice with 1x SSC that contained 0.1% SDS at 50°C. After autoradiography, plaques that gave hybridization signals on both replicate filters were purified and the inserts were rescued as

pBluescript SK^- , using helper phage R408 (Stratagene), for analysis with restriction enzyme analysis and sequencing.

DNA sequencing

DNA sequences were determined by the dideoxynucleotide chain-termination method (63) using the Sequenase version 2.0 sequencing system. The internal insert sequence was obtained from the series of nested deletions that were generated with a nested deletion kit .

Expression of the cDNA for the eel NPR-B receptor in COS-1 cells

A full-length cDNA (eNPR-B22) encoding the eel NPR-B receptor was ligated and subcloned into a mammalian expression vector, pcDL-SRα296 (79). COS-1 cells were maintained in DMEM that contained 10% fetal bovine serum, 10 mM HEPES, 50 U/ml penicillin and 50 μg/ml streptomycin in a controlled atmosphere of 5% CO_2/95% air at 37°C. Cells were harvested with trypsin from a 70% confluent culture, washed with Dulbecco's phosphate-buffered saline (2.7 mM KCl, 138 mM NaCl, 1.2 mM KH_2PO_4, 8.1 mM Na_2HPO_4; without Ca^{2+} and Mg^{2+} ions) and

suspended in phosphate-buffered saline (120.7 mM KCl, 30.8 mM NaCl, 1.46 mM KH_2PO_4, and 8.1 mM Na_2HPO_4) that contained 5 mM $MgSO_4$ for electroporation. Approximately 6 x 10^6 cells were electroporated with 16 μg plasmid DNA at 220 V and at a capacitance setting of 960 μF in a Bio-Rad Gene Pulser (Bio-Rad). After electroporation, the cells were seeded into 12-well culture plates for assays of the synthesis of cGMP.

Measurement of the intracellular accumulation of cGMP in COS-1 cells

COS-1 cells transfected with the eel NPR-B cDNA/pcDL-SRα296 construct and cultured in 12-well plates were washed with Eagle's minimum essential medium that contained 10 mM HEPES, pH 7.4, and incubated with 0.5 ml of the medium that contained 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) at room temperature for 30 min to inhibit phosphodiesterase activity. After a 15-min incubation with eANP, eCNP, or eVNP (10^{-10}-10^{-6} M) , 0.5 ml of 0.2 M HCl that contained 5 mM EDTA was added to stop the production of cGMP. Cell lysates were then assayed for levels of intracellular cGMP with a cGMP assay kit .

Northern blotting

Five μg of poly(A)-rich RNA obtained from eel gill was fractionated by electrophoresis on a 1% agarose gel that contained 2.2 M formamide, 20 mM MOPS, pH 7.0, 8 mM sodium acetate and 1 mM EDTA. After electrophoresis, the RNA was transferred to a Magnagraph nylon membrane (Micron Separations Inc.) and chemically cross-linked to the membrane with ultraviolet light. The membrane was incubated in a buffer that contained 50% formamide, 5x SSPE (SSPE contains 0.15 M NaCl, 15 mM NaH_2PO_4, pH 7.0, 1 mM EDTA), 5x Denhardt's solution and 1% SDS for 1 h at 42 °C and the RNA was allowed to hybridize for 16 h with the ^{32}P-labeled cRNA probe used for RNase protection analysis (see below). The membrane was washed twice with 2x SSC containing 0.05% SDS at room temperature for 5 min and twice with 0.1x SSC containing 0.1% SDS at 60 °C for 3 h and then it was exposed to X-Omat film with an intensifying screen at -80 °C.

Production of antiserum against recombinant eNPR-B protein

The pMAL-p1 expression vector was used for expression of eNPR-B

as a fusion protein with the maltose-binding protein of *E. coli.* The *Hin*cI-*Xba*I fragment of eNPR-B (corresponding to Asn98 through Leu248) was introduced in-frame into the expression vector. The resulting plasmid was used to transform XL-I Blue cells. The transformed bacteria were incubated with shaking in Luria-Bertani medium *plus* 50 μg/ml ampicillin at 37°C until a cell density of equal to an absorbance at 600 nm of 0.6 was reached. The synthesis of the maltose-binding protein-eNPR-B fusion protein was induced by addition of 0.3 mM IPTG. After a further incubation with 3 h shaking at 37°C, and the protein was purified essentially as described by the manufacturer of New England Biolab. A New Zealand White rabbit was immunized by intradermal injection with a homogenate that contained 1 ml of Freund's complete adjuvant and 1 ml of a solution of fusion protein (1 mg/ml). Two weeks later, the rabbit was given a booster injection of a homogenate that consist of 1 ml of Freund's incomplete adjuvant and 1 ml of the solution of fusion protein (1 mg/ml). Blood was collected and antiserum was prepared each week thereafter. Booster injections were given every two weeks until the titer of the antiserum ceased to increase.

Immunostaining of eel gill cells

An eel was anaesthetized in a solution of ethyl *m*-aminobenzonate (MS-222), adjusted to pH 7.4 with $NaHCO_3$ and perfused via ventral aorta with 20 ml of phosphate-buffered saline (PBS: 118 mM NaCl, 5 mM KCl, 10 mM Na_2PO_4, 1.2 mM KH_2PO_4, pH 7.4). The gill arch was embedded in OCT compound and cut into 6-μm-thick sections. The sections were sequentially incubated as follows: in 5% hydrogen peroxide in acetone at room temperature for 1 h, (ii) in 6% goat serum/PBS for 1 h; (iii) in antiserum against eNPR-B diluted in PBS (1: 1000 final dilution) at 4°C overnight; (iv) in a solution of biotinylated antibody raised in goat against rabbit IgG solution at 4°C for 3 h, (v) in avidin-biotin-peroxidase complex (ABC) at room temperature for 1 h; and finally (vi) in 0.1% diaminobenzidine, 0.02% hydrogen peroxide, 0.01 M imidazole in PBS for visualization of immunoreactions.

Staining of chloride cells in situ with a mitochondrion-specific dye, DASPMI

An eel was anaesthetized as described above. After injection of 0.2

μmol of DASPMI and 1,000 units of heparin in saline into the ventricle, the eel gill was incubated for 45 min at room temperature and then perfused via the ventral aorta to wash out the dye completely. Then the gill was removed and placed on a culture dish. Photographs were taken with a Kodacolor film (ISO 400; Kodak) under a fluorescence microscope (IMT-2; Olympus, Tokyo) with a excitation at 460 nm and emission at 581 nm.

Time course of changes in the levels of NPR-B mRNA after transfer of eels from freshwater to seawater, as determined by RNase protection assay

Eels were kept in freshwater for two weeks and then transferred to seawater in which they were allowed to adapt for 1, 3, 7, or 14 days. After adaptation, eels were anaesthetized and perfused and the gills were removed. The epithelial cells were scraped off as and the RNAs were purified by acid guanidium thiocyanate/phenol/chloroform method (19). An antisense cRNA probe [459 bp, corresponding to nucleotides -21 to 363 (384 bp) *plus* part of the multi-cloning site of pBluescript] was transcribed *in vitro* from pBluescript II SK^- using T7 RNA polymerase with [α-^{32}P]UTP (30 TBq/mmol). After transcription reaction, the template DNA was degraded by digestion with RNase-free DNase I, and

unincorporated [α-^{32}P]UTP was removed by ethanol precipitation. The radioactive cRNA (5 x 10^5 cpm) was allowed to anneal to 10 μg of total RNA, that was prepared from the epithelial cells, for 12 h at 45°C in 80% formamide, 40 mM PIPES, pH 6.4, 1 mM EDTA and 400 mM NaCl. Non-annealing RNA was then digested with ribonucleases A and T_1 at final concentrations of 40 μg/ml and 2 μg/ml , respectively, in a buffer that contained 10 mM Tris-HCl, pH 7.4, 300 mM NaCl and 5 mM EDTA, at 30°C for 1 h. The protected fragments were analyzed by electrophoresis in 5% polyacrylamide gel that contained 7 M urea and the gel was exposed to X-Omat films with an intensifying screen at -80°C.

Measurement of the accumulation of intracellular cGMP in eel gill epithelium

Freshwater- and seawater-adapted eels were anaesthetized and perfused as described above. The gill arches were removed and the filaments gently scraped with a razor blade. The resultant free cells were washed twice with PBS, suspended in PBS that contained 0.5 mM IBMX and incubated at 20°C for 20 min. After incubation with eANP, eCNP or

eVNP (10^{-10}-10^{-6} M) at 20°C for 20 min, 0.2 M HCl containing 5 mM EDTA was added to stop the production of cGMP. After boiling for 5 min, lysates were assayed for levels of intracellular cGMP as described above.

Tissue distribution of eNPR-B mRNA

Total RNA was isolated from selected eel tissues, the functions of which might be expected to be under the influence of natriuretic peptides, by the acid guanidium thiocyanate/phenol/chloroform method. The expression of mRNA for NPR-B was examined by the RNase protection assay as described above.

RESULTS

Isolation and characterization of cDNA clones for eel NPR-B

I and co-workers demonstrated previously that eel gill contains a large number of ANP-binding sites (61). Therefore, I constructed a cDNA library from eel gill poly(A)-rich RNA in λZAP II and screened it with a cDNA probe that corresponded to the GC-domain-encoding region of cDNA for bovine NPR-B cDNA under low-stringency conditions. Three positive clones (λeNPR-B11, λeNPR-B12 and λeNPR-B13) were isolated from among the 4×10^5 phages screened. The presence in the clones of cDNA for the eel homolog of the GC-containing NPR was confirmed by partial sequencing of the inserts. The sizes of the cDNA inserts of the three clones suggested that they were not long enough to cover the entire coding region (Fig. 4). Therefore, to obtain a full-length cDNA I rescreened the library with the eNPR-B12 cDNA under higher-stringency conditions. Screening of 1.3×10^6 plaques yielded 50 positive clones and 18 of these were plaque-purified. Restriction enzyme mapping with *Pst*I and *Hin*dIII indicated that these clones had similar restriction maps; two typical example of these clones are shown in Figure 4, namely, eNPR-B21 and eNPR-B22. The eNPR-B22 clone that had the longest cDNA insert

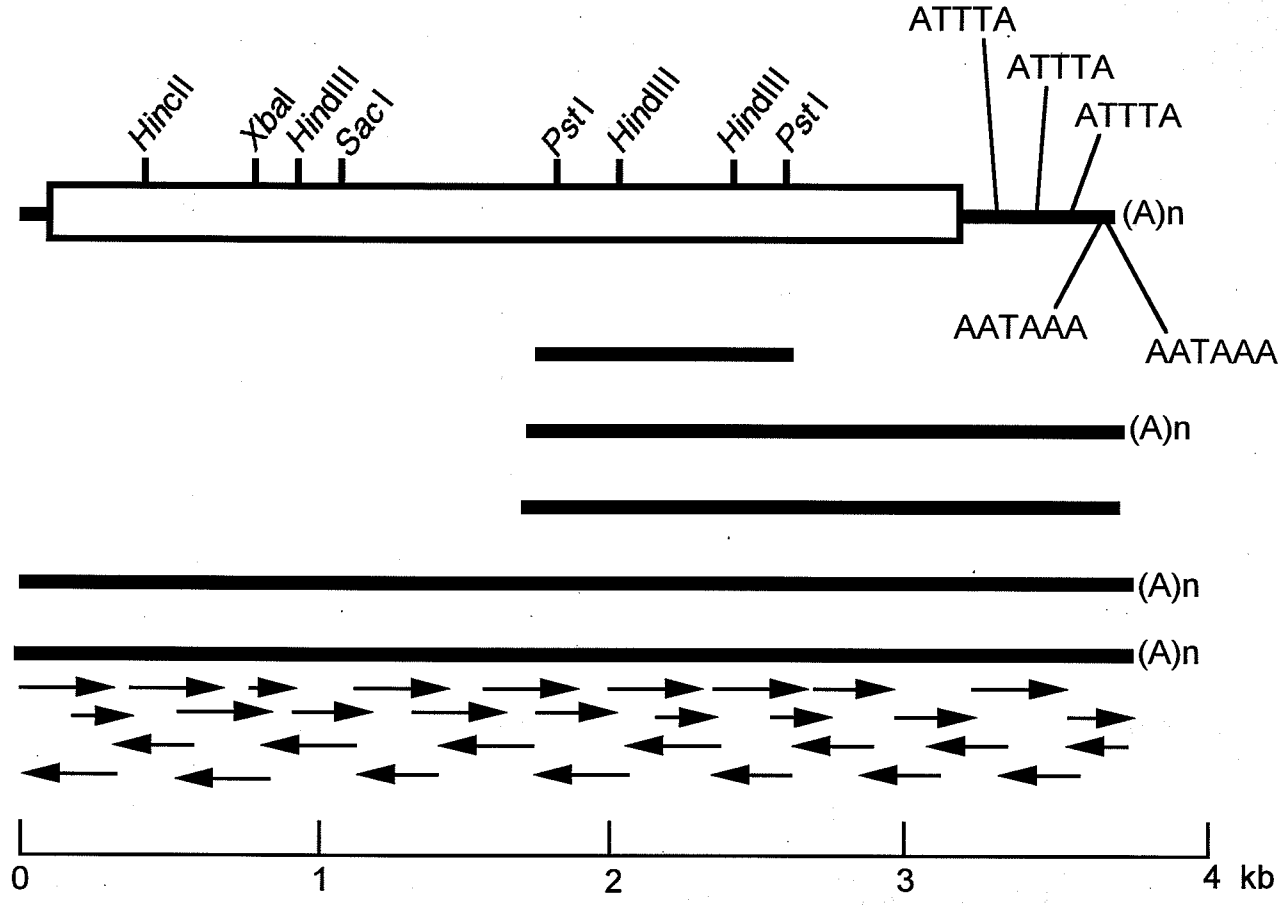

Fig. 4. **Partial restriction map and the sequencing strategy for cDNA for eel NPR-B.** The protein-coding region is represented by an open bar. Arrows indicate the directions and extents of sequencing reactions. Clones eNPR-B12, eNPR-B21 and eNPR-B22 had a poly(A) tail 31 bp downstream from the first polyadenylation signal AATAAA (22 bp downstream from the second one). The nucleotide sequence downstream of the stop codon contains three ATTTA motifs that could be involved in selective degradation of the mRNA.

was sequenced. Figure 5 shows the nucleotide sequence and deduced amino acid sequence. The cDNA insert contained 24 nucleotides that corresponded to the 5' untranslated region of eel NPR-B mRNA, 3,250 bases of the coding region, 523 bases of the 3' non-coding region and a poly(A) tail of 13 nucleotides. The eukaryotic polyadenylation signal AATAAA was found at two locations, namely, 22 and 31 bases upstream from the beginning of the poly(A) tail. The open reading frame encoded a protein of 1,026 amino acids with a calculated M_r of 119,856.

Comparison of eel NPR-B with its mammalian homologs

Figure 6 shows a comparison of the amino acid sequences of all forms of NPR-B characterized to date. The structural features of eel and human NPR-B receptors are summarized schematically in Figure 7. The comparison revealed a high degree of similarity within the cytoplasmic domain. However, as a whole, the eel and the mammalian sequences showed considerable divergence, exhibiting only 44% homology. Although the overall similarity in the extracellular domain was relatively low, certain regions appeared to be highly conserved, including the sequences immediately adjacent to the third and fourth cysteine residues, Cys236 and Cys339. Other features that were found to be conserved

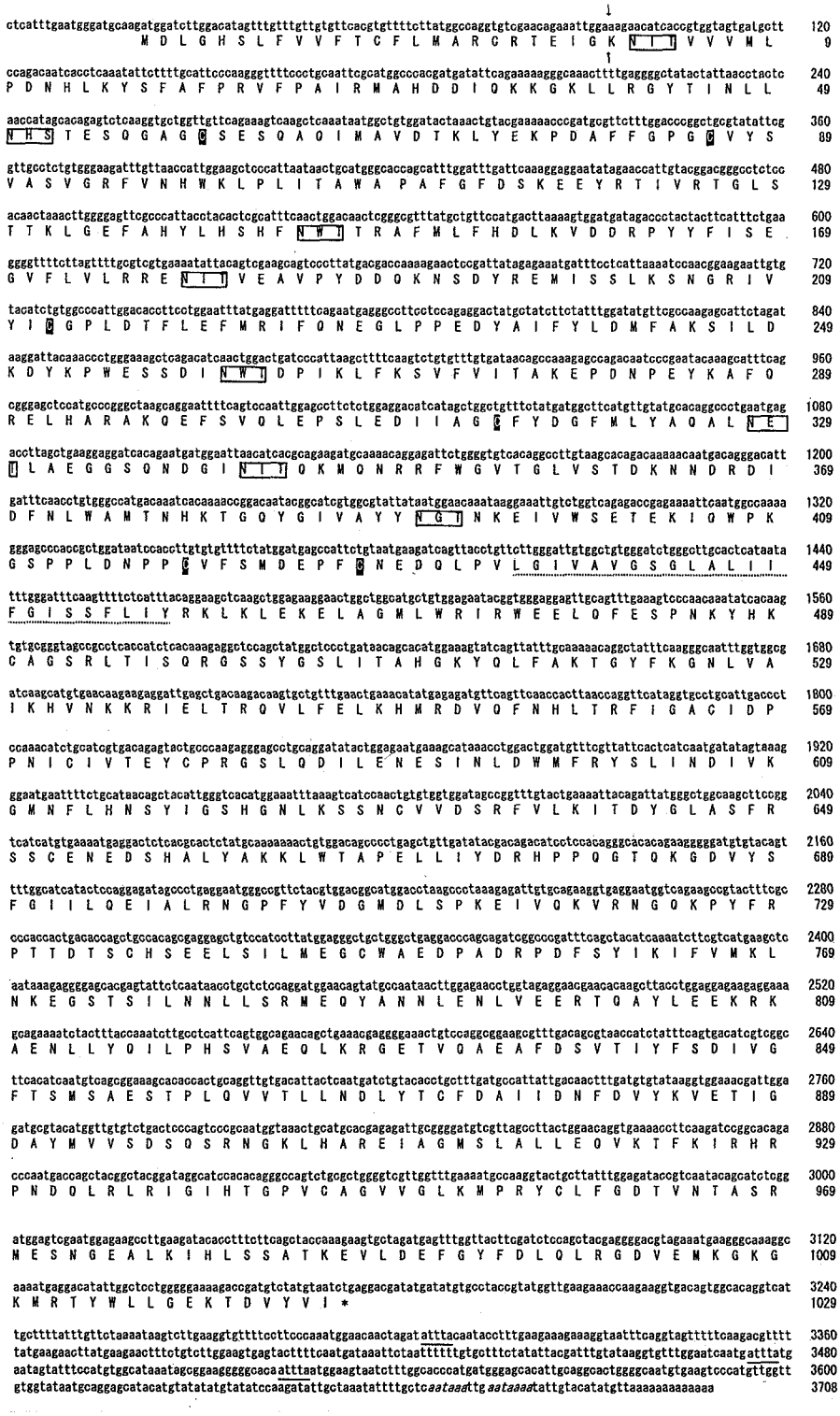

Fig. 5. **Nucleotide and deduced amino acid sequences of eel NPR-B.** Nucleotide and amino acid positions are shown on the right. The nucleotides are numbered in the 5'→3' direction, beginning with the first nucleotide of the cDNA clone eNPR-B22. Possible *N*-glycosylation sites are boxed. A putative transmembrane domain is indicated by the dotted line. Cysteine residues in the extracellular domain are black-boxed. Asterisk indicates a stop codon. The putative polyadenylation signal AATAAA is shown in italics, and the sequence ATTTA, a signal for rapid degradation of mRNA, is underlined. The arrow indicates the putative signal sequence cleavage site.

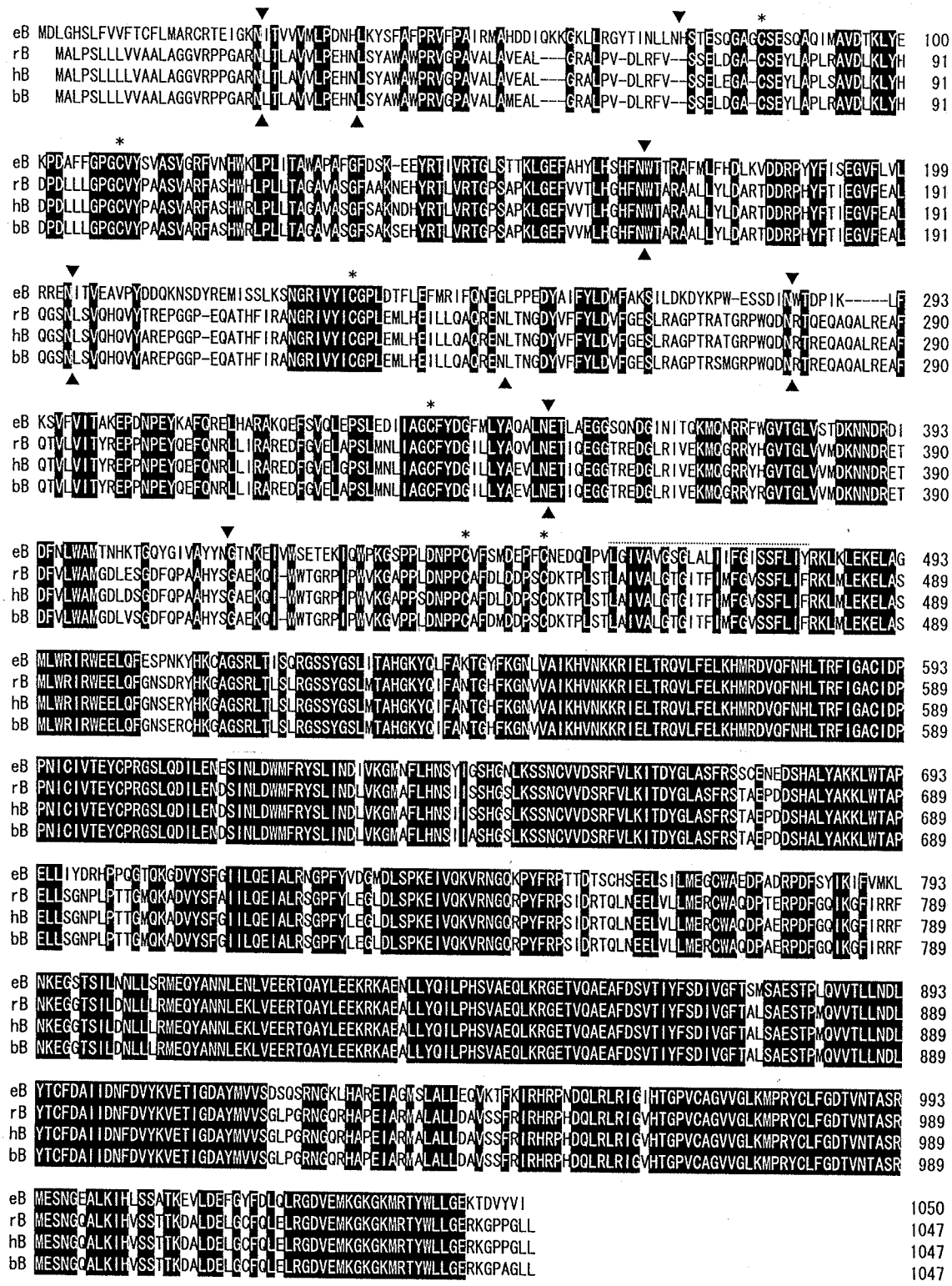

Fig. 6. **Alignment of the amino acid sequence of eel NPR-B with those of rat, human and bovine NPR-B.** The sequences of eel, rat and human NPR-B receptors were aligned by visual inspection with the assistance of SDC-Genetyx computer software (Software Development Co., Tokyo, Japan). The cysteine residues (asterisks) in the extracellular domain, *N*-glycosylation sites (closed triangles) and the membrane-spanning domain (dotted line) are marked

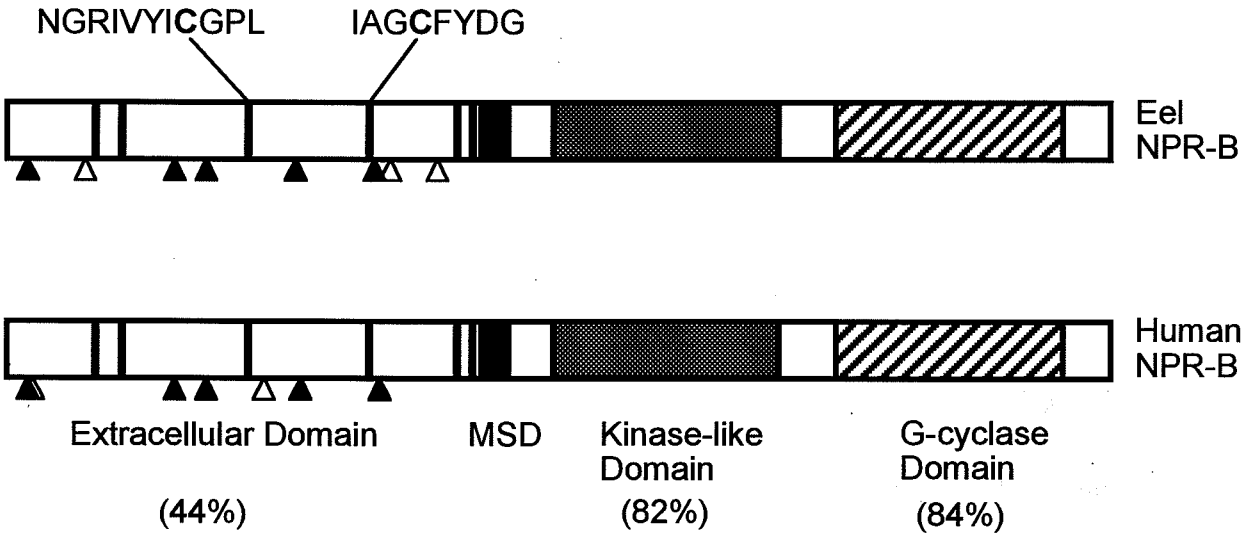

Fig. 7. **Structural comparison of eel and human NPR-B.** Vertical lines represent the positions of the six cysteine residues in the extracellular domain. Possible *N*-glycosylation sites are indicated by triangles; closed triangles represent conserved sites. Amino acid sequences surrounding the third and fourth cysteine residues, which are highly conserved, are also shown. MSD, membrane-spanning domain; G-cyclase, guanylyl cyclase. Values in parenthesis indicate the extent of homology.

between the eel and mammalian NPR-B receptors are the numbers and positions of the cysteine residues in the extracellular domain and those of the site of *N*-glycosylation; eel and mammalian NPR-B receptors contain eight and seven putative site of *N*-glycosylation, respectively, and five of their locations are conserved (Fig. 7). The extent of sequence homology between eel NPR-B and human NPR-A is 38% in the extracellular domain, 71% in the kinase-like domain, and 81% in the GC domain.

Northern hybridization

To estimate the size of the mRNA for eel NPR-B, Northern blot analysis was performed with poly(A)-rich RNA from the eel gill. One strong and one faint hybridization signal were detected at the positions that corresponded to mRNA of 4.0 kb and 5.7 kb, respectively (Fig. 8). The size of the predominant species was in agreement with that predicted from the analysis of the cDNA. The faint band of a 5.7-kb mRNA might represent the transcript of a related gene; there is a possibility that the band represents a subtype of eel NPR-B since we used relatively stringent hybridization conditions and, as a probe, a cDNA fragment that encoded a part of the extracellular domain in which there is a considerable degree of sequence divergence among the NPR subtypes.

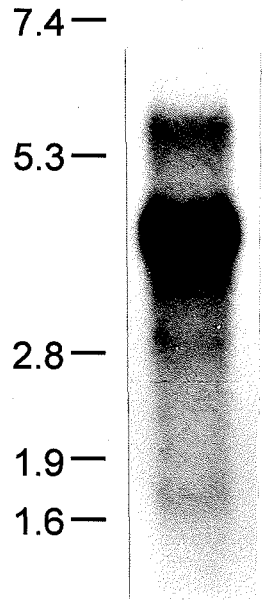

Fig. 8. **Northern blot analysis of mRNA for eel NPR-B.** Poly(A)-rich RNA was isolated from eel gill, subjected to electrophoresis on a 1% agarose gel under denaturing conditions, transferred to a nylon membrane and probed with a ^{32}P-labeled 459-bp cRNA probe. The positions and sizes (in kb) of RNA markers are shown on the left.

Expression of eel NPR-B in COS cells and demonstration of stimulation by CNP of its GC activity

An expression plasmid that directed the synthesis of eel NPR-B was constructed by inserting the full-length cDNA for NPR-B into the expression vector pSRα. COS-1 cells were transfected with either the construct (pSRα/eNPR-B) or the control vector (pSRα) and assayed for GC activity. As expected, the CNP-induced intracellular accumulation of cGMP occured in the transfected cell that carried the pSRα/eNPR-B plasmid exhibited while no such accumulation of cGMP was observed in the mock-transfected cells. The order of potency of ligands in stimulating the GC activity was eCNP > eVNP > eANP (Fig. 9); eCNP was at least 500 times more potent than eANP.

Dense localization in chloride cells of eNPR-B

After immunohistochemical staining, the chloride cells of gill epithelium of freshwater-adapted eels were heavily stained with the antiserum against eNPR-B. Figure 10 A shows the dense staining at the base of two secondary lamellae. The chloride-cell-specific fluorescent dye DASPMI also stained the same sites (Fig. 11 A). DASPMI was originally used for staining mitochondria (12). The dye stains the

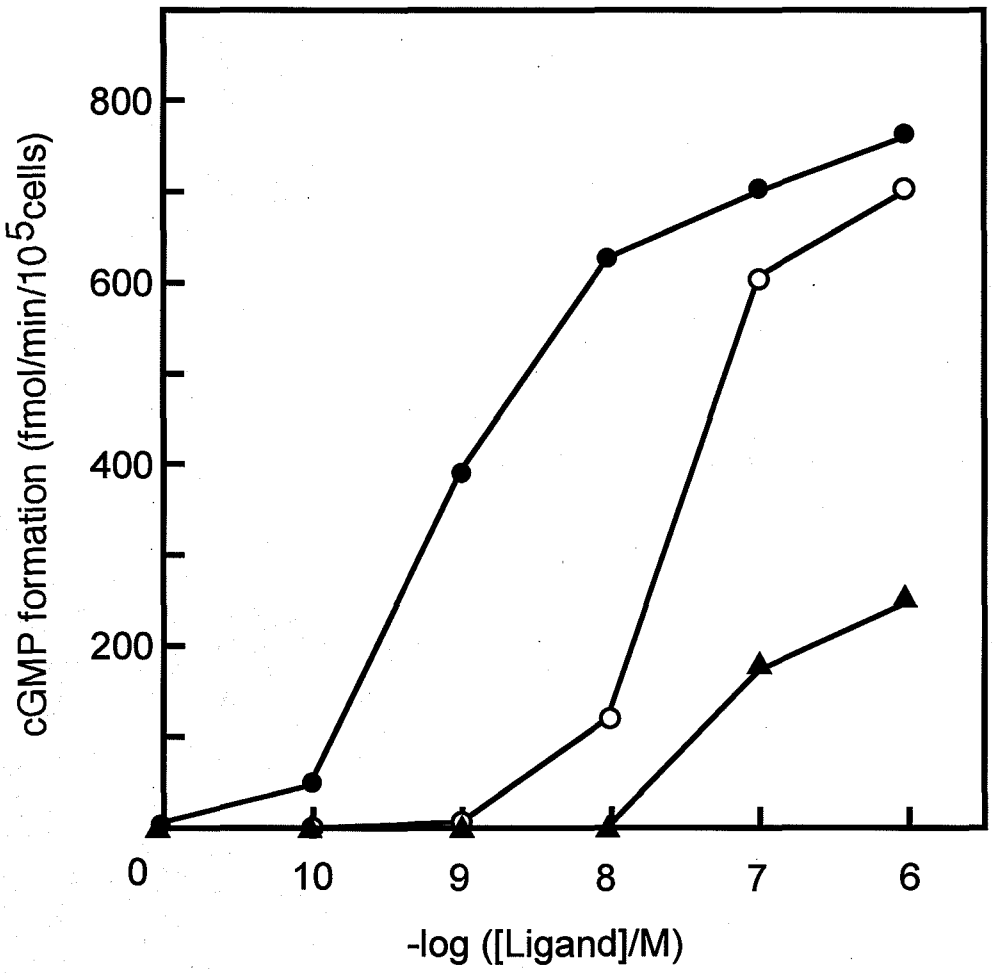

Fig. 9. **Stimulation of guanylyl cyclase activity of the eel NPR-B receptor expressed in COS-1 cells by eCNP, eVNP and eANP.** COS-1 cells were transfected with pcDL-SRα296/eNPR-B22 by electroporation and cultured for 48 h. The transfectant cells were then assayed for guanylyl cyclase activity in the presence of varying concentrations of eCNP (●), eVNP (○), or eANP (▲).

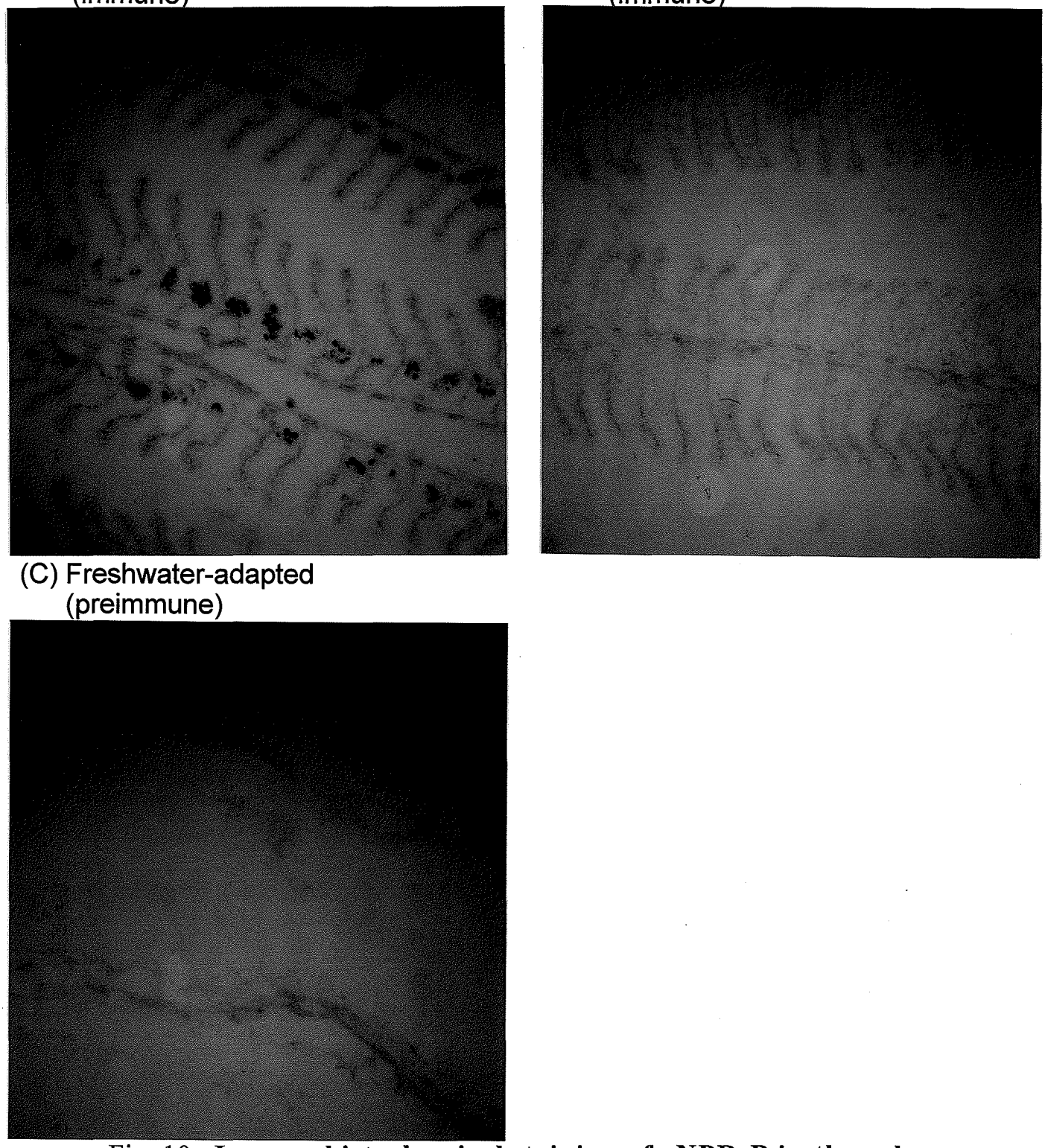

Fig. 10. **Immunohistochemical staining of eNPR-B in the eel gill.** Sections of gills of both freshwater-adapted (A) and seawater-adapted (B) eels were probed with an antiserum raised against eNPR-B and stained with avidin-biotin-peroxidase system. In panel C, a section from a freshwater-adapted eel was probed with preimmune serum and then processed in the same way as the section in A and B. Bars, 200 μm.

(A) Fluorescence (DASPMI)

(B) Phase-contrast image

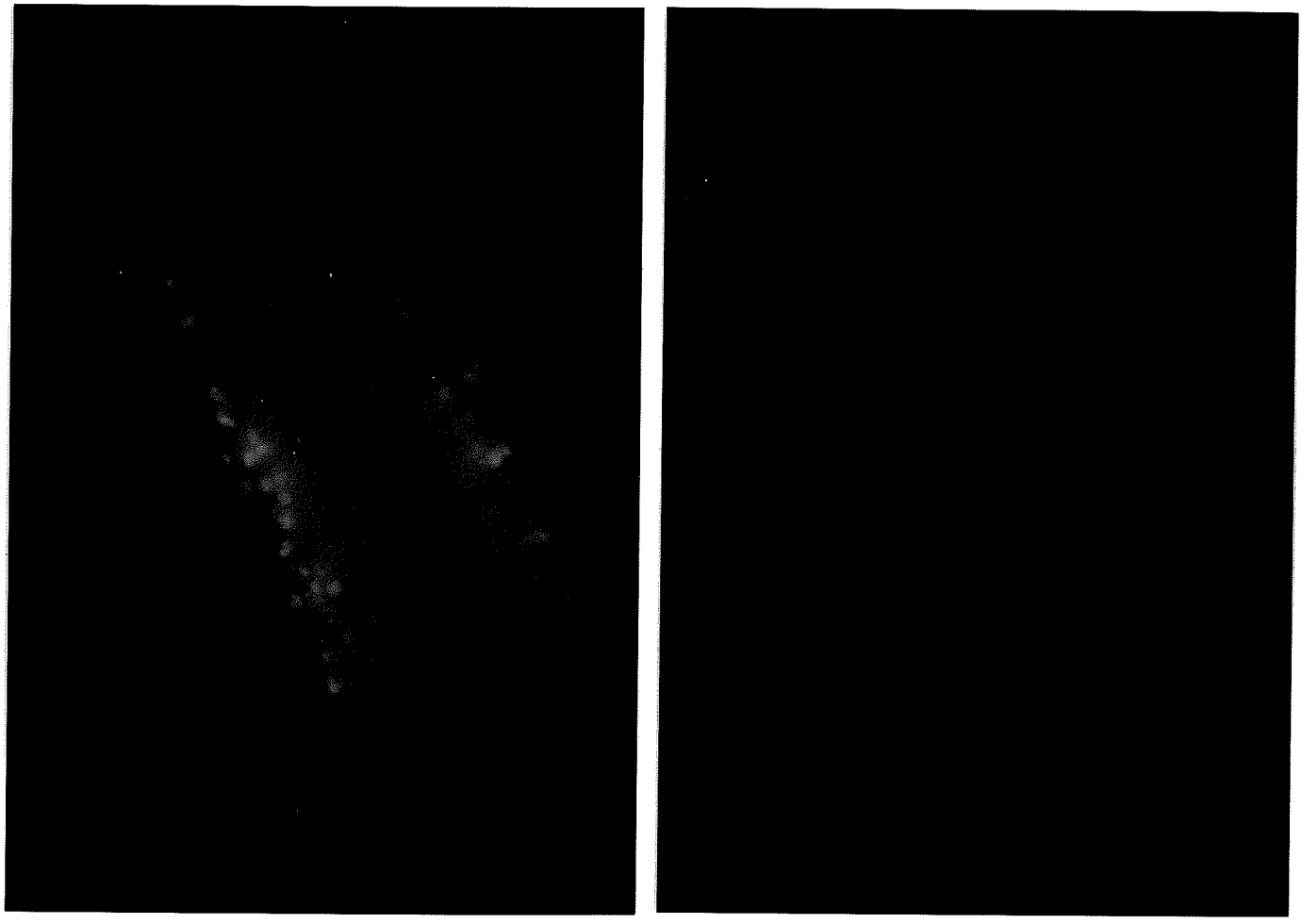

Fig. 11. **Staining of chloride cells with the mitochondrion-specific dye DASPMI.** Eel gills were perfused with PBS and stained with DASPMI. The staining was visualized with an Olympus IT-2 fluorescence microscope with an G520 filter. A: 100x, fluorescence. B: 100x, phase contrast image.

chloride cells which are very rich in mitochondria (35). These organelles produce the ATP required for pumping large amounts of ions across cell membranes. The colocalization of the products of the two different staining reactions indicated that NPR-B is localized and highly expressed on the chloride cells. On the other hand, in seawater-adapted eel, no specific staining was found, even at the base of two secondary lamellae (Fig. 10 B). This result is consistent with the results of the RNase protection analysis described above and also with the fact that the plasma concentration of CNP is higher in eels adapted to freshwater than in those adapted to seawater.

Reduced levels of NPR-B mRNA after transfer of eels from freshwater to seawater , as revealed by an RNase protection assay

The level of mRNA for eNPR-B fell dramatically as freshwater eels became adapted to seawater (Fig. 12). The level of this mRNA in the gill epithelium fell by about 50% within only one day, and by more than 90% within 7 days.

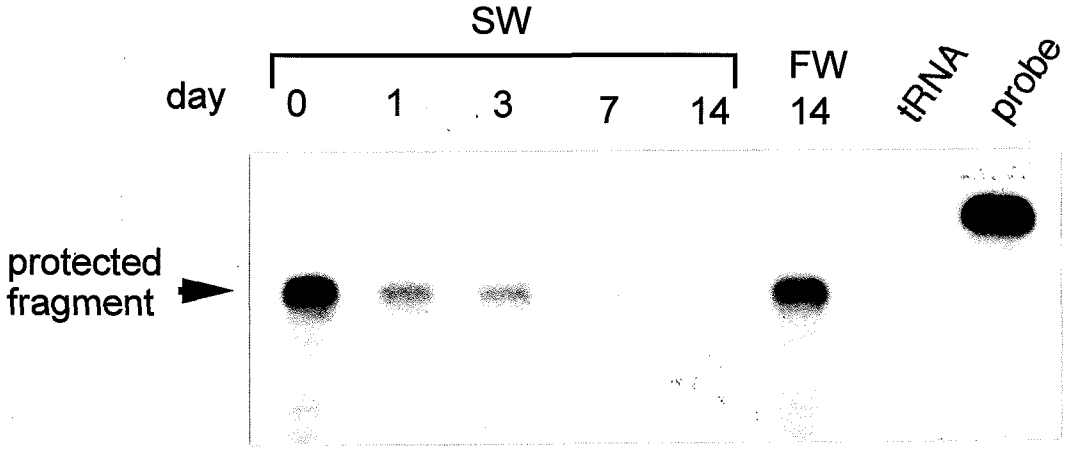

Fig. 12. **Time course of mRNA expression of NPR-B after transfer of eels from freshwater to seawater examined by an RNase protection assay.** Total RNA, as from eels adapted to seawater (SW) for the indicated number of days was annealed with a 459-bp ^{32}P-cRNA probe, digested with RNases A and T_1, and the protected fragments (384 bp, arrows) were analyzed by electrophoresis and fluorography as described under "Experimental Procedures". The pattern represents a typical result from one of three separated experiments. FW, freshwater.

Comparison of freshwater-adapted to seawater-adapted eels with respect to guanylyl cyclase activity in the gill epithelium

Figure 13 shows the production in response to various ligands of cGMP in the eel gill epithelium of freshwater-adapted and seawater-adapted eels. In the gill epithelium of freshwater-adapted eels, CNP stimulated the guanylyl cyclase activity but no other natriuretic peptides did so. This result indicates that only NPR-B, and not NPR-A, is expressed on the chloride cells of freshwater-adapted eels. In the seawater-adapted eels, consistent with the results described above, CNP no longer stimulated the production of cGMP; however, to our surprise, VNP stimulated the production of cGMP. This unexpected result indicates that some other receptor for natriuretic peptide, for which no evidence has previously been presented, appears on the gill epithelium of seawater-adapted eel.

Tissue distribution of mRNA for NPR-B in freshwater- and seawater-adapted eels

To determine the tissue distribution of mRNA for eel NPR-B and to examine whether levels of the mRNA are regulated by the osmotic pressure of the environment, an RNase protection analysis was performed

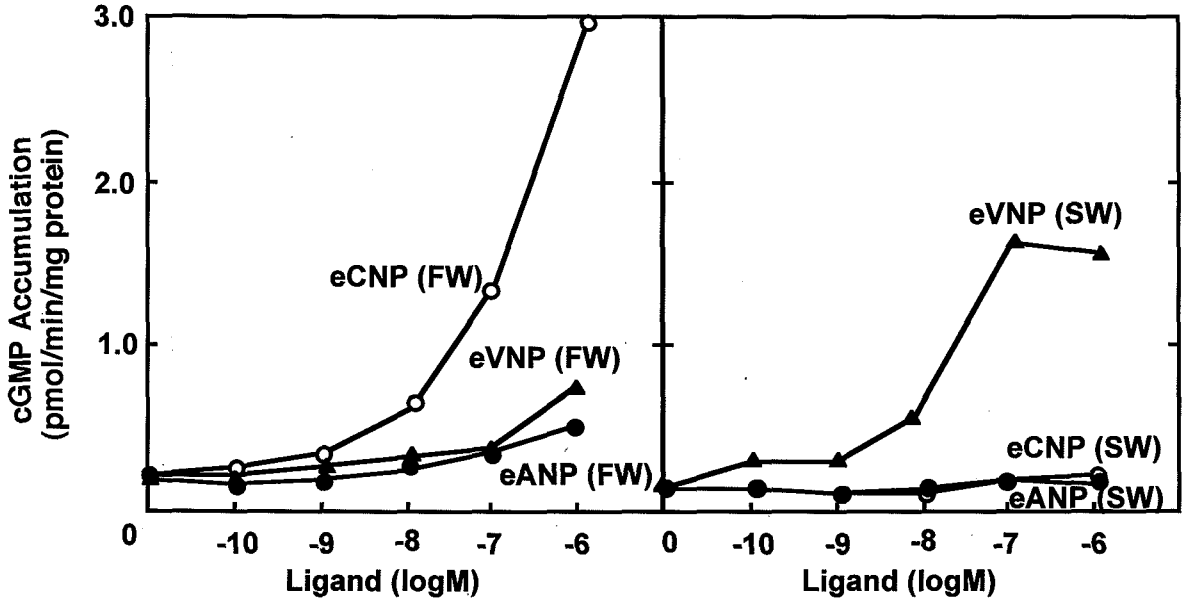

Fig. 13. **Comparison of guanylyl cyclase activity in the gill epithelium of freshwater-adapted (A) to that in seawater-adapted (B) eels.** The epithelial cells were removed from the gill arches with a razor blade. The eANP-(●), eCNP-(○), and eVNP-(▲) induced accumulation of cGMP was measured with a radioimmunoassay kit. Each point represents the result of triplicate determinations from three preparations.

with both freshwater-adapted and seawater-adapted eels (Fig. 14). In eels adapted to freshwater, NPR-B mRNA was detected at high levels in the liver, atrium and gill, at moderate levels in the brain and ventricle, at low levels in the esophageal sphincter, stomach, posterior intestine and kidney, and at trace levels in the esophagus, anterior intestine and head kidney (Fig. 14 A) The levels of NPR-B mRNA in the liver, atrium, and gill decreased markedly when eels were exposed to seawater. By contrast, the level in the ventricle remained unchanged (Fig. 14 B).

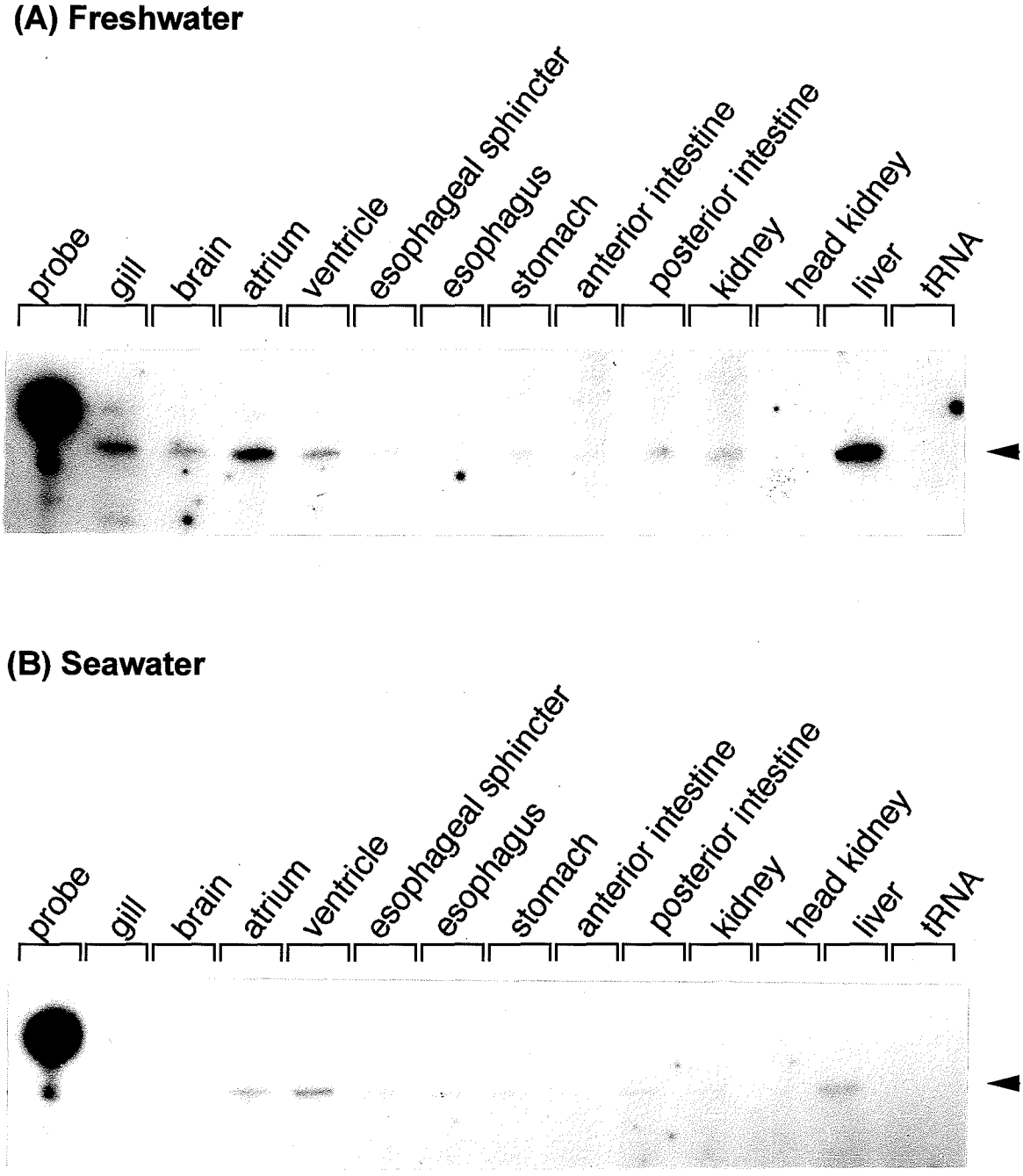

Fig. 14. **Levels of NPR-B mRNA in various eel tissues, as measured by an RNase protection analysis and their changes during adaptation to salinity.** Total RNA (10 μg) isolated from the indicated tissues was allowed to hybridize to the indicated probe as described under "Experimental Procedures". The arrow indicates the protected fragments. The pattern represents a typical result from of five separate experiments.

DISCUSSION

As part of an effort to clarify the physiological and adaptive roles of the natriuretic peptide system throughout the vertebrates species, molecular characterization was initiated of this system in the eel, *A. japonica,* which can tolerate a wide range of salinities, by cloning one of the GC-coupled receptors. The cDNA clone was isolated by cross-hybridization with a fragment of the cDNA for bovine NPR-B that encoded the GC domain. During the early stages of the study it was, however, unclear whether the isolated clone represented NPR-B or the other subtype, NPR-A, because the GC domains of the two types were expected to have very similar sequences, as demonstrated in the mammalian counterparts. The clone was unequivocally identified as a clone for NPR-B from the conserved locations of six cysteine residues in the encoded extracellular domain and the ligand specificity. The cloned receptor that was transiently expressed in COS-1 cells respond to CNP preferentially. Alignment of the amino acid sequence of eel NPR-B with the known mammalian sequences should help me to identify conserved sequences that merit future attention in attempts to understand the structure/function relationships. The high degree of sequence homology among the kinase-like domains as compared to the extracellular domains suggests that the kinase-like domains of the GC-coupled receptors are not

merely a link between the ligand-binding and GC domains but have, themselves, an important functions. Of interest also is the sequence homology in the vicinity of the third and fourth cysteine residues in the extracellular domain. By analogy to the disulfide-bonding pattern of the bovine NPR-C receptor which was recently determined in our laboratory (32), the third and fourth cysteine residues are expected to form a disulfide-bond. The two conserved sequences (NGRIVY236CGPL and IAG339CFYDG) may, therefore, be in close proximity and constitute an essential part of the ligand-binding site.

Biochemical and functional characterization of the shark natriuretic peptide system has demonstrated that in this chronically salt-loaded fish, CNP seems to be the only circulating hormone involved in the control of hemodynamics and osmoregulation (66, 77). For example, CNP has been shown to be much more potent than ANP in producing vasodilation (22) and the secretion of chloride ions from the rectal gland, (24, 34, 72) which expresses NPR-B that includes a GC domain and is highly specific for CNP. In mammals, by contrast, CNP is not detectable in the plasma and, when administered exogenously, it has only limited cardiovascular effects. CNP is also present in the plasma at high concentrations (82, 84). The amino acid sequence of eel CNP differs from those of shark and rat CNP at five and four positions among a total of 22 residues, respectively. Despite the similarity between eel and shark CNP in terms of their

pressure in the circulation eel NPR-B exhibits greater similarity to mammalian NPR-B than to the shark receptor in its ligand selectivity: eel NPR-B, resembling its the mammalian counterpart, is highly sensitive to CNP, sensitive to VNP, and weakly sensitive to ANP, whereas shark NPR-B is insensitive to natriuretic peptides other than CNP (29). The structural basis for the difference must await molecular cloning of the shark receptor. Such extensive comparative studies should also help us to gain some insight into the evolution of the natriuretic peptide systems.

Transfer of eels from freshwater to seawater has been shown recently, to cause a considerable decrease strongly in levels of CNP, as compared with those of ANP and VNP (84). Immunohistochemical staining revealed that similar changes occur also at receptor level in the gill epithelium, namely, in the chloride cells. These cells are thought to take up small quantities of ions in freshwater and secret large amount of salts in seawater (25, 55). RNase protection analysis demonstrated that the levels of the mRNA falls rapidly and the mRNA almost disappears within a week. In freshwater, an eel takes up various ions through chloride cells exclusively, because it does not drink sufficient water to allow it to take up ions via the gut (58). Thus, the role of CNP is, at least in chloride cells, to control the active uptake of ions, for example sodium, potassium and calcium ions, from the freshwater to eel plasma. By contrast, in seawater, the eel drinks so much seawater that water is taken into its body

and it can absorb ions from the seawater via the gut (58). The phenotype of chloride cells turns from "α and β" to hypertrophied "α" only (Fig. 15) (54, 71). Consistent with this change, the cells begin to excrete sodium, potassium, and chloride ions, acting in the opposite way to chloride cells in freshwater. (The "chloride cell" was originally named because this chloride-excretion activity (40).) NPR-B is now no longer necessary in these functions, and begins to disappear. After complete adaptation, CNP no longer stimulates the production of cGMP, but VNP is able to stimulate the production of cGMP in eel gill epithelia. This observation indicates the existence of a new member of natriuretic peptide receptor family which is affected by VNP only. The structure, tissue distribution and physiological properties of this receptor will be revealed after the molecular cloning of this receptor.

RNase protection analysis revealed that the mRNA for NPR-B is widely expressed in eel tissues and that the levels of its expression are controlled in a tissue-specific manner. The mRNA was found predominantly in the liver, atrium and gill. The highest level of expression in the liver was an unexpected finding. Although the functional significance of the hepatic NPR-B has yet to be determined, a report by Nair et al. (52) that the number of binding sites for natriuretic peptides and particulate GC activity are markedly increased in

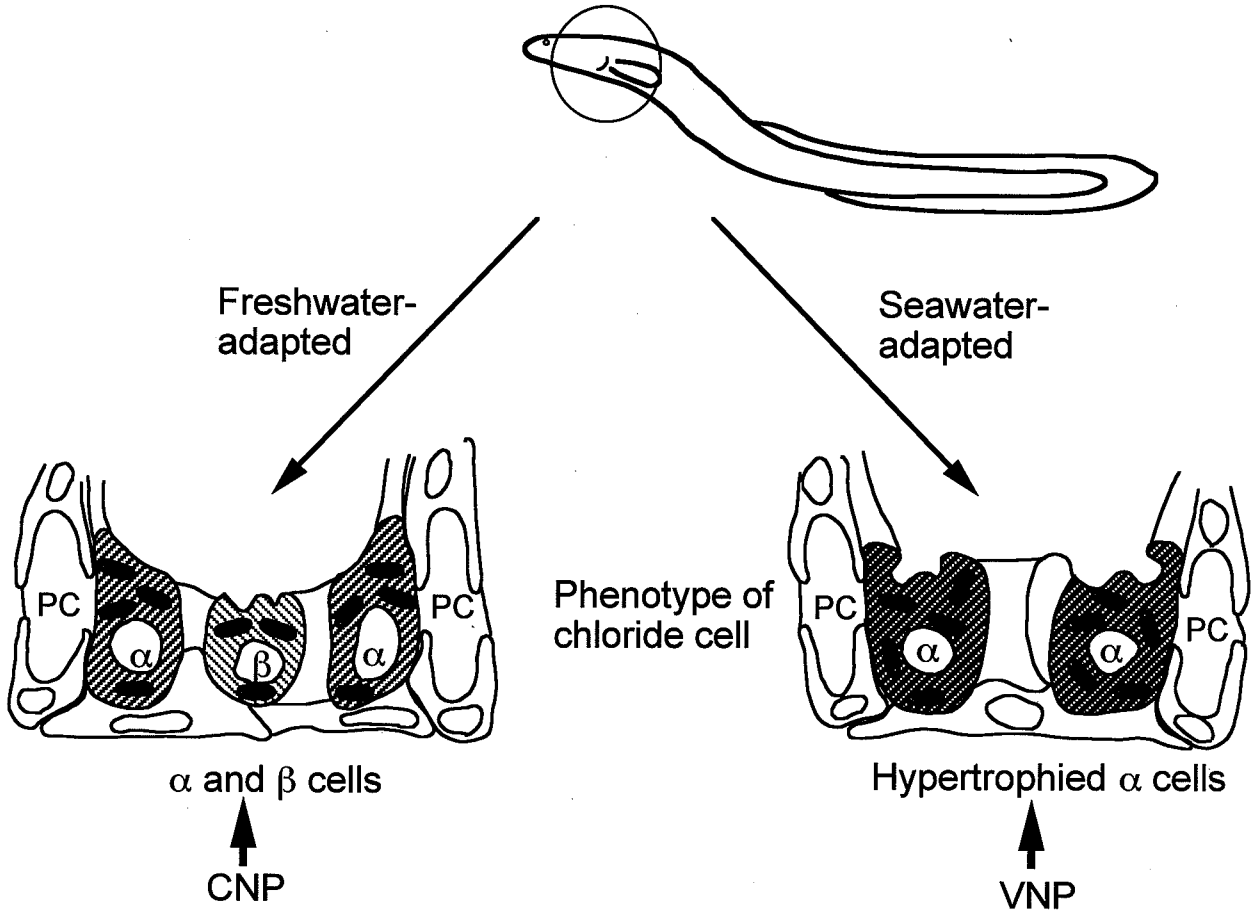

Fig. 15. **Schematic representation of the interlamellar space in freshwater- and seawater-adapted eels.** In freshwater, the ß-chloride cell is seen at the center between two α–chloride cells close to the basement membranes of the pillar capillaries. In seawater, the ß-chloride cell has disappeared and the entire space is occupied by two fully hypertrophied α–chloride cells.

regenerating liver in the rat suggests that the eel hepatic NPR-B identified here might also be involved in regulation of the growth and metabolic functions of the liver.

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REFERENCES

1. Aburaya, M., Minamino, N., Hino, J., Kangawa, K. and Matsuo, H. (1989) Distribution and molecular forms of brain natriuretic peptide in the central nervous system, heart and peripheral tissue of rat. *Biochem. Biophys. Res. Commun.* **165** 880-887.
2. Aburaya, M., Minamino, N., Kangawa, K., Tanaka, K. and Matsuo, H. (1989) Distribution and molecular forms of brain natriuretic peptide in porcine heart and blood. *Biochem. Biophys. Res. Commun.* **165** 872-879.
3. Akizuki, N., Kangawa, K., Minamino, N. and Matsuo, H. (1991) Cloning and sequence analysis of complementary DNA encoding a precursor for chicken natriuretic peptide. *FEBS Lett.* **280** 357-362.
4. Anand-Srivastava, M. B., Sairam, M. R. and Cantin, M. (1990) Ring-deleted analogs of atrial natriuretic factor inhibit adenylate cyclase/cAMP system. Possible coupling of clearance atrial natriuretic factor receptors to adenylate cyclase/cAMP signal transduction system. *J. Biol. Chem.* **265** 8566-8572.
5. Anand-Srivastava, M. B., Srivastava, A. K. and Cantin, M. (1987) Pertussis toxin attenuates atrial natriuretic factor-mediated inhibition of adenylate cyclase. Involvement of inhibitory guanine nucleotide regulatory protein. *J. Biol. Chem.* **262** 4931-4934.
6. Anand-Srivastava, M. B. and Trachte, G. J. (1993) Atrial natriuretic factor receptors and signal transduction mechanisms.

Pharmacol. Rev. **45** 455-497.

7. Appel, R. G., Wang, J., Simonson, M. S. and Dunn, M. J. (1986) A mechanism by which atrial natriuretic factor mediates its glomerular actions. *Am. J. Physiol.* **251** F1036-F1042.
8. Ardaillou, N., Nivez, M. P. and Ardaillou, R. (1985) Stimulation of guanylate cyclase by atrial natriuretic factor in isolated human glomeruli. *FEBS Lett.* **189** 8-12.
9. Arimura, J., Minamino, N., Kangawa, K. and Matsuo, H. (1991) Isolation and identification of C-type natriuretic peptide in chicken brain. *Biochem. Biophys. Res. Commun.* **174** 142-148.
10. Ballermann, B. J., Hoover, R. L., Karnovsky, M. J. and Brenner, B. M. (1985) Physiologic regulation of atrial natriuretic peptide receptors in rat renal glomeruli. *J. Clin. Invest.* **76** 2049-2056.
11. Bennett, B. D., Bennett, G. L., Vitangcol, R. V., Jewett, J. R., Burnier, J., Henzel, W. and Lowe, D. G. (1991) Extracellular domain-IgG fusion proteins for three human natriuretic peptide receptors. Hormone pharmacology and application to solid phase screening of synthetic peptide antisera. *J. Biol. Chem.* **266** 23060-23067.
12. Bereiter-Hahn, J. (1976) Dimethylaminostyrylmethylpyridiniumiodine (DASPMI) as a fluorescent probe for mitochondria in situ. *Biochim. Biophys. Acta* **423** 1-14.
13. Bourgoignie, J. J., Gavellas, G. and Hwang, K. H. (1986) Renal effects of atrial natriuretic factor in primate. *Am. J. Physiol.* **251**

F1049-F1054.

14. Burnett, J. C. J., Granger, J. P. and Opgenorth, T. J. (1984) Effects of synthetic atrial natriuretic factor on renal function and renin release. *Am. J. Physiol.* **247** F863-F866.

15. Chabrier, P. E., Roubert, P., Lonchampt, M. O., Plas, P. and Braquet, P. (1988) Regulation of atrial natriuretic factor receptors by angiotensin II in rat vascular smooth muscle cells. *J. Biol. Chem.* **263** 13199-13202.

16. Chang, M. S., Lowe, D. G., Lewis, M., Hellmiss, R., Chen, E. and Goeddel, D. V. (1989) Differential activation by atrial and brain natriuretic peptides of two different receptor guanylate cyclases. *Nature* **341** 68-72.

17. Chinkers, M., Garbers, D. L., Chang, M. S., Lowe, D.G., Chin, H. M., Goeddel, D. V. and Schulz, S. (1989) A membrane form of guanylate cyclase is an atrial natriuretic peptide receptor. *Nature* **338** 78-83.

18. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979) Isolation of biologically active ribonucleic acid from sourses enriched in ribonuclease. *Biochemistry* **18** 5294-5299.

19. Chomczynski, P., and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162** 156-159.

20. Chrisman, T. D., Schulz, S., Potter, L. R. and Garbers, D. L. (1993) Seminal plasma factors that cause large elevations in cellular cyclic GMP are C-type natriuretic peptides. *J. Biol. Chem.* **268** 3698-3703.

21. de-Bold, A. J. (1985) Atrial natriuretic factor: a hormone produced

by the heart. *Science* **230** 767-770.

22. Evans, D. H., Toop, T., Donald, J., and Forrest, J. N. J. (1993) C-type natriuretic peptides are potent dilators of shark vascular smooth muscle. *J. Exp. Biol.* **265** 84-87.

23. Fenrick, R., Babinski, K., McNicoll, N., Therrien, M., Drouin, J. and De-Lean, A. (1994) Cloning and functional expression of the bovine natriuretic peptide receptor-B (natriuretic factor R1c subtype). *Mol. Cell. Biochem.* **137** 173-182.

24. Forrest, J. N. J., Kelley, G.G., Opdyke, D., Schofield, J. P., and Aller, C. (1992) Synthetic shark CNP based on the amino acid sequence of cloned pre-pro shark heart CNP potentially stimulates chloride secretion in the perfused shark rectal gland. *Bull. Mt. Desert. Isl. Biol. Lab.* **31** 71-72.

25. Foskett, J. K., and Scheffey, C. (1982) The chloride cell: definitive identification as the salt-secretory cell in teleosts. *Science* **215** 164-166.

26. Fuller, F., Porter, J. G., Arfsten, A. E., Miller, J., Schilling, J. W., Scarborough, R. M., Lewicki, J. A. and Schenk, D. B. (1988) Atrial natriuretic peptide clearance receptor. Complete sequence and functional expression of cDNA clones. *J. Biol. Chem.* **263** 9395-9401.

27. Furuya, M., Yoshida, M., Hayashi, Y., Ohnuma, N., Minamino, N., Kangawa, K. and Matsuo, H. (1991) C-type natriuretic peptide is a growth inhibitor of rat vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* **177** 927-931.

28. Gauquelin, G., Garcia, R., Carrier, F., Cantin, M., Gutkowska, J., Thibault, G. and Schiffrin, E. L. (1988) Glomerular ANF receptor

regulation during changes in sodium and water metabolism. *Am. J. Physiol.* **254** F51-F55.

29. Gunning, M., Cuero, C., Solomon, R. and Silva, P. (1993) C-type natriuretic peptide receptors and signaling in rectal gland of *Squalus acanthias*. *Am. J. Physiol.* **264** F300-F305.
30. Hagiwara, H., Sakaguchi, H., Itakura, M., Yoshimoto, T., Furuya, M., Tanaka, S. and Hirose, S. (1994) Autocrine regulation of rat chondrocyte proliferation by natriuretic peptide C and its receptor, natriuretic peptide receptor-B. *J. Biol. Chem.* **269** 10729-10733.
31. Hirata, Y., Emori, T., Ohta, K., Shichiri, M. and Marumo, F. (1989) Vasoconstrictor-induced heterologous down-regulation of vascular atrial natriuretic peptide receptor. *Eur. J. Pharmacol.* **164** 603-606.
32. Itakura, M., Iwashina, M., Mizuno, T., Ito, T., Hagiwara, H. and Hirose, S. (1994) Mutational analysis of disulfide bridges in the type C atrial natriuretic peptide receptor. *J. Biol. Chem.* **269** 8314-8318.
33. Kangawa, K., Tawaragi, Y., Oikawa, S., Mizuno, A., Sakuragawa, Y., Nakazato, H., Fukuda, A., Minamino, N. and Matsuo, H. (1984) Identification of rat gamma atrial natriuretic polypeptide and characterization of the cDNA encoding its precursor. *Nature* **312** 152-155.
34. Karnaky, K. J. J., Valentich, J. D., Currie, M. G., Oehlenschlager, W.F. and Kennedy, M. P. (1991) Atriopeptin stimulates chloride secretion in cultured shark rectal gland cells. *Am. J. Physiol.* **260** C1125-C1130.
35. Karnaky, K. J. J., Degnan, K. J., Garretson, L. T. and

Zadunaisky, J. A. (1984) Identification and quantification of mitochondria-rich cells in transporting epithelia. *Am. J. Physiol.* **246** R770-R775.

36. Katafuchi, T., Hagiwara, H., Ito, T. and Hirose, S. (1993) A dramatic pH-dependent alteration in ANP receptor density: a note for using cultured cells. *Am. J. Physiol.* **264** C1345-C1349.
37. Katafuchi, T., Mizuno, T., Hagiwara, H., Itakura, M., Ito, T. and Hirose, S. (1992) Modulation by NaCl of atrial natriuretic peptide receptor levels and cyclic GMP responsiveness to atrial natriuretic peptide of cultured vascular endothelial cells. *J. Biol. Chem.* **267** 7624-7629.
38. Katafuchi, T., Takashima, A., Kashiwagi, M., Hagiwara, H., Takei, Y. and Hirose, S. (1994) Cloning and expression of eel natriuretic-peptide receptor B and comparison with its mammalian counterparts. *Eur. J. Biochem.* **222** 835-842.
39. Kato, J., Lanier-Smith, K. L. and Currie, M. G. (1991) Cyclic GMP down-regulates atrial natriuretic peptide receptors on cultured vascular endothelial cells. *J. Biol. Chem.* **266** 14681-14685.
40. Keys, A. B., and Willmer, E. N. (1932) "Chloride secreting cells" in the gills of fishes with special reference to the common eel. *J. Physiol.* **76** 368-378.
41. Kishimoto, I., Nakao, K., Suga, S., Hosoda, K., Yoshimasa, T., Itoh, H. and Imura, H. (1993) Downregulation of C-receptor by natriuretic peptides via ANP-B receptor in vascular smooth muscle cells. *Am. J. Physiol.* **265** H1373-H1379.
42. Kojima, M., Minamino, N., Kangawa, K. and Matsuo, H. (1990) Cloning and sequence analysis of a cDNA encoding a precursor for

rat C-type natriuretic peptide (CNP). *FEBS Lett.* **276** 209-213.

43. Kojima, M., Ohyama, Y., Miyamoto, K., Minamino, N., Kangawa, K. and Matsuo, H. (1994) Cloning and characterization of a novel natriuretic peptide in frog (*Rana catesbeiana*). *J. Biol. Chem.* **269** 13136-13140.

44. Koller, K. J., Lowe, D. G., Bennett, G. L., Minamino, N., Kangawa, K., Matsuo, H. and Goeddel, D.V. (1991) Selective activation of the B natriuretic peptide receptor by C-type natriuretic peptide (CNP). *Science* **252** 120-123.

45. Lazure, C., Ong, H., McNicoll, N., Netchitailo, P., Chretien, M., De-Lean, A. and Vaudry, H. (1988) The amino acid sequences of frog heart atrial natriuretic-like peptide and mammalian ANF are closely related. *FEBS Lett.* **238** 300-306.

46. Lowe, D. G., Camerato, T. R. and Goeddel, D. V. (1990) cDNA sequence of the human atrial natriuretic peptide clearance receptor. *Nucleic. Acids. Res.* **18** 3412.

47. Lowe, D. G., Chang, M. S., Hellmiss, R., Chen, E., Singh, S., Garbers, D. L. and Goeddel, D. V. (1989) Human atrial natriuretic peptide receptor defines a new paradigm for second messenger signal transduction. *EMBO J.* **8** 1377-1384.

48. Lynch, D. R., Braas, K. M. and Snyder, S. H. (1986) Atrial natriuretic factor receptors in rat kidney, adrenal gland, and brain: autoradiographic localization and fluid balance dependent changes. *Proc. Natl. Acad. Sci. USA* **83** 3357-3361.

49. Maack, T., Suzuki, M., Almeida, F. A., Nussenzveig, D., Scarborough, R. M., McEnroe, G. A. and Lewicki, J. A. (1987) Physiological role of silent receptors of atrial natriuretic factor.

Science **238** 675-678.

50. Minamino, N., Aburaya, M., Ueda, S., Kangawa, K. and Matsuo, H. (1988) The presence of brain natriuretic peptide of 12,000 daltons in porcine heart. *Biochem. Biophys. Res. Commun.* **155** 740-746.
51. Minamino, N., Makino, Y., Tateyama, H., Kangawa, K. and Matsuo, H. (1991) Characterization of immunoreactive human C-type natriuretic peptide in brain and heart. *Biochem. Biophys. Res. Commun.* **179** 535-542.
52. Nair, B. G., Steinke, L., Yu, Y. M., Rashed, H. M., Seyer, J. M. and Patel, T. B. (1991) Increase in the number of atrial natriuretic hormone receptors in regenerating rat liver. *J. Biol. Chem.* **266** 567-573.
53. Pandey, K. N. and Singh, S. (1990) Molecular cloning and expression of murine guanylate cyclase/atrial natriuretic factor receptor cDNA. *J. Biol. Chem.* **265** 12342-12348.
54. Pisam, M., Caroff, A., and Rambourg, A. (1987) Two types of chloride cells in the gill epithelium of freshwater-adapted euryhaline fish: *Lebistes reticulatus*; their modifications during adaptation to seawater. *Am. J. Anatomy.* **179** 40-50.
55. Pisam, M., and Rambourg, A. (1991) Mitochondria-rich cells in the gill epithelium of teleost fishes: an ultrastructural approach. *Int. Rev. Cytology* **130** 191-232.
56. Porter, J.G., Arfsten, A., Fuller, F., Miller, J.A., Gregory, L. C. and Lewicki, J.A. (1990) Isolation and functional expression of the human atrial natriuretic peptide clearance receptor cDNA.

Biochem. Biophys. Res. Commun. **171** 796-803.

57. Price, D. A., Doble, K. E., Lee, T. D., Galli, S. M., Dunn, B. M., Parten, B. and Evans, D. H. (1990) The sequencing, synthesis and biological actions of an ANP-like peptide isolated from the brain of the killfish *Fundulus heteroclitus*. *Biol. Bull. (Woods Hole)* **178** 279-285.
58. Prosser, C. L. (1973): *Comparative Animal Physiology.* Saunders, Philadelphia.
59. Rosenzweig, A. and Seidman, C. E. (1991) Atrial natriuretic factor and related peptide hormones. *Annu. Rev. Biochem.* **60** 229-255.
60. Saito, Y., Nakao, K., Itoh, H., Yamada, T., Mukoyama, M., Arai, H., Hosoda, K., Shirakami, G., Suga, S., Minamino, N. and et al. (1989) Brain natriuretic peptide is a novel cardiac hormone. *Biochem. Biophys. Res. Commun.* **158** 360-368.
61. Sakaguchi, H., Katafuchi, T., Hagiwara, H., Takei, Y. and Hirose, S. (1993) High-density localization of ANP receptors in chondrocytes of eel gill cartilage. *Am. J. Physiol.* **265** R474-R479.
62. Sakata, J., Kangawa, K. and Matsuo, H. (1988) Identification of new atrial natriuretic peptides in frog heart. *Biochem. Biophys. Res. Commun.* **155** 1338-1345.
63. Sanger, F., Nicklen, S. and Coulson, A. R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74** 5463-5467.
64. Sarzani, R., Paci, V. M., Dessi-Fulgheri, P., Espinosa, E. and Rappelli, A. (1993) Comparative analysis of atrial natriuretic peptide receptor expression in rat tissues. *J. Hypertens. Suppl.*

11 S214-S215.

65. Schiffrin, E. L., Deslongchamps, M. and Thibault, G. (1986) Platelet binding sites for atrial natriuretic factor in humans. Characterization and effects of sodium intake. *Hypertension* **8** II6-I10.

66. Schofield, J. P., Jones, D. S. and Forrest, J. J. (1991) Identification of C-type natriuretic peptide in heart of spiny dogfish shark (*Squalus acanthias*). *Am. J. Physiol.* **261** F734-F739.

67. Schulz, S., Singh, S., Bellet, R. A., Singh, G., Tubb, D. J., Chin, H. and Garbers, D. L. (1989) The primary structure of a plasma membrane guanylate cyclase demonstrates diversity within this new receptor family. *Cell* **58** 1155-1162.

68. Schweitz, H., Vigne, P., Moinier, D., Frelin, C. and Lazdunski, M. (1992) A new member of the natriuretic peptide family is present in the venom of the green mamba (*Dendroaspis angusticeps*). *J. Biol. Chem.* **267** 13928-13932.

69. Seilhamer, J. J., Arfsten, A., Miller, J. A., Lundquist, P., Scarborough, R. M., Lewicki, J. A. and Porter, J. G. (1989) Human and canine gene homologs of porcine brain natriuretic peptide. *Biochem. Biophys. Res. Commun.* **165** 650-658.

70. Shimonaka, M., Saheki, T., Hagiwara, H., Ishido, M., Nogi, A., Fujita, T., Wakita, K., Inada, Y., Kondo, J. and Hirose, S. (1987) Purification of atrial natriuretic peptide receptor from bovine lung. Evidence for a disulfide-linked subunit structure. *J. Biol. Chem.* **262** 5510-5514.

71. Shirai, N., and Utida, S. (1970) Development and degeneration of the chloride cell during sea water and fresh water adaptation of the

Japanese eel: *Anguilla japonica. Z. Zellforsch* **103** 247-264.

72. Solomon, R., Protter, A., McEnroe, G., Porter, J. G. and Silva, P. (1992) C-type natriuretic peptides stimulate chloride secretion in the rectal gland of *Squalus acanthias. Am. J. Physiol.* **262** R707-R711.
73. Sudoh, T., Kangawa, K., Minamino, N. and Matsuo, H. (1988) A new natriuretic peptide in porcine brain. *Nature* **332** 78-81.
74. Sudoh, T., Maekawa, K., Kojima, M., Minamino, N., Kangawa, K. and Matsuo, H. (1989) Cloning and sequence analysis of cDNA encoding a precursor for human brain natriuretic peptide. *Biochem. Biophys. Res. Commun.* **159** 1427-1434.
75. Sudoh, T., Minamino, N., Kangawa, K. and Matsuo, H. (1990) C-type natriuretic peptide (CNP): a new member of natriuretic peptide family identified in porcine brain. *Biochem. Biophys. Res. Commun.* **168** 863-870.
76. Suga, S., Nakao, K., Itoh, H., Komatsu, Y., Ogawa, Y., Hama, N. and Imura, H. (1992) Endothelial production of C-type natriuretic peptide and its marked augmentation by transforming growth factor-beta. Possible existence of "vascular natriuretic peptide system". *J. Clin. Invest.* **90** 1145-1149.
77. Suzuki, R., Takahashi, A., Hazon, N. and Takei, Y. (1991) Isolation of high-molecular-weight C-type natriuretic peptide from the heart of a cartilaginous fish (European dogfish, *Scyliorhinus canicula*). *FEBS Lett.* **282** 321-325.
78. Suzuki, R., Takahashi, A. and Takei, Y. (1992) Different molecular forms of C-type natriuretic peptide isolated from the brain and heart of an elasmobranch, *Triakis scyllia. J. Endocrinol.*

135 317-323.

79. Takebe, Y., Seiki, M., Fujisawa, J., Hoy, P., Yokota, K., Arai, K., Yoshida, M., and Arai, N. (1988) SRα promoter: an efficient and versatile mammalian cDNA expression system composed of the simian virus 40 early promoter and the R-U5 segment of human T-cell leukemia virus type 1 long terminal repeat. *Mol. Cell. Biol.* **8** 466-472.
80. Takei, Y., Takahashi, A., Watanabe, T. X., Nakajima, K. and Sakakibara, S. (1989) Amino acid sequence and relative biological activity of eel atrial natriuretic peptide. *Biochem. Biophys. Res. Commun.* **164** 537-543.
81. Takei, Y., Takahashi, A., Watanabe, T. X., Nakajima, K. and Sakakibara, S. (1991) A novel natriuretic peptide isolated from eel cardiac ventricles. *FEBS Lett.* **282** 317-320.
82. Takei, Y., Takahashi, A., Watanabe, T. X., Nakajima, K., Sakakibara, S., Takao, T. and Shimonishi, Y. (1990) Amino acid sequence and relative biological activity of a natriuretic peptide isolated from eel brain. *Biochem. Biophys. Res. Commun.* **170** 883-891.
83. Takei, Y., Ueki, M. and Nishizawa, T. (1994) Eel ventricular natriuretic peptide: cDNA cloning and mRNA expression. *J. Mol. Endocrinol.* **13** 339-345.
84. Takei, Y.and Balment, R. J. (1993) Biochemistry and physiology of a family of eel natriuretic peptides. *Fish. Physiol. Biochem.* **11** 183-188.
85. Tanaka, I., Misono, K. S. and Inagami, T. (1984) Atrial natriuretic factor in rat hypothalamus, atria and plasma:

determination by specific radioimmunoassay. *Biochem. Biophys. Res. Commun.* **124** 663-668.

86. Ueda, S., Minamino, N., Aburaya, M., Kangawa, K., Matsukura, S. and Matsuo, H. (1991) Distribution and characterization of immunoreactive porcine C-type natriuretic peptide. *Biochem. Biophys. Res. Commun.* **175** 759-767.
87. Wilcox, J. N., Augustine, A., Goeddel, D. V. and Lowe, D. G. (1991) Differential regional expression of three natriuretic peptide receptor genes within primate tissues. *Mol. Cell. Biol.* **11** 3454-3462.
88. Winquist, R. J., Faison, E. P., Waldman, S. A., Schwartz, K., Murad, F. and Rapoport, R. M. (1984) Atrial natriuretic factor elicits an endothelium-independent relaxation and activates particulate guanylate cyclase in vascular smooth muscle. *Proc. Natl. Acad. Sci. USA* **81** 7661-7664.
89. Yoshihara, A., Kozawa, H., Minamino, N., Kangawa, K. and Matsuo, H. (1990) Isolation and sequence determination of frog C-type natriuretic peptide. *Biochem. Biophys. Res. Commun.* **173** 591-598.