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Cloning and Sequence Analysis of cDNAs Encoding Precursors of Urotensin II-α and -γ

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The primary structures of the precursors of urotensin II (UII)-α and -γ, neuropeptide hormones of the caudal neurosecretory system of the carp, *Cyprinus carpio,* have been determined by analyzing the nucleotide sequences of cloned DNAs complementary to the mRNAs encoding them.

A cDNA library, constructed with poly(A)$^+$RNA in the preterminal spinal cord, was screened using ^{32}P-labeled synthetic oligonucleotides representing all possible cDNA sequences corresponding to the pentapeptide common to all forms of carp UII (UII-α, -β, and -γ). Twenty out of 39 positive clones were analyzed with the restriction endonucleases, *Hind*III and *Pvu*II, and classified into 4 groups. Nucleotide sequence analysis of 4 clones representing each group revealed that 2 clones encode the precursor of UII-α and the other 2 that of UII-γ. Both precursors consist of 125 amino acid residues, and UII-α and -γ exist at the carboxyl-termini preceded by Arg-Lys-Arg. The homology in both nucleotide and amino acid sequences between the precursors of UII-α and -γ is more than 90%, suggesting that the genes were generated from a common ancestral gene by duplication. There is no sequence homology between the precursors of UII and urotensin I, another peptide hormone of the caudal neurosecretory system, nor between the precursors of UII and somatostatin-14. RNA transfer blot analysis indicated that mRNAs encoding the precursors of UII-α and -γ are present in the spinal cord but not in the brain, intestine, liver, or kidney of the carp. *In situ* hybridization using ^{32}P-labeled synthetic oligonucleotide complementary to common sequence of mRNAs for both UII-α and -γ has detected the UII-producing neurons in the caudal spinal cord of the carp.

The caudal neurosecretory system in the posterior spinal cord of elasmobranch and teleost fishes, defined by Enami (1959), synthesizes and releases at least 2 neurohormones, urotensins I and II (UI and UII). Although the architecture of this system, similar to the hypothalamoneurohypophysial system, and its universal presence in fish suggest that these peptides have an important role in their physiology, a coherent function of caudal neurosecretion has not been established. Urotensin I is a 41 amino acid residue peptide (Ichikawa et al., 1982; Lederis et al., 1982) homologous to mammalian corticotropin-releasing factor (CRF; see Ichikawa, 1985). The elucidation of the primary structure of the precursor of carp UI (Ishida et al., 1986) has shown a sequence homology, as well as an organizational similarity, between the precursors of UI and CRF (Furutani et al., 1983; Shibahara et al., 1983), suggesting that they are evolutionarily related. The primary structure of a single UII peptide from the goby, *Gillichthys mirabilis,* was determined by Pearson et al. (1980), followed by the identification of 2 forms of UII (UII$_A$ and UII$_B$) from the sucker, *Catostomus commersoni* (McMaster and Lederis, 1983), and 3 forms of UII (UI-α, -β, and -γ) from the carp, *Cyprinus carpio* (Ichikawa et al., 1984). All forms of UII so far isolated are cyclic dodecapeptides that share an identical 6 residue disulfide-bridged ring, while multiple substitutions occur in amino-terminal sequences. An array of spasmogenic, hypophysiotropic, osmoregulatory, and metabolic effects has been ascribed to UII (see Bern et al., 1985). Recent immunohistochemical studies have demonstrated the coexistence of UI and UII in the same neurons in the spinal cord of several fishes (see Bern et al., 1985; H. Kobayashi et al., 1986).

Characterization of the precursors of UII peptides would help to elucidate the evolutionary relationships among different forms of UII, between UII and UI, and between UII and somatostatin, which has been shown to be partially homologous to UII (Pearson et al., 1980); it may also provide information on the putative carrier proteins, urophysins (Berlind et al., 1972; Lederis et al., 1974, 1981; Moore et al., 1975). We report here the cloning and nucleotide sequence of cDNAs that encode the precursors of UII-α and -γ of the carp.

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Materials and Methods

Materials

Terminal deoxynucleotidyltransferase, *Escherichia coli* DNA polymerase and *E. coli* RNase H were obtained from Takara Shuzo (Kyoto, Japan); *E. coli* DNA, poly(A), and dG-tailed Okayama linker from Pharmacia P-L Biochemicals; reverse transcriptase from Bio-Rad; ^{32}P-ATP (3000 Ci/mmol) and ^{32}P-dCTP (400 Ci/mmol) from Amersham; proteinase K from Merck; and Vectastain ABC kit from Vector Laboratories.

Construction of cDNA library from carp spinal cord

Total RNA, from the preterminal region of the spinal cord corresponding to the sixth to the second preterminal vertebrae of the carp (*C. carpio*), was extracted in 4 M guanidine thiocyanate buffer as described by Chirgwin et al. (1979). Poly(A)$^+$RNA was purified from the total RNA by oligo(dT)-cellulose column chromatography (Aviv and Leder, 1972). A cDNA library was constructed by the method of Okayama and Berg (1982) with 5 μg of poly(A)$^+$RNA and 1.6 μg of vector/primer DNA as described by Ishida et al. (1986). After transformation of *E. coli* HB 101 (Morrison, 1979), the cells were plated on ampicillin-containing agar plates and replicated on nitrocellulose filters.

Identification of UII precursor cDNAs

A total of 6000 transformants was screened by colony hybridization using a mixture of ^{32}P-labeled synthetic oligonucleotides, 5′-TA(TC)TTCCA(AG)AA(AG)CA-3′, representing all possible cDNA sequences that corresponded to the peptapeptide sequence Cys-Phe-Trp-

Lys-Tyr, amino acid residues 6–10 common to UII-α, -β, and -γ. The probe was 5′-end-labeled to a specific activity of 1.1 × 10^8 cpm/μg. Prehybridization was performed at 55°C for 12 hr in 1 M NaCl/50 mM Tris-HCl, pH 8.0/10 mM EDTA/10 × Denhardt's solution (1 × Denhardt's = 0.02% BSA/0.02% Ficoll/0.02% polyvinylpyrolidone)/*E. coli* DNA (100 μg/ml). The filters were hybridized with the probe at 33°C for 46 hr in 4 × SSC (1 × SSC = 0.15 M NaCl/15 mM sodium citrate, pH 7.4)/10 × Denhardt's solution/*E. coli* DNA (100 μg/ml), and washed twice at 0°C for 5 min and twice at 33°C for 20 min in 4 × SSC.

Nucleotide sequence analysis

Plasmid DNAs from the positive clones were analyzed by digestion with restriction enzymes. Restriction fragments were subcloned into the pUC 18 vector, and their nucleotide sequences were determined by the dideoxy-termination method (Sanger et al., 1977). Since portions of cDNA could not be analyzed in this way, plasmid DNAs carrying artificial deletions were prepared by the kilosequence method (Frischauf et al., 1980).

RNA transfer blot analysis

Poly(A)$^+$RNAs from the spinal cord, brain, intestine, liver, and kidney of the carp were electrophoresed in a 1.4% agarose/formaldehyde gel and transferred to a nitrocellulose filter (Maniatis et al., 1982). The filter was prehybridized at 42°C for 4 hr in 50% (vol/vol) formamide/5 × SSC/5 × Denhardt's solution/0.1% SDS/denatured salmon sperm DNA (100 μg/ml). It was then hybridized at 42°C for 18 hr in the same solution containing ^{32}P-labeled nick-translated whole plasmid (pUII-16 for UII-α or pUII-10 for UII-γ), washed at 68°C in 0.1 × SSC/0.5% SDS, and subjected to autoradiography.

In situ *hybridization*

A 22mer-nucleotide probe, 5′-TTCCAGAAGCAATCTGCACCGC-3′, complementary to common sequence of mRNAs for both UII-α and -γ encoding the octapeptide sequence Gly-Gly-Ala-Asp-Cys-Phe-Trp-Lys, was synthetized and 5′-end-labeled to a specific activity of 1 × 10^8 cpm/μg.

The preterminal region of the spinal cord of the carp (800–1000 g) was rapidly dissected out upon decapitation. The tissue was fixed by immersion in Bouin's solution without acetic acid at 4°C for 16 hr. The tissue was then dehydrated through graded ethanols and embedded in soft paraffin. Sagittal sections were cut serially at 10 μm and collected on gelatinized slides. An appropriate section was subjected to *in situ* hybridization, and the next section was processed for immunohistochemistry of UII.

After rehydration, tissue sections were digested with proteinase K (1 μg/ml in 0.1 M Tris-HCl, pH 7.5/50 mM EDTA) at 37°C for 30 min, washed in distilled water, and air-dried. The slides were then placed in a moist chamber and the ^{32}P-labeled probe diluted to 10^4 cpm/μl with the hybridization solution [0.9 M NaCl/6 mM EDTA/10 × Denhardt's solution/denatured salmon sperm DNA (100 μg/ml)/0.1 M Tris-HCl, pH 7.5] was pipetted directly onto the tissue sections. Sections were coverslipped and incubated at 30°C for 16 hr. The hybridized slides were washed for 15 min in 6 × SSC, 3 × SSC, 1 × SSC, dehydrated through graded ethanols, dipped in Sakura NR-M2 emulsion, and exposed at 4°C for 2 weeks.

Tissue sections for immunohistochemistry were stained by the avidin-biotin-peroxidase complex (ABC) method (Hsu et al., 1981) using Vectastain ABC kit. The staining procedure consisted of the following incubation steps: (1) anti-goby UII serum (kindly provided by Professor H. Kobayashi, Toho University) diluted 1:200 with 0.1 M phosphate buffer, pH 7.4, containing 0.9% NaCl and 0.1% Triton X-100 (PBS-T) for 2 hr; (2) biotinylated anti-rabbit IgG diluted 1:200 with PBS-T for 1 hr; (3) complex of avidin and biotinylated HRP diluted 1:100 with PBS-T for 30 min; and (4) 0.05% 3,3′-diaminobenzidine tetrahydrochloride and 0.01% H_2O_2 in 0.1 M phosphate buffer, pH 7.4, for 10 min. Incubations were carried out at room temperature. The sections were rinsed for 15 min with PBS-T between each step. The specificity of the antiserum was described elsewhere (Owada et al., 1985).

Results

Cloning of cDNAs encoding carp UII precursors

Six thousand transformants were screened with the 5′-^{32}P-labeled synthetic oligonucleotide probe and 39 positive clones were obtained. Of 39 clones, 20 with strong hybridization were analyzed with the restriction endonucleases, *Hind*III and *Pvu*II, and classified into 4 groups according to the restriction patterns (Fig. 1). A single clone had only a *Hind*III site (pUII-3); 5 clones had both *Hind*III and *Pvu*II sites (pUII-10); 7 clones had neither a *Hind*III nor a *Pvu*II site (pUII-16); and 7 clones had only a *Pvu*II site (pUII-18).

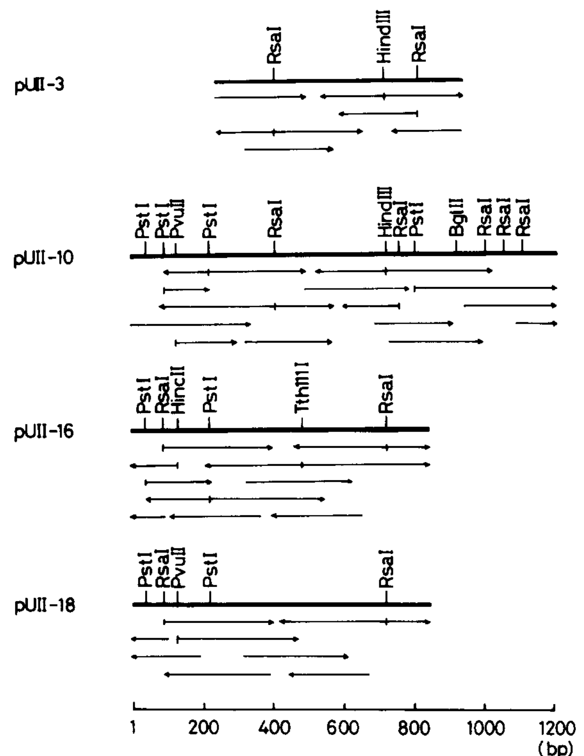

Figure 1. Restriction maps and sequence analysis strategies of 4 clones of UII cDNAs. Two clones, pUII-16 and -18, encoded precursor of UII-α and the other two, pUII-3 and -10 UII-γ precursor. *Horizontal arrows* indicate the direction and extent of sequence determination. *bp,* base pairs.

Structural analysis of carp UII precursors

The strategies for determining the nucleotide sequences of 4 clones are shown in Figure 1. Sequence analysis revealed that pUII-16 and -18 encoded UII-α precursor, and pUII-3 and -10 encoded UII-γ precursor. The nucleotide sequences of mRNAs encoding the precursors of UII-α (pUII-18) and -γ (pUII-10) and deduced amino acid sequences are shown in Figure 2*A*. Both pUII-16 and -18 consisted of 793 nucleotides, excluding the poly(A) sequences at the 3′-termini. There were 7 nucleotide substitutions between them; one in the protein coding region at position 83 and the others in the 3′-untranslated region (data not shown). On the other hand, pUII-3 and -10 consisted of 657 and 1162 nucleotides, respectively. The pUII-3 was apparently an incomplete cDNA lacking 237 nucleotides at the 5′-terminus. There were no nucleotide substitutions in protein coding region, but a large number of substitutions occurred between them in the 3′-untranslated regions (data not shown).

The translation initiation site was tentatively assigned to the methionine codon AUG at positions 1–3, which was the first AUG triplet encountered within all of the mRNA sequences analyzed. The sequence of nucleotide residues at positions 340–375 corresponded precisely with the amino acid sequence of UII-α or -γ. The sequence encoding either UII-α or UII-γ was preceded by AGGAAGAGA (Arg-Lys-Arg) and followed by the

A

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pUII-18                      5'---AUUCACCAAAGCCUGACCUGAAAGGACCUUCAACCUGCUGCAGAG-1
pUII-10                                                C

          Met Met Cys Asn Leu Leu Leu Ser Phe Ser Val Leu Leu Leu Ser Cys Thr His Leu Val 20
pUII-18   AUG AUG UGU AAC CUG CUC CUG UCC UUC UCU GUC CUC CUG CUC UCU UGU ACU CAU CUG GUA 60
pUII-10                         U          G                        C   C  G        U U
                                          Cys                             Ser         Leu

          Ala His Pro Val Thr Asp Thr Ala Asp Met Thr Tyr Ser Gly Pro Asp Ser Val Glu Glu 40
pUII-18   GCA CAU CCU GUU ACG GAC ACA GCU GAC AUG ACU UAC AGC GGC CCU GAU UCA GUG GAA GAG 120
pUII-10                     A                               U

          Ala Gly Gly Val Ser Pro Asp Asp Phe Ala Val Ser Asp Leu Asn Asp Leu Leu Gln Arg 60
pUII-18   GCC GGA GGC GUC AGU CCA GAU GAU UUC GCU GUC UCU GAU CUC AAU GAU CUG CUG CAG AGG 180
pUII-10             G      A                  U                         A  AC
                          Asn                 Ser                     Glu His

          Ala Ala Val Val Gly Tyr Ser Pro Leu Leu Ser Arg Glu Asn Ile Lys Val Pro Gly Gln 80
pUII-18   GCG GCA GUC GUA GAA UAU UCC CCG CUG CUC AGC CGA GAG AAU AUC AAA GUG CCU GGG CAG 240
pUII-10                C   G             A     U        A
                      Ala                     Phe     Gln

          Ile Pro Lys Glu Ala Leu Arg Glu Leu Leu Leu Glu Lys Pro Tyr Arg Leu Ile Pro Pro 100
pUII-18   AUU CCU AAA GAG GCU CUU AGA GAG UUA CUG UUA GAA AAA CCG UAU CGC CUC AUU CCU CCC 300
pUII-10

          Ser Gly Leu Trp Gly Ser Arg Arg Gln Phe Arg Lys Arg Gly Gly Gly Ala Asp Cys Phe 120
pUII-18   AGC GGU CUG UGG GGC AGC AGG AGA CAG UUC AGG AAG AGA GGC GGC GGU GCA GAU UGC UUC 360
pUII-10   C C
          Arg

          Trp Lys Tyr Cys Val END
pUII-18   UGG AAA UAC UGU GUU UGA GGCAUUCGUGACAUACAGAACUGAGUCACAGGGCUUUUAUAGCUAUUCCUACCAG
pUII-10         G         A         GGCAUUUGUGACAUCACUGAACUGAGUCACAGGGCUUUUAUAGCUUUUCCUACCA 433
                          Ile

pUII-18   UAUGGACACUCUCUUUCACACACACCCACACAAUAAUACACACAAUACAAAAGGAAAAAUAUACAGGACACAUUUAUAA
pUII-10   GUUUGGACUCUCUCUCUCUGUCUCUCUCUCUUUCUCUCUCUCUCUCUCUCUCUCUCACACACACACACACACA 512

pUII-18   AAACUAUUGUGCAAAACAGUUGCUUCACAGAGAGACUGACGUAAACCACUAAAAUAAAATAUCUUUUUUUCAACAUAUC
pUII-10   CACACACACACACACACACACACACACACACACACACACACACACACACAUAUAUAUAUAUAUAUAUAUAUAUAUAUAG 591

pUII-18   AUCAGCAUUUAUGUGUCUGUAAAUUUUGAAUGACAUAAUUCUGGAAUAUUUUAAAUAAGUCUUUAUAGAAGAGACCACA
pUII-10   GACAGUUAUAAAAACAUGUGCAAAACAGUUGCUCCACAGAGAAGCUGAUAUUAACCACUAAACGUAAAAUAAUUUUCUA 670

pUII-18   AUAGCUGUACUGUAUAAGACAUAUAACUGUAAACUAUAAAUCAAUGCAAAAAUAAACUAAUAAAAAAAAAGAAAUAAAA n
pUII-10   CAUAUCUUAAGCUUUUAUGUGUACUGUGUGUGUGUGUAUAUGUACUAUGUGUGAAGCAACAGAGCCGAAUAUAUCCU 749

pUII-18   ---3'
pUII-10   GAUGUCUGCUGCAGAGAUGAUCUGUAACCUGCUUCUGCCUGCUCUGUCCUCCUGCUCUCCUGCAGACACCGUGUUUAAC 828

pUII-10   CUGAUUUACCAAGGAACGGGAAGGAGAGAAUCUAGCUUUCCGAAAUGACAGAUCUCUAUAAAGAAAACCAACUACAGAG 907

pUII-10   UGUGUGUGUGUGUGUGUGUGUGUGAGUGAGUGAGUGUGAAGGACUGGAAUAAGCUGUACUCUCUUAACCGGUGAUCAUUUG 986

pUII-10   UGUGGUCAAUCUUGGUUUUACAGAUUGUACUGGUGUUGGCUGUGUGCUUACCUGUUGUUAUACCUCAAGCUGCUCUCUG 1065

pUII-10   UACUGUUUGACUCAUGCAUGUGUUGAACUCCAAUAAAACAUACCGAGAUGGUA n ---3'
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B

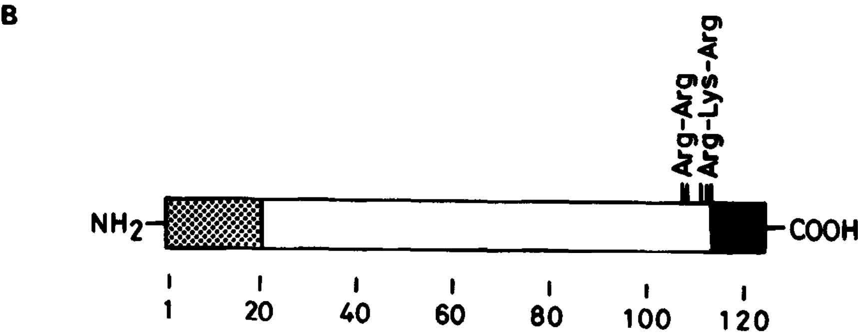

Figure 2. Structure of mRNAs for carp UII-α and -γ and organization of the precursor. *A*, Primary structure of mRNAs for UII-α and -γ. Nucleotide sequences of mRNAs are deduced from the sequences of cDNA inserts (pUII-18 for UII-α and pUII-10 for UII-γ). Nucleotide residues are numbered in the 5′ to 3′ direction starting with the first residue of the AUG triplet (methionine) as no. 1. The deduced amino acid residues are numbered beginning with the initiating methionine. UII sequences are boxed. Blank spaces in pUII-10 indicate the sequence identity with pUII-18. AAUAAA sequences in the 3′-untranslated region are underlined. *B*, Schematic representation of the structure of the precursor of either UII-α or -γ. UII sequence is indicated by a *black box* and the putative signal peptide is indicated by a *stippled box*. Proteolytic sites, Arg-Arg and Arg-Lys-Arg, are shown. Amino acid numbers are given at the bottom. Only 12 amino acid substitutions are observed between the precursors of UII-α and -γ.

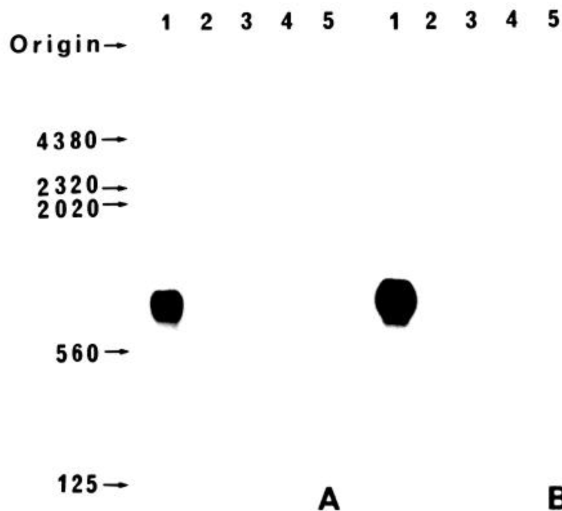

Figure 3. RNA transfer blot analysis. Poly(A)+RNAs from the spinal cord, 2 μg; brain, intestine, liver, and kidney, 5 μg, respectively (lanes 1–5), of the carp were hybridized with either UII-α (*A*) or -γ (*B*) probe. ^{32}P-labeled λ wild-type phage DNA digested with *Hind*III was used as a size marker.

translation termination codon, UGA. The poly(A) tail of mRNA for UII-α was preceded by 4 copies of polyadenylation signal (AAUAAA) and by 1 copy in UII-γ mRNA. There was a high homology in nucleotide sequences between pUII-18 and -10 in the 5′-untranslated regions, in the protein coding sequences and 55-nucleotide sequences in the 3′-untranslated regions following the protein coding sequences. The nucleotide sequence homology in the protein coding regions was 93.6%.

The precursors of both UII-α and -γ consisted of 125 amino acid residues, including a putative signal peptide (Fig. 2*B*). The signal peptides were tentatively assigned to the amino-terminal 21 residues in both UII-α and -γ precursors. The UII peptides were preceded by the amino acid sequence, Arg-Lys-Arg, which apparently represents a proteolytic processing site (Steiner et al., 1980). An additional paired amino acid sequence, Arg-Arg, was observed at positions 107 and 108. The amino acid sequences of the precursors deduced from pUII-3 and -10 were identical, although 64 amino-terminal residues were absent in pUII-3. In the UII-α precursors there was 1 amino acid substitution at position 28, where alanine in pUII-18 was replaced by valine in pUII-16. A remarkable similarity in amino acid sequence existed between the precursors of UII-α and -γ. There were only 12 amino acid substitutions in the sequences deduced from pUII-18 and -10; 10 amino acids showed single base changes and the substituted amino acids were chemically related (Dayhoff et al., 1979) except for leucine/histidine at position 57 and valine/alanine at 64. Two amino acids represented double base changes. The homology in amino acid sequence was 90.4%.

RNA transfer blot analysis

Analysis of the carp spinal cord poly (A)+RNA by blot hybridization under high stringency conditions with pUII-16 and -10 whole plasmid probes each revealed a single band, corresponding to 800–900 nucleotides (Fig. 3, lane 1). Identical results were obtained using pUII-18 and -3 probes (data not shown). Poly(A)+RNA isolated from the brain, intestine, liver, and kidney of the carp (Fig. 3, lanes 2–5, respectively) hybridized with neither pUII-16 nor -10 probes. Southern blot hybridization was performed with genomic DNA from carp liver after digestion with restriction endonucleases *Eco*RI, *Bam*HI, or *Kpn*I. Six bands were detected with both pUII-16 and -10 probes, indicating the presence of multiple genes for the UII family or polymorphism of UII genes (data not shown).

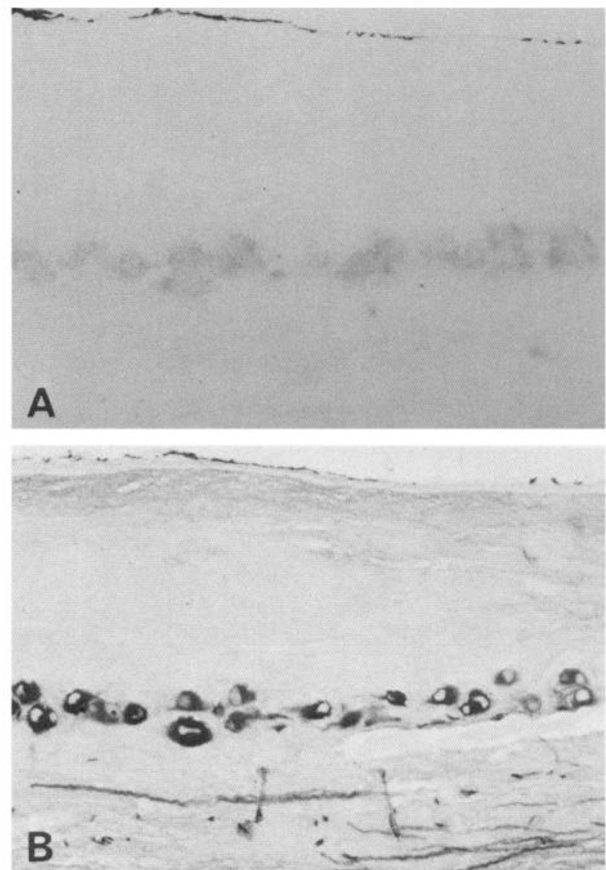

Figure 4. In situ hybridization and immunohistochemical localization of UII expressing neurons in the caudal spinal cord of the carp. *A, In situ* hybridization using ^{32}P-labeled synthetic oligonucleotide complementary to common sequence of mRNAs for UII-α and -γ. Autoradiographic grains were localized over the cell bodies. Note the absence of grains in the white matter (×80). *B,* UII-immunoreactive neurons in the section contiguous to that shown in *A*. Note the colocalization of UII-mRNA and UII peptide in the same neurons.

In situ *hybridization*

Autoradiographic silver grains, which would represent probe-mRNA hybrids, were localized over the cell bodies in the caudal spinal cord of the carp (Fig. 4*A*). No grain was observed in the white matter. UII-immunoreactive neurons were detected in the next section (Fig. 4*B*). Most of the hybridization-positive cells corresponded with UII-immunoreactive ones, indicating the colocalization of UII-mRNA and its corresponding peptide within the same cells. There was no conspicuous difference in the density of grains among the labeled neurons, whereas the intensities of their immunoreactivity varied.

Discussion

The present study reports the cloning and characterization of the cDNAs for the mRNAs encoding the precursors of carp UII-α and -γ, and demonstrates UII-producing neurons in the caudal spinal cord of the carp by *in situ* hybridization. Both pUII-16 and -18 clones encode the precursor of UII-α, consist of 793 nucleotides, and show 7 nucleotide substitutions. A substitution at position 83 in pUII-16 results in a loss of the *Pvu*II site. On the other hand, pUII-10 encodes the full-length cDNA for UII-γ, consisting of 1162, whereas pUII-3 is an incomplete cDNA, lacking the 5′-region. The 3′-untranslated regions are

markedly different in their nucleotide sequences between pUII-3 and -10. The presence of 2 different mRNAs for UII-α and -γ, respectively, could be due to polymorphism of the genes inasmuch as spinal cords from many carp were pooled. Three types of repeated sequences (UC, AC, and AU) are observed in the 3′-untranslated regions of pUII-3 and -10, and another (GU) in the latter. Such repeated sequences (AU and AC) have been found in the 3′-noncoding regions of mRNAs for catalase of rat liver (Osumi et al., 1984) and for peroxisomal enoyl-CoA: hydratase-3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme (Osumi et al., 1985). The alternating purine–pyrimidine sequences have been reported to be characteristic of DNA with Z-forming potential (Arnott et al., 1980; Wang et al., 1979).

The precursors of both UII-α and -γ contain 125 amino acid residues. The amino-terminal 21 residues in both UII-α or -γ precursors could represent the signal peptide. These regions exhibit typical features characteristic of the signal peptides of secretory proteins (Blobel and Dobberstein, 1975), a region rich in hydrophobic amino acids with long side chains in the central portion and terminating in a residue having a small neutral side chain such as alanine, glycine, or serine (Steiner et al., 1980). Therefore, a possible site for cleavage of the signal peptide seems to exist after the alanine residue at position 21 in each precursor. The precursors of UII-α and -γ contain Arg-Arg at positions 107 and 108 in addition to Arg-Lys-Arg, which precedes UII peptides. It is therefore plausible that both precursors yield a peptide consisting of 85 amino acid residues and a dipeptide Gln-Phe, as well as the signal peptide and UII. The former may represent a putative urotensin carrier protein, urophysin (Berlind et al., 1972; Lederis et al., 1974, 1981; Moore et al., 1975). We have isolated such peptides from the urophysis of the carp, and we found that one of the peptides is indistinguishable in amino acid composition from the 85-residue peptide mentioned above. Identification of this novel peptide and binding studies with UII will clarify whether the peptide is a carrier protein. The dipeptide, Gln-Phe, may also have an unknown physiological role in fishes.

More than 90% homology in amino acid as well as nucleotide sequences between precursors of UII-α and -γ suggests that the genes encoding both precursors have been generated from a common ancestral gene by duplication. Although a partial homology in amino acid sequence has been indicated previously (Pearson et al., 1980), there is homology in neither amino acid nor nucleotide sequence between the precursors of UII and somatostatin from the rat (Montominy et al., 1984). There is also no homology in amino acid or nucleotide sequences between the precursors of UII and urotensin I (Ishida et al., 1986), indicating that urotensins I and II have evolved from separate genes.

In RNA transfer blot analysis of poly(A)$^+$RNA from the spinal cord of the carp, a single band corresponding to 800–900 nucleotides was observed using either UII-α or -γ probe. The size of the mRNA detected by the UII-α probe is in good agreement with that of the cDNA. However, there is a difference in size between the mRNA hybridized with the UII-γ probe and that of pUII-10 itself. The pUII-3 is an incomplete cDNA of UII-γ, which, if complete, would have 894 nucleotides and coincide with the size of mRNA. At the present time, it is unclear why the UII-γ probe does not detect the mRNA with 1162 nucleotides. The mRNA of this size might be low in quantity or be inseparable from smaller mRNA on electrophoresis. The presence of UII-immunoreactivity in the brain of fishes has been controversial (Y. Kobayashi et al., 1986; Owada et al., 1985). We could not detect UII mRNA in the carp brain by RNA transfer blot hybridization.

By applying *in situ* hybridization technique or UII immunohistochemistry to alternate sections, we demonstrated the colocalization of UII mRNA and its peptide in the same neurons in the caudal spinal cord of the carp. The observations that there is no conspicuous difference in the density of mRNA but varied intensity of immunoreactivity among neurons suggest that the turnover rate of peptides may differ among them. It is not clear whether this difference reflects the presence of 2 types of neurons, one producing UII-α and the other UII-γ. *In situ* hybridization with probes specific to mRNAs for UII-α and -γ, respectively, will give an answer to this question.

In the present study, we could not find any cDNA clone encoding UII-β precursors, although 14 clones for UII-α and 6 clones for UII-γ were isolated out of 6000 transformants. There may be several explanations for the negative result. (1) Since the quantity of UII-β, a mixture of equal amounts of 2 components, is about half of either UII-α or -γ (Ichikawa et al., 1984), mRNA encoding either component of UII-β might be lower in quantity compared with that of UII-α or -γ. (2) UII-immunoreactive neurons have been shown to be located in the spinal cord both anterior and dorsal to the urophysis in the carp (Owada et al., 1985). We extracted mRNA from the spinal cord anterior to the urophysis. If the UII-β-producing neurons were to exist only in the spinal cord dorsal to the urophysis, our cDNA library would not contain UII-β precursor. We detected at least 6 bands on Southern blot hybridization of genomic DNA, some of which might encode UII-β precursor. It should be possible to isolate clones for UII-β from a carp genomic library.

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