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# Comparative distribution of the mRNAs encoding urotensin I and urotensin II in zebrafish

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ABSTRACT

The neural neurosecretory system of fishes produces two biologically active neuropeptides, *i.e.* the corticotropin-releasing hormone paralog urotensin I (UI) and the somatostatin-related peptide urotensin II (UII). In zebrafish, we have recently characterized two UII variants termed UIIα and UIIβ. In the present study, we have investigated the distribution of UI, UIIα and UIIβ mRNAs in different organs by quantitative RT-PCR analysis and the cellular localization of the three mRNAs in the spinal cord by *in situ* hybridization (ISH) histochemistry. The data show that the UI gene is mainly expressed in the caudal portion of the spinal cord and, to a lesser extent, in the brain, while the UIIα and the UIIβ genes are exclusively expressed throughout the spinal cord. Single-ISH labeling revealed that UI, UIIα and UIIβ mRNAs occur in large cells, called Dahlgren cells, located in the ventral part of the caudal spinal cord. Double-ISH staining showed that UI, UIIα and UIIβ mRNAs occur mainly in distinct cells, even though a few cells were found to co-express the UI and UII genes. The differential expression of UI, UIIα and UIIβ genes may contribute to the adaptation of Dahlgren cell activity during development and/or in various physiological conditions.



## 1. Introduction

The terminal segment of the spinal cord of fishes displays a neurosecretory system that is anatomically and functionally similar to the hypothalamo-neurohypophysial complex [4,16,17]. In teleosts, the caudal neurosecretory system (CNSS) consists of magnocellular neurons, called Dahlgren cells, located in the ventral part of the posterior spinal cord [14], which project their axons into a neurohemal organ, the urophysis [18,19,22]. Dahlgren cells are neurosecretory neurons that synthesize and release several regulatory substances, including two bioactive neuropeptides, termed urotensins.

Urotensin I (UI) is a 41-amino acid peptide initially isolated from the urophysis of the sucker *Catostomus commersoni* [26] which belongs to the corticotropin-releasing hormone (CRH)/urocortin superfamily (Fig. 1A) [6,28]. Urotensin II (UII) is a cyclic peptide originally characterized from the urophysis of the goby *Gillichthys mirabilis* [3,36] that has been recently shown to be a member of the somatostatin/cortistatin superfamily (Fig. 1B) [41–43].

Dahlgren cells are not the unique source of urotensins. For instance, in the fish brain, the presence of UI-expressing cells has been observed in several nuclei of the telencephalon, diencephon and mesencephalon [1]. Similarly, in the fish

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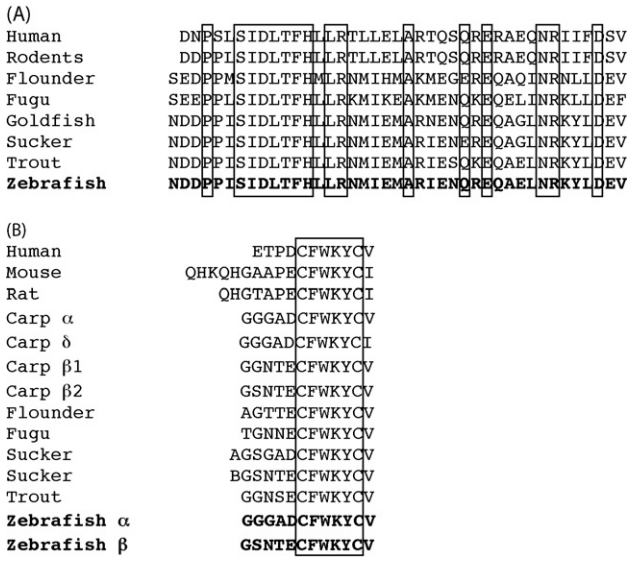


**Fig. 1 – Primary structures of urotensin I (or urocortin 1) (A) and urotensin II (B) from different vertebrate species, including zebrafish (in bold characters). Conserved residues are boxed.**

spinal cord, a second UII-producing system composed of cerebrospinal fluid (CSF)-contacting neurons has been described [50,51]. In tetrapods and notably in mammals, urocortin 1, the ortholog of UI [47], is primarily expressed in the Edinger–Westphal nucleus [29] while UII and its paralog urotensin II-related peptide (URP) [40] are expressed in motoneurons of the brainstem and spinal cord [7,8,11–13,15,37,38]. The physiological role of urotensins and thereby the functions of the CNSS have not been fully elucidated [33,49]. UI has been shown to participate in osmoregulation and stress response, and to regulate cardiovascular functions [24,30,32] while UII appears to be involved in the control of gastrointestinal, reproductive and cardiovascular activities, as well as in osmoregulatory and metabolic functions [10,31,49].

The zebrafish appears to be a powerful model to study genetic control of vertebrate neurodevelopment [25]. We have recently started to use this model to investigate the morphogenesis and physiology of the urophysis. In particular, we have performed an immunohistochemical and ultrastructural analysis of the CNSS in adult zebrafish [35] and we have characterized the sequence of the cDNAs encoding two distinct UII variants in zebrafish, namely UIIα and UIIβ [42]. In the present study, the distribution of UI, UIIα and UIIβ mRNAs was investigated in different organs of adult zebrafish using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and the cellular distribution of UI, UIIα and UIIβ mRNAs was determined by *in situ* hybridization (ISH) histochemistry.

## 2. Materials and methods

### 2.1. Animals

Adult male and female zebrafishes (*Danio rerio*) ($n = 100$; from 2.5 to 3.5 cm) were obtained from local suppliers and kept in tap water at 28 °C, under a 12 h/12 h light/dark photoperiod, for at least one week before use. Animals were anaesthetized in 0.02% MS 222 (Sandoz, Levallois-Perret, France) and killed by decapitation. The different organs used for RT-PCR experiments were carefully dissected and immediately frozen in liquid nitrogen. After removal of the skin, the caudal part of the body, just posterior to the dorsal fin, was separated and immersed in 4% paraformaldehyde (PFA) for 24 h at room temperature. Forty-μm thick parasagittal sections of the spinal cord were cut with a Sartorius–Werke freezing microtome and kept as floating sections in 0.05 M phosphate buffer (PBS). Animal manipulations were carried out according to the recommendations of the French Ethical Committee and performed under the supervision of authorized investigators.

### 2.2. Quantitative real-time RT-PCR

Total RNAs from zebrafish muscle, heart, liver, kidney, intestine, gas bladder, gill, ovary, testis, brain, and rostral and caudal parts of spinal cord were extracted using Tris-Reagent (Sigma–Aldrich, Saint-Quentin Fallavier, France) and the RNeasy Minikit (Qiagen, Courtaboeuf, France). Contaminating genomic DNA was removed by treatment with DNase I (Qiagen). About 1 μg of treated RNA was converted into first-strand cDNA using ImPromII™ Reverse Transcription System (Promega, Charbonnnière, France) with 0.5 μg oligo(dT)$_{15}$ primer and 20 units RNAsin (Promega).

qRT-PCR was carried out in an ABI 7000 SDS (Applied Biosystems, Courtaboeuf, France) using a SYBR Green PCR Mastermix. Three RT reactions were performed for each sample and each cDNA sample was analyzed in duplicate along with standard and no-template controls. The incubation media contained 1:100 of each RT reaction in 1× Mastermix containing pre-set concentrations of dNTPs, $MgCl_2$ and buffer, along with 300 nM forward and reverse primers, and the SYBR Green reporter dye. PCR parameters were 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 s, and 60 °C for 1 min.

The levels of expression of each gene were calculated by the comparative threshold cycle (Ct) method and expressed as $2^{-\Delta\Delta Ct}$ using the qBase v1.3.4 program [21]. mRNA levels were determined by relative quantification using geometric mean of the Ct of two reference genes, namely the β-actin and phosphoglycerate kinase 1 (PGK) genes. These two genes

**Table 1 – Sequences of the oligonucleotides used for PCR amplifications**

| Name | Sequence (5′-3′) |
|---|---|
| β-Actin for | 5′-TCA CCA CCA CAG CCG AAA G-3′ |
| β-Actin rev | 5′-GGT CAG CAA TGC CAG GGT A-3′ |
| PGK1 for | 5′-AGG AGG GCA AGG GCA AAG-3′ |
| PGK1 rev | 5′-CAG GGA GGC TCG GAA AGC-3′ |
| UI for | 5′-TTC CAC CTG CTC AGA AAC ATG A-3′ |
| UI rev | 5′-GTT CCG CCT GTT CCC TTT G-3′ |
| UIIα for | 5′-GCA GCC GCA GCA CAT TC-3′ |
| UIIα rev | 5′-AAG ACC ACT GGG AGG AAC CA-3′ |
| UIIβ for | 5′-AGT CGG CGG AGA TGA GCT T-3′ |
| UIIβ rev | 5′-GCG GCA TCA TGG GAG AGA TA-3′ |

were selected according to [46]. All data were normalized to value of genes of interest in the caudal part of the spinal cord to ensure a direct comparison of gene expression levels. Product purity was confirmed by dissociation standard curve and agarose gel electrophoresis. Primers (Proligo, Paris, France) were designed from the following GenBank zebrafish cDNA sequences: UI (NM_001030180), UIIα (NM_212848), UIIβ (NM_205591.1), β-actin (AF025305) and PGK1 (NM_213387) (Table 1) using the ABI Prism Primer Express 2.0 program (Applied Biosystems). Data from replicate experiments were analyzed with the Prism 4.0 software (GraphPad Software, San Diego, CA) and expressed as mean ± S.E.M.

### 2.3. *In situ hybridization*

#### 2.3.1. *Probe labeling*

Three sets of complementary oligonucleotide probes were used for each zebrafish UI, UIIα and UIIβ mRNA precursor (Table 2). All oligonucleotide probes were 3′-end labeled with digoxigenin-11-dUTP (DIG-dUTP) or with fluorescein-12-dUTP (fluorescein-dUTP): 100 pmol of each probe was incubated in a total volume of 20 μl containing 4 μl of reaction buffer consisting of 1 M potassium cacodylate, 125 mM Tris–HCl, 1.25 mg/ml bovine serum albumin (BSA) pH 6.6 at 25 °C (Roche Diagnostics), 2 μl of 25 mM $CoCl_2$, 1 μl of DIG-dUTP or 1 μl of fluorescein-dUTP (1 mM each; Roche Diagnostics), 1 μl of dATP (10 mM; Roche Diagnostics), 1 μl of terminal transferase (400 U/μl in 60 mM potassium phosphate pH 7.2, 150 mM KCl, 1 mM 2-mercaptoethanol, 0.5 M Triton X-100; Roche Diagnostics), 50% glycerol and 10 μl of sterile water. After 45 min at 37 °C, the reaction was stopped with 1 μl of 0.2 M EDTA and 5 μg/μl yeast tRNA (Sigma, Saint-Louis, MO) and precipitated with 75 μl of cold absolute ethanol and 2.5 μl of 4 M LiCl. Probes were stored at −20 °C overnight, and centrifuged (7000 × *g*) at 4 °C for 30 min. The pellets were dried and resuspended in 100 μl of 1 mM Tris base and 0.16 mM EDTA buffer, pH 8.

#### 2.3.2. *Hybridization procedures*

*2.3.2.1. Single-in situ hybridization using digoxigenin-labeled probes.* Floating sections were incubated for 1 h at 37 °C in a prehybridization solution containing 4% standard saline citrate (SSC; 0.6 M NaCl and 0.015 M sodium citrate; Sigma), 1% Denhardt's solution (Sigma), and 1% dextran sulfate (Sigma) in double-distilled water. Sections were then incubated for 60 h at 37 °C in the hybridization buffer (50% deionized formamide, 1% dextran sulfate, 600 mM NaCl, 80 mM Tris–HCl, pH 7.5, 4 mM EDTA, 0.05% disodium-pyrophosphate, 0.05% tetrasodium pyrophosphate, 0.2% N-lauryl-sarcosine, 500 μg/ml salmon testes DNA; Sigma) containing three oligonucleotides corresponding to each mRNA: 2 nM of digoxigenin-labeled UI probe, or digoxigenin-labeled UIIα probe or digoxigenin-labeled UIIβ probe.

Two kinds of control experiments were performed with oligonucleotide probes in order to test the specificity of the hybridization signal: (1) incubation with an excess (50 times) of the unlabeled oligonucleotides and (2) addition in the hybridization buffer of 2 nM of the corresponding unlabeled sense probes before deposition on sections.

After hybridization, sections were rinsed twice in 1× SSC at 42 °C for 15 min and in 0.1× SSC at room temperature for 30 min. To reveal the digoxigenin signal, sections were preincubated for 1 h with 2% BSA and 0.1% Triton X-100 (Merck, Darmstadt, Germany) in 0.05 M PBS at room temperature and then incubated overnight at 4 °C in the same solution with alkaline phosphatase-conjugated anti-digoxigenin F(ab) fragment (1:500; Roche Diagnostics). Sections were rinsed at room temperature three times for 10 min in 0.1 M Tris, pH 7.5, 1 M NaCl and 5 mM $MgCl_2$, 10 min in 0.1 M Tris, pH 9.5, 1 M NaCl and 5 mM $MgCl_2$, and 10 min in 0.1 M Tris, pH 9.5, 0.1 M NaCl, 5 mM $MgCl_2$. Alkaline phosphatase activity was developed by incubating the sections in 20 μl/ml of nitro blue tetrazolium chloride, 5-bromo-4-chloro-3-indolyl phosphate (NBT-BCIP, toluidine salt) mix (Roche Diagnostics) for 3 h. Sections were mounted on gelatin-coated slides in Mowiol (Calbiochem, La Jolla, CA), coverslipped, and examined on a Leica DMRB microscope (Wetzlar, Germany) equipped with a Micropublisher Q imaging camera. All 40-μm thick parasagittal sections prepared according to this procedure from four (UI + UIIα probes), eight (UI + UIIβ probes) and nine (UIIα + UIIβ probes) animals were used for counting of single- and double-positive cells.

*2.3.2.2. Single-in situ hybridization using fluorescein-labeled probes.* In order to test the efficiency of incorporation of fluorescein in the probe, floating sections were treated with the same procedures as described in Section 2.3.2.1, except that the alkaline phosphatase-conjugated anti-digoxigenin F(ab) fragment (1:500) was replaced by the alkaline phosphatase-conjugated anti-fluorescein F(ab) fragment (1:100; Roche Diagnostics).

**Table 2 – Sequences of the oligonucleotides used as probes for *in situ* hybridization experiments**

| Name | Sequence (5′-3′) |
|---|---|
| UI 45 mers | 5′-GTCTCCTCCTCCTCCTTGACGGCCGAGCAGCAGGTCGTCGAG-3′<br>5′-AGCAGGACCAATGGGACGGGCTTCATGCTGGACACAGTCGCGCA-3′<br>5′-ATTGAGATCGCGGGGTCGGCAGAAGCTCAGCGGGATGTGGCTCA-3′ |
| UIIα 35 mers | 5′-CGGCCTCTTCCACTGAATCCGGGCCAATGTTGGTC-3′<br>5′-GTAACGGGATGAGCAGAAAGGAGACTGCAGGACAG-3′<br>5′-GCGTCTTTAGAAATCTGCCCAGACATTTTGATACT-3′ |
| UIIβ 35 mers | 5′-GCACCACCGGGTGACCGAGCAGAGGTTCGAGGACG-3′<br>5′-ACGGGCCTGCCGAAGCTCATCTCCGCCGACTGCAC-3′<br>5′-CCGAACCCTGCGGCATCATGGGAGAGATACGCCTG-3′ |

2.3.2.3. *Double labeling in situ hybridization using two distinct probes*. Floating sections were incubated in the same pre-hybridization solution as for single hybridization and then incubated at 37 °C for 60 h with 2 nM of fluorescein-labeled UI and digoxigenin-labeled UIIα probes, or fluorescein-labeled UI and digoxigenin-labeled UIIβ probes, or digoxigenin-labeled UIIα and fluorescein-labeled UIIβ probes. Probes were diluted in the same buffer as that used for single hybridization. After hybridization, sections were rinsed in 1× SSC at 42 °C for 15 min followed by 0.1× SSC at room temperature for 30 min.

The sections were first treated for visualization of the fluorescein probes: they were preincubated for 1 h with 2% BSA and 0.1% Triton X-100 in 0.05 M PBS at room temperature and then incubated overnight at 4 °C in the same solution with alkaline phosphatase-conjugated anti-fluorescein F(ab) fragment (1:100). Sections were rinsed three times for 10 min in washing buffer (0.1 M Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20; Amersham Biosciences, Piscataway, NJ) and twice in developing buffer (0.1 M Tris, pH 8, 400 mM NaCl, 10 mM $MgCl_2$). Alkaline phosphatase activity was visualized by incubating the sections three times for 30 min in a 2-hydroxy-3-naphtoic acid-2′-phenylanilide phosphate (HNPP) fluorescent detection set solution (Roche Diagnostics): 10 μl HNPP (2-hydroxy-3-naphtoic acid-2′-phenylanilide phosphate, 10 mg/ml in dimethylformamide) with 10 μl Fast Red TR solution (25 mg/ml 4-chloro-2-methylbenzenediazonium hemi-zinc chloride salt in double-distilled water), diluted in 1 ml developing buffer. The enzymatic reaction was stopped by two rinses in wash buffer.

For visualization of the digoxigenin probes, sections were preincubated for 1 h with 0.5% blocking reagent (tyramide signal amplification (TSA) fluorescence systems, PerkinElmer, Boston, MA) in 0.1 M Tris, pH 7.5, 150 mM NaCl at room temperature and then incubated overnight at 4 °C in the same solution with peroxidase-conjugated anti-digoxigenin F(ab) fragment (1:100; Roche Diagnostics). Sections were rinsed three times for 5 min in washing buffer, and peroxidase activity was revealed by incubating the sections three times for 10 min with 40 μl coumarin tyramide diluted in 2 ml tyramide working solution (TSA fluorescence systems; PerkinElmer). The enzymatic reaction was stopped by three rinses in washing buffer and double-distilled water. Sections were mounted on gelatin-coated slides in Mowiol, coverslipped and examined on a Leica TCS SP confocal microscope or a Zeiss Axiovert 200 M microscope (Göttingen, Germany) equipped with an Axiocam MRm camera.

## 3. Results

### 3.1. *Tissue distribution of UI, UIIα and UIIβ mRNAs*

qRT-PCR was used to determine the distribution of UI, UIIα and UIIβ mRNAs in different zebrafish organs. As shown in Fig. 2, the highest amounts of UI and UII mRNAs were measured in the caudal part of the spinal cord. UI mRNA was also detected in the brain, but at a much lower level. In all other tissues tested, the expression of the UI, UIIα and UIIβ genes was extremely low or undetectable.

### 3.2. *Cellular distribution of UI, UIIα and UIIβ mRNAs in spinal cord*

3.2.1. *Single labeling in situ hybridization*

ISH with digoxigenin-labeled UI, UIIα and UIIβ probes revealed intense labeling in the caudal part of the zebrafish spinal cord at the level of the 4–5 last vertebrae, rostrally to the urophysis (Figs. 3A, 4A and 4B). No labeling was observed in the urophysis itself and the axial muscles (Figs. 3A, 4A and 4B). Positive cells corresponding to Dahlgren cells, were observed in the ventro-lateral gray matter. The largest Dahlgren cells (about 20 μm in diameter) were located more rostrally and the smallest cells (less than 10 μm in diameter) were located more caudally, although small Dahlgren cells were also observed at all levels

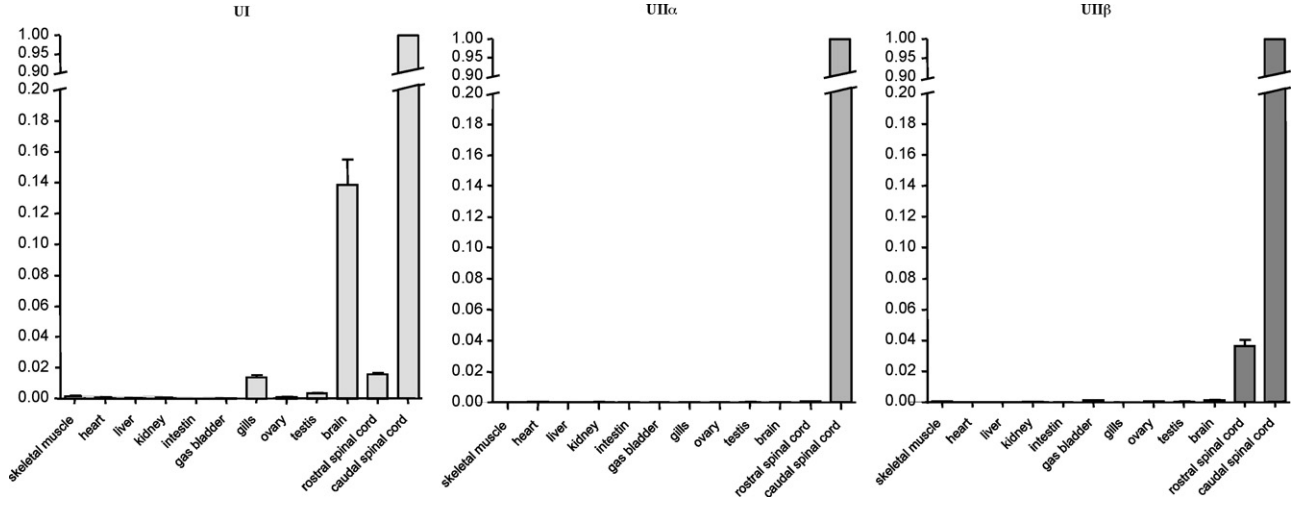


**Fig. 2 – Relative concentrations of UI, UIIα and UIIβ mRNAs in various zebrafish tissues as measured by quantitative RT-PCR. The expression levels of mRNAs were normalized by using β-actin and PGK1 mRNAs as reference standards using the qBase v1.3.4 program [46]. Data, expressed as arbitrary units (caudal spinal cord = 1), are mean ± S.E.M. of triplicates from each mRNA sample.**

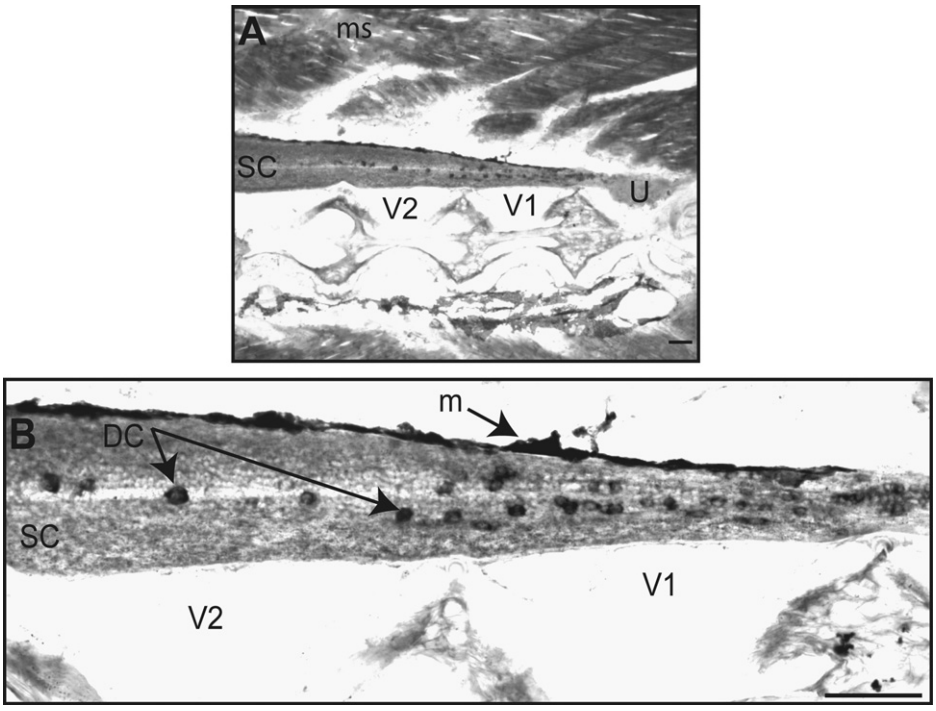


**Fig. 3 – Parasagittal sections of the zebrafish tail illustrating the distribution of UI mRNA in the caudal spinal cord. Tissue sections were incubated with an antisense digoxigenin-labeled UI probe. Hybridizing probe was detected using anti-digoxigenin–alkaline phosphatase conjugate with NBT/BCIP as chromogen substrate. (A) Low-magnification photomicrograph showing the caudal spinal cord (SC) with the attached urophysis (U). (B) Higher magnification illustrating the distribution of intensely labeled Dahlgren cells (DC) in the posterior extremity of the SC: m, melanocytes; ms, skeletal muscle; V1 and V2, preterminal vertebra 1 and 2. Scale bars: 100 μm.**

of the CNSS up to the urophysis and even caudally to it in the filum terminale. The average size of positive cells was about 10–15 μm in diameter.

The perikarya displaying each of the three mRNAs were intensely labeled over the soma and the onset of the processes (Figs. 3B, 4C and 4D). With the UI probe, small intensely labeled Dahlgren cells were numerous at the proximity of the urophysis (Fig. 3A and B). With the UIIα probe, numerous medium-sized or large Dahlgren cells with a round or ovoid shape were labeled (Fig. 4C). These cells, localized in two central and ventral bands on frontal sections (not illustrated) were seen either alone or grouped in small clusters (Fig. 4). Dahlgren cells labeled with the UIIβ probe exhibited the same shape and the same localization (Fig. 4D) as those labeled with the UIIα probe. Similar results were obtained with the fluorescein-labeled probes revealed by alkaline phosphatase and NBT-BCIP (Fig. 5) and thus, the fluorescein probes could be used together with the digoxigenin probes for double-ISH labeling.

Control experiments performed using labeled probes preincubated with the corresponding sense probes did not show any hybridization signal (Fig. 6). Similarly, preincubation with a cocktail of unlabeled antisense probes totally suppressed the labeling (data not illustrated).

*3.2.1.1. Double labeling in situ hybridization.* Double ISH of caudal spinal cord sections with the digoxigenin-labeled UI probe and the fluorescein-labeled UIIα or UIIβ probe revealed that UI mRNA-containing cells were about 2.5–3 times more numerous than UIIα or UIIβ mRNA-containing cells. Only a few UI/UIIα double-labeled cells were observed. The proportion of UI/UIIβ double-labeled cells was about 2% of the total number of labeled cells (Table 3). The color of double-labeled cells containing UI and UIIβ mRNAs varied from blue to red indicating different expression levels of the corresponding genes. Double labeling with the fluorescein-labeled UIIα probe and the digoxigenin-labeled UIIβ probe revealed that the numbers of UIIα- and of UIIβ-expressing neurons are approximately the same. About 10% of the labeled cells were double labeled (Table 3). Double-labeled cells (UI/UIIα, UI/UIIβ and UIIα/UIIβ), which were essentially medium-sized and large Dahlgren cells, were mainly localized at the level of vertebrae 2 and 3 rostrally to the urophysis (not illustrated). UI/UIIβ (Fig. 7A) and UIIα/UIIβ (Fig. 7B) double-labeled cells were almost exclusively seen grouped in small clusters.

## 4. Discussion

We have previously described the distribution of UI- and UII-like immunoreactivity in the CNSS of adult zebrafish [35]. However, soon after this study was published, the existence of two distinct isoforms, termed UIIα and UIIβ, has been reported [42]. Since the sequences of zebrafish UIIα and UIIβ only differ by four residues, immunohistochemical approaches will probably not be appropriate to determine specifically the

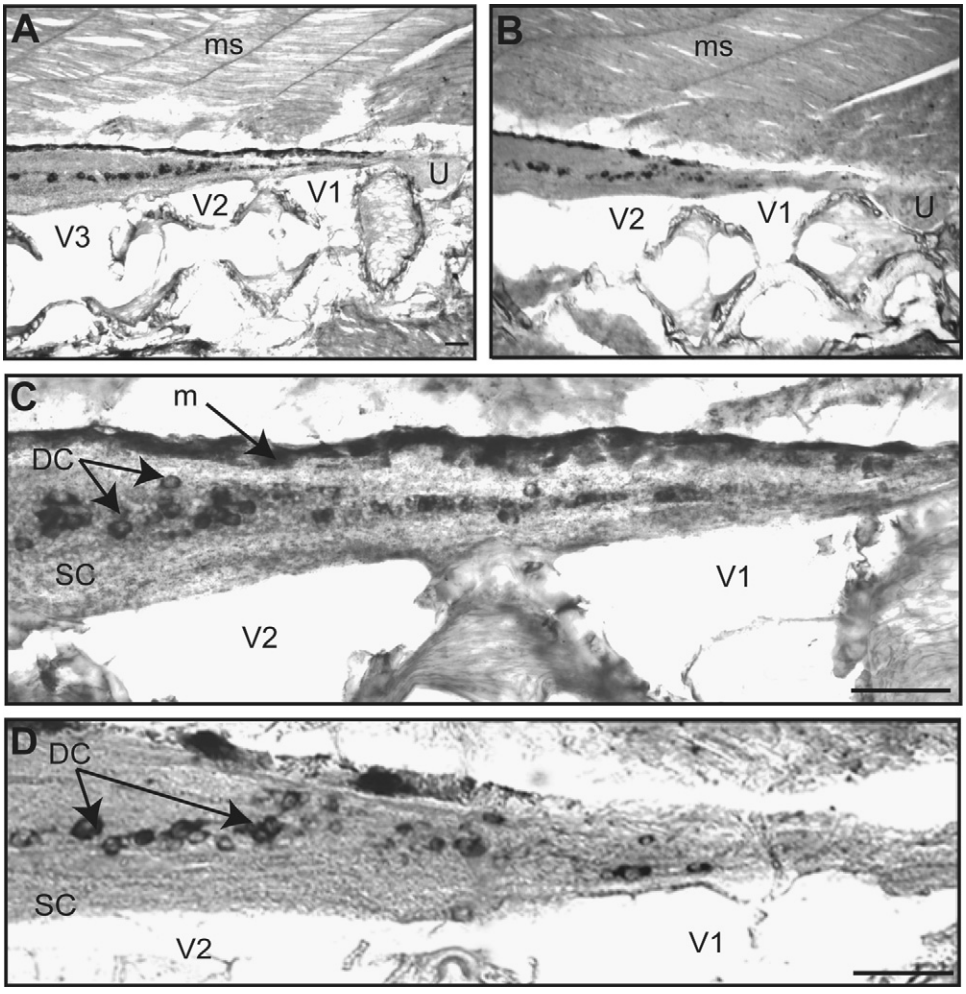


**Fig. 4 – Parasagittal sections of the zebrafish tail illustrating the distribution of UIIα and UIIβ mRNAs in the caudal spinal cord. Tissue sections were incubated with an antisense digoxigenin-labeled UIIα (A and C) or UIIβ (B and D) probe. Hybridizing probes were detected using anti-digoxigenin–alkaline phosphatase conjugate with NBT/BCIP as chromogen substrate. (A and B) Low-magnification photomicrographs showing the caudal spinal cord (SC) with the attached urophysis (U). (C and D) Higher magnification illustrating the distribution of intensely labeled Dahlgren cells (DC) in the posterior extremity of the SC: m, melanocytes; ms, skeletal muscle; V1 and V2, preterminal vertebra 1 and 2. Scale bars: 100 μm.**

localization of each UII variant. In the present study, we have thus investigated the distribution UI, UIIα and UIIβ mRNA in the zebrafish spinal cord by qRT-PCR analysis and ISH histochemistry.

While UI mRNA was detected by qRT-PCR in the zebrafish brain and spinal cord, UIIα and UIIβ mRNAs were only expressed in the spinal cord, mainly in its caudal portion. In agreement with these observations, the expression of the UI gene has been recently reported in several nuclei of the zebrafish telencephalon, diencephalon and mesencephalon [1]. Although UII has been chemically identified from extracts of the whole brains of rainbow trout and long-nose skate [48], very few UII-immunoreactive neurons can actually be seen in the brain of freshwater and seawater fish [50,51]. These data strongly suggest that the UII peptides isolated from the trout and skate brains [48] are located in nerve fibers rather than in neurons. Therefore, in contrast to tetrapods, in which the UII gene is expressed in motoneurons of both the brainstem and the spinal cord [7,8,11–13,15,37,38], it appears that in fish the expression of the UIIα and UIIβ variants is confined to the spinal cord.

The distribution of UI-, UIIα- and UIIβ-expressing cells in the zebrafish spinal cord was investigated by single- and double-*in situ* hybridization labeling. Since high concentrations of biotin are present in zebrafish tissues [20], particularly in the spinal cord (unpublished observations), the antisense oligonucleotide probes were labeled with digoxigenin or fluorescein. In order to improve detection of low levels of UI and UII mRNAs, we used a cocktail of three non-overlapping digoxigenin or fluorescein oligonucleotide probes [44]. This procedure does not increase non-specific labeling and is adapted to both chromogenic stain and fluorescent detection systems [45]. The specificity of the hybridization signal was verified by control experiments performed with either pre-incubation with sense probes or with an excess of unlabeled antisense oligonucleotides. The most sensitive system

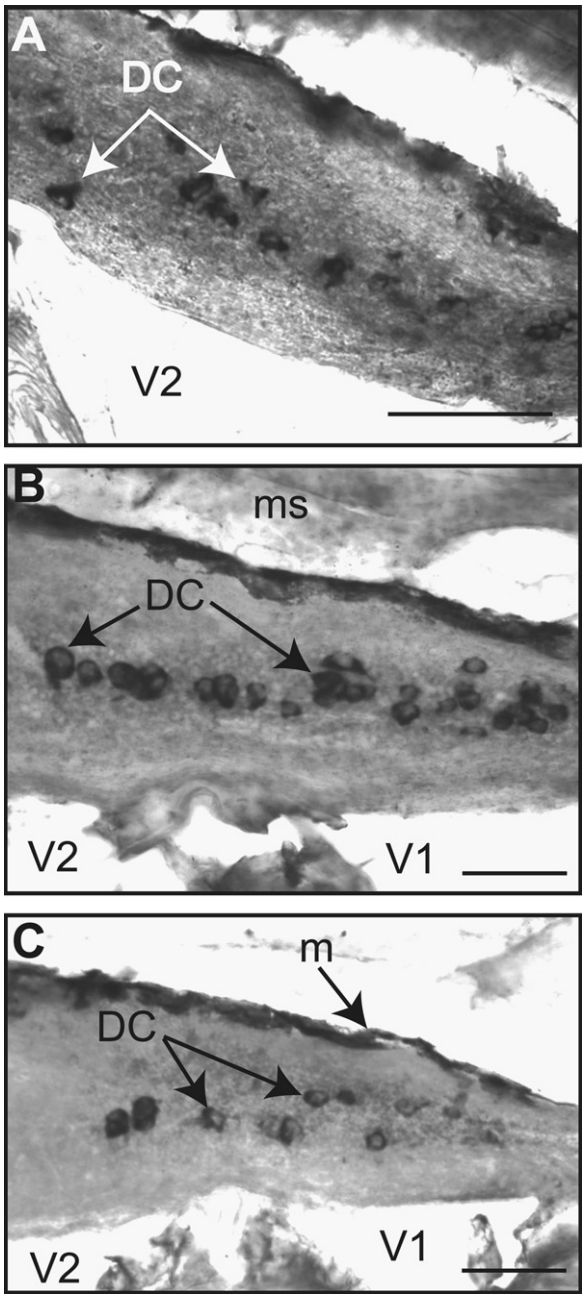


**Fig. 5 – Parasagittal sections of the zebrafish tail illustrating the distribution of UI, UIIα and UIIβ mRNAs in the caudal spinal cord using a fluorescein-labeled antisense UI (A), UIIα (B) or UIIβ (C) probe. Hybridizing probes were detected using anti-fluorescein-alkaline phosphatase conjugate with NBT/BCIP as chromogen substrate: DC, Dahlgren cells; m, melanocytes; ms, skeletal muscle; V1 and V2, preterminal vertebra 1 and 2. Scale bars: 100 μm.**

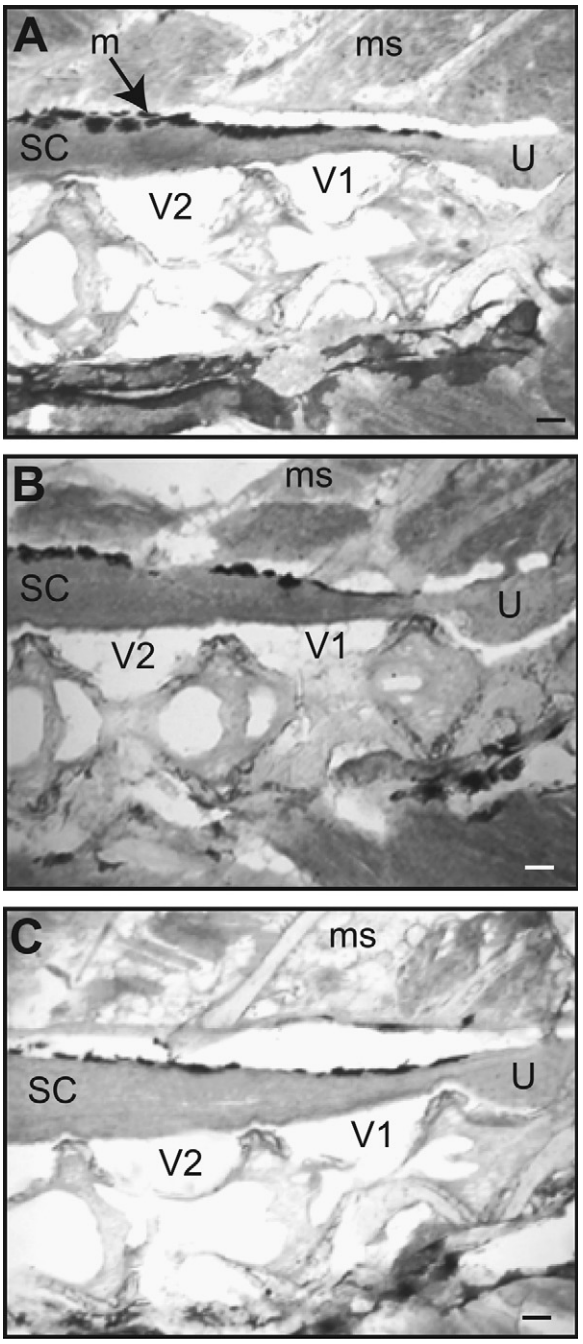


**Fig. 6 – Control experiments illustrating the specificity of the *in situ* hybridization labeling. Tissue sections were incubated with an antisense digoxigenin-labeled UI (A), UIIα (B) or UIIβ (C) probe preincubated with the corresponding UI, UIIα and UIIβ sense probes: m, melanocytes; ms, skeletal muscle; SC, spinal cord; V1 and V2, preterminal vertebra 1 and 2. Scale bars: 100 μm.**

appeared to be the digoxigenin–alkaline phosphatase complex. However, double ISH could be performed using the less sensitive fluorescent detection system.

Single-ISH labeling revealed that, in zebrafish, UI, UIIα and UIIβ mRNAs are exclusively expressed in Dahlgren cells, as previously observed in carp [23,34] and flounder [31,32]. The distribution and size of the positive cells were similar to those previously visualized by immunohistochemistry [35], except that ISH-labeled cells extended to the fifth instead of the fourth vertebrae.

Immunohistochemical labeling of zebrafish spinal cord using antibodies raised against flounder UI and UII had

**Table 3 – Quantitative analysis indicating the number of labeled and double-labeled cells containing UI, UIIα and/or UIIβ mRNA**

| | UI mRNA-containing cells | UIIα mRNA-containing cells | UIIβ mRNA-containing cells | Double-labeled cells |
|---|---|---|---|---|
| UI/UIIα probes (data from four animals) | 212 | 84 | – | 3 |
| UI/UIIβ probes (data from eight animals) | 659 | – | 203 | 17 |
| UIIα/UIIβ probes (data from nine animals) | – | 225 | 208 | 45 |

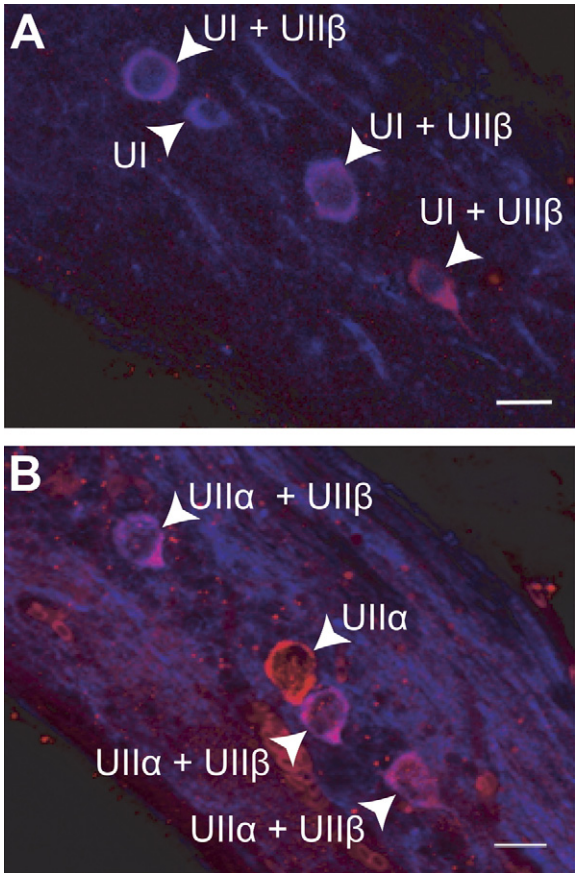


**Fig. 7 – Double-*in situ* hybridization labeling of UI-, UIIα- and UIIβ-expressing cells in the caudal portion of the zebrafish spinal cord. Parasagittal sections of the spinal cord were incubated with a fluorescein-labeled antisense UI probe and a digoxigenin-labeled antisense UIIβ probe (A) or a digoxigenin-labeled antisense UIIα probe and a fluorescein-labeled antisense UIIβ probe (B). Hybridizing fluorescein-labeled probes were vizualized using anti-fluorescein-alkaline phosphatase conjugate and the HNPP/Fast Red detection reaction. Positive cells are stained in red. Hybridizing digoxigenin-labeled probes were vizualized using anti-digoxigenin peroxidase conjugate and the coumarin/TSA. Positive cells are stained in blue. (A) Double-labeled cells containing UI and UIIβ mRNAs are of various colors, from blue to red, depending on the expression levels of the corresponding genes. (B) Double-labeled cells containing UIIα and UIIβ mRNAs appear in different colors, from red to blue. Scale bars: 20 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)**

previously suggested that UI-positive cells were much less numerous than UII-positive cells [35]. Single-ISH labeling now shows that the numbers of UI-, UIIα- and UIIβ-expressing cells are similar even though their distribution is different. The apparent discrepancy between IHC and ISH labeling of UI-positive cells can be ascribed to the lower sensitivity of the IHC technique especially when heterologous antibodies are used. As a matter of fact, while the sequence of the cyclic region of UII has been fully conserved from fish to man [9,27], the sequence of UI/urocortin 1 is more variable [28]. In particular, the sequences of flounder and zebrafish UI exhibit only 68% identity [6]. Indeed, using a new antibody raised specifically against zebrafish UI (Agrobio, La Ferté-Saint-Aubin, France), we have recently found that the number of UI-immunoreactive cells is similar to the number of UI-mRNA-expressing cells (data not shown).

Besides Dahlgren cells that clearly represent the major source of urotensins in teleosts [33], the existence of an extra-urophysial UII- (but not UI-)producing system has been described in several species of freshwater and seawater fishes [50,51]. This second UII-selective system consists of CSF-contacting neurons whose fibers project widely to the spinal cord and various brain regions. In agreement with previous ISH studies which did not confirm the occurrence of UII mRNA in CSF-contacting neurons [23,31,32,34], we did not detect UIIα and UIIβ mRNA expression in such cells, suggesting that the UII-immunoreactive peptide observed by Yulis and Lederis [50,51] in CSF-contacting neurons may not correspond to authentic UII but to a closely related peptide. In support of this hypothesis, we have recently identified from zebrafish genome databases a DNA sequence encoding the URP precursor (unpublished observations). Since UII and URP share the cyclic hexapeptide sequence Cys-Phe-Trp-Lys-Tyr-Cys [27], it is conceivable that the UII-immunoreactivity detected in CSF-contacting cells could be ascribed to the presence of URP in these cells.

Double-ISH labeling revealed the occurrence of six different types of Dahlgren cells according to their ability to express each of the urotensin genes: UI, UIIα, UIIβ, UI/UIIα, UI/UIIβ, and UIIα/UIIβ mRNA-containing cells. However, only a few cells appeared to express simultaneously the UI and the UII (either α or β isoforms) genes. Consistent with this observation, immunohistochemical labeling has previously shown that, in the zebrafish urophysis, most nerve fibers contain only UI- or UII-like immunoreactivity [35]. We observed that most cells that express simultaneously two urotensins were grouped in small clusters suggesting that the microenvironment of Dahlgren cells may orientate their phenotype. The aggregation of Dahlgren cells exhibiting the same phenotype may contribute to the synchronization of their secretory activity as previously demonstrated for vasopressinergic neurons [39].

The proportions of the different subtypes of Dahlgren cells (*i.e.* those expressing only UI, those expressing only UII and those expressing both) markedly differ from one species to the other. Thus, in carp, the UI, UIIα and UIIγ genes are simultaneously expressed in most Dahlgren cells [23] while, in flounder, most cells express the UI gene [32] but only 30–40% of them express the UII gene, alone or in combination with the UI and/or the CRH gene [31]. Consistent with the existence of various subpopulations of Dahlgren cells regarding their peptide content, electrophysiological studies have shown that these cells exhibit a wide diversity of electrophysiological activity patterns [2,5,33].

In conclusion, qRT-PCR analysis has shown that, in zebrafish, the UI gene is expressed both in the brain and spinal cord while the expression of UIIα and UIIβ genes is restricted to the caudal spinal cord. ISH labeling has revealed that most Dahlgren cells express only one of the urotensin genes although a few cells do express simultaneously the genes encoding UI and UIIα, or UI and UIIβ, or UIIα and UIIβ. Functional studies should help to elucidate whether the differential expression of the UI, UIIα and UIIβ gene is regulated during development and/or in response to environmental and physiological conditions.

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