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Cloning, sequence analysis and tissue distribution of the mouse and rat urotensin II precursors[1]

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Abstract Urotensin II (UII) is a cyclic neuropeptide initially isolated from the caudal neurosecretory system of teleost fish. The recent cloning of the UII precursor in frog and human has demonstrated that the peptide is not restricted to the fish urophysis but that it is also expressed in the central nervous system of tetrapods. Here, we describe the characterization of the cDNAs encoding prepro-UII in mouse and rat. A comparison of the primary structures of mouse and rat UII with those of other vertebrate UII reveals that the sequence of the cyclic region of the molecule (CFWKYC) has been fully conserved. In contrast, the N-terminal flanking domain of prepro-UII has markedly diverged with only 48% sequence identity between the mouse or rat and the human precursors. In situ hybridization histochemistry showed that the prepro-UII gene is predominantly expressed in motoneurons of the brainstem and spinal cord, suggesting that UII may play a role in the control of neuromuscular functions.

Key words: Urotensin II; Regulatory neuropeptide; Motoneuron; Molecular evolution

1. Introduction

The caudal portion of the spinal cord of teleost fish incorporates a neurosecretory organ called the urophysis [1]. Two neuropeptides, urotensin I (UI) and urotensin II (UII), have been isolated from extracts of urophyses on the basis of their ability to reduce blood pressure in rat [2] and to contract the isolated trout intestine [3], respectively. UI is a 41-amino acid residue peptide which belongs to a family of regulatory peptides including the frog skin peptide sauvagine [4] and the brain peptides corticotropin-releasing factor [5] and urocortin [6]. Fish UII is a 12-amino acid residue cyclic peptide which exhibits some structural similarities with somatostatin but is not homologous to any other regulatory peptide identified to date [3,7].

It has long been considered that UII was specifically produced by the fish urophysis [1,7]. However, the characterization of UII from the brain of a frog [8] has shown that a UII-encoding gene does also exist in certain species of tetrapods and that this gene is expressed not only in the caudal portion of the spinal cord but also in brain neurons. The observation that synthetic fish UII could exert various spasmogenic effects in rat and rabbit [9–11], together with the characterization of high affinity binding sites for fish UII in rat arteries [12], strongly suggested that a UII-related peptide also existed in mammals. Indeed, we have recently identified the cDNA encoding the UII precursor in human and we have found that the UII gene is intensely expressed in a subset of motoneurons in the human spinal cord [13]. This latter study has revealed that the amino acid sequence of the cyclic C-terminal region of UII has been totally preserved from fish to human [3,8,13,14] while the sequence of the N-terminus is highly variable [15,16]. Up to now, calcitonin gene-related peptide (CGRP) [17] and UII are the only neuropeptides found to occur in motoneurons of the spinal cord, suggesting that UII could exert modulatory activities at the neuromuscular junction as previously shown for CGRP [18].

The UII cDNA has now been cloned from human cells [13] but the sequence of UII has not yet been identified in other mammalian species. In the present study, we have characterized the cDNAs encoding the UII precursors in mouse and rat and we have determined the distribution of the UII mRNA in the central nervous system (CNS) and in peripheral organs.

2. Materials and methods

2.1. Cloning of mouse and rat prepro-urotensin II (prepro-UII) cDNA

Total RNAs from mouse and rat spinal cords were extracted by the acid guanidinium thiocyanate-phenol-chloroform procedure, using Tri reagent (Sigma) [19]. Poly(A)$^+$ RNAs were purified from total RNAs with the PolyATract mRNA Isolation System III,IV (Promega). 1 μg of each poly(A)$^+$ RNA was used to construct libraries of adapter-ligated double-strand cDNAs from mouse and rat spinal cords, using the Marathon cDNA Amplification kit (Clontech). The 5′-ends of mouse and rat prepro-UII cDNAs were amplified by polymerase chain reaction (PCR) using the adapter primer AP1 and UROM (Table 1), a degenerated primer designed from the conserved cyclic region of UII (CFWKYCV). All reactions were performed in a total volume of 50 μl PCR mixture containing 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 200 μM each dNTP, 1 μM each primer, 5% dimethylsulfoxide and 1.25 U Taq DNA Polymerase (Promega), using 1 μl of a 1:50 dilution of adapter-ligated ds-cDNA as target. Touchdown PCR was performed successively for five cycles (denaturation at 94°C for 5 s; annealing at 57°C for 3 min), five cycles (denaturation at 94°C for 5 s; annealing at 55°C for 3 min) and 25 cycles (denaturation at 94°C for 5 s; annealing at 53°C for 3 min), in a Perkin-Elmer Gene Amp PCR System 9700. The 5′-sequence information obtained was then used to design rat (RA3) and mouse (SOU3) prepro-UII specific primers (Table 1) in order to perform 3′-RACE reactions in the presence of the AP1 primer (denaturation at 94°C for 5 s and annealing at 55°C, for 30 cycles). The PCR products obtained were inserted into pGEM-T vector (Promega) and sequenced in both directions, using the Thermosequenase kit (Amersham) in an Automated 4000L DNA Sequencer (LI-COR).

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[1] The sequences reported in this paper have been deposited in the GenBank database [accession numbers AF172175 (mouse prepro-UII) and AF172174 (rat prepro-UII)].

Abbreviations: UII, urotensin II; prepro-UII, prepro-urotensin II

PII: S0014-5793(99)01003-0

Table 1
Sequences of the oligonucleotides used for PCR amplification

Primer	Sequence 5′→3′
AP1	CCATCCTAATACGACTCACTATAGGGC
UROM	AC(AG)CA(AG)TA(TC)TTCCA(AG)AA(AG)-CA(TC)TC
RA3	GACTGCCCCAGAATGTTTCTGGAAATATTG
SOU3	CTGCCCCAGAATGCTTTTGGAAGTATTGC
CCLLSOU	GCTGCCTGCTCTTCATAGGACTTCTGAA
TRALSOU	GTTCTTGCCAAGAGACGACTCAGTACAGTG
MDRVRA	ATGGACAGGGTGCCCTTCTGCTGCCTGCTC
LLRSRA	AAAAGACGGCTCAGTACAGTGTTAGAATCT
GAPDHS	TGCTGAGTAYGTCGTGGAGTC
GAPDHAS	TTGGTGGTGCAGGAKGCATTGC
SOINT	TCCCCTGCTTCCGTGCCCATGGTCTGACGC
RAINT	GTGCCCACGGTCTGGCGCAGCGTCTGCAGG

2.2. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Single-stranded cDNA was generated by reverse transcription (RT) of 5 μg total RNA extracted from several mouse and rat tissues. RT-PCR was conducted in a 20-μl final reaction mixture containing 25 mM Tris-HCl, pH 8.3, 37.5 mM KCl, 1.5 mM $MgCl_2$, 50 mM DTT, 25 μg/ml oligo-dT_{15} (Promega) and 200 U SuperscriptII (Gibco BRL). Aliquots (12.5%) of the RT reaction were then analyzed by PCR using primer pairs designed from mouse (CCLLSOU; TRALSOU) and rat (MDRVRA; LLRSRA) prepro-UII mRNA (Table 1). The PCR conditions were similar to those described above except that 30 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 40 s and extension at 72°C for 40 s were used. An internal control PCR reaction was performed in a separate tube, using two specific glyceraldehyde-3-phosphate dehydrogenase primers (GAPDHS, GAPDHAS). The amplified products were separated on 1.5% agarose gels, blotted on nylon membranes and hybridized with an UII internal oligonucleotide probe specific for mouse (SOINT) or rat (RAINT) prepro-UII (Table 1). The PCR products were subcloned into pGEM-T (Promega) and sequenced on both strands by using the Thermosequenase fluorescent labeled primer cycle sequencing kit (Amersham).

2.3. In situ hybridization histochemistry

Rats were killed with chloral hydrate and perfused transcardially with 4% paraformaldehyde. Frontal sections (12 μm thick) were cut in a cryostat and kept at −80°C until use. Sections were pretreated as previously described [20] and covered with pre-hybridization buffer (50% formamide, 0.6 M NaCl, 10 mM Tris-HCl, pH 7.5, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.1% BSA, 1 mM EDTA, pH 8.0;

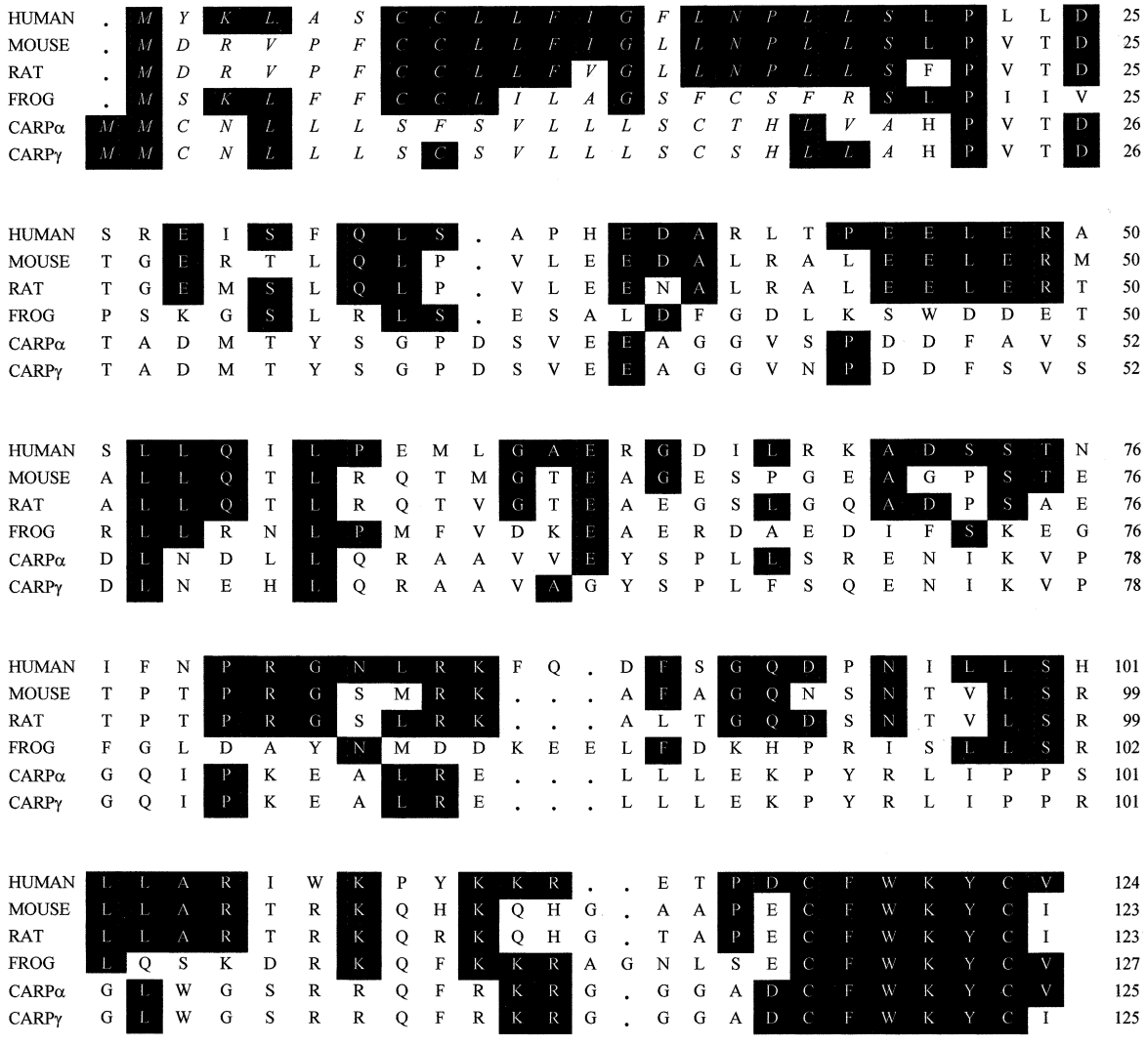

Fig. 1. Alignment of the amino acid sequences of human, mouse, rat, frog and carp prepro-UII. The putative signal peptide sequence is indicated in italics. Conserved amino acid residues are indicated in black. The dots in the sequences indicate gaps introduced for the alignment purpose. The human, frog and carp prepro-UII sequences are from [13,13,15], respectively.

Table 2
Percentage amino acid identity of prepro-UII from different species

Human						
Mouse	48%					
Rat	47%	82%				
Frog	25%	26%	26%			
Carp α	16%	19%	22%	12%		
Carp γ	16%	24%	25%	22%	89%	
	Human	Mouse	Rat	Frog	Carp α	Carp γ

The sequences of human and frog prepro-UII are from [13]. The sequences of carp α prepro-UII and carp γ prepro-UII are from [15]. Sequence identity was calculated using the Clustal Program (http://www.sanger.ac.uk/Software/EGCG/eclustalw.html).

550 μg/ml denatured salmon sperm DNA, 50 μg/ml yeast tRNA). Sense and antisense riboprobes were prepared by in vitro transcription of PCR products obtained with prepro-UII specific primers (CCLLSOU and TRALSOU for mouse; MDRVRA and LLRSRA for rat) extended at the 5′-end with the SP6 and T7 RNA polymerase promoters, in the presence of [^{35}S]UTP (Amersham). Hybridization was performed overnight, at 55°C, in pre-hybridization buffer supplemented with 10 mM dithiothreitol, 10% dextran sulfate and heat-denatured riboprobes. Post-hybridization washes were performed in 2×SSC for 30 min, at 60°C, followed by treatment with RNase A (50 μg/ml) for 1 h, at 37°C, and by five final stringent washes in 0.1×SSC. Sections were then dehydrated and apposed onto Hyperfilm β-max (Amersham) for 2 weeks. After exposure, the slides were dipped into Kodak NTB-2 liquid emulsion at 40°C for 15 days and developed. The tissue sections were counterstained with eosin and hematoxylin to identify neuroanatomical structures.

3. Results and discussion

3.1. Cloning and characterization of mouse and rat UII precursors

Previous studies have shown that human and frog UII genes are intensely expressed in motoneurons of the spinal cord [13,21]. Mouse and rat spinal cords were thus used to prepare libraries of adapter-ligated double-stranded cDNA from which full-length mouse and rat prepro-UII cDNAs were isolated. The overall organization of the two rodent UII pro-hormones is similar to those of other vertebrates (Fig. 1). The mouse 539-bp cDNA encompasses an open reading frame encoding a 123-amino acid prepro-protein including a putative 20-residue signal peptide [22]. Two potential cleavage sites for pro-hormone convertases [23] are present at positions Arg^{85}–Lys^{86} and Arg^{105}–Lys^{106}. Cleavage at the latter dibasic site would generate a 17-residue peptide containing the UII signature motif at its C-terminus. Two additional peptides containing 18 and 64 residues may be generated if the upstream Arg^{85}–Lys^{86} basic pair is used. Prepro-UII also possesses several potential monobasic cleavage sites. In particular, cleavage at the Lys^{109} position would have the potential to generate a shorter 14-residue UII variant. Although cleavage at single lysine residues is not common, the existence of a lysine-specific endopeptidase has been postulated [24].

The rat 529-bp cDNA encodes a 123-amino acid protein which includes a 20-amino acid hydrophobic signal peptide. Analysis of the putative processing sites revealed that the rat UII precursor may be cleaved at three basic doublets, Arg^{85}–Lys^{86}, Arg^{105}–Arg^{106} and Arg^{108}–Lys^{109}, and thus may generate two flanking peptides containing 64 and 18 residues, in addition to the 14-residue UII peptide. Alternatively, if the cleavage at Arg^{108}–Lys^{109} is not complete, a 17-residue form of UII, similar to mouse UII, can be generated.

3.2. Comparison of the structures of prepro-UII among vertebrates

Alignment of the sequence of the mouse and rat prepro-UII proteins with the human sequence showed an overall identity of only 48% and 47%, respectively (Table 2). In the UII region, the degree of similarity between the mouse or rat and the human sequences was 63%. In fact, the sequence of the N-terminal region of UII is highly variable while the cyclic domain located in the C-terminal region of the molecule has been fully conserved between fish and mammals [3,13,15]. Evolutionary pressure has also acted to conserve an acidic residue (Asp or Glu) upstream of the cyclic region and a hydrophobic residue (Val or Ile) at the C-terminus. Structure-activity relationship studies on isolated rat aorta have shown that the C-terminal region of UII, including the acidic and hydrophobic residues flanking the cyclic domain, is the minimum effective core which exhibits the same contractile potency as the intact UII peptide [12]. Mouse and rat UII possess, at their N-terminus, a Gln residue which is generally converted during posttranslational processing into a P-Glu residue by a glutaminyl cyclase [25,26]. The presence of an N-terminal P-Glu residue plays an important role for the biological activity of certain neuropeptides such as thyrotropin-releasing hormone and gonadotropin-releasing hormone [27] but is not implicated in the activity of others including corticotropin-releasing factor [5] and neurotensin [28]. Whether N-terminal pyroglutamyl modification of mouse and rat UII is required for the biological activity of the peptides is currently unknown.

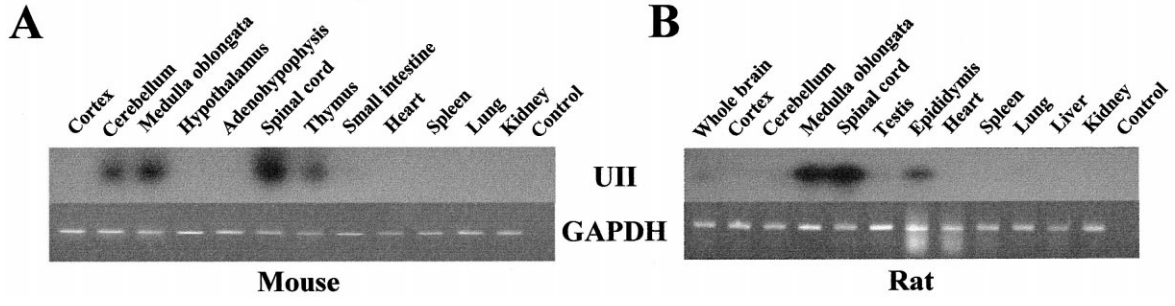

Fig. 2. Analysis of prepro-UII mRNA distribution in several mouse (A) and rat (B) tissues by RT-PCR. Total RNA was extracted and subjected to RT-PCR as described in Section 2. The PCR products were detected by hybridization with a specific mouse or rat ^{32}P-labeled oligonucleotide probe. Ethidium bromide staining of GAPDH PCR products showed that equivalent amounts of mRNA were reverse-transcribed.

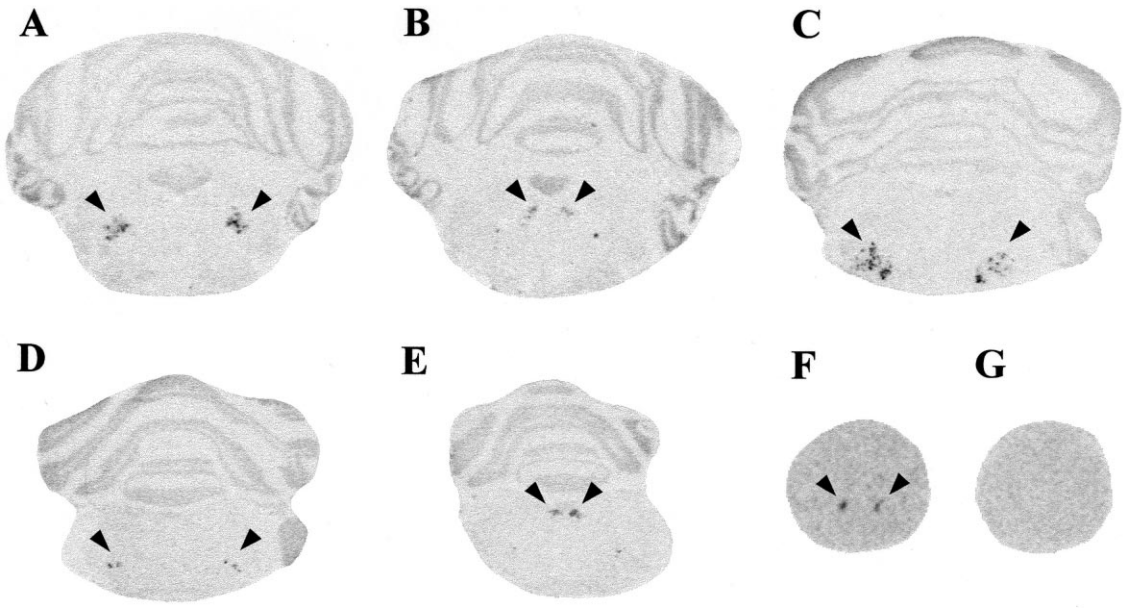

Fig. 3. X-ray autoradiographs showing the distribution of prepro-UII mRNA in the rat brainstem and spinal cord. Coronal sections were hybridized with the antisense (A–F) or sense (G) prepro-UII riboprobe, and apposed for 2 weeks onto X-ray films. Clusters of positive neurons were found in several motor nuclei (arrowheads) including the (A) motor trigeminal, (B) abducens, (C) facial, (D) ambiguus and (E) hypoglossal nuclei as well as (F) in the ventral horn of the spinal cord.

3.3. Distribution of prepro-UII mRNA

The expression of the prepro-UII gene in the CNS and in peripheral organs was studied by RT-PCR analysis. In both mouse and rat, the strongest hybridization signals were observed in the medulla oblongata and the spinal cord (Fig. 2). A less intense amplification signal was found in the mouse cerebellum (Fig. 2A). At the periphery, a weak signal was detected in the mouse thymus (Fig. 2A) and in the rat epididymis (Fig. 2B). The expression pattern of the prepro-UII gene in rodents is globally similar to that previously reported in human [13] and frog [13,21]. Notably, in all four species of tetrapods investigated so far, the highest concentrations of prepro-UII mRNA are found in the medulla oblongata and spinal cord. The occurrence of prepro-UII mRNA has also been detected in the human thymus [13], suggesting that UII, like many other neuropeptides, could be involved in the modulation of the immune response.

In situ hybridization histochemistry revealed that, in the rat CNS, UII mRNA exclusively occurs in motoneurons located in several nuclei of the medulla oblongata, including the trigeminal, abducens, facial, ambiguus and hypoglossal nuclei (Fig. 3A–E), as well as in the ventral horn of the spinal cord (Fig. 3F,G). Specific expression of the prepro-UII gene in motoneurons of the human and frog brainstem has already been described [13] and, in frog, the UII peptide has been characterized in spinal cord extracts [21], indicating that translation of the mRNA and processing of the precursor to generate mature UII actually occur in motoneurons. So far, CGRP is the only other neuropeptide which has been detected in adult motoneurons [29]. It has been shown that CGRP is located in cholinergic terminals at the neuromuscular junction [30]. It has also been reported that CGRP may act at the presynaptic level by stimulating acetylcholine release from motoneurons [31] and at the postsynaptic level by enhancing the expression of the α-subunit of the nicotinic receptor [18]. The specific expression of the prepro-UII gene in motoneurons of the brainstem and spinal cord suggests that UII, like CGRP, may exert modulatory activities at the neuromuscular junction.

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