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Full Length Research Paper

Ribin-like proteins expression in the chaetognath *Spadella cephaloptera*

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In chaetognaths, a marine phylum of approximately 120 species, paralogous ribosomal protein genes and two classes of both *18S* and *28S rRNA* gene sequences have been evidenced. Moreover, differential and specific expression of the *rRNA* genes has been demonstrated suggesting implications of regulatory mechanisms in the synthesis of the ribosome constituents. Results of *in situ* hybridization of *ribin-like* mRNAs in *Spadella cephaloptera* were compared with immunofluorescence localization of the corresponding protein. Previous studies showed that in rat Ribin protein is encoded on the antisense strand of the *28S rRNA* gene and known as rRNA promoteur binding protein. In chaetognaths, expression of *ribin-like* gene(s) is restricted to oocytes similarly to both *18S* and *28S* class II genes and to gut, whereas Ribin-like proteins have been identified in the nervous system, oocytes and gut. Chaetognath nuclear regions, homologous to rat *ribin* mRNA have been sequenced; however, no complete open reading frame can be reached. Analysis of DNA databases reveals that deduced sequences of several animal nuclear sequences exhibit a region having a great level of conserved amino-acids with the COOH-part of the rat Ribin, suggesting a high selection pressure in this region. Moreover, analysis of EST libraries shows that *Ribin-like* genes are expressed in both animals and plants. In addition, in invertebrates, these transcripts are principally found during the first stages of development or in nervous tissues or in infected/stressed organisms. The selective expression and translation of *Ribin-like* genes added to their large evolutionary distribution suggest important physiological roles of the corresponding proteins.

Key words: Ribin, chaetognath, nervous system, oocytes, gut.

INTRODUCTION

Chaetognaths are a small marine phylum living in various habitats, but most of them are among the most abundant planktonic organisms (Feigenbaum and Maris, 1984). Their body is constituted of three parts, the head, trunk and tail, separated by septa (Casanova, 1999). These animals are protandric hermaphrodites; the ovaries lie in the trunk on both sides of the gut, while the testis are in the tail. Their phylogenetic position remains enigmatic, although recent molecular analyses suggest a protostome affinity (Shimotori and Goto, 2001; Faure and Casanova, 2006; Matus al., 2006; Marletaz et al., 2006, 2008). Casanova et al. (2001) showed that chaetognaths can be considered as a model animal.

In eukaryotes, the ribosomal RNA (rRNA) transcription unit usually exists in tandemly repeated arrays containing three *rRNA* genes (18S, 5.8S and 28S), separated by spacer sequences (Prokopowich et al., 2003). Eukaryotic cells contain several hundred *rRNA* genes (rDNA) (Grummt, 2007); however, significant intra-individual variation in the sequences of the rDNA genes is highly unusual in animal genomes. In chaetognaths, two classes of both *18S* and *28S rRNA* gene sequences have been detected (Telford and Holland, 1997; Papillon et al., 2006). Interestingly, a wide distribution of both 18S and 28S classes has been found in the phylum Chaetognatha. Moreover, the results of phylogenetic analyses showing a separation between Class I and II sequences supported by high bootstrap va-

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lues (Telford and Holland, 1997; Barthélémy et al., 2006; Papillon et al., 2006), strongly plead for an ancestral duplication of the whole ribosomal gene cluster prior to the radiation of extant chaetognaths. In addition, recently, for the first time in a metazoan, differential expressions of rRNA pa-ralog genes have been shown, suggesting an ubiquitous role of one of the rRNA Class (Class I) and one or more tissue-specific implication(s) for the variant of the other Class (Barthélémy et al., 2007a, c; 2008). All these results provide support for the hypothesis that each type of rDNA paralog is important for specific cellular functions and is under the control of selective factors. Moreover, added to the two rRNA classes, numerous paralogous ribosomal protein genes have also been found in chaetognath (Barthélémy et al., 2007b). Probably each paralogous protein has specific functions, one of the paralogs plays its role in the ribosome, while the other could have specific extra-ribosomal functions in cells; however, a ribosome heterogeneity where each ribosome is constituted by a class of rRNA associated with a class of ribosomal protein paralogs could not be excluded, and even the two mechanisms could co-exist.

Moreover, in muridae a region homologous to the complementary strand of region homologous to *28S rRNA* genes could encode a protein named Ribin (Kermekchiev and Ivanova, 2001). This protein bound to the rRNA promoter and stimulated its activity. Moreover, Ribin contains two predicted nuclear localization sequence elements and green fluorescent protein-ribin fusion proteins were localized in the nucleus. One main goal of our study was to search if products of the Ribin gene could be one of the selective factors of one of the classes of chaetognath *28S rRNA* genes. Furthermore, with these data, we wanted to contribute to the knowledge on the histological distribution of the Ribin in this taxon.

MATERIALS AND METHODS

Specimens

Adult specimens of the benthic species *S. cephaloptera* have been caught during the spring and summer 2006 in a marine meadows east of Marseilles (Brusc lagoon, France). In the laboratory, samples were kept in aquaria containing natural sea water and placed in a constant temperature at 21 ± 1°C where they were maintained under natural light cycle.

DNA extraction, amplification of *ribin*-like regions and DNA analyses

DNAs from 3 adult individuals have been extracted separately using CTAB method (Barthélémy et al., 2007c). Then, *ribin-like* regions were amplified by polymerase chain reaction (PCR) using several sets of primer couples designed from *ribin*, and chaetognath *28S rDNA* consensus sequences have been used; however, only some of the last primer couples have given significantly consistent results. These primer sequences with their positions on the *28S rDNA* gene of the chaetognath *Sagitta elegans* (AF342799) are respectively 28S23: 5'-TCCCCGGAACGAGCGAAAG-3' (nt1801-nt1819), 28S23B: 5'-GGAATCCGGTCAATATTCCGG-3' (nt1820-nt1840), 28S31: 5'-GATTTCTGCCCAGTGCTCTGAATG-3' (nt2526-nt2549), 28S31R: 5'-CATTCAGAGCACTGGGCAGAAATC-3' (nt2549-nt2526), 28S35R 5'-GATGTACCGCCCCAGTCAAACTCC-3' (nt3006-nt2983). The 25 µl PCR reaction mix contained 100 ng template DNA, 2.5 µl *Taq* DNA polymerase buffer 10×, 1 µl dNTP mix (50 µM), 1 µl of each primer (20 µM), and 1U *Taq* DNA polymerase (Promega). Samples were amplified during 30 cycles under the following regime: 94°C for 1 min, 45 - 60°C for 30 s (according the primer couples), and 72°C for 1.5 min. Using the single-stranded DNA as a template, the nucleotide sequence was determined with an automated DNA sequencer (Macrogen, Seoul, South-Korea). The nucleotide and predicted protein sequences were analyzed online using NCBI-BLAST server (http://blast.ncbi.nlm.nih.gov). Multiple alignments were performed with CLUSTAL X (1.83).

In situ hybridizations

In the laboratory, whole mature specimens were embedded in Tissue-Tek O.C.T. compound and frozen in liquid nitrogen. Samples were then sectioned serially at –20°C using a cryostat microtome. 12 µm frontal sections were collected onto twice gelatine-coated slides, dried on a slide warmer and kept at -70°C.

Concerning the specific probes, the nucleotide position numbers are given by reference to *28S rDNA* of the chaetognath *Sagitta elegans* (accession n°: AF342799). The *ribin* probe was issued from the *S. cephaloptera 28S rDNA* sequenced in this present work (5'-GGTAAGGGAAGTCGGCAAAATGGATCCGTAACTTCGGG-3', from nt2160 to nt2197). Two other probes have been used as controls, one corresponding to a part of the D2 domain of *S. cephaloptera* 28S rDNA gene (Telford and Holland 1997) (5'-CTCTGAAGAGAGAGTTCAATAGGACGTGAACCCGTC-3', from nt390 to nt425) and the other to the 3' end part of this same gene (5'- GGCGAGAGGTGTCAGAAAAGTTACCACAGGGAT-3', from nt3172 to nt3204). Moreover, all these probes exhibit a relatively high level of homology with almost all their respective *28S rDNA* metazoan regions. The respective positions of these probes versus both the *ribin-like* region and chaetognath *28S* gene are shown in Figure 1. A 45-mer scrambled oligonucleotide has been used as a negative control.

In situ hybridization was performed as previously described (Barthélémy et al., 2007a, c). Briefly, the sections were warmed at room temperature and fixed with 4% formaldehyde in PBS, pH 7.2. After two washes in PBS, they were placed in 0.25% acetic anhydride in 0.1M triethanolamine 0.9% NaCl, pH 8, for 10 min and delipidated in ethanol and chloroform. They were hybridized with 50 µl buffer containing 2xSSC (1xSSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.2), 50% formamide, 1x Denhardt's solution, 600 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA, 10% (w/v) dextran sulphate, 0.5 mg/ml tRNA, 0.5 mg/ml denatured salmon sperm DNA and $3x10^7$ d.p.m./ml of 35S-labelled oligoprobe with under a glass coverslip. After 20 h incubation in moist sealed chamber at 37°C, coverslips were removed in 1xSSC, 30 min at room temperature and then slides were successively washed in 2xSSC 50% formamide four times at 40°C and 30 min in 1xSSC at room temperature. Sections were exposed to X-ray films (Biomax-MR; Kodak, Rochester, NY, USA) for 24 h and subsequently dipped in nuclear emulsion (1:1 in water, K5; Ilford, Saint-Priest, France) for 6 days (*ribin* probe) or 3 days (three other probes). After development, sections were counterstained with nuclear fast red.

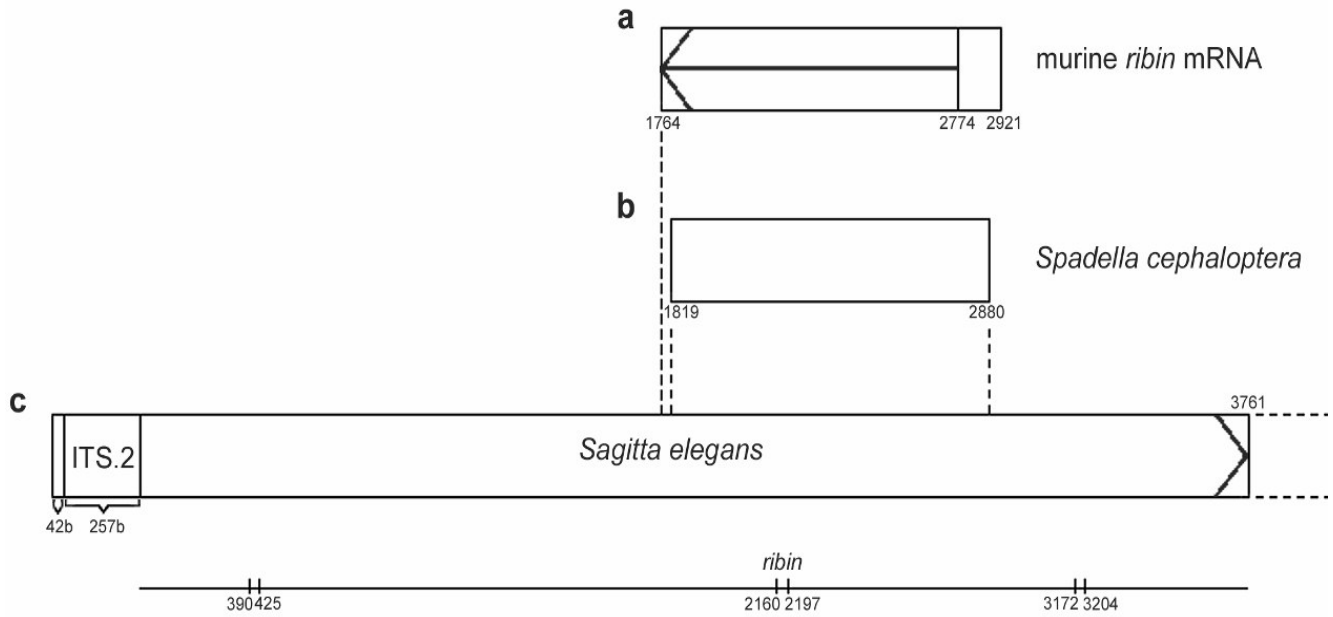

Figure 1. Chaetognath DNA regions homologous to rat *ribin* mRNA. (A) Rat *ribin* mRNA with its orientation (arrow corresponding to the coding region) (Acc. n°: NM_147136). (B) *Spadella cephaloptera* sequenced region homologous to rat *ribin* mRNA. (C) *Sagitta elegans* internal transcribed spacer 2 (ITS.2) and *28S rRNA* partial sequence, the orientation of the *rRNA* gene has been schematized by an arrow (Acc. n°: AF342799). Under this last scheme, the nucleotide positions of the DNA probes using for *in situ* hybridization are given. All the nucleotide position numbers are given by reference to *28S rDNA* of *S. elegans*.

IMMUNOHISTOCHEMISTRY

For immunodetection of Ribin, a polyclonal antibody raised in a rabbit against bacterially expressed and gel-purified Ribin protein (Kermekchiev and Ivanova, 2001) has been used. Sections were first incubated in 10% normal goat serum in PBS (0,1 M), with 0,6% Triton X-100 and 2% bovine serum albumin (PBS buffer), for 1h at room temperature, before being incubated overnight at 4°C with Ribin antibody or preimmune serum diluted 1/500 in the same PBS buffer. After being rinsed three times, sections were incubated for 1h into the dark at room temperature with TRITC-coupled anti-rabbit IgG, diluted 1:400, rinsed in PBS and mounted in a medium containing antifading (Gel/MountR, Bibmeda, Foster City, CA, USA). F-actin is labeled with diluted Phalloidin-FluoProbes® 647 (0.16 nmol/ml final concentration in PBS) for 20 min - 1 h at room temperature.

Tissue sections were examined under a confocal scanning laser microscope (Leica, 4D, Heidelberg, Germany), using a krypton/ argon laser. The control immunostaining with preimmune serum remained negative.

RESULTS

Sequence

Several primer couples spanning the *ribin* cDNA previously described (Kermekchiev and Ivanova, 2001) or designed specifically for this study was used for amplification. However, only some of the primer couples complementary to chaetognath *28S rDNA* consensus sequences have given positive results. Various regions homologous to rat *ribin* cDNA have been sequenced, but all the sequences harbor frameshift mutations and premature stop codons in their apparent reading frame. Only the sequence which exhibits the greatest homology level with those of *Rattus norvegicus* has been used for our analyses and has been deposited in GenBank (Accession number FJ932763). Moreover, after translation only homologies with the rat Ribin COOH-part has been found (Figure 2). Interestingly, *TBLASTN* analyses, which allow comparing a protein query sequence against a nucleotide sequence database dynamically translated in all reading frames, showed evidence that in animal DNA databases the homologies are principally found with the Ribin COOH-part (significant examples are shown in the Figure 2). Moreover, many eukaryotic mRNAs contain sequences that resemble segments of 28S and 18S rRNAs, and these rRNA-like sequences are present in both the sense and antisense orientations. Some are similar to highly conserved regions of the rRNAs, whereas others have sequence similarities to expansion segments (Mauro and Edelman, 1997; Kong et al., 2008). *TBLASTN* analyses against expressed sequence tag (EST) libraries show evidence that regions homologous to rat *ribin* mRNA are expressed in both animals and plants; however, as in several EST libraries, no data concerning the position of the polyadenylated regions are given, we should be suspicious of any assignment based on the orientation criterion in EST databases, as the 3' and 5' designations on ESTs (particularly the 3') are sometimes reported as the reverse-complement (Table 1).

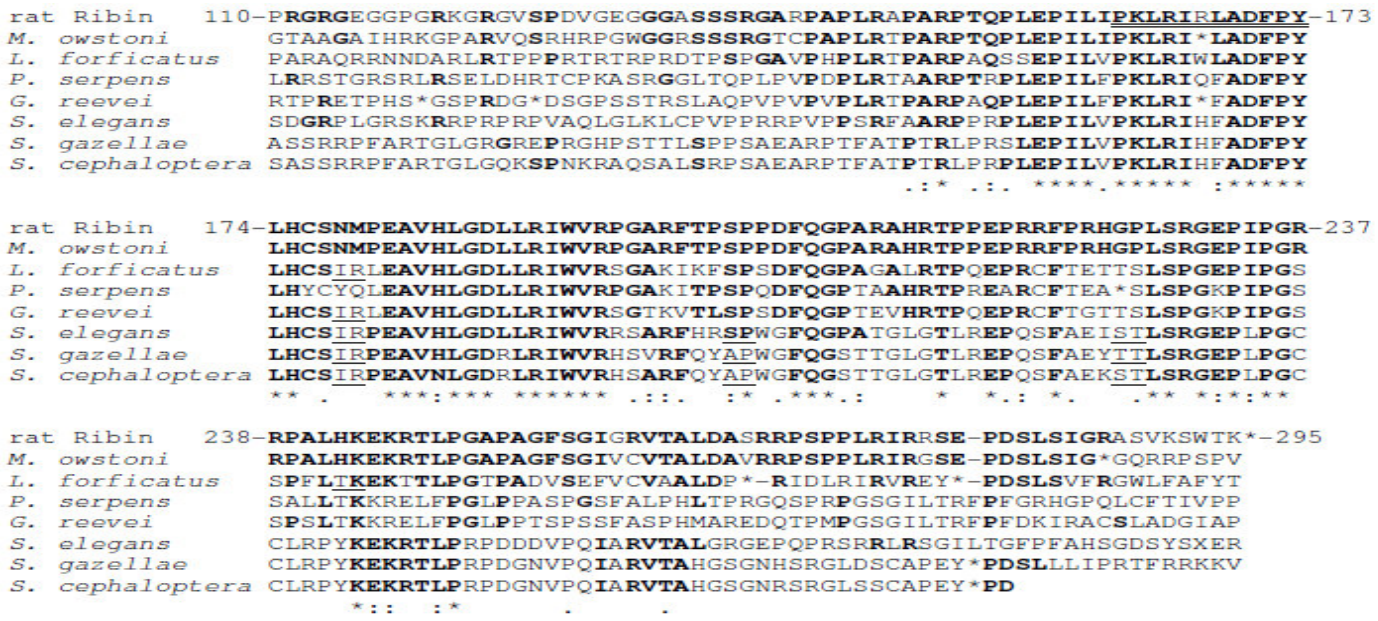

Figure 2. Amino acid alignments of rat Ribin with the corresponding region deduced from various animal nuclear genes. Numbering has been given *Rattus norvegicus* Ribin (Acc. n° NM_147136). But for the chaetognath *S. cephaloptera*, the other sequences have been deduced for DNA sequences extracted from GenBank, *M. owstoni* (*Mitsukurina owstoni* ; Vertebrata; Chondrichthyes ; Acc. n° AY049839), *L. forficatus* (*Lithobius forficatus* Arthropoda; Myriapoda; Acc. n° EF199984), *P. serpens* (*Poecilochaetus serpens*; Annelida; Acc. n° EU418869), *G. reevei* (*Glycymeris reevei*; Mollusca; Acc n° AB101609), *S. gazellae* (*Sagitta gazellae*; chaetognath; acc n° EU375500) *S. elegans* (*Sagitta elegans*; chaetognath; acc n° AF342799). Amino acid identities are shown below the alignments: *, identity; :, high similarity; ., low similarity. Sites of insertion/deletion are marked by a solid line. A star indicates a stop codon, a solid line indicates a inserted gap. Identical amino acids versus those of rat Ribin are shown in bold letters. The region corresponding to the DNA probe is double underlined.

Interestingly, in invertebrates, the deduced products of ESTs which exibit the higher homology level with the murine Ribin are principally found in nervous tissues or during the first stages of development or in infected/ stressed organisms (Table 1). ESTs homologous to *ribin* gene, but polyadenylated and in the opposite orientation to *ribin* gene, have not been included in our analysis; however, even if doubts persist concerning most of the other EST sequences, the length and the high degrees of homology found between deduced amino acid sequences suggest functional open reading frames (Figure 3). However, BLAST analyses against EST databases have also shown that the sequence homology corresponds only to the COOH-part of the murine Ribin, see e.g., the examples shown in the Figure 3.

In situ hybridization

Four various probes have been used; one of them is complementary to a part of the corresponding to the COOH-region of the Ribin protein, two others are in the *ribin* flanking regions, upstream or downstream the region homologous to ribin-like sequence (Figure 1), and finally a scrambled oligonucleotides control (negative control) has also been used. *In situ* hybridizations evidenced the ribin-like genes have two distinct localizations with a strong and specific expression at the level of the ova inside the ovaries and a less intense but positive signal in the gut epithelium (Figures 4 a and c). Which differ from the hybridization results using two other probes against transcripts complementary to either an 5' region of the *28S* gene or a 3' segment ; the first probe allow to reveal a strong expression in the gut whereas the second shows a weak labeling in the entire animal (data not shown). In addition, *in situ* hybridization with negative control exhibits no signal pattern, even after long times of exposure which evidenced that the signal is specific (data not shown).

IMMUNOCHEMICAL LOCALIZATION OF RIBIN-LIKE PROTEINS IN THE WHOLE ANIMAL

Immunolocalization of Ribin-like proteins shows an immunoreactivity more or less marked according to the organs (Figures 4 b, d and e). A strong Ribin-like proteins localization is obvious in the cerebral and ventral ganglia and also in some nerves linking them to the rest of the nervous system, and notably the large paired fibre tracts ending posteriorly the ganglion. Sensory receptors scattered on the epithelium also exhibit reactivity. A less

Table 1. List of ESTs which the deduced sequences show homologies with rat Ribin. But for Deuterostomia, only the EST sequences which have more than 58% of identical amino acid residues in a contigous region of at least 85 residues are given. For Deuterostomia, more than 810 ESTs exhibiting more than 95% of identical amino acid residues in a contigous region of at least 140 residues have been found, also the ESTs which deduced sequences showing the highest homology with Ribin are only given for the three species for which more than 200 ESTs meet these last criteria. 1) in the same EST library, only the accession number of the sequence (or one of the sequences) exhibiting the highest homology level is given. 2) Libraries could be normalised or not, and some of them contain redondant sequences. 3) Start codon signifies that an ATG codon has been found upstream the sequence which exhibits homology. 4) FS: frameshift. 5) internal codon found in the region exhibiting sequence homology. 6) # signifies that no stop codon has been found after the region exhibiting sequence homology. The orientation is noted + and – for same and opposite orientation respectively.

Species and taxon	Accession numbers[1]	Number of *ribin-like* EST/ total EST number[2]	Orientation / published sequence	Start codon[3]	FS[4]	Internal stop codon[5]	Cell, tissue type and/or stade	% identity /Ribin[6]	Miscellaneous
Bilateria									
Deuterostomia									
Mus musculus - Craniata	CF581302	11/8486	-	no	0	0	Pancreas	98% 255/258aa	A total of 272 ESTs have been found
Homo sapiens - Craniata	CT004804	62/30,630	-	no	0	0	T-Lymphocytes - adult	98% 156/158aa#	A total of 308 ESTs have been found
Canis lupus familiaris - Craniata	DN418188	57/9742	-	no	0	0	Skeletal muscle	98% 153/155aa	A total of 203 ESTs have been found
Protostomia									
Laupala kohalensis - Insecta	EH628931	1/ 14,502	-	1	0	0	Total nerve cord - juvenile at instars 5 to 8	82.1% 119/145aa#	
Culex quinquefasciatus - Insecta	FF227666	11/108,734	-	no	0	0	N.D.	95.1% 77/81aa#	Infected with filarial worms
Mytilus californianus – Mollusca	GE750243	2/42,354	-	no	1	1	Gill and adductor muscle	68.4% 80/117aa#	Exposion to a variety of environmental challenges
Mytilus galloprovincialis – Mollusca	AJ624874	4/18,788	+	no	1	0	Mix of various organs	68.4% 67/98aa#	stressed animals
Helobdella robusta - Annelida	EY359785	13/25,208	-	no	1	0	Embryo st. 7-11	59.8% 73/122aa#	
Rhipicephalus appendiculatus - Chelicerata	CD792249	2/9844	-	no	1	0	Salivary glands	63.4% 71/112aa#	Infected with the protozoa *Theileria parva*
Alvinella pompejana - Annelida	GO231699	5/50,693	+	1	1	1	Posterior end - adult	66.6% 68/102aa#	
Crassostrea virginica - Mollusca	CD649324	1/1693	-	no	1	1	Gonad	62.1% 64/103aa	Exposure to mercury

Table 1. Contd.

Aplysia californica - Mollusca	EB269739	5/175,000	-	no	1	0	Cerebral ganglion : metacerebral neuron	68.5% 61/89aa#	Also found in another *A. californica* library (GD229562)
Amblyomma variegatum - Chelicerata	BM291830	1/3992	-	no	1	0	Salivary glands - adult	59.8% 64/107aa	
Other invertebrates									
Nippostrongylus brasiliensis - Nematoda	EB185721	2/716	-	no	0	0	Development stage: L3, activated	97% 97/100aa#	
Viridiplantae									
Fragaria vesca	EX666285	6/8884	-	no	0	0	Seedlings	98.7% 156/158aa	Heat stressed
Pinus taeda	DN452388	1/39,503	-	no	2	2	Somatic embryos	72.6% 85/117aa	

Figure 3. Amino acid alignments of murine Ribin with the corresponding region deduced from various ESTs. Numbering has been given *Rattus norvegicus* Ribin (Acc. n° NM_147136). Characteristics of the ESTs are in Table I. For both protostomia (*Laupala kohalensis*, Acc. n° EH628931) and viridiplantae (*Fragaria vesca*, Acc. n° EX666285) only the sequences which have the highest level of homology with murine Ribin are given, whereas three vertebrae sequences are given (*Mus musculus*, Acc. n° CF581302; *Homo sapiens*, CT004804; *Canis lupus*, Acc. n° DN418188). Amino acid identities are shown below the alignments: *, identity; :, high similarity; ., low similarity. Identical amino acids versus those of rat Ribin are shown in bold letters. Non sequenced regions are marked by a solid line. A star indicates a stop codon.

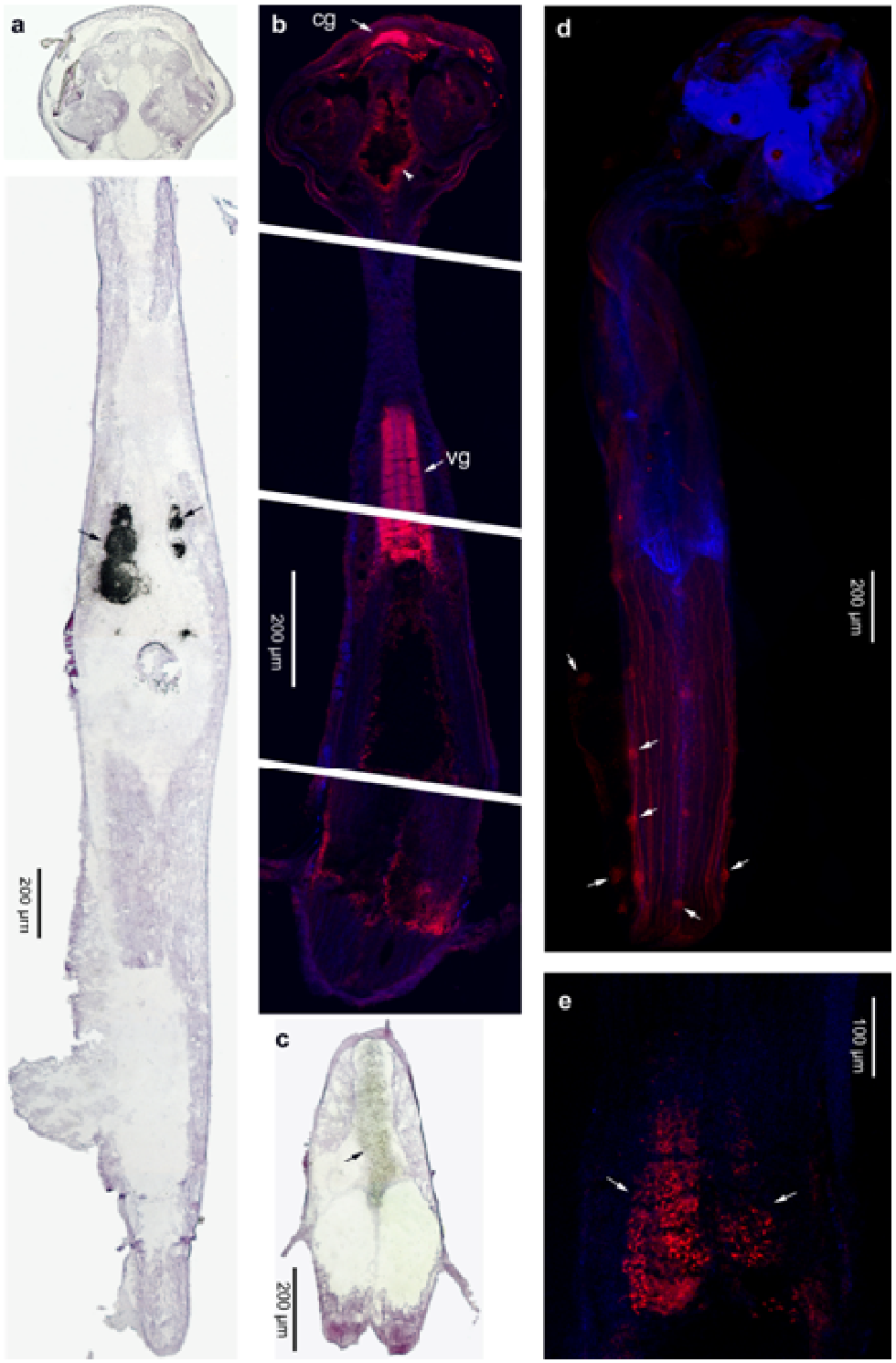

Figure 4. *In situ* hybridization of *ribin-like* mRNAs and Immunolocalization of Ribin-like proteins. (a & c) Localization of the expression of the *ribin-like* genes by *in situ* hybridization on frontal sections of two different specimens. Note the strong expression of the *ribin* at the level of the ovaries (a) and more weakly in the gut epithelium (c). Small spots represent the positive signals, while the grey background is the result of the coloration process using nuclear fast red. (b, d & e) Confocal photomicrographs of Ribin-like expression. (b & e) Frontal sections showing a great Ribin-like immunoreactivity in the cerebral and ventral ganglions (b) and in the ovaries (e, arrows). Note also the reactivity of the gut epithelium (arrowhead). (d) Confocal photomicrographs of an entire specimen. Note the labelling of the sensory receptors (arrows). cg – cerebral ganglion, vg – ventral ganglion.

intense labelling is also evident in ova and in the gut epithelium.

DISCUSSION

In spite of the use of several set of primers, amplification of a chaetognath *ribin-like* region without frameshift mutations and premature stop codons has been unsuccessful. However, in chaetognaths *in situ* hybridizations show that one or more *ribin-like* genes are expressed in specific tissues whereas immunohistochemistry analyses have revealed the presence of Ribin-like proteins in particular tissues suggesting that these proteins are functional. Moreover, BLAST analyses on DNA databases have shown that a region corresponding the rat Ribin COOH-part has been found in the opposite strand of animal nuclear genome (principally in *28S rRNA* genes) and in spite of the evolutionary distance a great level of conserved amino acid has been found. Eukaryotic cells contain several hundred *rRNA* genes and pseudogenes of rRNA and mRNA-derived pseudogenes that reside in rDNA are well known (Gonzalez and Sylvester, 1997). This suggests that functional *ribin* sequences could be only found in rRNA pseudogenes due to the lost of the selection pressure on the strand encoding the rRNA; an alternative hypothesis is that specific mRNA-derived pseudogenes incorporated in rDNA could encode Ribin. Moreover, various mRNA can contain small regions of rRNA sequences (Kong et al., 2008); however, analyses of deduced products of EST sequences exhibiting high homology levels with rat Ribin have shown that regions flanking the Ribin domain do not have sequence homology with 28S rDNA suggesting that Ribin genes could be independent of the rRNA genes. In addition, PCR experiments and BLAST analyses strongly suggest that genes encoding Ribin or more especially only a region homologous to COOH-part of the rat Ribin are rare in the genome, comparatively to the number of rRNA genes. Owing the lost of the COOH-region, these proteins do not exhibit the nuclear localization sequence suggesting a cytoplasmic localization. Moreover, analyses of ribin-like EST sequences made in GenBank suggest that the corresponding genes could be expressed during stress due to infections or addition of chemical products. This suggests that stress could induce change of ribin-like gene expression. Moreover, ribin genes have a large evolutionary distribution including plants suggesting important physiological roles of the corresponding proteins.

In situ hybridizations on frontal sections of the chaetognath *Spadella cephaloptera* shown evidence that sequences homologous to rat *ribin* gene products are expressed principally in oocytes with also a weaker signal in the gut. Moreover, experiments using probes both upstream and downstream the *ribin* region have revealed that this last region is overexpressed versus its flanking sequences and that the pattern of expression is also quite different. This provides strong evidence that ribin-like mRNAs are specifically produced in chaetognath. Moreover, our previous analyses have shown selective expression of the two classes of both 18S and 28S rDNA, whereas the Class I genes correspond probably to “housekeeping” genes, the expression of those of Class II being restricted to oocytes. These observations led to the hypothesis that both Ribin-like proteins and class II rRNAs could play physiological role(s) in oocytes.

Use of anti-Ribin antibodies has revealed that Ribin-like proteins are present in the nervous system, oocytes and gut. Due to in situ hybridization results, the presence of Ribin in ova and gut is not surprising but its strong expression in the nervous system was unexpected. The cerebral and ventral ganglia, which are the two main nervous centres, are particularly labelled. The typical architecture of the latter is well evidenced by the labelling. Indeed, it is built upon a serial arrangement first described more than a century ago by both Hertwig (1880 a, b) and Grassi (1883), and detailed since then by numerous authors for different species (see for review Harzsch and Müller, 2007; Harzsch et al., 2009).

The two "classical" neuron somata zones flanking the central fibre zone or neuropil core are well seen (Figure 4 b). Interestingly, all these three longitudinal areas are serially and regularly arranged in compartments, as already reported in *Sagitta setosa* Müller (1847) by Bone and Pulsford (1984) for transverse fibres crossing the neuropile core and recently by Harzsch and Müller (2007) who detected ca. 80 transverse microcompartments in the whole ganglion by synapsin immunolocalization. According to the latter authors, these microcompartments are not visible at a slightly more ventral focus, so that their experiment more evokes our labelling of the ventral ganglion in ventral view. The neuropil labelling of Ribin-like proteins in *Spadella cephaloptera* probably corresponds to the synaptic contacts evidenced by Harzsch and Müller (2007).

Interestingly again, there are ca. a dozen of "macrocompartments" that might correspond to the "temporal segmentation in the ventral ganglion" highlighted by Goto et al. (1992) relating the increasing apparition of regularly arranged paired RfaLI neurons during the postembryonic development of *Paraspadella gotoi* Casanova, 1990, and that might also corresponds to the twelve pairs of nerves that radiate laterally from the ventral ganglion (Bone and Goto, 1991). Such a localization of the ribin-like ESTs in the whole nervous system, including the sensorial epidermic organs, has already been reported for an insect, *Laupala kohalensis* (Table 1). As a consequence of this fact, the Ribin-like proteins might be used as a marker of nervous tissues in animals.

Surprisingly, *in situ* hybridizations and immunohistochemical analyses give only partially congruent results; indeed, no hybridization has been found in nervous system whereas a great level of protein is detected in the same tissue. This suggests that, whereas a correlation between *ribin-like* mRNA expression and protein abun-

dance is found in oocytes, in nervous system, the *ribin-like* mRNA level is under our detection limit suggesting that they are translated with a higher efficiency. Moreover, it is well known that a little fluctuation in mRNA half-life or protein half-life could have significant effects on steady-state levels of mRNA or protein (references therein Shu and Hong-Hui, 2004). In addition, the *ribin-like* mRNAs produced in the nervous system could be different to those found in oocytes.

In chaetognaths, proteins have been detected using Ribin antibodies in the axons suggesting a cytoplasmic localization; this is congruent with BLAST analyses suggesting that only the COOH-part of the Ribin could be produced inducing a lost of the nuclear localization signals located in the rat Ribin NH_2-part, but not with the previous study of Kermekchiev and Ivanova (2001) which have shown evidence that Ribin has a nuclear localization and could be a rRNA promoter binding protein playing a role as enhancer of the rRNA transcription and cell proliferation. Indeed, this protein is known to interact with RNA polymerase I (Pol I) and the nucleolar transcription factor UBF (=upstream binding factor). Our results suggest that Ribin-like proteins have a different function to that shown by these authors; added to BLAST analyses, they suggest that Ribin-like proteins could play many physiological roles in the nervous system, during development (from oocytes to juveniles) and also during a large variety of stresses including infections.

Interestingly, it is known that some rRNAs may process to become mature mRNAs, which may have specific functions or may encode functional proteins. For example, the Humanin mRNA is 100% homologous to *16S rRNA* gene and encodes a short polypeptide (24 residues). Humanin has been shown to rescue neuronal cell death caused by multiple different types of familial Alzheimer's disease genes and the rat protein homolog also displays protective activity against excitotoxic neuronal death (rewiewed in Kong et al., 2008). Owing to these last studies on Humanin and our immunochemistry results and Blast analysis results, the role of Ribin in some neurodegenerative disease processes must be considered.

In conclusion, the physiological role(s) of Ribin-like protein(s) remain unknown; however, evidences of both differential gene expression and selective translation added to the relatively conserved regions found in the animal Kingdom suggest that these proteins play essential role(s) in animals. Moreover, as antibodies against rat Ribin could recognize proteins in an invertebrate protostomian also suggest a high level of sequence conservation of these proteins linked to a major physiological role. In the future, experiments will be carried out to investigate the Ribin role in neuronal tissues that could be fruitful in neurological investigations.

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