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Short communication

THE DIFFERENTIAL EXPRESSION OF RIBOSOMAL 18S RNA PARALOG GENES FROM THE CHAETOGNATH *Spadella cephaloptera*

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Abstract: Chaetognaths constitute a small marine phylum of approximately 120 species. Two classes of both 18S and 28S rRNA gene sequences have been evidenced in this phylum, even though significant intraindividual variation in the sequences of rRNA genes is unusual in animal genomes. These observations led to the hypothesis that this unusual genetic characteristic could play one or more physiological role(s). Using *in situ* hybridization on the frontal sections of the chaetognath *Spadella cephaloptera*, we found that the 18S Class I genes are expressed in the whole body, with a strong expression throughout the gut epithelium, whereas the expression of the 18S Class II genes is restricted to the oocytes. Our results could suggest that the paralog products of the 18S Class I genes are probably the "housekeeping" 18S rRNAs, whereas those of class II would only be essential in specific tissues. These results provide support for the idea that each type of 18S paralog is important for specific cellular functions and is under the control of selective factors.

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Abbreviations used: EDTA – ethylene diamine tetra-acetic acid; mRNA – messenger ribonucleic acid; nt – nucleotide; OCT – optimal cutting temperature; PBS – phosphate buffered saline; RER – rough endoplasmic reticulum; rRNA – ribosomal ribonucleic acid; RT-PCR – reverse transcription-polymerase chain reaction; SSC – sodium chloride sodium citrate; tRNA – transfer ribonucleic acid

Key words: 18S, Chaetognath, *Spadella cephaloptera*, *In situ* hybridization, Duplication, Expression pattern, rRNA paralogs

INTRODUCTION

Chaetognaths are a small marine phylum of about 120 species, subdivided into two orders, Phragmophora and Aphragmophora [1]. The chaetognath body is constituted of three parts, the head, trunk and tail, separated by septa. The anatomical level is simple. There are four longitudinal muscle masses (the body wall) in the trunk and tail. The gut has no differentiated regions; the anus opens at the trunk-tail limit. These animals are protandric hermaphrodites, and cross-fertilization is considered to be the more usual mechanism in nature; the ovaries lie in the trunk on both sides of the gut, while the testis are in the tail [1]. The testis are not always well individualized, but often a patch of germinal cells that produce spermatogonia more or less continually during spermatogenesis; mature spermatozoa are stored in external posterior seminal vesicles. They live in various marine habitats, but most species are among the most abundant planktonic organisms [2].

The phylogenetic position of the chaetognaths remains enigmatic, although recent molecular analyses suggest a protostome affinity [3, 4]. Casanova *et al.* [5] showed that they can be considered as a model animal, a view based on about 30 years of research on the phylum. For example, chaetognaths constitute a good model as an indicator of stress responses for experimental studies at the level of the whole organism [6]. Moreover, the benthic species *Spadella cephaloptera* Busch, 1851, and *Paraspadella gotoi* Casanova, 1990, are well-adapted to the methods of *in situ* analysis of immunodetection or gene expression [6, 7], because the eggs, embryos and adults are totally transparent; and most of the members of the family Spadellidae are easy to collect and to breed in the laboratory [8].

In eukaryotes, the ribosomal RNA transcription unit usually exists in tandemly repeated arrays containing three rRNA genes, 18S, 5.8S and 28S RNA genes, with the genes separated by spacer sequences. The primary transcript of the rRNA genes is a large 45S pre-rRNA which contains the three rRNA types. Non-transcripted spacers separate tandem copies of the entire transcripted unit. The 5S rRNAs are coded separately.

In Metazoa, multiple copies of a given ribosomal RNA gene family undergo concerted evolution, in such a way that sequences of all the gene copies are virtually identical within a species, although they diverge normally between species [9]. However, the individuals of some species have two or more types of 18S genes exhibiting the mean divergence value, generally > 5%, whereas the values found in congeneric interspecific comparisons between the 18S rRNA sequences of different species are mostly < 4%. To the best of our knowledge, the only animals in which intraindividual variation of the 18S rRNA genes has been observed are apicomplexans [10], *Acanthamoeba* [11], *Trypanosoma cruzi*

[12], platyhelminthes Dugesiidae [13], cephalopods [14], sturgeon [15, 16], and chaetognaths [17]. Interestingly, a wide distribution of both 18S and 28S classes has been found across the phylum of chaetognaths. Together with the results of phylogenetic analyses (separation between 18S Class I and II sequences supported by very high bootstrap values), this fact strongly supports an ancestral duplication of the whole ribosomal gene cluster prior to the radiation of extant chaetognaths, suggesting that the two classes of both 18S and 28S rRNA genes are expressed and functional [17, 18]. Although this has yet to be experimentally verified, there is little data to evidence that the rRNA paralogs are functional genes. To date, the sole strongest and most conclusive evidence of the functionality of the rRNA paralogs has been shown in some species of the protist *Plasmodium*, where at least two different types of 18S rDNA exist, with their expression linked to different stages of the parasitic life cycle of these organisms [19, 20]. These last results provide support for the idea that some of the variants are not pseudogenes and are important for proper cellular function.

In this study, the spatial pattern of 18S rDNA paralogous gene expression was examined by *in situ* hybridization on *Spadella cephaloptera*. Our results reveal that the 18S Class I genes are expressed throughout the bodies of animals, with a stronger expression in the whole gut epithelium than in the rest of the body, whereas the 18S Class II genes are restricted to the oocytes.

MATERIALS AND METHODS

Specimens

Adult specimens of the benthic species *Spadella cephaloptera* were collected in spring 2006 from a marine meadow east of Marseille (Brusc lagoon, France). In the laboratory, whole specimens (two during spermatogenesis and 8 during ovogenesis) were embedded in Tissue-Tek O.C.T. compound and frozen in liquid nitrogen. The samples were then sectioned serially at -20ºC using a cryostat microtome. 12-µm frontal sections were collected onto twice gelatine-coated slides, dried on a slide warmer and kept at -70ºC.

In situ hybridizations

An almost complete sequence of the *S. cephaloptera* 18S Class I gene has been sequenced [17], but for Class II paralogs, only about half of the gene has been sequenced. Therefore, we performed numerous PCR in order to sequence the rest of this gene, but unsuccessfully. Interestingly, the analyses of the chaetognath sequences published by Papillon *et al.* [17] reveal that of 14 18S Class I sequences, all but 2 are almost complete, while 8 out of 12 18S Class II sequences represent only approximately half of the gene. These sequencing results, added to those of Telford and Holland [18], who reported that only 25% of the clones represent 28S Class II genes, strongly suggest that it is more difficult to obtain Class II than Class I sequences. This may be due to a lower number of Class II than Class I genes and/or to a greater level of nucleotide

divergence within Class II genes than within their paralogs. Thus, we used two 18S probes, an autologous probe for 18S Class I genes and a chimeric probe for its paralogs. The 18S Class I probe was issued from a *S. cephaloptera* sequence (DQ351884, from nt745 to nt711), 5'-ACAAGCAAGGTGGAACCCCAGCG ACAGCTAGGGAC-3'. The other probe has its origin in the analyses of Papillon *et al.* [17], who evidenced that the two 18S paralogs arose by a gene (cluster) duplication in a common ancestor of extant chaetognaths. So, as the sequence of the *S. cephaloptera* 18S Class II gene is partial (DQ351897) and as the most divergent regions between the two classes of genes, which are in the first third of the gene, are not contained in this partial sequence, we used a chimeric probe using a part of the 5' end of the *S. cephaloptera* sequence (from nt19 to nt1, underlined nt) linked to a sequence of another Phragmophora (*Eukrohnia fowleri,* DQ351889, from nt704 to nt688) 5'-CATGATACCAG GGACCATCTGGGTGAGGCCCCAACAG-3'. In addition, the *E. fowleri* region exhibits more than 76% identity with the consensus region of the three Aphragmophora species sequenced to date (*Krohnitta pacifica*, *Sagitta bipunctata*, *Sagitta lyra*). A 45-mer scrambled oligonucleotide was used as a negative control.

In situ hybridization was performed as previously described [21], with some modifications in accordance with the use of oligoprobes. 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 0.1 M triethanolamine 0.9% NaCl, pH 8, for 10 min, and delipidated in ethanol and chloroform. They were hybridized with 50 µl buffer containing 2 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.2), 50% formamide, 1 x 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 3 x 10^7 d.p.m./ml of 35S-labelled oligoprobe under a glass coverslip. After 20 h incubation in a moist sealed chamber at 37ºC, the coverslips were removed in 1 x SSC, for 30 min at room temperature, and then the slides were successively washed in 2 x SSC 50% formamide four times at 40ºC, and for 30 min in 1 x SSC at room temperature. The sections were exposed to X-ray films (Biomax-MR; Kodak, Rochester, NY, USA) and subsequently dipped in nuclear emulsion (1:1 in water, K5; Ilford, Saint-Priest, France). Depending to the intensity of the signal on the X-ray film and in order to get a sufficient but not overexposed signal, tissue sections were exposed for 6 h, ten weeks after hybridization for 18S class I, and for three weeks, a few days after hybridization for 18S class II. After development, the sections were counterstained with nuclear fast red.

RESULTS AND DISCUSSION

After as little as 4 hours of exposure, X-ray film evidenced that the 18S Class I paralog genes are strongly expressed from the head to the tail of the animals,

including the ova (data not shown). When the radioactivity had sufficiently decreased, i.e. ten weeks after *in situ* hybridization, it was possible to highlight some differences. The dipped slides show a strong labeling in the gut cells, from the pharynx to the end of intestine, suggesting the strongest expression of the 18S Class I in this tissue in comparison to the other parts of the body (Fig. 1A and B). In addition, *in situ* hybridization with the negative control yielded no signal pattern, even after a long exposure, indicating that the signal is specific (data not shown).

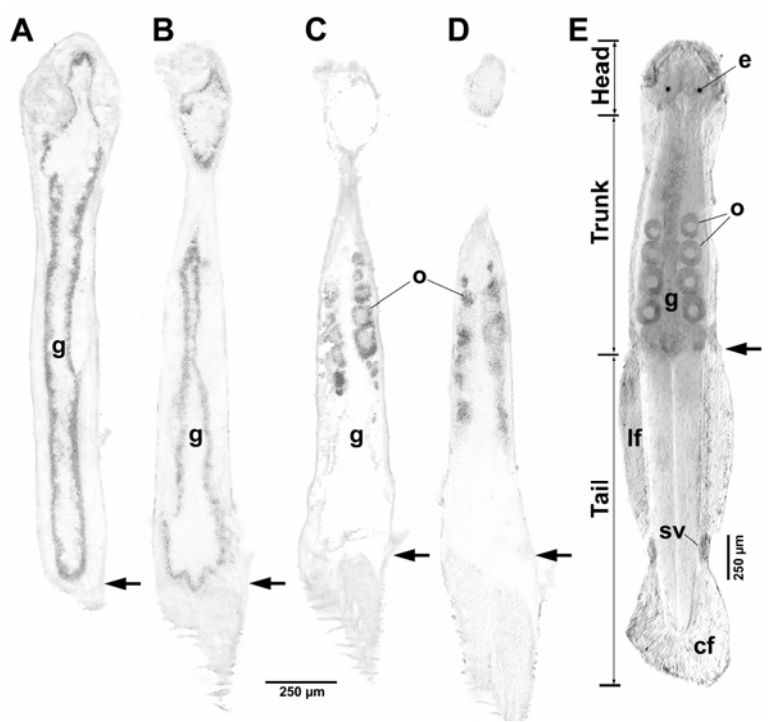

Fig. 1. *In situ* hybridization of 18S rRNA paralogs. A-D – Localization of the 18S rRNA paralogs by *in situ* hybridization using an anti-sense 18S Class I (A and B, one specimen) and an anti-sense 18S Class II (C and D, another specimen). Note in A and B the strong expression of the 18S Class I in the whole gut epithelium, and in C and D that 18S Class II are expressed only in the cytoplasm of the oocytes. Small spots represent the positive signals, while the grey background is the result of the coloration process using nuclear fast red. E – Photograph in light microscopy of entire animal stained with methylene blue in view to see the anatomy. Note the oocyte cytoplasm around the nucleus as in C and D. Arrow – transverse septum, cf – caudal fin, e – eye, lf – lateral fin, g – gut, o – oocyte, sv – seminal vesicle.

Contrarily to the 18S Class I experiment, the expression of the 18S Class II genes began to be visible only after a week of exposure. The dipped slides show that this class of genes is specifically expressed in the cytoplasm of the vitellogenic oocytes of individuals during ovogenesis (Fig. 1C and D), whereas

no specific signal was identified in the individuals during spermatogenesis (data not shown). Moreover, the low percentage identity between each probe and the homologous region of the other class (< 50%), taken together with the relatively high stringency hybridization conditions, strongly suggest that the two hybridization patterns are specific, i.e. the pattern observed in Fig. 1C and D could not be a weak version of the pattern in Fig. 1A and B. However, the evidence for restricted-level transcription of Class II rDNA does not prove processing, folding, and involvement in protein synthesis, although, since this specific pattern has been found in all the individuals investigated, this could suggest that the two classes of genes are expressed in the oocytes and functional.

To date, only four almost complete sequences of chaetognath 18S Class II have been obtained, and three of these belong to the same order (Aphragmophora). However, the analyses of the percentage of nucleotide differences show that the Class II genes evolve more rapidly than their paralogs: within the Class I genes, intra-genus (~2%), inter-genus but belonging to the same order (3-5%), inter-genus belonging to different orders (6-8%); within the Class II genes: inter-genus but belonging to the same order (4-7%), inter-genus belonging to different orders (10-11%). On the other hand, the percentage of difference between Class I and II genes belonging to the same species is 8-9%. When comparisons were made with sequences for which the length is approximately 1000 nt, the percentages of difference were a little weaker because most of the differences are in the first 700 nt. For example, the percentage of difference between *S. cephaloptera* Class I and II genes is ~8%. Together with our phylogenetical investigations using indications of secondary structure elements which showed the longest branches for Class II paralogs versus Class I (data not shown), this data showed that Class II 18S genes evolved more rapidly than Class I genes. Moreover, comparisons of the predicted secondary structures of 18S chaetognatha sequences hitherto published (although for approximately one third of them only 1000 nt have been sequenced) suggest they are globally similar, and sequence comparisons of the two classes issuing from the same species show that substitutions and nucleotide indels (insertion and/or deletions) are almost always found in hyper variable regions, i.e. regions in which the most interspecies divergence occurs. This is particularly highlighted for the two species (*Krohnitta pacifica* and *S. lyra*) for which almost complete Class I and II gene sequences have been published (data not shown).

To explore further the functionality of the Class II genes, a secondary structure model was used to localize in the sequences some positions previously defined as functionally important for small-subunit rRNA in prokaryotes [22]. These regions have also been compared to those of some members of the platyhelminthes Dugesiidae, where two 18S classes have been found, and which is, to date, the only Metazoan taxon for which the expression of the two rRNA classes has been evidenced [13]. This allowed us to identify unambiguously in the chaetognath 18S rRNA sequences 6 positions that possibly act as tRNA or

Tab. 1. The alignments of the six 18S rRNA regions that contain conserved tRNA or mRNA contact sites.

Nucleotide positions			554-568	962-968	1269-1276	1660-1669	1724-1734	1896-1889
18S of Dugesiidae[a]	Class I		CAGCAGCCGCGGTAA	GTGAAA	ACTCAACA	TGAACGAGGA	ACACACCGCCC	TGAA
	Class II		.....	.....	.....	.....	.....	....
18S of Chaetognatha	Class I[b]	Consensus	.....	.....	.....	.....	.....	....[c]
		Variants	.....T.....[d]	.....-[d]		.....	.....	
	Class II[e]	Consensus	.....[f]	.....	.....	.....	.....	....[g]
		Variants	.....A.A....[h]		----....[i]	.....T...[i]	.....A.....[j]	
							....G.....[k]	
16S of *E. coli*			.....	.....	.TG...CG	.....TC...	.....	G...

Each nucleotide fragment corresponds to a single-stranded region in the secondary structure of the 18S rRNA; for the chaetognath sequences, we aligned the sequences using the indications of secondary structure elements of the chaetognath *Paraspadella gotoi* sequence (D14362), http://www.psb.ugent.be/rRNA/ssu/data/PargotD1.EAN. The positions suggested in *E. coli* to be possible tRNA and/or mRNA contacts are underlined [22]. [(a)]18S of five species of the family Dugesiidae (Platyhelminthes) [13]. The corresponding Class I sequence are used as a reference; nucleotides differing from these sequences are indicated, with dots indicating identity and dashes (-) deletions. The numbers correspond to the positions in the 18S Class I sequence of *S. cephaloptera* (DQ351884). References for the chaetognath 18S sequences are in [17]. List of the species: [(b)]18S Class I sequences, *Aidanosagitta neglecta*, *Eukrohnia bathypelagica*, *Eukrohnia hamata*, *Flaccisagitta enflata*, *Krohnitta pacifica*, *Mesosagitta decipiens*, *Parasagitta megalophthalma*, *Paraspadella gotoi*, *Sagitta lyra*, *Pterosagitta draco*, *Sagitta elegans*, *Sagitta bipunctata*, *Sagitta crassa*, *Serratosagitta tasmanica*, *Spadella cephaloptera*, *Spadella ledoyeri* and *Xenokrohnia sorbei*; in [(c)] on this list, species which names have been underlined do not contain the sequence. In addition, a species which does not contain consensus sequences is indicated independently [(d)] *Serratosagitta tasmanica*; [(e)] 18S Class II sequences, *Eukrohnia bathypelagica*, *Eukrohnia fowleri*, *Krohnitta pacifica*, *Mesosagitta decipiens*, *Parasagitta megalophthalma*, *Parasagitta setosa*, *Pseudosagitta lyra*, *Pterosagitta draco*, *Sagitta bipunctata*, *Spadella cephaloptera*, *Spadella ledoyeri* and *Xenokrohnia sorbei*, except in [(f and g)] which, due to shorter sequences, contain respectively (*Krohnitta pacifica*, *Pseudosagitta lyra* and *Sagitta bipunctata*) and (*Eukrohnia fowleri* and *Krohnitta pacifica*). Moreover, the species which do not contain consensus sequences are indicated independently [(h)] *Eukrohnia fowleri*, [(i)] *Krohnitta pacifica*, [(j)] *Eukrohnia bathypelagica* and [(k)] *Xenokrohnia sorbei.*

mRNA contacts; moreover, most of the Class I and Class II chaetognath sequences are similar to the planarian sequences (Tab. 1). These regions have also been found in the 18S sequences of animals for which two or more classes of genes have been found and expression shown; this result is particularly interesting because the level of nucleotide conservation between chaetognath 18S and other animals bearing two classes of these genes is relatively low, between 61 to 78% (data not shown). In chaetognaths, the number of variants

versus consensus sequences is very low. Tab. 1 shows that most of the variants have been found in Class II sequences. As it is more difficult to amplify and to obtain a large quantity of Class II genes than Class I genes, PCR and/or sequencing errors could not be excluded in the case of Class II. However, several cases of low intraindividual variations have been reported in numerous taxa, including human, and the polymorphisms which have been detected are not functionally constrained and seem to exist beneath the level of selection [references in 9]. This suggests that the low intra-phylum variations observed may only reflect species differences. As shown for 28S chaetognath genes [18], this could also reflect different rates of molecular evolution between the two classes, with the 18S Class II sequences evolving more rapidly than Class I paralogs. The *in situ* hybridization results, together with the fact that most of the functionally important positions for small-subunit rRNA in prokaryotes have been conserved, indicate that the Class II genes are probably functional.

Our results show that in chaetognaths, one class of 18S genes is expressed in the whole animal with a strong signal in the gut; this is likely to correspond to housekeeping genes (genes involved in basic functions needed for the sustenance of the cell and which are constitutively expressed), whereas the Class II gene is expressed in lower amounts in the oocytes specifically. Interestingly, in chaetognaths, the gut and oocytes are the cell types which require the greatest translational activities. Intestinal cells, either secretory or absorptive, are characterized by an abundant rough endoplasmic reticulum (RER) forming long parallel cisternae covered with many ribosomes. No other somatic tissue of the body (mainly constituted by muscles) exhibit cells with such a high quantity of RER [23]. Furthermore, according to Ghirardelli [24], the greatest amount of ribonucleoproteins generally occurs in the cytoplasm of smaller oocytes always situated near the oviductal complex running along the outer side of the ovaries; this may support the idea that the oviductal complex may provide oocytes with metabolites issued from the intestine in view to support oogenesis. It is known that during oogenesis there is an increase in the levels of materials containing RNA in the cytoplasm. Later, during vitellogenesis, the RNA is less abundant. Moreover, in chaetognaths [24], as in other animal taxa [25], the induction of maturation in oocytes results in an increase in the rate of protein synthesis. It is well known that chaetognaths lay a cluster of eggs every day for several days in succession [24]. The 18S Class II rRNA could allow the translation of specific mRNA or maintain a high and continuous level of translation.

Interestingly, in *Plasmodium*, the two 18S variants each have a specific role at different stages of the life cycle [26] and in the platyhelminthes Dugesiidae, analysis of the secondary structure has suggested that both 18S Classes could be functional, but only very low levels of transcription of the Class II genes has been detected by RT-PCR [13]. The authors have hypothesized that Class II genes were expressed in very low amounts in some minority tissues or cell types in intact non-regenerating organisms. Moreover, in the amphibian *Xenopus laevis* and the teleostean *Misgurnus fossilis,* it has been reported that there are

two classes of 5S rRNA transcripts that are specific to either somatic or oocyte ribosomes [27]. Our results suggest that cell-specific genes (Class II) could evolve faster than their ubiquitously expressed paralogs (Class I). Recent studies on protein gene families have evidenced that shorter branch lengths are correlated with ubiquitous tissue expression [28], and that such expression tends to evolve rapidly for genes that are expressed in only a limited number of tissues, whereas tissue expression can be conserved for a long time for genes expressed in a large number of tissues [29]. This is in agreement with our hypothesis.
The functionality of both paralog genes will be further investigated by determining if the two classes of chaetognath 18S RNAs are structural components of cytosolic ribosomes. Moreover, a putative differential transcription of the paralogs during the various stages of chaetognath development will be also investigated. Comparisons between animals which encode several classes of rRNA genes could also be fruitful. To conclude, an intriguing question remains open: how can regulatory mechanisms generate the expression of one variant and not the other?

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