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SELECTIVE EXPRESSION OF TWO TYPES OF 28S RRNA PARALOGOUS GENES IN THE CHAETOGNATH *SPADELLA CEPHALOPTERA*

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Abstract – Significant intra-individual variation in the sequences of the ribosomal RNA (rRNA) genes is highly unusual in animal genomes; however, two classes of both 18S and 28S rRNA gene sequences have been detected in chaetognaths, a small phylum of marine invertebrates. One species, *Spadella cephaloptera* Busch, 1851, is well-suited to the methods of *in situ* analysis of gene expression, since it is totally transparent. To test our hypothesis of a possible functional division of the two classes of genes, we carried out *in situ* hybridization. Our results indicated that 28S class II genes are expressed intensively in the oocytes of chaetognaths. In contrast, hybridization using an heterologous probe of 28S class I genes revealed only a single and relatively weak signal in a distinct area of intestinal cells. Our results suggest that the *S. cephaloptera* genome contains at least three different types of rRNA 28S genes; however, those which are expressed during housekeeping conditions could not be detected in our experiments.

Key words: 28S, chaetognath, *Spadella cephaloptera*, *in situ* hybridization, rRNA paralogs

INTRODUCTION

Ribosomes are the sites of protein synthesis in all living creatures. These organelles basically consist of two subunits (large and small) composed of ribosomal RNA (rRNA) and proteins, whose size (molecular weight) varies between taxa. In eukaryotes, a small subunit contains a 16-18S rRNA molecule (small-subunit rRNA); a large subunit contains a 5S, 5.8S, and a 25-28S rRNA molecule (large-subunit rRNA). The genes encoding 5.8S, 18S, and 28S rRNA are generally found together in one or more tandem arrays of a repeat unit (rDNA) that consists of the three coding regions separated by intergenic spacers (ITS). The copy number of rDNA repeats can vary from as few as one copy per haploid genome in *Tetrahymena* (Alveolata) to hundreds or thousands of copies in several vertebrates, and it is positively correlated with genome size (1). A high degree of sequence homogeneity is observed among rDNA copies within individuals and throughout entire species despite the divergence of these sequences between species. This pattern of sequence homogeneity, which was first described in the rDNA of *Xenopus* (2), is known as concerted evolution and is thought to be the result of molecular mechanisms such as unequal crossing over, gene conversion, and gene amplification, which have collectively been termed molecular drive (3). Even so, variation among rDNA copies within species and within individuals has been observed, with respect to both nucleotide substitutions and length heterogeneity, the latter often being the result of variable numbers of subrepeats in the intergenic spacers. However, intra-individual variations of the 18S and/or 28S rRNA genes have been observed in some animals, such as apicomplexans (3), *Acanthamoeba* (4), the euglenozoan *Trypanosoma cruzi* (5), platyhelminthes of the family Dugesiidae (6), chaetognaths (7,8), cephalopods (9) and sturgeon (10,11). The number of paralog types varies according to the taxa, but usually only two or three sequence variants are detected; in addition, generally all the variants can be grouped into two great classes, even if the number is very high, as has been found in sturgeon (10).

For studying rRNA paralogs, chaetognaths are a particularly interesting model. They are

among the most abundant marine planktonic organisms (12), and are subdivided in two orders, Phragmophora and Aphragmophora, totalling approximately 120 nominal species. In the last decade, their relationships within the metazoans have been strongly debated because of embryological and morphological features shared with the two main branches of Bilateria, the deuterostomes and the protostomes (13). Recent use of the mitochondrial genome, a powerful phylogenetic marker, indicates close relationships with the protostomes (14,15,16). Casanova et al. (17) have shown that chaetognaths can be effectively used as a model animal, a view based on about 30 years of research on the phylum. One of the benthic species, *Spadella cephaloptera* Busch, 1851, is well-adapted to the methods of *in situ* analysis of immunodetection or gene expression (18, 19), because the eggs, embryos and bodies are totally transparent; in addition, species of the family Spadellidae are easy to collect and to breed in the laboratory (20). Moreover, analyses of chaetognath rRNA genes have shown that both 18S and 28S sequences can be allocated to two different classes (named I and II) (7,8), which are correlated with the absence or a very low rate of gene conversion (21). The broad distribution of this phenomenon within the phylum, along with phylogenetic analyses, have led to the hypothesis that these molecular patterns are the result of duplication of an ancestral ribosomal gene cluster. It has been suggested that the two classes of chaetognath 18S and 28S rRNA are expressed and functional (7,8); however, this was not verified experimentally. Similarly, in other taxa, few data, using principally RT-PCR or Northern-blots, have demonstrated the expression of one (6) or two of the paralogs (10). According to these last authors, these results suggest that some of the variants are unimportant for proper cellular function or are pseudogenes. Here we use *in situ* hybridization to visualize the expression patterns of two classes of 28S genes in a chaetognath.

MATERIALS AND METHODS

Experimental design

Specimens of *S. cephaloptera* were collected during spring and summer 2006 in a marine meadow east of Marseille (Brusc Lagoon, France) where they lived on *Posidonia oceanica* at a depth of 0.5–5 m. In the laboratory, samples were kept in aquaria containing natural sea water and placed at a constant temperature of 21±1°C under a natural light cycle. The specimens used for experiments were at the same stage of sexual maturity (mean total length ~4 mm) with well-developed ovaries and were fed daily with *Artemia salina* nauplii. After a week period of acclimation, a randomized set of mature specimens without gut contents was captured, embedded in tissue-Tek O.C.T. (Optimal Cutting Temperature) and sectioned at 12 µm using a cryostat. Ventral sections were mounted on to coated slides.

In situ *hybridization*

Two 28S probes complementary to a specific region of the D2 domain for each class were used. The 28S class II probe was obtained from a *S. cephaloptera* sequence (Z77129, from nt194 to nt156), 5-CCTACTACCGCCCTCACGGTTTTAGACGCAGCCTA AACC-3. This region exhibits more than 76.9% identity with respectively the consensus sequence issuing from other Phragmophora sequences (all from *Eukrohnia fowleri*) and 65.3% identity with Aphragmophora sequences (7). Concerning the 28S class I probe, no *S. cephaloptera* sequence has been published to date; however, as the analyses of Telford and Holland (7) strongly suggested that the two 28S paralogs arose by a cluster duplication in a common ancestor of extant chaetognaths, a region of another Phragmophora was chosen (*E. fowleri*, Z77103,from nt394 to nt360), 5-CACACATTTCATGGCTCCAGCCCAGTCGGACCGAC-3. This region exhibits more than 88.2% and 71.3% of identity with respectively the consensus sequence issuing from the other Phragmophora sequences and all the Aphragmophora sequences (7). A 45-mer scrambled oligonucleotide was used as a negative control. *In situ* hybridization was performed by the methodology of Grino and Zamora (22) adapted to oligonucleotide probes previously 3'-end labelled by incubation with [^{35}S]deoxy-ATP and terminal deoxynucleotidyl transferase. Slides with the ventral sections of *S. cephaloptera* 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 0.15M NaCl, 0.015M 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 ^{35}S-labelled oligoprobe under a glass coverslip. After 20 h of incubation in moist sealed chamber at 37°C, coverslips were removed in 1xSSC, held 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) and subsequently dipped in nuclear emulsion (1:1 in water, K5; Ilford, Saint-Priest, France). After development, sections were counterstained with nuclear fast red.

RESULTS

The spatial patterns of 28S paralog expression have been examined by *in situ* hybridization on ventral sections (Fig. 1). 28S class II genes are strongly and specifically expressed in the oocytes (Fig.1C) whereas use of a heterologous probe suggests that the 28S class I

genes are weakly expressed; indeed, the time needed for visualization of the hybridization signal in the X-ray film was 4 h for the 28S class II versus 6 days with the class I probe. Contrary to expectations, the 28S class I gene products are located in a restricted area which is at the end part of the gut (Fig. 1B). This area corresponds to the central of the three distinct areas of intestinal cells, which still contain dark granules, remaining from digestion, 24 h after feeding (Fig 1A). These areas are always present at approximately the same location in all specimens, and the aspect of cells along the intestine suggests that they correspond to mitotic sites where renewal of intestinal cells takes place (23,24). However, as *in situ* hybridization with the negative control shows no signal pattern even after the same time of exposure (data not shown), that indicates that all the 28S signals observed are specific.

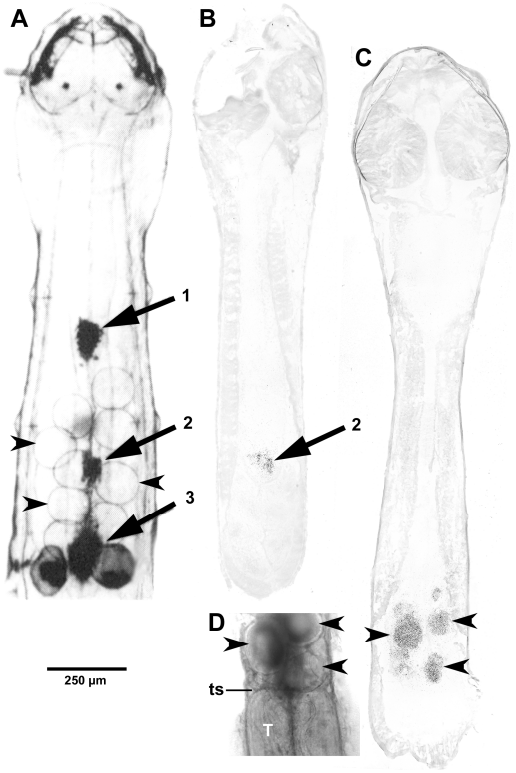

Figure 1. Photomicrographs of four different specimens of *S. cephaloptera.* (A) and (D): living specimen showing the three mitotic regions of intestinal cells (arrows with numbers 1 to 3) and oocytes (arrowheads). (B) and (C): spatial expression of 28S rRNA paralogs after *in situ* hybridization using an anti-sense 28S class I and an anti-sense 28S class II. Note in (B), expression at the level of the second mitotic region of the intestine (arrow), and in (C) at the level of oocytes. T = tail, ts =transverse septum. Fig. A is modified from (24).

DISCUSSION

In this study, using for the first time *in situ* hybridization to reveal the spatial patterns of rDNA paralog expression, we have found that 28S paralog genes have differential expressions. One of the classes of genes (class II) is expressed specifically in oocytes. Owing to the pattern observed for this class of 28S genes, we expected to observe an intense hybridization signal using the class I probe. Indeed, the translational mechanism needs a high transcription level of each type of rRNA gene (5S, 5.8S, 18S and 28S), and we hypothesized that one of the paralogs is intensely expressed in all the parts of the animal during housekeeping conditions. Unfortunately, use of a heterologous probe of the 28S class I genes detected only a weak, though specific, signal. Although the percent identity of this probe with homologous sequences of both Phragmophora and Aphragmophora species is relatively high suggesting that the corresponding genes could display ubiquitous transcription, our results suggest that the products detected with this probe play only a minor role in chaetognaths. Interestingly, in the family Dugesiidae (platyhelminthes), analyses of the secondary structure have suggested that both 18S classes could be functional; however, only type I transcripts have been detected by Northern blot analysis, whereas the type II genes were shown to be transcribed, though at very low levels, only by RT-PCR (6). These last authors have hypothesized that one type of genes was expressed in very low amounts in a minority of tissues or cell types in organisms. In our experiments in chaetognaths, the expression of one of class of paralog is restricted to the oocytes whereas the other is only expressed in a restricted area at the end of the gut. These two paralog genes are expressed in these cell types in which increased rates of protein synthesis are especially important (23,24). Moreover, the low percent identity between the two probes is ~44% added to the relatively high stringency hybridization conditions suggest strongly that the two hybridization patterns are specific, *i.e.*, the pattern observed in the figure 1B could not be a weak version of the pattern in the figure 1C. As expression of a type of 28S paralog genes in all the parts of the organism during housekeeping conditions is essential for the survival of the animal, our study suggests that at least three types of 28S genes in two classes are expressed in the chaetognath *S. cephaloptera.* This should

not be surprising since four 28S class II variants have been observed in *E. fowleri*, and two 28S class I variants have been found in three other species (a Phragmophora, *Eukrohnia hamata*, and two Aphragmophora, *Sagitta macrocephala* and *Sagitta setosa)*. The percent identity within the variants for each species varies from 79.1 (89.0 % if not including insertions-deletions) to 94.7% for *E. fowleri* and from 97.9 to 98.4 % within the 28S class I variants of the three other species. In addition, in an individual of the lake sturgeon *Acipenser fulvescens*, at least 17 different 18S sequence variants have been identified although RT-PCR experiments indicated that only one of them was expressed in major quantities (10). Probably, other 28S variant genes could exist in the *S. cephaloptera* genome, since, in their experiments, Telford and Holland (7) have isolated class I clones from 15 divergent species (41 in 55 clones), and class II clones from five divergent species (14 in 55 clones). However, in only three species, both classes were readily found. This suggests two interesting conclusions. First, it is not easy to amplify the two classes from an individual; unfortunately, it is the case for *S. cephaloptera*. Second, as the clones corresponding to class I are most numerous, this could suggest that 28S class I genes or some of them encode the majority of housekeeping 28S products. On the other hand, in many species of the protist phylum Apicomplexa, rRNA gene copies are structurally and functionally heterogeneous, owing to distinct requirements for rRNA-expression patterns at different developmental stages (25). Similarly, our results show that the 28S class II gene products play a role in ovogenesis. Interestingly, it had been seen that in *Xenopus laevis* and in the teleostean *Misgurnus fossilis* there are two multigene families of 5S RNA genes: the oocyte-type 5S RNA genes which are expressed only in oocytes and the somatic-type 5S RNA genes which are expressed throughout development (26).

In the future, using RT-PCR, all the 28S rRNA variants of *S. cephaloptera* will be sequenced. In addition, the spatial expression of all these genes and the roles of each of their products will be investigated.

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