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DNA-methylation dependent regulation of embryo-specific 5S ribosomal DNA cluster transcription in adult tissues of sea urchin *Paracentrotus lividus*

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ABSTRACT

We have previously reported a molecular and cytogenetic characterization of three different 5S rDNA clusters in the sea urchin *Paracentrotus lividus* and recently, demonstrated the presence of high heterogeneity in functional 5S rRNA. In this paper, we show some important distinctive data on 5S rRNA transcription for this organism. Using single strand conformation polymorphism (SSCP) analysis, we demonstrate the existence of two classes of 5S rRNA, one which is embryo-specific and encoded by the smallest (700 bp) cluster and the other which is expressed at every stage and encoded by longer clusters (900 and 950 bp). We also demonstrate that the embryo-specific class of 5S rRNA is expressed in oocytes and embryonic stages and is silenced in adult tissue and that this phenomenon appears to be due exclusively to DNA methylation, as indicated by sensitivity to 5-azacytidine, unlike *Xenopus* where this mechanism is necessary but not sufficient to maintain the silenced status.

1. Introduction

5S ribosomal RNA (rRNA) is a component of the large ribosomal subunit in all ribosomes. Its genes have been studied extensively in animals and plants, especially in relation to species or population characterization [1,2], evolutionary relationships [3–9] genome structuring [10–18], and functional analysis of their transcripts [19,20].

Genomic 5S ribosomal DNA (rDNA) is in the majority of living organisms, from Archaea to higher Eukaryotes. Though very distant from ancestral 5S rDNA clusters, they have maintained the tandem-repeat organization, displaying repetitions in a head-to-tail arrangement [10–12]. These 5S rDNA clusters may be localized on one or several chromosomal *loci* and are generally separated from genes which encode the "large" rRNAs (28S, 18S and 5.8S). *Taxon* analysis of 5S rDNA repeat units reveals a highly variable non-transcribed spacer (NTS), both in length and sequence. The 5S rRNA-transcribed region, in contrast, shows a high degree of homology between organisms belonging to unrelated *taxa* (for example, over 80% in sea urchins and humans) [19,20].

Also remarkable are the conserved secondary structures formed by these molecules in different *taxa*, which are always referable to RNA with five stems and five loops. During evolution, mutations are conserved only if the secondary structure and the functional nucleotides are maintained [19]. The coexistence of more than one 5S rDNA cluster in the same genome, varying in NTS length and sequence (and not only), is extremely interesting and can be seen as evidence of the dynamism of these genes [21].

Although 5S genes are among the most extensively studied genes, little data is available on sea urchins, on which only studies of the structure and/or localization of these genes are conducted. [22–25]. In the sea urchin *Paracentrotus lividus*, the copy number of 5S rDNA is 120–130 units for the haploid genome [26]. In our laboratory, we have demonstrated the existence of three clusters of repeat units in this organism that encodes for 5S rRNA, differing in length of NTS. These units present a 121 bp transcribed region and they are around 700 bp, 900 bp and 950 bp with NTS (their nucleotide sequences were registered in the EMBL-Bank database with accession numbers AJ417697, AJ417698, and AJ417699 respectively) and have been molecularly and cytogenetically characterized. The longest repeats (900 bp and 950 bp) are characterized by similar NTS where the 950 bp unit spacer displays additional CT di-nucleotide repeats, whereas the shorter repeat (700 bp) exhibits a spacer sequence which diverges completely from the longer repeats [24].

Abbreviations: rRNA, ribosomal RNA; rDNA, ribosomal DNA; NTS, non-transcribed spacer; SSCP, single-strand conformation polymorphism; 5-AZA, 5-azacytidine; VGGE, voltage gradient gel electrophoresis; bp, base pair; CCM, cell culture medium; MCF, millipore-filtered coelomic fluid.

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In a recent paper, we have also demonstrated the existence of five 5S rRNA variants of 121 nucleotides (nt), two major transcripts (700 bp 5S RNA and 900/950 bp 5S rRNA) and three minor transcripts (Minor Transcript 1, Minor Transcript 2 and Minor Transcript 3) (EMBL-Bank accession numbers FM242579.1, FM242580.1, FM242581.1, FM242582.1 and FM242583.1) and the first example of high heterogeneity in the animal kingdom. We have also associated each individual variant with its related cluster, demonstrating that the "900/950 bp 5S rRNA" sequence is the sole variant found in the larger two clusters, while the "700 bp 5S rRNA" and all minor variants are exclusively associated with the 700 bp cluster through SSCP analyses [25].

In this paper, using SSCP analysis, we report the identification of two classes of 5S rRNA, encoded by these three clusters with a different stage-specificity, in the sea urchin *P. lividus*.

We also show that the expression of the 700 bp cluster is associated with the methylation status of these genes in non-canonical CpG islands in adult tissue.

2. Results

To discriminate between the different 5S rRNA forms of sea urchin *P. lividus*, we used the SSCP protocol described in Materials and methods which proved ideal for this analysis, as demonstrated in a recent published paper [25]. SSCP is a technique that permits the detection of point mutations detecting differences in electrophoretic mobility of short (200 nucleotides or less) mutated single strand molecules due to dissimilar secondary conformations.

Eluted RT-PCR products of 5S rRNA obtained from RNA extracted from isolated ribosomes of oocytes, eggs, post-hatching blastula, *pluteus*, muscle of Aristotle's lantern, intestine and coelomocytes were analyzed by means of the SSCP protocol, using genomic 5S rDNA clones (2 different genomic clones per cluster) as controls. The results of this experiment are shown in Fig. 1.

This analysis indicates that the 700 bp cluster is expressed exclusively in oocytes and during early development, while the other two clusters (900 bp and 950 bp clusters) are expressed in every stage. Indeed, bands corresponding to different 5S rRNAs in the 700 bp cluster are present exclusively in oocytes and the developmental stage, while they are absent in adult tissue (muscle of Aristotle's lantern, intestine and coelomocytes) where only the 900/950 bp cluster variant is present.

To test whether the silencing of the 700 bp cluster was dependent on methylation status, we conducted an analysis of the methylation status of rDNA in oocytes, advanced embryo stages and adult tissues. No differences were observed in the methylation status of the 900 bp and 950 bp rDNA clusters in oocytes and advanced embryo stages, while adult tissue was always hypomethylated (less than 5% of methylation in a small number of positions, see Table 1). In contrast, our analysis of the 700 bp cluster showed a high methylation state exclusively in adult tissue. In the DNA of adult tissue, we found a hypermethylation status in the transcribed sequence and spacer region; indeed, the cytosines were methylated in specific positions in 95% of cases. In the same positions we found only 5% of methylation in DNA extracted from oocytes and embryos (see Table 1 and Fig. 2). The silenced status of the 700 bp cluster seems to be correlated to methylation status.

To test whether the silenced status is really correlated with methylation status, we carried out different coelomocyte vital suspensions treated with different concentrations of 5-azacytidine (5-AZA) (1 μM, 2 μM, 5 μM, 10 μM, 50 μM and 100 μM) for 24 h. 5-AZA is a methylation-decreasing molecule which causes inhibition of DNA methyltransferase activity, causing hypomethylation of DNA. The highest concentrations of 5-AZA (50 μM and 100 μM) were found to be lethal for the cells. The analysis of methylation status of 700 bp cluster of coelomocyte DNA treated with 5-AZA (10 μM), confirms the full demethylations (Table 2).

The results of the SSCP analysis carried out on eluted RT-PCR products of 5S rRNA obtained from the RNA of ribosomes of coelomocytes treated with different non-lethal concentrations of 5-AZA (1 μM, 2 μM, 5 μM and 10 μM) are shown in Fig. 3. This SSCP analysis shows that at the 2 μM concentration of 5-AZA the major transcript variant of the 700 bp cluster is synthesized, and that at the 10 μM concentration the expression of all 700 bp cluster variants is reactivated.

To confirm the effective presence of 700 bp cluster variants, we carried out the sequencing of cloned RT-PCR of 5S rRNA isolated from untreated coelomocytes and coelomocytes treated with 5-AZA (10 μM). The result is showed in Table 3.

3. Discussion

In a previous paper, we showed the characterization of three 5S rDNA clusters (700 bp, 900 bp and 950 bp respectively) in the sea urchin *P. lividus* and demonstrated that these clusters were mapped in different chromosomal *loci* [24]. The existence of a cluster that exhibited

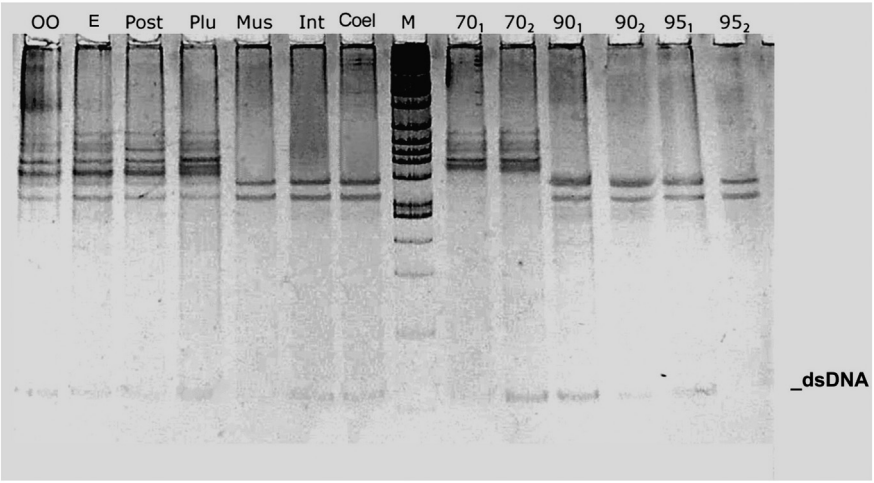

Fig. 1. SSCP analysis of RT-PCR and cluster-specific 5S rDNA PCR. We have analyzed RT-PCR performed on RNA extracted from oocytes (OO), eggs (E), post-hatching blastula (Post), *pluteus* (Plu), muscles of Aristotle's lantern (Mus), intestine (Int) and coelomocytes (Coel). Controls 5S rDNA of 700 bp cluster, obtained by two different "700 bp cluster genomic clones", are indicated as 70_1 and 70_2. Controls 5S rDNA of 900 bp cluster, obtained by two different "900 bp cluster genomic clones", are indicated as 90_1 and 90_2. Controls 5S rDNA of 950 bp cluster, obtained by two different "950 bp cluster genomic clones", are indicated as 95_1 and 95_2. dsDNA indicates the double strand form of re-annealed 5S rDNA. "GeneRuler™ DNA Ladder Mix" as molecular mass marker, is indicated with M.

t1.1 **Table 1**
t1.2 Percentage of methylation observed in the 700 bp, 900 bp and 950 bp clusters (*, CpG site; #, CHG site; §, CHH site). In brackets are showed the number of found clones with specific
t1.3 methylated positions. The positions of the 5S rRNA transcribed regions are shown in gray.

Position in 700 bp cluster		20 #	68 §	70 *	83 §	95 §	118 §	134 §	187 *
DNA methylation rates (%)	Oocytes and embryos (47 clones)	0	2.1 (1)	0	2.1 (1)	4.2 (2)	0	0	0
	Adult tissue (51 clones)	88.2 (45)	96 (49)	86.2 (44)	100 (51)	100 (51)	86.2 (44)	88.2 (45)	88.2 (45)

Position in 700 bp cluster		212 *	235 *	255 §	266 *	292 §	297 #	317 §	338 #
DNA methylation rates (%)	Oocytes and embryos (47 clones)	0	0	2.1 (1)	0	0	0	0	0
	Adult tissue (51 clones)	86.2 (44)	86.2 (44)	96 (49)	86.2 (44)	88.2 (45)	86.2 (44)	86.2 (44)	88.2 (45)

Position in 700 bp cluster		358 *	371 #	385 §	403 §	416 *	429 #	452 §	467 §
DNA methylation rates (%)	Oocytes and embryos (47 clones)	0	0	4.2 (2)	0	0	0	0	4.2 (2)
	Adult tissue (51 clones)	86.2 (44)	86.2 (44)	98 (50)	90.2 (46)	86.2 (44)	88.2 (45)	86.2 (44)	98 (50)

Position in 700 bp cluster		504 §	524 §	546 §	559 *	619 §	627 *	687 §	–
DNA methylation rates (%)	Oocytes and embryos (47 clones)	0	0	0	0	0	0	0	
	Adult tissue (51 clones)	90.2 (46)	92.1 (47)	90.2 (46)	86.2 (44)	88.2 (45)	88.2 (45)	96 (49)	

Position in 900 bp cluster		67 §	69 *	133 *	333 *	617 *	847 *	–	–
DNA methylation rates (%)	Oocytes and embryos (50 clones)	2 (1)	4 (2)	0	2 (1)	2 (1)	0		
	Adult tissue (48 clones)	2.08 (1)	4.16 (2)	2.08 (1)	2.08 (1)	4.16 (2)	2.08 (1)		

Position in 950 bp cluster		67 §	69 *	138 *	383 *	676 *	905 *	920 *	–
DNA methylation rates (%)	Oocytes and embryos (46 clones)	2.17 (1)	4.35 (2)	0	4.35 (2)	2.17 (1)	2.17 (1)	0	
	Adult tissue (49 clones)	2.04 (1)	6.12 (3)	2.04 (1)	2.04 (1)	4.08 (2)	2.04 (1)	2.04 (1)	

differences either in the spacer or in the transcribed region might have led to the conclusion that this cluster was a pseudo-gene, as has been demonstrated in several organisms [27–30]. More interestingly, an alternative way to interpret these data is to hypothesize that the cluster synthesizes its product in a specific developmental stage only, in a similar manner to *Xenopus* [31]. Indeed, in a recent paper describing a study of the sea urchin *P. lividus*, we demonstrated not only that all clusters are transcribed, but also that the 700 bp cluster exhibited a high level of heterogeneity in terms of sequence [25]. We also demonstrated that a regulation exists during the developmental stage and in adult tissue, as reported in *Xenopus laevis* [31–34]. In this organism, in particular, the somatic 5S rRNA variant (encoded by somatic 5S rDNA gene family with 400 copies of these genes) is present only in the early developmental stages through the adult tissue stage, but is not detectable during oogenesis (although its genes are able to be transcribed), while the oocytes variant (encoded by the oocyte gene family with 20,000 copies of 5S rRNA genes) is present only in oocytes. Moreover, its expression is silenced during the early developmental stage, where the oocyte variants are substituted by somatic–specific ones.

In contrast, in the sea urchin *P. lividus* we found an "embryo-specific" cluster (the 700 bp rDNA cluster). Indeed, we observed "700 bp 5S rRNA" variants (major and minor transcripts) only in oocytes, and in all early

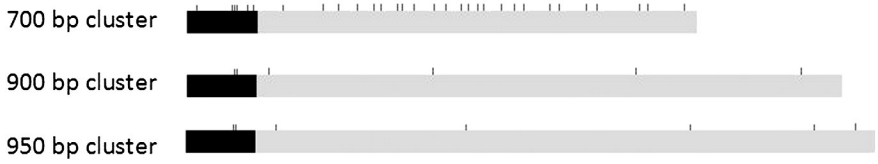

Fig. 2. Schematic drawing of methylation positions of 5S rDNA clusters. The transcribed region of 5S rDNA is indicated in black, the non-transcribed spacer (NTS) is indicated in gray.

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t2.1 **Table 2**
t2.2 Percentage of 700 bp DNA methylation observed in coelomocytes treated with 10 μM 5-azacytidine and referred to the same positions analyzed in untreated ones (*, CpG site; #, CHG site;
t2.3 §, CHH site). In brackets are showed the number of found clones with specific methylated positions. The positions of the 5S rRNA transcribed regions are shown in gray.

Position in 700 bp cluster	20 #	68 §	70 *	83 §	95 §	118 §	134 §	187 *
DNA methylation rates (%) of treated coelomocytes (21clones)	0	0	4.7 (1)	4.7 (1)	9.5 (2)	0	0	0

Position in 700 bp cluster	212 *	235 *	255 §	266 *	292 §	297 #	317 §	338 #
DNA methylation rates (%) of treated coelomocytes (21clones)	0	0	0	4.7 (1)	0	0	0	0

Position in 700 bp cluster	358 *	371 #	385 §	403 §	416 *	429 #	452 §	467 §
DNA methylation rates (%) of treated coelomocytes (21clones)	0	0	0	0	4.7 (1)	0	0	9.5 (2)

Position in 700 bp cluster	504 §	524 §	546 §	559 *	619 §	627 *	687 §	–
DNA methylation rates (%) of treated coelomocytes (21clones)	0	0	0	4.7 (1)	0	4.7 (1)	0	

stages (through at least the *pluteus* stage). In contrast, the "900/950 bp 5S rRNA" variant transcribed by the larger rDNA clusters was present in all stages, including adult tissues, where it is the only existing form. For this reason we termed the 900 bp and 950 bp clusters "constitutive" because they were observed to be always expressed in every stage. Moreover, in our previous papers we also showed that there is an asymmetrical transcription activity for the three 5S rRNA clusters [25] and in particular increased transcription for the 700 bp cluster (65%) compared with the other two (17.5% for each cluster).

With the data reported, we may now state that DNA methylation is a cluster activity regulation mechanism; this is observed in particular in the 700 bp cluster. Our analysis of DNA methylation status for all clusters indicated that the silencing of the "embryo-specific" (700 bp) cluster in adult tissues was induced by DNA hypermethylation. In keeping with this finding, the 900 bp and 950 bp rDNA clusters, expressed in every stage, were likewise observed to be consistently hypomethylated.

We believe that this epigenetic regulation takes place in the sea urchin because the 700 bp cluster must be more active than the other two; its activity, in oogenesis and the early stages of sea urchin development, is driven by the need for a high amount of 5S rRNA in order for ribosome synthesis to take place. Indeed, as is well known, a regulation mechanism for ribosome synthesis exists in *P. lividus*. Conversely, large amounts of 5S rRNA synthesis are not required in adult tissues, and the 700 bp cluster is silenced. Since we found that most (approximately 71%) of methylated DNA sequences are non-canonical CpG islands (in particular about 55% is CHH sites, where H are all nucleotides except G, and about 16% is CHG sites, see Table 1 and Fig. 2) and since it is widely known that DNA hypermethylation causes gene silencing and DNA hypomethylation gene expression [35], we performed a vital suspension of coelomocytes treated with 5-azacytidine for 24 h, a known methylation-decreasing molecule, in order to demonstrate that the association between DNA hypomethylation and gene reactivation also applies to these DNA sequences. Under this forced DNA demethylation, confirmed by analysis of methylation status of 700 bp cluster of DNA treated with 10 μM 5-AZA, where is shown an high decrease of methylation in all positions (see Table 2), we obtained a reactivation of transcription for the 700 bp cluster as indicated by the detection of embryo-specific 5S rRNA variants illustrated in Fig. 3. These experiments confirm that also in the sea urchin the hypermethylation status of this particular DNA region, which can be described as a non-canonical CpG island, is related to the silenced status of the cluster and that the methylation-decreasing action of 5-azacytidine was able to reactivate the synthesis of 5S rRNA variants of 700 bp cluster, as demonstrate by the sequencing analysis of RT-PCR performed by ribosome

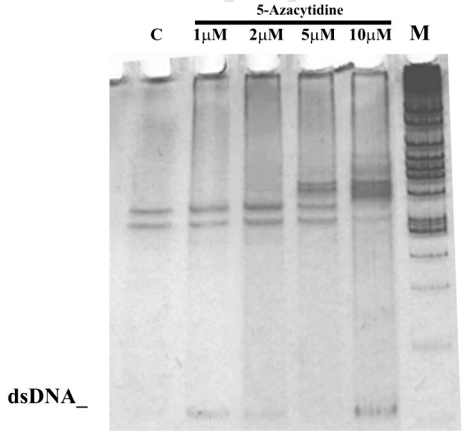

Fig. 3. SSCP analysis of RT-PCR performed on RNA extracted from cultured coelomocytes treated with 5-azacytidine (1 μM, 2 μM, 5 μM and 10 μM) and related control (untreated). dsDNA indicates the double strand form of 5S rDNA re-annealed.

Table 3 t3.1
Percentage of clones of all 5S rRNA sequences found in untreated and treated coelomocytes t3.2
(5-azacytidine 10 μM). In brackets are indicated the number of found clones. MT is the t3.3
acronym used for the three minor transcripts. t3.4

5S rRNA sequences	Found clones per variant (%) (total clones n = 41)		t3.5
	Control (untreated coelomocytes) (n = 16)	Treated coelomocytes (5-AZA 10 μM) (n = 25)	t3.6
900/950 bp 5S rRNA	100 (n = 16)	28 (n = 7)	t3.7
700 bp 5S rRNA	0 (n = 0)	32 (n = 8)	t3.8
5S rRNA MT1	0 (n = 0)	12 (n = 3)	t3.9
5S rRNA MT2	0 (n = 0)	16 (n = 4)	t3.10
5S rRNA MT3	0 (n = 0)	12 (n = 3)	t3.11

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isolated from untreated and 5-AZA-treated ceoleomocytes (see Table 3). Finally, our findings underline the epigenetic similarity of these features as they occur in mammals and consequently the difference from other organisms, in particular in *X. laevis*, where gene silencing seems to be mainly due to selective synthesis of the specific H1 histone variant [32].

4. Materials and methods

4.1. Sea urchin oocyte purification and embryo cultures

Sea urchin oocytes were purified from female gonads using sucrose gradient [36] as described by G. Giudice et al. [37], in order to extract DNA and RNA. After collection, the *P. lividus* female gametes were purified through two subsequent steps of sedimentation of eggs, removal of supernatant, and resuspension in millipore-filtered sea water. Embryos were reared from fertilization at 10,000/ml in a thermostatic chamber at the physiological temperature of 10 °C by stirring with rotating propeller (25 rpm) in artificial sea water (425 mM NaCl, 9 mM $CaCl_2$, 25.5 mM $MgSO_4$, 23 mM $MgCl_2$, and 2 mM $NaHCO_3$) containing 10 mM Tris, pH 8.0, 50 μg/ml streptomycin sulphate (Sigma), 30 μg/ml penicillin G potassium salt (Merck), and 10 μg/ml sulfadiazine (Sigma). Once the embryos had reached the desired developmental stage, we extracted RNA and DNA from them as indicated below.

4.2. Vital suspension of coelomocytes and treatment of cells with 5-azacytidine

Coelomic fluid was collected from different individual organisms through the peristomium, using a 10-ml syringe with a 21-gauge needle. The collected liquid was added to an isosmotic anti-coagulant solution (ISO–EDTA: 20 mM Tris, 0.5 M NaCl, 70 mM EDTA, pH 7.5) to prevent clotting.

The coelomic fluid was centrifuged for 5 min at 1000 ×*g* at RT to collect the coelomocytes.

The resulting cell pellet was resuspended in coelomic fluid which was micro-filtered through a 0.2 μm millipore membrane coelomic fluid (MCF). We progressively replaced MCF by cell culture medium (CCM: NaCl 0.5 M, $MgCl_2$ 5 mM, EGTA 1 mM, HEPES 20 mM, pH 7.2, as indicated in Henson et al. [38]) supplemented with a 10% L-15 medium (Sigma L4386), 5% fetal bovine serum (GIBCO 10270) and 100 units penicillin, and streptomycin 0.1 mg/ml.

5-AZA (1 μM, 2 μM, 5 μM, 10 μM, 50 μM and 100 μM) was added to cell-culture plates and the cells obtained were harvested 24 h later; DNA and RNA were extracted using the procedure described below.

4.3. DNA-methylation analysis

The extracted DNA was treated using a "MethylCode™ Bisulfite Conversion kit" in accordance with the instructions of the manufacturer (Invitrogen™), converting all of the unmethylated cytosines into uracils. In the sequencing analysis of amplified clusters, which was carried out as described previously, the uracils were read as thymines.

4.4. Nucleic acid isolation

Genomic DNA was extracted as described previously from oocytes, different embryonic stages (post-hatching blastula and *pluteus*) and adult tissues (muscle of Aristotle's lantern, intestine and coelomocytes) [39].

Ribosomes from the sea urchin *P. lividus* — oocytes, eggs, different embryonic stages (post-hatching blastula and *pluteus*), adult tissues (muscle of Aristotle's lantern, intestine and coelomocytes) and cultured coelomocytes (treated with 5-azacytidine and untreated) were isolated as previously described [40]. RNA from ribosomes was extracted by acid guanidinium-thiocyanate-phenol–chloroform extraction [41]. The RNA was quantified by the spectrophotometer "Life Science UV/Vis Spectrophotometer DU 730" (Beckman Coulter), and the quantity of RNA was confirmed by electrophoretic analysis.

4.5. cDNA synthesis

500 ng of total RNA (extracted as described previously) was used for retro-transcription of the 5S rRNA transcribed region using an oligonucleotide Cod5S rev (see Table 2) as a primer. Retro-transcription reaction was performed using SuperScript™ II Reverse Transcriptase in accordance with the instructions of the manufacturer (Invitrogen™). The cDNA was precipitated with ethanol at −20 ° C for 20 min, and then pelleted using centrifugation at 12,000 rpm for 15 min [42].

4.6. PCR amplifications

Cluster-specific genomic clones (two different "cluster-specific genomic clones" for each cluster) and cDNA obtained from specific reverse transcriptions of 5S rRNA and bisulfite-treated DNAs were used as templates in polymerase chain reactions (PCR) amplification. Negative controls of RT-PCRs were affected under the same conditions using RNA without reverse transcription reactions. Q7

The transcribed regions were amplified using Cod5S dir and Cod5S rev, while specific repeating units (for methylation analyses) were amplified using the same direct primer (Cod5S dir) and, as the reverse process, a cluster-specific primer (700 bp-rev, 900 bp-rev or 950 bp-rev) (Table 4). Q8

All of the oligonucleotides used are listed in Table 2, and were built on the 5S rRNA gene sequence of the sea urchin *P. lividus* (EMBL-Bank accession numbers AJ417697, AJ417698 and AJ417699). PCR conditions for all amplifications were as follows.

The reaction was initially denatured at 95 °C for 2 min, and followed by 30 cycles of: denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. When required (in the sequencing of clones for methylation analysis), the final extension step at 72 °C was extended to 30 min, in order to complete the extremities and create the overhangs — dATP is required for cloning in the TOPO-TA vector (Invitrogen™, USA). Q10 Q9

4.7. Elution of DNA fragments from agarose gel

Amplified fragments were fractionated using the modified voltage gradient gel electrophoresis (VGGE) technique [43] and then eluted as described elsewhere [44].

4.8. Cloning and sequencing of amplicons

Eluted PCR amplified bisulfite-treated genomic DNAs are cloned in TOPO-TA plasmid using the TOPO-TA Cloning Kit, in accordance with the instructions of the manufacturer (Invitrogen™). Sequencing of recombinant plasmids was performed using the Sanger procedure [45], in the presence of T7 DNA polymerase (Invitrogen™). Q11

Table 4 t4.1
Oligonucleotides used in cDNA synthesis and PCR amplification. t4.2

Name	Sequence (5′ to 3′)	t4.3
Cod5S dir	gcctacgaccataccat	t4.4
Cod5S rev	agcctacaacacccggta	t4.5
700 bp-rev	tcttgctttaaaaaagtt	t4.6
900 bp-rev	ccttgcaatcggatttcta	t4.7
950 bp-rev	cgcaatcctaattctaccc	t4.8

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4.9. Single-strand conformation polymorphism (SSCP) analysis

SSCP analysis was performed using horizontal polyacrylamide gels (Acrylamide/Bis 37.5:1, 20% w/v solution, 1 × TBE, pH 8.3, 2.5% glycerol) as described by Izzo et al. [46].

The samples, in 1 × loading buffer (10 × loading buffer contains 0.25% bromophenol blue, 0.25% xylene cyanol, and 50% glycerol) were denatured at 95 °C for 5 min, and rapidly chilled in ice blocks for 2 min, before loading. Electrophoretic fractionation was performed at 7 V/cm in 1 × TBE buffer (89 mM Tris, 89 mM boric acid, and 2 mM EDTA, pH 8.0) for 7 h. After electrophoresis, the gel was colored for silver staining as described herein. The gel was processed overnight with a fixing solution (50% methanol, 12% acetic acid, 0.05% formalin). It was washed three times in a wash solution (35% ethanol) for 20 min and then processed with a sensitizing solution (0.02% $Na_2S_2O_3$) for 2 min. After three washes with distilled water for 5 min, the gel was processed with silver stain solution (0.2% $AgNO_3$, 0.076% formalin) for 20 min. The gel was washed again twice in distilled water for 1 min and then processed with developing solution (6% Na2CO3, 0.05% formalin, 0.0004% $Na_2S_2O_3$) until the bands were compared. The coloration processing was stopped with staining stop solution (50% methanol, 12% acetic acid) for 5 min. The gel was conserved in 1% acetic acid at 4 °C overnight, before photography was performed.

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