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Genome methylation of the marine annelid worm *Chaetopterus variopedatus*: methylation of a CpG in an expressed H1 histone gene

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Abstract Hydrolysis by methylation-dependent restriction enzymes shows that the genomic DNA of the polychaete annelid worm *Chaetopterus variopedatus* is methylated. Electrophoretic analyses of the digestion products indicate that the degree of methylation is lower in adult tissues than in sperm and embryonic DNA. 5-Methylcytosine was identified by HPLC, absorption spectroscopy and mass spectrometry analyses of free bases obtained by acid hydrolysis of the DNA. An average value of 1.6% methylated cytosines was determined in sperm DNA. Partial methylation was also found in an actively expressed H1 histone gene. This is the first time that genomic DNA methylation is demonstrated to occur in a worm.

Key words: *Chaetopterus variopedatus*; Annelid polychaete; Invertebrate; DNA methylation; Histone H1 gene

1. Introduction

DNA methylation is an epigenetic modification that occurs in a wide range of eukaryotic organisms. This modification is performed by the enzyme *S*-adenosyl DNA-methyltransferase that introduces a methyl group in position C5 of the cytosines of CpG dinucleotides [1,2]. The functional role of this DNA modification is not yet clearly established although its fundamental importance for embryonic development has been demonstrated in the mouse where it has been shown that a null mutation of the enzyme causes embryonic lethality [3]. In addition, DNA of mouse embryos is demethylated in the preimplantation stages [4] and de novo methylated after implantation [5]. The control of DNA methylation during development and in different cell lineages has been demonstrated in several other organisms [6]. Studies of DNA methylation by methylation-sensitive restriction nucleases has shown that genomic DNA of vertebrates has the highest level of cytosine methylation with values ranging between 60 and 90% of the existing CpGs [7,8]. The genomes of invertebrates show lower levels, in the range of about 2–3% methylated cytosines. Methylation is reported to be absent in the DNA of the nematode worm *Caenorhabditis elegans* [9] and in the DNA of the insect *Drosophila melanogaster* [10]. Data have been presented that in the sea urchin methylated and unmethylated sequences retain their identity in the embryo and in the adult tissues indicating stability of the methylation state of the different DNA regions [11,12]. We report here the results of our investigation on the methylation of genomic DNA of the polychaete annelid marine worm *Chaetopterus variopedatus* showing, for the first time for a worm, that the DNA of this organism is methylated and that methylation is lower in adult than in embryo or sperm DNA. In addition, our previous finding of a H1 histone gene containing a cluster of seven CCGG sites in the genome of *C. variopedatus* provided a convenient opportunity to analyze the methylation state of the DNA in a CpG island, that is a region usually not methylated even in organisms with high levels of methylation [13]. We show that a specific CpG of the cluster of CCGG sites present in the somatic H1 histone gene is partially methylated when the gene is expressed.

2. Materials and methods

2.1. DNA isolation and preparation of embryos

Annelid worms *C. variopedatus*, kindly provided by the Zoological Station of Naples (Italy), were collected in the bay of Naples in the months of July and August when they are sexually active. Sperm cells and unfertilized eggs were obtained by cutting the parapods of the worms as described [14]. Sperm cells of single males were purified by repeated centrifugation in Millipore-filtered sea water (MFSW). DNA was prepared as described [15] from sperm cells lysed in 0.5 M EDTA, 2% SDS.

Pooled unfertilized eggs of several females were freed of immature cells by repeated sedimentation in MFSW and used for the preparation of embryo cultures. A few drops of a diluted suspension of sperm cells obtained from several males were added to the suspension of unfertilized eggs under gentle agitation. Embryonic preparations were used for the experiments only if, following sperm addition, at least 90% of the eggs showed formation of the fertilization membrane. Embryos were grown at 15°C with slow and constant stirring at a concentration of about 5000/ml for 17 h, until the blastula stage when they were collected by centrifugation, lysed in 20 volumes of 0.5 M EDTA, 2% SDS and the DNA was extracted and purified as indicated above.

Single adult specimens were either used immediately or quick-frozen on dry ice and stored at −80°C for delayed experiments. DNA of adult tissues was prepared as described [15] from the worm tails.

2.2. Restriction endonuclease digestion analysis

High molecular weight genomic DNA (1–2 μg) was incubated and digested to completion at 37°C for 16–18 h with 5 U/μg DNA of the isoschizomers *Hpa*II or *Msp*I nucleases in the conditions specified by the manufacturer (Boehringer Mannheim). Digestion products were fractionated by electrophoresis on 1% (w/v) agarose gels in TBE buffer (0.089 M Tris, 0.089 M sodium borate, 0.009 M EDTA, pH 8.3), stained with ethidium bromide and photographed.

2.3. Southern blot hybridization analysis

The electrophoretic patterns of DNA fragments obtained on slab gels were transferred to nylon-N membrane (Amersham) in 20×SSC (0.150 M NaCl, 0.005 M Na-citrate). After UV cross-linking and prehybridization, each filter was hybridized in sequence with probes A, A1 and A2 (see Fig. 3C) each radiolabeled with [α-^{32}P]dCTP (3000 Ci/mmol, Amersham) using the Multiprime Labeling Kit (Amersham). The hybridization procedure was as follows: filters were prehybridized for 6 h at 65°C in 5×SSPE (0.15 M NaCl, 0.01 M Na-phosphate, 0.001 M EDTA pH 7.4), 5×Denhardt's solution (0.2% w/v Ficoll, 0.2% w/v polyvinylpyrrolidone, 0.2% w/v bovine serum al-

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bumin), 0.1% sodium dodecyl sulfate (SDS), 0.1 mg heat-denatured herring sperm DNA per milliliter. Hybridization was performed by adding the labeled probe to a freshly made prehybridization solution and incubating for 16–18 h at 65°C. Filters were then washed at room temperature twice in 2×SSPE, 0.1% SDS and sequentially, at 65°C, with 1×SSPE, 0.1% SDS and twice more with 0.1×SSPE, 0.1% SDS before the exposure to Fuji RX film for autoradiography at −80°C. After each hybridization experiment, filters were dehybridized at 45°C for 30 min with 0.4 M NaOH and neutralized by washing with 0.1×SSC, 0.1% SDS, 0.2 M Tris-HCl pH 7.5, and then used for hybridization with another probe.

2.4. HPLC analysis of free bases

DNA was purified from the sperm cells of three different male worms and was individually hydrolyzed at 60°C for 24 h in 10 volumes of 70% perchloric acid to produce free bases that were purified as described [16]. Hydrolyzed and purified free bases were analyzed by HPLC (Perkin Elmer Model 410 Bio with Perkin Elmer 235 Diode Array detector) on a Beckman Ultrasphere C (5 μm) column of 4.6 mm×25 cm as described [16] with the modification that the equilibration buffer was 20 mM ammonium acetate, pH 4.0 to permit mass spectrometry analysis of the molecules eluting with the same retention time of standard 5-methylcytosine (5MeC). Peaks were eluted from the column with a three solvent gradient (the equilibration buffer, methanol and water) (16 min duration) at a flow rate of 1 ml/min. Peaks were detected by UV absorption at 254 nm. Spectroscopic analyses of the peaks were performed on a model Lambda 17 Perkin Elmer spectrophotometer to characterize each eluted free base. Standard free bases, including 5MeC, were from Sigma (Milan, Italy).

2.5. Mass spectrometry analysis

The gas chromatography/mass spectrometry (GC/MS) analysis was performed on a TRIO 2000 quadrupole mass spectrometer equipped with a GC 8000 gas chromatograph (Micromass, Manchester, UK). The reference standard and the sample were derivatized with *N,O*-bis(trimethylsilyl)-trifluoroacetamide according to [17] and directly injected into the GC/MS. The injector was kept at 250°C and the samples were eluted by increasing the temperature from 50°C to 280°C at 4°C/min using a DB5 column (30 m, 0.25 mm i.d.).

2.6. Northern blot analysis

Total RNA was isolated from *C. variopedatus* embryos at the blastula stage, following the described procedure [18]. 10 μg of RNA was fractionated on 1.2% agarose-formaldehyde gel and transferred to nylon N Hybond membrane (Amersham). Hybridization with probe A (Fig. 3A) was performed in 50% formamide at 42°C as described [19]. 30 ng of probe A was radiolabeled as described in Section 2.3.

3. Results and discussion

3.1. Analysis of DNA by isoschizomer restriction nuclease digestions

High molecular weight genomic DNA, prepared from sperm, blastula embryos and cells of adult tissues, were digested with the two isoschizomer restriction nucleases *Hpa*II and *Msp*I. Both enzymes recognize CCGG but *Hpa*II does not hydrolyze that site when the inner cytosine is methylated (C^mCGG). For this reason the presence of methylated cytosines in those sites results in a different degree of DNA hydrolysis with the two enzymes. Hydrolyzed *C. variopedatus* DNA samples were fractionated in adjacent lanes on 1% agarose slab gel in TBE buffer (Fig. 1). The sperm and embryonic DNAs appear highly fragmented upon digestion with *Msp*I with no main component in the high molecular weight region. The digestion patterns with *Hpa*II show, in contrast, the presence of major DNA fragments in the high molecular weight region, not observed in the digests with *Msp*I. This suggests the occurrence of CpG methylation in CCGG sites and the presence of long DNA regions in which CpGs are all methylated and, consequently, not hydrolyzed by *Hpa*II. Unlike the embryonic DNA, the adult DNA gives patterns almost identical with the two enzymes indicating that it is scarcely methylated in those sites. In the same figure, the digestion patterns of *C. variopedatus* sperm DNA are also shown side by side with those of the sea urchin *P. lividus* sperm DNA, which is a reference organism for DNA methylation [11]. It is apparent that the differences between *Hpa*II and *Msp*I digestion patterns are very similar in the two organisms. Since the DNA of the sea urchin has been shown to contain about 0.8% methylated cytosines [11], this result provides an approximate value of the percent 5MeC in the genome of *C. variopedatus*. An accurate evaluation is performed by direct 5MeC determination by HPLC of the free bases prepared from purified DNA.

3.2. HPLC and mass spectrometry analysis of 5MeC in C. variopedatus DNA

DNA extracted from sperm cells was purified and hydrolyzed to free bases in acid condition. Commercial free bases, including 5MeC, were used as reference molecules to calibrate the chromatographic patterns optimized to separate minor bases in the elution region of 5MeC. As apparent (Fig. 2), the DNA of *C. variopedatus* sperm cells contains a nucleic acid base that elutes with the same retention time as standard commercial 5MeC. In addition, when the DNA hydrolyzate is mixed with a known amount of 5MeC, the amplitude of the 5MeC band increases proportionally. The absorption spectrum of the eluted band has a maximum absorbance value at 278 nm, corresponding to that of 5MeC.

Unambiguous identification of 5MeC in the HPLC band that is eluted with the same retention time of standard 5MeC was achieved by GC/MS analysis. The EI mass spectrum of the derivatized sample showed the molecular ion at *m/z* 269 and the main fragment ion at *m/z* 254 originated from the loss of a methyl group [20]. This spectrum coincided perfectly with that of real 5MeC used as a standard. The analysis was also carried out using the selected ion monitoring technique by monitoring the ions at *m/z* 269, 254, 238 and 184, confirming the identification.

The average percent value of 5MeC was determined by HPLC analyses making reference to the peak area of the other free bases and to the peak area of known amounts of authentic 5MeC fractionated on the same column in mixture with the hydrolyzed DNA and alone. The values of 5MeC were found to be 1.8, 1.45 and 1.6% of total cytosines, respectively, in the DNA of three different males, with an average value of 1.6. This is about 50% of the value reported for 5MeC in sea urchin DNA [11]. As an additional difference, the hydrolysis patterns with *Msp*I and *Hpa*II of sperm, adult and embryo sea urchin DNA appear invariant while those of *C. variopedatus* are clearly different for the adult and for the sperm or embryo DNA (Fig. 1).

3.3. Hybridization analysis of the hydrolyzed patterns

An important point concerning the presence of 5MeC in the genomic DNA is its possible occurrence in the regions containing expressed genes. To check this point, the DNA of *C. variopedatus* blastula embryos was restricted with *Hpa*II and *Msp*I, fractionated on agarose slab gel, blotted and hybridized with a series of probes corresponding to different regions of a somatic H1 histone gene that we identified and sequenced in this organism [21] (sequence data have been deposited with GenBank under accession number U96764). As reported in

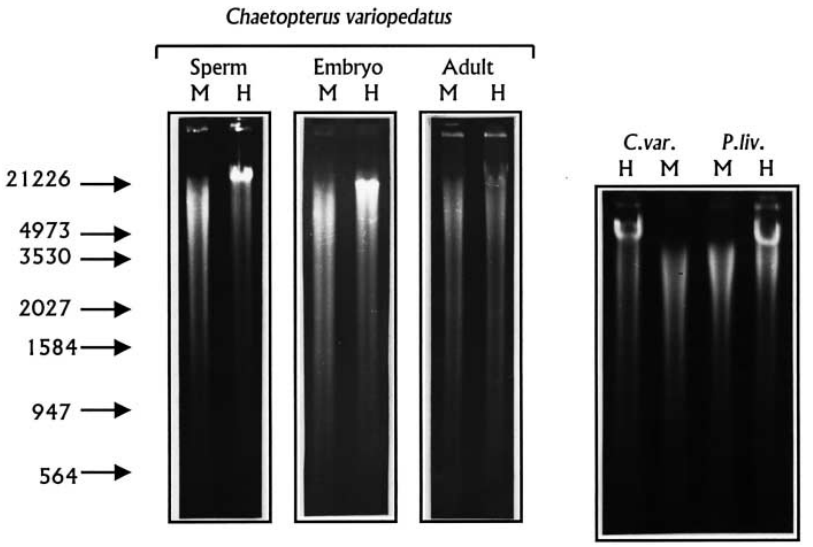

Fig. 1. Fragmentation patterns of total genomic DNA of *C. variopedatus* hydrolyzed with *Msp*I (M) and *Hpa*II (H). The patterns of the size distributions of the DNA fragments are different for the two enzymes indicating the occurrence of methylated CCGG restriction sites. DNA sources and positions of molecular weight markers are indicated. The fragmentation patterns of the sea urchin sperm DNA, as a reference organism for DNA methylation, are reported side by side with those of *C. variopedatus* to show the type of patterns to be expected when DNA is methylated.

Fig. 3A, six CCGG sites are present in the H1 gene and another site is outside the gene immediately after the termination triplet forming a cluster of seven sites potentially hydrolyzed by the restriction enzymes. Two other sites, 35 bp distant from each other, are present at about 1000 bp downstream, in the H2B gene. The blot was hybridized with each of the three probes indicated in Fig. 3C. The results of these experiments (Fig. 3A) show that the CCGG site at position 669, immediately after the termination triplet of the H1 histone gene occurring at position 666, is partially methylated in the DNA of the embryo at the blastula stage. In fact, when the pattern is hybridized with probe A, corresponding to the region between position 1 and 710, enclosing the entire H1 gene and 43 bp of the 3′ and 57 bp of the 5′ UTRs, a band of about 1200 bp is present in the lane of the digest with *Hpa*II and is absent in the lane of the digest with *Msp*I. Two other bands, one corresponding to small fragments and the other to 850 bp, are present in both lanes. Inspection of the linear gene map (Fig. 3C) gives the following indication about the origins of the different bands. The 850 bp band can only derive from the 848 bp fragment between the CCGG sites at positions 669 and 1517 (or 1552). The smaller band can only derive from the fragments of the clustered sites between positions 88 and 669. The 1200 bp band can only derive from the joining of the mentioned 848 bp fragment with the adjacent 319 bp fragment. This requires that the CCGG site at position 669 be methylated. Support for this conclusion derives from the hybridization results with the two other probes. Indeed, probe A1, spanning the 5′ region of probe A up to position 88, hybridizes with none of the three smaller bands. This indicates that the bands hybridizing with probe A do not concern the DNA regions upstream the initial CCGG site at position 88. Probe A2, spanning the 3′ region of probe A up to the CCGG site at position 669, hybridizes with the bands corresponding to the small fragments and to 1200 bp but not with the 850 bp band of the hydrolyzed genomic DNA. This is expected because, as reported in the gene map (Fig. 3C), this probe does not overlap the 848 bp fragment and cannot detect it while it hybridizes with the 1200 bp band that is formed when the CCGG site at position 669 is methylated and, hence, not hydrolyzed by *Hpa*II. The very faint hybridization signal present at the 850 bp position in the digest with *Msp*I (Fig. 3A) is very likely the result of the low sequence homology between the two DNA sequences under analysis.

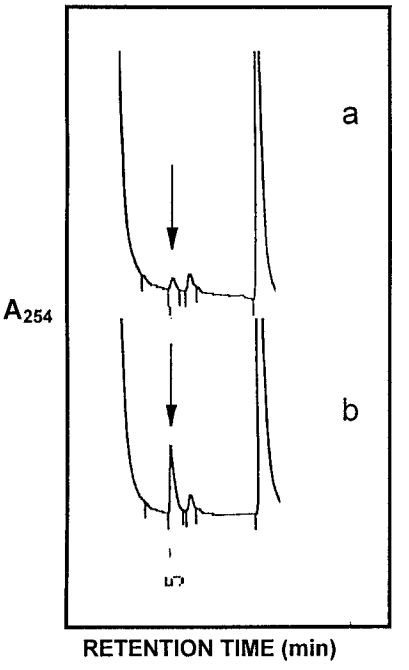

Fig. 2. HPLC patterns of free bases produced from acid hydrolysis of 10 μg *C. variopedatus* sperm DNA. The region corresponding to the peak position of 5MeC is reported. a and b are, respectively, the patterns of sperm and sperm DNA plus 3 nmol of commercial 5MeC. The arrows indicate the position of 5MeC.

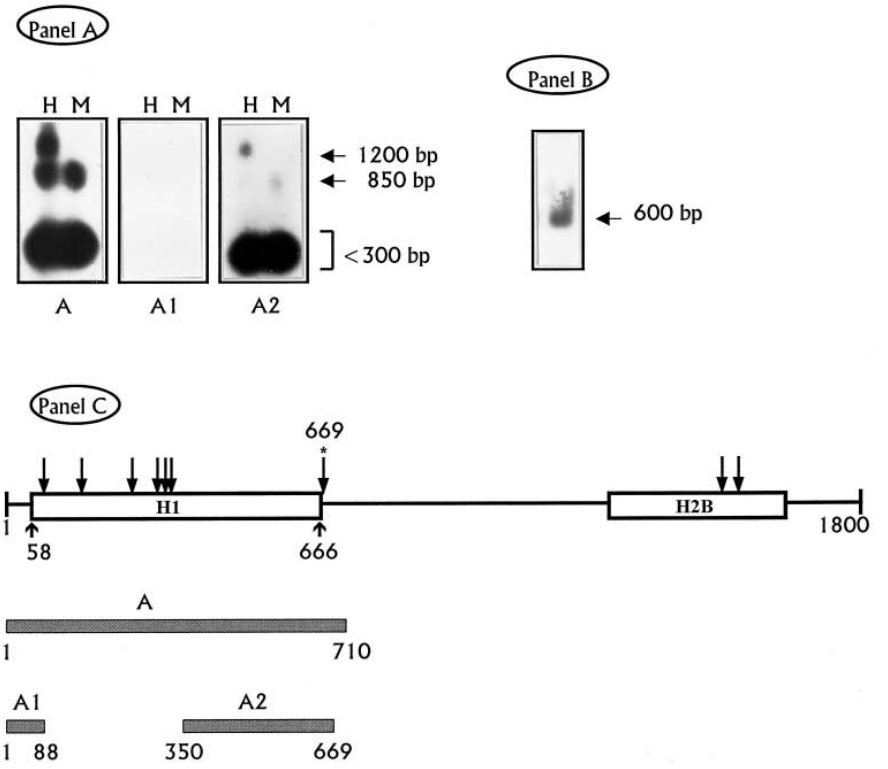

Fig. 3. Identification of a methylated CCGG site in the DNA coding for an expressed H1 histone gene of *C variopedatus*. A: Hybridization patterns of blastula embryo DNA, hydrolyzed with *Msp*I (M) and *Hpa*II (H), obtained with probes corresponding to different regions of the H1 gene as shown in C. The presence of a 1200 bp DNA band hybridizing with probe A only in the *Hpa*II digest indicates the occurrence of CpG methylation. The position of the methylated site at 669 bp results from the comparison of the hybridization patterns obtained with probes A, A1 and A2. For details see text. B: Northern blot of the RNA of blastula stage showing positive hybridization signal with probe A of C, indicating that the mRNA of H1 histone gene is present. C: Linear map of the 5′ region of the cluster of histone genes of *C. variopedatus*, enclosing the H1 histone gene investigated in A. Down-pointing arrows indicate the positions of the CCGG sites at 88, 157, 267, 314, 341, 350, 669, 1517 and 1552 bp. Up-pointing arrows indicate the positions of the start and stop codons of the H1 gene. The asterisk marks the position of the methylated CpG in the 669 bp CCGG site. Filled boxes represent the DNA probes used for hybridization in A. Probes: A, *Pst*I-*Dra*II fragment enclosing the entire H1 histone gene with 57 bp of the adjacent 5′ UTR and 43 bp of the 3′ UTR; A1 and A2 are subfragments of probe A. A1, *Pst*I-*Bgl*I fragment corresponding to the 5′ UTR and the initial 30 H1-coding nucleotides; A2, *Hpa*II-*Hpa*II fragment corresponding to the terminal 316 coding nucleotides plus the triplet after the stop codon.

3.4. Expression of the H1 histone gene

Northern blot analysis was performed on total RNA extracted and purified from *C. variopedatus* embryos at the blastula stage. The RNA was fractionated on an agarose slab gel, blotted and hybridized with probe A, which contains the entire H1 gene. The result of the Northern blot (Fig. 3B) shows the presence of a strong band of about 600 bp, typical of histone mRNAs [22], demonstrating that the H1 gene is expressed.

4. Conclusions

The results presented here show that the genomic DNA of the marine annelid worm *C. variopedatus* is methylated providing the first demonstration that methylation also occurs in a worm. In fact, it has been reported that in nematodes, the only worms so far analyzed for DNA methylation, 5MeC is not present or is below 0.01%, the limit of detection [9]. In *C. variopedatus* methylation is less evident in the adult tissues than in embryo or sperm DNA as revealed by methylation-dependent restriction analyses. High molecular weight fragments, corresponding to long regions of the genome, become apparent on the electrophoretic patterns of the DNA hydrolyzed with the nuclease *Hpa*II (Fig. 1). This has also been observed in other organisms and is considered to indicate the occurrence of two DNA compartments, one corresponding to inactive DNA and the other to actively transcribed DNA regions [12]. Our results clearly show that in *C. variopedatus* partial methylation occurs in a DNA region encoding a H1 histone gene that is actively transcribed, as demonstrated by Northern blot analysis (Fig. 3B). This is in line with the results showing that partial methylation is observed in the histone genes of the sea urchin [23]. In that organism it has also been shown that hypermethylation of DNA does not alter the timed expression of late histone genes and even stimulates the expression of early histone genes [24]. In both cases, however, embryos failed to develop correctly. Methylation in the cluster of CpGs of the H1 histone gene of *C. variopedatus* appears to be specific since methylation concerns a particular cytosine (Fig. 3A,C). It should be noted that the CCGG site at position 669 is only partially methylated. In fact, the pattern of the DNA hydrolyzed with *Hpa*II (Fig. 3A) and hybridized with probe A reveals, in addition to the 1200 bp DNA band, also the 850 bp DNA band. This should not occur if the CCGG site at position 669 were totally methylated. Moreover, the hybridization results with probe A2 are also in line with this interpretation because the intensity of the small fragment band is much higher than that of the 1200 bp band.

In conclusion, methylation is also present in a worm and with properties similar to those already described for the sea urchin. It is therefore possible that the absence of methylation in some organisms, such as *C. elegans* and *D. melanogaster*, might not directly concern phylogenetic mechanisms.

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