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Effects of 5-aza-2'-deoxycytidine on the Gene Expression Profile During Embryogenesis of the Ascidian *Ciona intestinalis*: A Microarray Analysis

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DNA methylation is an important epigenetic factor that participates in silencing genes. Genomic approaches to studying DNA methylation promise to be particularly fruitful, since DNA methylation is involved in global control of gene expression in many organisms. With its draft genome completed and a large quantity of available cDNA data, *Ciona intestinalis* is newly emerging as an invaluable model organism for investigating genome-wide gene expression and function. Here we examine the effects of 5-aza-2'-deoxycytidine (5-aza-CdR), a chemical that blocks CpG methylation, on the gene expression profile of early *C. intestinalis* embryos, using oligonucleotide-based microarray analysis. Embryos treated with 5-aza-CdR show delayed gastrulation and are developmentally arrested at the neurula stage. They subsequently lose cellular adhesion and finally die. Apoptosis was not detected in these embryos by TUNEL staining at 12 h, indicating that the defects observed did not result from 5-aza-CdR-induced apoptosis. Gene expression profiles of 12-h-old 5-aza-CdR-treated embryos compared to wild-type revealed 91 upregulated genes and 168 downregulated genes. Although nearly half of these encoded proteins with unknown functions, several encoded cell-signaling molecules and transcription factors. In addition, genes associated with the stress response and cell defense were upregulated, whereas genes involved in cell adhesion were downregulated.

Key words: *Ciona intestinalis*, embryogenesis, gene expression profiles, DNA methylation, 5-aza-CdR, microarray

INTRODUCTION

With the recent progress in compiling genome project data, the ascidian *Ciona intestinalis* has become an attractive experimental organism for studying developmental genomics, particularly for genome-wide approaches to studying the molecular mechanisms underlying embryonic cell specification (Satoh *et al.*, 2003). The draft *C. intestinalis* genome predicts 15,852 protein-coding genes (Dehal *et al.*, 2002), with those encoding transcription factors and cell-signaling molecules benefiting from precise annotation (*e.g.*, Satou *et al.*, 2003a; Wada *et al.*, 2003). Together with the availability of a large quantity of expressed sequence tags (ESTs) and cDNA data (Satou *et al*., 2005), this information has facilitated characterization of gene expression profiles during early *C. intestinalis* embryogenesis (Imai *et al.*, 2004, 2006). Furthermore, an oligonucleotide-based microarray covering more than 85% of transcripts expressed during *Ciona* embryogenesis has been generated (Yamada *et al.*, 2005) and has been used to examine zygotic gene expression profiles up to the 64-cell stage (Hamaguchi *et al.*, 2007).

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The molecular mechanisms responsible for epigenetic control of gene expression in early ascidian embryos are still poorly understood. DNA methylation of dinucleotide 5'-CpG sequences and histone acetylation are two major mechanisms known to be involved in global regulation of gene expression (Bird, 2002). The genome of *C. intestinalis* is reportedly a stable mosaic of methylated and non-methylated domains (Simmen *et al.*, 1999). For instance, multiple copies of an apparently active long terminal repeat retrotransposon, as well as a long interspersed element and a large fraction of abundant short interspersed elements, have been shown to be non-methylated. In contrast, genes are predominantly methylated, indicating that cytosine methylation in *C. intestinalis* may be preferentially directed to genes (Simmen *et al.*, 1999).

The effects of DNA methylation on biological processes can be studied using 5-aza-2'-deoxycytidine (5-aza-CdR), a chemical that blocks methylation and thus causes demethylation of CpG sequences (Taylor and Jones, 1979). In *C. intestinalis*, 5-aza-CdR was observed to produce general, non-specific effects during embryogenesis (Puccia *et al.,* 1986), but no studies examining its effects on ascidian embryogenesis have since been reported. Here we investigate the effects of 5-aza-CdR on gene expression profiles in *Ciona* embryos using microarray analysis.

MATERIALS AND METHODS

Maintenance of animals

Ciona intestinalis were cultivated at the Maizuru Fisheries Research Station of Kyoto University, Maizuru Bay, Kyoto, Japan. Eggs and sperm were surgically obtained from the gonoduct. After fertilization, they were dechorionated chemically with seawater containing 0.1% actinase E (Kaken Pharmaceutical Co., Tokyo) and 1.3% sodium thioglycolate (Wako Pure Chemical Industries, Osaka). Embryos were allowed to develop at about 18°C on 1% agar-coated plastic dishes filled with Millipore-filtered seawater containing 50 µg/ml streptomycin sulfate.

5-aza-2'-deoxycytidine (5-aza-CdR) treatment

5-aza-CdR (Sigma), a cytosine analog, was directly dissolved in seawater immediately before use. Fertilized eggs were incubated in seawater containing various concentrations of 5-aza-CdR.

TUNEL staining

TUNEL staining was basically performed as described previously (Chambon *et al.*, 2002). Embryos treated with 5-aza-CdR were fixed 12 h after fertilization with 3.7% formaldehyde in Millipore-filtered seawater at room temperature for 20 min. As a control, normal embryos were collected and fixed at the same time. Embryos were washed for 5 min in phosphate-buffered saline (PBS) with 0.2% Triton-X100 (PBSTx) three times and twice with PBS. For positive controls, normal and experimental embryos were incubated with 0.5 µM DNase I in PBS for 10 min. After two washes in PBSTx and one wash in TdT buffer, embryos were incubated in 10 µM biotin-16-dUTP (Roche Molecular Biochemicals) and 0.6 µg/µl TdT (Promega) in TdT buffer for 1 h at 37°C. Embryos were subsequently washed for 10 min in PBSTx three times, then incubated for 45 min in 1:200 FITC-streptavidin at room temperature. Finally, embryos were washed in PBSTx, and mounted in 80% glycerol/N-propyl-gallate for visualization.

Oligonucleotide-based microarray

Ciona intestinalis oligonucleotide-based microarray version 2 was custom-made by Agilent Technologies. Oligonucleotide probes (60-mer) were synthesized on a base chip by the inkjet-based Sure Print technology. The 44,290 spots on the chip consisted of three types of features or probes: one negative control distributed evenly on the chip at 314 spots, 57 positive controls at 1,942 spots, and 39,523 gene-specific probes at 42,034 spots. These specific probes represented 19,964 genes predicted from cDNA and EST sequence information (http://ghost.zool.kyoto-u.ac.jp//indexr1.html) as well as genome information from the JGI ver.1 assembly (Dehal *et al.*, 2002). One to ten probes were designed for each predicted gene.

RNA extraction, labeling, and hybridization

Embryos treated with 5-aza-CdR and control embryos were collected, quickly frozen in liquid nitrogen, and stored at –80°C. Total RNA was isolated by the acid guanidium thiocyanate-phenol-chloroform (AGPC) method (Chomczynski and Sacchi, 1987). The quality and quantity of the total RNA were tested using a NanoDrop microscale spectrophotometer (NanoDrop Technologies).

Five µg of total RNA from each sample were amplified linearly and used as a template to generate labeled cRNA for a microarray probe. The amplified target product of each sample was labeled with fluorescently tagged cyanine 3-CTP or cyanine 5-CTP (Perkin Elmer/NEN), using an Agilent Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies). The quality and size of the probe were tested using an RNA 6000 Nano Lab-on-chip (Agilent Technologies) and an Agilent 2100 Bioanalyzer, and the quantity was determined using a NanoDrop microscale spectrophotometer. For dye swapping, the same procedure was performed using reciprocal pairings of labeled CTP and total RNA.

A set of 1-µg targets from two samples was used for competitive hybridization on the *C. intestinalis* microarray version 2 with an In Situ Hybridization Kit Plus (Agilent Technologies). After hybridization, chips were washed and dried according to the manufacturer's protocol and scanned with a G2565BA Agilent DNA Microarray Scanner.

Microarray data analysis and annotation of gene function

The signal intensity of each fluorescent dye was extracted from scanned microarray images and normalized using Agilent G2567AA Feature Extraction Software (version 7.5). The local background method was used as a background subtraction algorithm, and other algorithms and parameters were used at default settings. Some spots that exhibited non-uniformity or saturated or low signal intensity were excluded from the following analysis. Genes that gave an average two-fold change in processed signal intensity were extracted, and those that showed this differential signal intensity in both sets of dye swap data were collected. Because low signal intensity often led to non-reproducible results, we excluded genes whose average processed signal was below 1,000.

Because the genes used to design our microarray were predicted from pre-existing sequence data, we were able to confirm which genes corresponded to each probe. A genomic sequence corresponding to each probe was found on the genome browser of the Ghost Database (http://ghost.zool.kyoto-u.ac.jp/indexr1.html). We preferentially used Kyoto Grail 2005 gene models (*e.g.*, KYOTOGRAIL2005.1.1.1), which had high consistency with EST data because they were derived from RNA fragments with poly(A) sequences. When no appropriate Kyoto Grail 2005 gene model was found, appropriate cDNAs (*e.g.*, Fis_citb001a01), clusters of assembled ESTs (*e.g.*, CLSTR00001r1), or JGI gene models predicted from *Ciona intestinalis* draft genome ver.1 (*e.g.*, ci0000000001) were adopted. When assembled sequences from more than two clusters were used as gene models, cluster names were connected by an underscore (*e.g.*, CLSTR00001r1_CLSTR00002r1). When no gene model exactly matched the probes, but the open reading frame of a predicted gene appeared to be correct, that gene model was adopted. More details of other sequence data are available from the corresponding author of this paper.

Sequence similarity searches of gene models were performed using the BLASTX algorithm (Altschul *et al.*, 1997) against four protein databases to annotate for gene function as accurately as possible. We searched the *Homo sapiens* protein database with Uniprot/SPTrEMBL ID based on the NCBI36 genome assembly (http://www.ensembl.org/Homo_sapiens/index.html), the *Drosophila melanogaster* protein database with Uniprot/SPtrEMBL ID based on the BDGP4.3 genome assembly (http://www.ensembl.org/Drosophila_melanogaster/index.html), the NCBI refseq database (November 1, 2006 release, http://www.ncbi.nlm.nih.gov/RefSeq/), and the NCBI non-redundant protein database (November 1, 2006 release). Parameters were set at default values, and hits with expected values lower than $1e^{-15}$ were used to identify genes with similarities. When two or more hits were found, the best hit with a clear annotation of gene function was used. Hits with significant similarity to *C. intestinalis* proteins were picked up at the same time. To exclude the possibility that gene models corresponded to non-coding RNA fragments, a search against the NCBI non-redundant RNA database was performed for those with no hits against four protein databases, but no significant similarities were found.

Following the similarity searches, gene models were classified based on Lee *et al.* (1999) into 17 categories. The basic classification criteria are described in Fig. 3C. Gene ontology (http://www.geneontology.org/index.shtm) was used for a reference. In cases in which function could be classified in more than two groups, we used the following criteria: transmembrane transporters of ions and small molecules were classified to AI, transmembrane complements to AVII, transmembrane signaling receptors to BI, and other transmembrane proteins to AIV. Proteins involved in protein folding

were assigned to AV, those involved in wound recovery (*e.g.*, blood coagulation) to AVII, and proteases other than those involved in intracellular signaling to AVIII. *Ciona intestinalis* transcription factors were assigned to CI, and zinc finger proteins to DI. When other hits with clear annotations were found for gene models, they were categorized to class DI. Molecules without definite assignment to DI and with no similarity were categorized as DII.

Relative EST number as a measure of temporal expression level

In previous studies, we performed large-scale EST analysis of transcripts expressed at several *C. intestinalis* developmental stages (*e.g.*, Satou *et al.*, 2002). Because these libraries were not normalized or amplified, the number of cDNA clones from each library accurately reflected the quantity of the transcripts of the corresponding genes (Satou *et al.*, 2003b). Thus, the relative number of ESTs corresponding to any given gene model at different developmental stages can be used to trace the spatio-temporal expression level of the predicted gene.

RESULTS AND DISCUSSION

Effects of 5-aza-CdR on *Ciona* embryogenesis

When fertilized *C. intestinalis* eggs were dechorionated and allowed to develop in seawater containing 1 μM 5-aza-CdR, their early cleavage steps appeared to be normal, both in terms of timing and pattern of cleavage. However, development of 5-aza-CdR-treated embryos was delayed at the onset of gastrulation. Twelve hours after fertilization, when control embryos had reached the mid tailbud stage (Fig. 1A), experimental embryos appeared to be at the neurula stage (Fig. 1C). Sixteen hours after fertilization, when normal embryos had developed to the late tailbud stage (Fig. 1B), experimental embryos remained arrested at the neurula stage (Fig. 1D). At 19–21 hours post-fertilization, cells of the outermost epidermal layer began to detach from 5-aza-CdR-treated embryos (data not shown). Subsequently, most cells in the experimental embryos became dissociated, all cells were disconnected by 2 days of development, and the embryos finally died.

TUNEL staining of 5-aza-CdR-treated embryos

Treatment with 5-aza-CdR is known to induce nitric acid production, which causes death in pre-implantation mouse embryos (Athanassakis *et al*., 2000). Moreover, 5-aza-CdR treatment causes abnormal mitosis and apoptosis of neural progenitor cells in the fetal rat brain (Ueno *et al*., 2006). It is likely that similar damage occurred in *Ciona* embryos treated with 5-aza-CdR for more than 16 hours. However, the delayed gastrulation and neurulation arrest observed in these embryos suggests that 5-aza-CdR has different effects at earlier stages. To rule out the possibilities that induction of apoptosis by 5-aza-CdR accounts for these defects and that noise from the RNA of dead cells disturbs the expression profile, we performed TUNEL staining on 12-h-old 5-aza-CdR-treated embryos. As a control, apoptosis was induced in both 5-aza-CdR-treated and untreated embryos by DNase-I treatment. As shown in Fig. 2A, untreated tailbud-stage embryos showed no TUNEL staining, while control embryos treated with DNase I showed distinct TUNEL staining (Fig. 2B). Similarly, 12-h-old 5-aza-CdR-treated embryos showed no TUNEL staining (Fig. 2C), while those treated with DNase I did (Fig. 2D). These results indicate that apoptosis does not occur in 5-aza-CdR-treated embryos at 12 h of development.

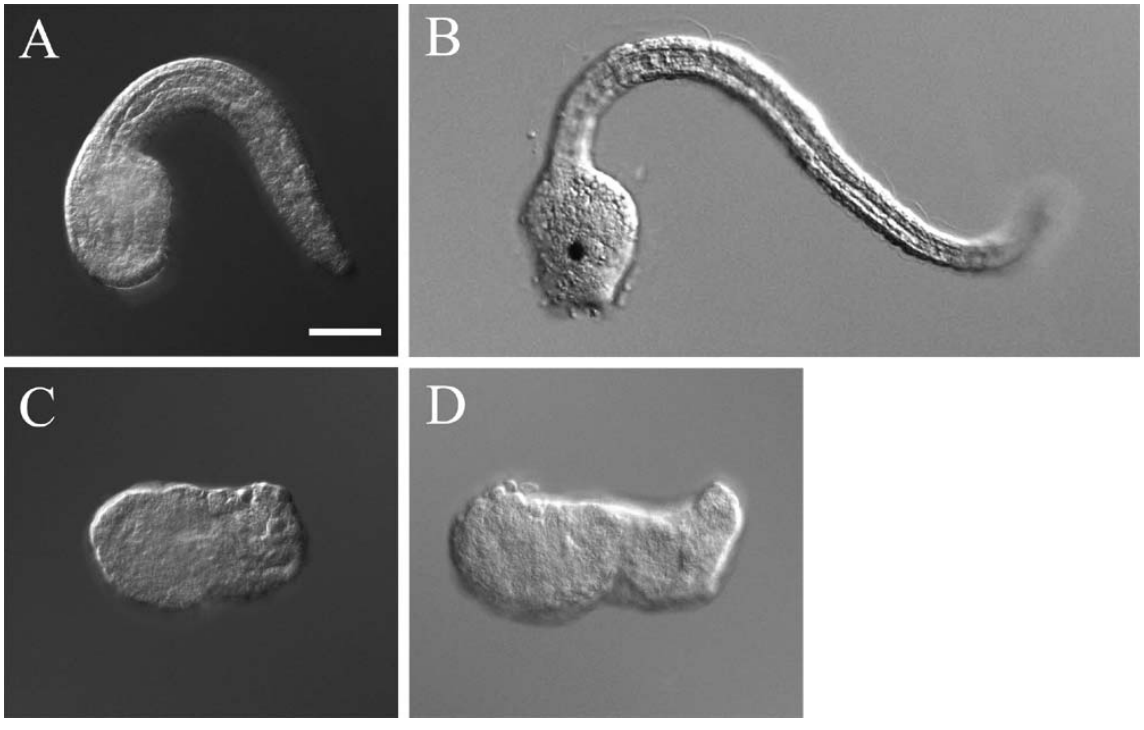


Fig. 1. Effects of 5-aza-CdR on embryogenesis of *Ciona intestinalis.* Fertilized and dechorionated eggs were allowed to develop in seawater that contained 1 μM 5-aza-CdR. **(A, B)** Control embryos and **(C, D)** experimental embryos, at 12 hrs **(A, C)** and 16 hrs of development **(B, D)**. Scale bar, 50 μm.

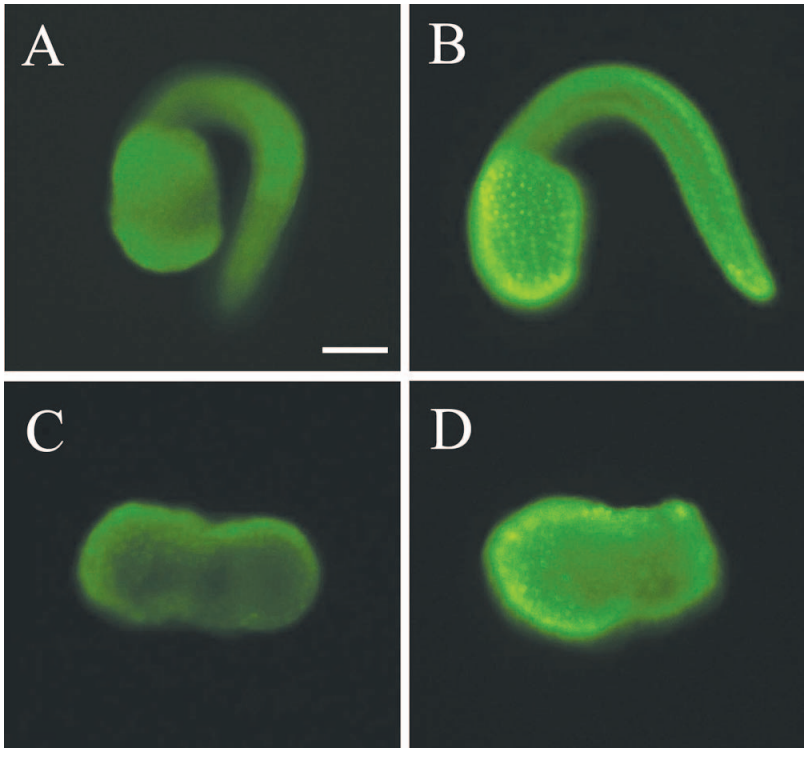
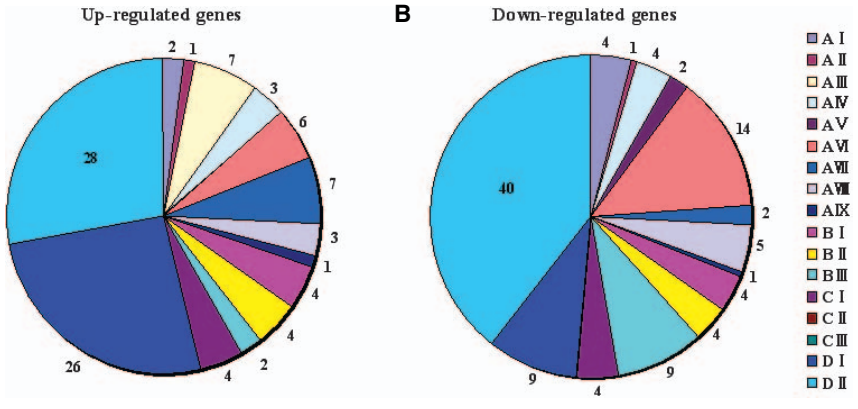


Fig. 2. TUNEL staining of embryos at 12 hours of development. **(A)** Normal control embryo and **(B)** control embryo treated with DNase I. **(C)** Experimental embryo treated with 5-aza-CdR and **(D)** that treated with DNase I. Scale bar, 50 μm.



C

Class	
	A. Functions common to many kind of cells
AI	Transportation and binding proteins for ions and other small molecules
AII	RNA processing, polymerizing, splicing and binding proteins and enzymes
AIII	Cell replication, histones, cyclins and allied kinases, DNA polymerases, topoisomerases, DNA modification
AIV	Cytoskeleton and membrane proteins
AV	Protein synthesis cofactors, tRNA synthetases, ribosomal proteins
AVI	Intermediary synthesis and catabolism enzymes
AVII	Stress response, detoxification and cell defense proteins
AVIII	Protein degradation and processing proteases
AIX	Transport and binding proteins for proteins and other macromolecules
	B. Cell-cell communication
BI	Signaling receptors, including cytokine and hormone receptors, and signaling ligands
BII	Intracellular signal transduction pathway molecules including kinases and signal intermediates
BIII	Extracellular matrix proteins and cell adhesion
	C. Transcription factors and other gene regulatory proteins
CI	Sequence-specific DNA binding proteins
CII	Non-DNA binding proteins that perform positive or negative roles
CIII	Chromatin proteins with regulatory function other than AIII
	D. Not enough information to classify
DI	Not enough information to classify
DII	No significant similarities to known proteins

Fig. 3. Classification of genes which are **(A)** upregulated or **(B)** downregulated by treatment of embryos with 5-aza-CdR. Classification was carried out according to Lee *et al.* (1999), as shown in **(C)**. The numbers with each pie show the rounded percentage of genes in each model.

Microarray analysis of changes in gene expression profiles in 5-aza-CdR-treated embryos

To examine the global effects of 5-aza-CdR on gene expression, total RNA was isolated from 5-aza-CdR-treated or control embryos, labeled with either Cy-3 or Cy-5, and hybridized to a 60-mer oligonucleotide-based microarray. Dye swapping was carried out to reduce false-positive data. Genes represented by probes or features were characterized carefully, as described in the Materials and Methods. Transcripts that were enriched in experimental embryos were selected by using a threshold value of 2.0 for the ratio of experimental to control expression. Ninety-one genes were identified as upregulated (Supplementary Table 1), while 168 genes appeared to be downregulated (Supplementary Table 2).

Because the EST library we previously generated was not normalized or amplified, the relative number of ESTs corresponding to any given gene can be used to trace spatiotemporal expression levels (see Materials and Methods). Supplementary Tables 1 and 2 show EST data for up- and downregulated genes, which are classified according to function. These data indicate that 54 of the 91 upregulated genes were expressed actively in normal embryos at the neurula and/or tailbud stage, whereas the other 37 genes were not expressed during these stages. Of the 168 downregulated genes, 106 were expressed in normal neurulae and/or tailbud embryos, while the other 62 genes were not expressed during these stages. It is likely that the upregulation of the 37 genes and the downregulation of the 62 genes which were not expressed in normal neurulae and/or tailbud embryos were indirect effects of 5-aza-CdR.

Genes upregulated in 5-aza-CdR-treated embryos

As shown in Fig. 3A, about 53% (48/89) of upregulated genes fell into Class D, whose protein functions are unknown. Twenty-seven genes were in Class A, 10 in Class B, and 4 in Class C. We could not identify appropriate gene sequences for two genes in the Ghost Database, so these were not included in this analysis. Two conspicuous groups among Class-A genes were subclass AIII, which includes genes associated with cell replication and DNA modification, and AVII, which includes genes associated with stress response and cellular defense. It is possible that 5-aza-CdR somehow affects cell division (Ueno *et al*., 2006), leading to delayed development and arrest at the neurula stage, accompanied by a failure of tail elongation. Upregulation of stress-response and cellular-defense genes is likely due to general damage caused by 5-aza-CdR treatment.

In addition, 10 genes associated with cell signaling and four genes involved in transcription machinery were upregulated (Fig. 3A; Table 1). This suggests that 5-aza-CdR is not simply toxic, but also has specific effects on the transcription of certain genes. EST data suggest that all four genes encoding transcription factors, KYOTOGRAIL2005.258.1.1, KYOTOGRAIL2005.73.38.1, *snail* (*Ci-sna*), and *Distal-less* (*Ci-Dll-B*) (Table 1), are expressed in wild-type *Ciona* embryos. Although the functions of the first two are unknown, *Ci-sna* encodes a transcriptional repressor that inhibits *Brachyury* (*Ci-Bra*) expression in muscle cells by binding to a 434-bp enhancer in the promoter region of *Ci-Bra,* thus acting as a boundary repressor to restrict *Ci-Bra* expression to the neighboring notochord cells (Corbo *et al*., 1997, Fujiwara *et al*., 1998). In addition, the *C. intestinalis* genome contains three *Distal-less* genes, *Ci-Dll-A*, *Ci-Dll-B* and *Ci-Dll-C*, which are presumably derived from a single ancestral gene by duplication (Caracciolo *et al*., 2000) but which show different expression patterns. Future studies should focus on whether upregulation of these genes is accompanied by ectopic expression in cells of other lineages or whether upregulation affects expression of other genes.

Genes downregulated in 5-aza-CdR-treated embryos

As shown in Fig. 3B, 49% (81/167) of downregulated genes fell in Class D, which comprises proteins of unknown function (Supplementary Table 2). Of the remaining genes, 52 were in Class A, 27 in Class B, and 7 in Class C. Among Class A genes, a conspicuous group was subclass AVI, which includes genes associated with intermediate enzymes in synthetic and catabolic pathways, suggesting that 5-aza-CdR affected negatively the activity of these important intermediate enzymes. We could not find the appropriate gene model sequence for one gene in the Ghost Database, so it was not included in further analysis.

As shown in Table 1, Class B genes that were downregulated in experimental embryos included precursors of integrin β4, collagen α-1 (XIV) chain, spondin-1, P-selectin, and fibulin-2. The gene ontology (GO) suggests that these genes are involved in the interaction of cells with the extracellular matrix (ECM). Downregulation of these genes may contribute to the loss of cell-ECM adhesion and the gradual disintegration of 5-aza-CdR-treated embryos. In addition, the plakin family member plectin-1, which is a component of the desmosome (Huber, 2003; Litjens *et al*., 2006), and the actin-binding protein Shroom, which is essential for neural tube morphogenesis in vertebrate embryos (Hildebrand, 2005), were included in Class B. It is likely that 5-aza-CdR treatment suppresses the activity of these genes involved in maintenance of cell architecture and embryo shape.

Among seven downregulated transcription factors was *Ci-Hox5*, which is first expressed at the early tailbud stage in the anterior nerve cord in an anterior-to-posterior gradient, as well as in the lateral trunk (Ikuta *et al.,* 2004). Future studies should examine the mechanisms of *Ci-Hox5* downregulation and its effects on the expression of other genes.

One downregulated Class-D gene was *Ci-META2*, which was originally identified in a differential screen for genes expressed in metamorphosing juveniles, but not in swimming larvae (Nakayama *et al*., 2001). However, the function of *Ci-META2* is unknown.

Conclusion

The spectrum of methylation levels and patterns is very broad among animals (Bird, 2002; Colot and Rossignol, 1999). For instance, 5-methylcytosine (m5C) has not been detected in the DNA of *Caenorhabditis elegans,* whose genome does not contain a conventional DNA methyltransferase. *Drosophila melanogaster,* whose DNA was long thought to be devoid of methylation, in fact does have a DNA methyltransferase-like gene (Tweedie *et al.*, 1999) and has very low m5C levels (Lyko *et al.*, 2000). In contrast, vertebrate genomes have the highest levels of m5C found in the animal kingdom, and methylation is dispersed over

Table 1. Class B and Class C genes up- and down-regulated in *Ciona intestinalis* embryos treated with 5-aza-CdR.

No.	Exp/ cont	Gene model	Accession No	Description	Organism	E value	Data base	Class	Cluster ID	EST count gastrulae & neurulae /23475 clones	EST count tailbud embryos /31209 clones
Up-regulated genes in Class B											
12	3.19	KYOTOGRAIL2005.315.3.1	P16109	P-selectin precursor	Homo sapiens	4e-36	H	BIII	CLSTR16267r1	0	0
32	2.64	KYOTOGRAIL2005.184.18.1	P49796	Regulator of G-protein signaling 3	Homo sapiens	5e-18	H	BII	CLSTR14768r1	2	2
46	2.45	KYOTOGRAIL2005.60.16.1	O00292	Transforming growth factor beta-4 precursor (Lefty-A protein)	Homo sapiens	8e-35	H	BI	CLSTR04063r1	2	1
53	2.36	KYOTOGRAIL2005.210.13.1	Q02817	Mucin-2 precursor (Intestinal mucin-2)	Homo sapiens	8e-94	H	BIII	CLSTR16721r1	0	0
									CLSTR38130r1	0	0
55	2.36	KYOTOGRAIL2005.1436.1.1	Q04721	Neurogenic locus notch homolog protein 2 precursor (Notch 2)	Homo sapiens	3e-55	H	BI	CLSTR32101r1	0	0
64	2.28	KYOTOGRAIL2005.757.3.1	NP_989716	Regulator of G-protein signalling 4	Gallus gallus	6e-16	R	BII	CLSTR33802r1	0	0
									CLSTR44432r1	0	0
68	2.26	KYOTOGRAIL2005.43.16.1	NP_055336	SH3-domain binding protein 4	Homo sapiens	2e-46	H	BII	CLSTR03812r1	7	1
74	2.23	KYOTOGRAIL2005.238.4.1	Q9NR61	Delta-like protein 4 precursor (Drosophila Delta homolog 4)	Homo sapiens	1e-136	H	BI	CLSTR04120r1	0	0
									CLSTR06818r1	5	2
									CLSTR42679r1-CLSTR42681r1	0	0
77	2.22	KYOTOGRAIL2005.68.36.1	P63000	Ras-related C3 botulinum toxin substrate 1 precursor	Homo sapiens	1e-69	H	BII	CLSTR03940r1	1	2
87	2.11	KYOTOGRAIL2005.195.5.1	Q01973	Tyrosine-protein kinase transmembrane receptor ROR1 precursor	Homo sapiens	1e-138	H	BI	CLSTR02441r1	*0	*1
									CLSTR12714r1	3	5
Up-regulated genes in Class C											
26	2.76	KYOTOGRAIL2005.258.1.1	BAE06603	Transcription factor protein	Ciona intestinalis	2e-172	N	CI	CLSTR02175r1	0	2
59	2.34	KYOTOGRAIL2005.2.32.1	O43623	Zinc finger protein SLUG (Snail homolog 2)	Homo sapiens	4e-52	H	CI	CLSTR02928r1	9	0
60	2.34	KYOTOGRAIL2005.10.5.1	NP_001027672	Distal-less	Ciona intestinalis	3e-91	R	CI	CLSTR11516r1	5	1
									CLSTR13912r1	0	0
83	2.16	KYOTOGRAIL2005.73.38.1	BAE06608	Transcription factor protein	Ciona intestinalis	0.0	N	CI	CLSTR03172r1	0	0
									CLSTR05423r1	20	2
									CLSTR13358r1	0	0
									CLSTR34655r1	0	0
									CLSTR44441r1-CLSTR44443r1	**0	**0
Down-regulated genes in Class B											
3	6.26	CLSTR04861r1	P16144	Integrin beta-4 precursor	Homo sapiens	4e-24	H	BIII	CLSTR04861r1	0	5
									CLSTR15522r1	*0	*0
6	5.30	KYOTOGRAIL2005.61.8.1	O60241	Brain-specific angiogenesis inhibitor 2 precursor	Homo sapiens	3e-21	H	BI	CLSTR03442r1	0	3
									CLSTR05106r1	*0	*0
									CLSTR15478r1	*0	*0
									CLSTR32257r1	0	0
10	4.70	KYOTOGRAIL2005.49.25.1	Q92736	Ryanodine receptor 2	Homo sapiens	0.0	H	BI	CLSTR04951r1	2	1
17	4.16	KYOTOGRAIL2005.159.22.1	P46531	Neurogenic locus notch homolog protein 1 precursor (Notch 1)	Homo sapiens	5e-55	H	BI	CLSTR33147r1	0	2
									CLSTR44709r1	0	0
23	3.87	Fis_ciad017c21	O60241	Brain-specific angiogenesis inhibitor 2 precursor	Homo sapiens	2e-23	H	BI	CLSTR00568r1	0	4
25	3.75	KYOTOGRAIL2005.17.44.1	Q05707	Collagen alpha-1(XIV) chain precursor	Homo sapiens	2e-22	H	BIII	CLSTR03352r1	0	2
									CLSTR06764r1	0	1
									CLSTR08662r1	*1	*0
									CLSTR11247r1	*0	*0
									CLSTR32203r1	0	0
35	3.29	KYOTOGRAIL2005.46.32.1	Q9HCB6	Spondin-1 precursor (F-spondin)	Homo sapiens	1e-141	H	BIII	CLSTR05046r1	0	0
48	2.85	KYOTOGRAIL2005.20.60.1	P16109	P-selectin precursor	Homo sapiens	1e-56	H	BIII	CLSTR03278r1	0	7
									CLSTR44483r1-CLSTR44493r1	**0	**0
56	2.76	KYOTOGRAIL2005.530.7.1	Q14CC2	NEXN protein	Homo sapiens	3e-19	H	BIII	CLSTR00419r1	*0	*0
									CLSTR30452r1	0	1
57	2.76	KYOTOGRAIL2005.26.81.1	P36383	Gap junction alpha-7 protein (Connexin-45)	Homo sapiens	3e-49	H	BIII	CLSTR10009r1	0	1
									CLSTR14612r1	0	0
59	2.72	KYOTOGRAIL2005.756.2.1	P16066	Atrial natriuretic peptide receptor A precursor	Homo sapiens	0.0	H	BI	CLSTR04809r1	0	0
60	2.72	KYOTOGRAIL2005.410.2.1	NP_001027800	Preprogonadotropin-releasing hormone 2	Ciona intestinalis	5e-87	R	BI	CLSTR07965r1	0	0
65	2.65	KYOTOGRAIL2005.52.32.1	Q15149	Plectin-1	Homo sapiens	1e-105	H	BIII	CLSTR06691r1	*0	*2
									CLSTR31023r1	0	0
									CLSTR32629r1	0	0
74	2.55	KYOTOGRAIL2005.781.9.1	Q96PC2	Inositol hexaphosphate kinase 3	Homo sapiens	2e-46	H	BII	CLSTR13695r1	0	3
81	2.48	KYOTOGRAIL2005.23.55.1	P98095	Fibulin-2 precursor	Homo sapiens	1e-89	H	BIII	CLSTR13475r1	0	1
									CLSTR16623r1	0	0
									CLSTR34405r1	0	1
83	2.48	Fis_ciad009o03	Q969Q1	Ubiquitin ligase TRIM63	Homo sapiens	5e-39	H	BII	CLSTR01565r1	*0	*0
									CLSTR01594r1	*2	*1
									CLSTR37778r1	0	0
85	2.45	CLSTR16596r1	P16109	P-selectin precursor	Homo sapiens	7e-18	H	BIII	CLSTR03977r1	*0	*0
									CLSTR16596r1	0	0
88	2.42	KYOTOGRAIL2005.143.32.1	P16109	P-selectin precursor	Homo sapiens	1e-66	H	BIII	CLSTR00772r1	*0	*0
									CLSTR12566r1	1	0
89	2.41	KYOTOGRAIL2005.257.11.1	P98095	Fibulin-2 precursor	Homo sapiens	6e-60	H	BIII	CLSTR11246r1	0	2
									CLSTR15764r1	0	0
									CLSTR15862r1	0	0
									CLSTR43569r1-CLSTR43570r1	**0	**0

To be continued.

Table 1. (continued)

No.	Exp/ cont	Gene model	Accession No	Description	Organism	E value	Data base	Class	Cluster ID	EST count gastrulae & neurulae /23475 clones	EST count tailbud embryos /31209 clones
93	2.39	KYOTOGRAIL2005.182.26.1	P07996	Thrombospondin-1 precursor	Homo sapiens	0.0	H	BIII	CLSTR02685r1	0	0
									CLSTR06794r1	0	0
									CLSTR36839r1	0	0
113	2.29	KYOTOGRAIL2005.77.37.1	P35556	Fibrillin-2 precursor	Homo sapiens	6e-77	H	BIII	CLSTR12135r1	*0	*0
									CLSTR12291r1	0	0
138	2.17	KYOTOGRAIL2005.1032.1.1	NP_065910	Shroom family member 3 protein	Homo sapiens	1e-44	H	BIII	CLSTR05640r1	0	0
									CLSTR09512r1	*0	*1
139	2.16	KYOTOGRAIL2005.230.23.1	O95411	TGFB1-induced anti-apoptotic factor 1	Homo sapiens	0.0	H	BII	CLSTR03977r1	*0	*0
									CLSTR05457r1	*0	*0
									CLSTR07716r1	0	0
									CLSTR13102r1	6	2
									CLSTR13452r1	0	0
148	2.13	KYOTOGRAIL2005.693.7.1	NP_057125	Der1-like domain family, member 2	Homo sapiens	5e-90	H	BII	CLSTR03070r1	*0	*1
154	2.10	Fis_cilv005a20_20	P42336	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha isoform	Homo sapiens	1e-105	H	BII	CLSTR07929r1	0	0
157	2.08	KYOTOGRAIL2005.71.24.1	Q14993	Collagen alpha-1(XIX) chain precursor	Homo sapiens	9e-29	H	BIII	CLSTR14750r1	1	4
159	2.08	KYOTOGRAIL2005.322.1.1	NP_726435	Sphingomyelin phosphodiesterase precursor	Homo sapiens	1e-106	H	BII	CLSTR04766r1	0	0
									CLSTR16845r1	*0	*0
Down-regulated genes in Class C											
63	2.67	KYOTOGRAIL2005.210.11.1	NP_996112	Limpet CG32171-PE, isoform E	Drosophila melanogaster	1e-79	D	CI	CLSTR02711r1	*1	*0
64	2.66	KYOTOGRAIL2005.765.2.1	P09067	Homeobox protein Hox-B5	Homo sapiens	1e-32	H	CI	No cluster found	–	–
69	2.59	KYOTOGRAIL2005.91.21.1	NP_997309	Forkhead box I2	Homo sapiens	3e-50	H	CI	CLSTR30003r1	0	0
90	2.41	KYOTOGRAIL2005.87.10.1	P31264	Homeotic protein proboscipedia	Drosophila melanogaster	6e-22	R	CI	CLSTR06810r1	0	0
									CLSTR10816r1	0	0
126	2.24	KYOTOGRAIL2005.500.2.1	Q9H334	Forkhead box protein P1	Homo sapiens	1e-78	H	CI	CLSTR01826r1	*0	*0
									CLSTR02755r1	0	4
									CLSTR03977r1	*0	*0
									CLSTR44905r1	0	0
130	2.21	ci0100154756	P52954	Transcription factor LBX1	Homo sapiens	4e-21	H	CI	No cluster found	–	–
142	2.16	KYOTOGRAIL2005.142.17.1	Q8IXZ3	Transcription factor Sp8	Homo sapiens	7e-37	H	CI	CLSTR00285r1	3	5
									CLSTR07449r1	*0	*0

Footnotes for Table 1. Exp/cont shows the mean fold change of the processed fluorescent intensity of the experimental samples treated with 5-aza-CdR and the control samples. Accession No., Description, Organism and E value show results of the similarity search (described in the Material and method in detail). Data base shows the database used to the search; H is the *Homo sapience* protein database, D is the *Drosophila melanogaster* protein database, R is the NCBI refseq and N is the NCBI non-redundant protein database. Class shows the classification of the gene function. Cluster ID shows the cDNA assembled cluster corresponding to the probes of the gene models. EST counts of the cluster are shown for temporal expression of the genes. *shows that only some clones of the cluster correspond to the gene model. **shows that a number of cluster containing only one clone are put together.

much of the genome. Most other invertebrate genomes have moderately high levels of methyl-CpG concentrated in large domains of methylated DNA that are separated by similarly large domains of unmethylated DNA (Bird *et al*., 1979). This mosaic methylation pattern has been confirmed at higher resolution in *Ciona intestinalis* (Simmen *et al.*, 1999). Since ascidians occupy a unique phylogenetic position as basal chordates, bridging the two major animal groups (invertebrates and vertebrates), the investigation of DNA methylation and its role in gene regulation in *Ciona* embryos is of special interest.

Our study is the first attempt to examine the relationship between DNA methylation and gene expression in *Ciona* embryos. It is unclear whether the changes in transcription we observed are directly associated with the state of DNA methylation, and we plan to address this in future studies.

The supplementary data for this article can be found online at “http://dx.doi.org/10.2108/zsj.24.648.s1”.

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