The Role of DNA Methylation in Invertebrates: Developmental Regulation or Genome Defense?

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Cytosine methylation is widely distributed in multicellular organisms. We present a comprehensive survey of the existing data on the phylogenetic distribution of DNA methylation in invertebrates, together with new data for the crustacean *Penaeus semisulcatus,* the annelid *Aporrectodea caliginosa trapezoides,* and the parasitic platyhelminth *Schistosoma mansoni.* Two alternative hypotheses addressing the function of cytosine methylation in invertebrates are evaluated: (1) cytosine methylation is an ancient regulatory mechanism which was lost in species with low rates of cell turnover, and (2) cytosine methylation is primarily a defense mechanism against genomic parasites and is expected to be present in all species with large genomes. We discuss the role of DNA methylation in the evolution of development in light of these hypotheses and conclude that gene control and cell memory are important and primitive functions of DNA methylation.

Introduction

In mammals, DNA methylation is associated with the regulation of gene expression and the maintenance of the differentiated state in cell lineages (Riggs 1990). Methylation is also present in other phylogenetic groups, and it has been suggested that it is the major cell memory system of multicellular organisms (Holliday 1990). However, DNA methylation is not found in all species. It is absent or extremely rare in invertebrates such as *Drosophila* and *Caenorhabditis elegans,* whereas it has been found in sponges, some insects, sea urchins, and invertebrate chordates, as well as in all vertebrates and plants so far examined.

Since cell memory is important in the development of all multicellular organisms, three overlapping questions about the phylogenetic distribution of DNA methylation arise: (1) Are there any features of different groups that are correlated with the extent of DNA methylation? (2) Is there an evolutionary explanation for the presence of DNA methylation in some species and its absence in others? (3) What type of cell memory system is used in organisms lacking DNA methylation? Cell memory systems, or epigenetic inheritance systems (EISs), include steady-state systems, in which self-sustaining regulatory loops maintain particular functional states; structural inheritance systems, in which the architecture of cell structures is transmitted to daughter cells; and chromatin-marking inheritance systems (including the methylation system), in which the nonobligatory parts of chromosomes are transmitted to daughter cells by hitchhiking on the semiconservative replication of DNA (Jablonka and Lamb 1989, 1995, pp. 79–110; Jablonka, Lachmann, and Lamb 1992).

Abbreviations: EIS, epigenetic inheritance system; 5-mC, 5-methyl cytosine.

Key words: DNA methylation, evolution of development, epigenetic inheritance, cell memory, genome size, invertebrates.

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Mol. Biol. Evol. 15(7):880–891. 1998

Two main hypotheses have been offered to explain the distribution and extent of DNA methylation in animals and the evolution of its functions. According to the first, DNA methylation is primarily associated with genome size. Bestor (1990) suggested that the role of DNA methylation in the regulation of gene expression evolved from its part in the restriction-modification system of bacteria. This system, which could originally have been a bacterial genome defense system or a selfish addictive genetic element (Naito, Kusano, and Kobayashi 1995), evolved into a new role in primitive eukaryotes, silencing newly introduced genomic parasites through DNA methylation. Bestor (1990) argued that the phylogenetic increase in genome size was associated with a further change in the role of DNA methylation: it became a general repressor of endogenous repeated sequences and, eventually, of tissue-specific genes. Thus, methylation prevents unscheduled gene expression and transposition in large parts of the expanded genomes of higher eukaryotes. Bird (1995) elaborated on Bestor's hypothesis. He accepted that DNA methylation acts as a repressor of genomic parasites and repeated sequences in nonvertebrates, but suggested that the role of DNA methylation as a regulator of individual endogenous genes was an innovation that occurred in the protovertebrates. Bird argued that the transition from protovertebrates to vertebrates was causally related to the novel use of methylation to control the activity of endogenous genes. By reducing unscheduled gene expression, methylation was instrumental in allowing an increase in gene number, which enabled the increase in complexity seen in vertebrates. Hence, in multicellular nonvertebrates, methylation is expected to be correlated with genomic parasite load and genome size, while in vertebrates its presence is explained by a change in function leading to an increase in the number of genes.

The second hypothesis to explain the phylogenetic distribution of DNA methylation focuses on the correlation between methylation and mode of development. Bestor (1990) suggested that the lack of detectable methylation in *Drosophila* is associated primarily with its small genome, but also with its early tissue development and lack of long-lived lines of stem cells. Ja-

Table 1
Methylation Levels, Genome Sizes, and Adult Cell Turnover Levels in Invertebrate Groups

Organism	Adult Cell Turnover	% 5-Methyl Cytosine	Genome Size (haploid, pg)	% Repetitive Sequences[a]
Porifera		ND–9.4	0.055–0.06	
Clathrina coreacea	√	6.7		
Chondrosia reniformis	√	3		
Ancorina cerebrum	√	ND		
Geodia	√	ND		
Tethya limski	√	5		
Tethya aurantium	√	ND		
Suberites domuncula	√	6.7		
Axinella polypoides	√	ND		
Hemimycale columella	√	ND		
Mycale massa	√	9.4		
Mycale tunicata	√	6.4		
Crella rosea	√	8.4		
Dysidea avara	√	7.9	0.055[b]	
Hippospongia communis	√	ND		
Cacospongia scalaris	√	3.2		
Cnidaria		4.6	0.33–0.73	
Metridium senile	√	4.6		
Actinia equina	√	4.6		
Platyhelminthes		ND	0.26–14.8	
Schistosoma mansoni	√	ND	0.26	35–40
Opisthorchis viverrini	√	ND		
Spirometra mansonoides	√	ND	1.65	45
Fasciola gigantica	√	ND		
Fasciola hepatica	√	ND		
Gigantocotyle siamensis	√	ND		
Nemertea		√	1.4	
Lineus longissimus	√	√		
Nematoda		ND	0.05–2.5	
Meloidogyne hapla	—	ND	0.05[b]	20
Meloidogyne incognita	—	ND		
Panagrellus redivivus	—	ND	0.07	17–20[c]
Caenorhabditis elegans	—	ND	0.088	14
Nippostrongylus brasiliensis	—	ND		
Brugia malayi	—	ND		
Ascaris lumbricoides	—	ND	0.32 (germ line) 0.25 (soma)	25 (germ line) 10 (soma)
Priapulida		√	0.28	
Priapulus caudatus	?	√		
Mollusca		2.5–4.9	0.43–5.4	
Mytilus edulis	√	√	1.6	
Mytilus galloprovincialis	√	√		
Patella	√	4.9		
Pecten islandicus	√	2.5	2.1[b]	
Helix pomatia	√	2.9		
Annelida		8.4–13[d]	0.36–7.2	
Aphrodita aculeata	√	√		
Nephtys ciliata	√	8.4	2.2–7.2[b]	
Apporectodea caliginosa trapezoides	√	13[d]		
Arthropoda				
Crustacea		ND–4.5[d]	0.2–22.6	
Penaeus semisulcatus	√	4.5[d]	2.4–3.0[b]	
Cancer pagurus	√	ND	1.7[b]	
Artemia	—	ND	1.5–3.0	55
Orthoptera		0.96–3	0.85–12.65	
Locusta migratoria	+/−	0.96	6.35	30
Eyprepocnemis plorans	+/−	√		
Pyrgomorpha conica	+/−	√		
Gryllotalpa fossor	?	3	0.85	
Baetica ustalata	?	√		

Table 1
Continued

Organism	Adult Cell Turnover	% 5-Methyl Cytosine	Genome Size (haploid, pg)	% Repetitive Sequences[a]
Homoptera		0.36–1.75	0.18–0.96	
Planococcus lilacinus	—	1.75		
Pseudococcus calceolariae	—	0.36–0.68[e]		
Pseudococcus obscurus[f]	—	1.26–1.44[e]		
P. obscurus[g]	—	1.09–1.21[e]		
Megoura viciae	—	√	0.96	
Myzus persicae	—	√	0.32	
Coleoptera		ND	0.15–3.69	
Tribolium castaneum	—?	ND	0.21	37
Diptera		ND or little	0.1–1.85	
Drosophila melanogaster	—	ND[h]	0.18	24
Drosophila virilis	—	ND	0.24	41
Sciara coprophila	+/−	ND[h]		
Musca domestica	—	ND	1.04	34
Sarcophaga bullata	—	ND	0.6	15
Culex bitaeniorhynchus	+/−	√[i]	0.23–1.85[b]	23
Chironomus plumosus	+/−	0.2	0.2[b]	10[b]
Anopheles maculipennis	+/−	√	0.23–0.29[b]	20–33[b]
Aedes albopictus	+/−	0.17[i]	0.41–2.8[j]	20–57[k]
Lepidoptera		ND–0.19	0.42–1.0	
Bombyx mori	—?	0.15–0.19	0.53	45
Hyalophora cecropia	—?	ND		
Antheraea pernyi	—?	ND	1.0	14
Spodoptera frugiperda	—?	ND		
Hymenoptera		ND	0.166–0.35	
Apis mellifica	—	ND	0.35[b]	6–10
Psithyrus	—	ND		
Bryozoa		√		
Membranipora membranacea	√	√		
Echinodermata		3.29–6.6	0.67–1.9	
Echinometria mathaei		26–29[d]	0.83	
Strongylocentrotus purpuratus	√	~20[d]	0.89	27
Echinus esculentus	√	√		
Paracentrotus lividus	√	6.6	0.7	
Psammechinus miliaris	√	√		
Asterias rubens	√	√		
Thyone fusus	√	√		
Cucumaria frondosa	√	5.7		
Stichopus japonicus	√	3.29	0.99[b]	
Ophiopholis	√	√		
Ophiothrix fragilis	√	√		
Cephalochordata		~15[d]	0.6	
Branchiostoma lanceolatum		~15[d]	0.6	17
Urochordata		4.9–20[d]	0.16–0.2	
Halocyntia aurantium	√	4.9		
Ciona intestinalis	√	~20[d]	0.2	30

NOTE.—√ = significant levels; — = none; —? = probably none; +/− = only in a few adult cell types; ? = unknown levels; ND = not detected. References are as follows: Porifera—Shapiro (1970*a*); Vanyushin, Tkacheva, and Belozersky (1970); Breter, Hundt, and Zahn (1976); Sparrow and Nauman (1976). Cnidaria—Shapiro (1970*a*, 1970*b*); Vanyushin, Tkecheva, and Belozersky (1970); Goldberg et al. (1975); Bird and Taggart (1980). Platyhelminthes—Simpson, Sher, and McCutchan (1982); Pellicciari et al. (1986); Martens, Curini-Galletti, and Van-Oostveldt (1989); Cox, Phares, and Schmidt (1990); Hebert and Beaton (1990); Sermswan, Mongkolsuk, and Sirisinha (1991); Musto et al. (1994). Nemertea—Goldberg et al. (1975); Tweedie et al. (1997). Nematoda—Shapiro (1970*b*); Sin and Pasternak (1970); Sulston and Brenner (1974); Moritz and Roth (1976); Sparrow and Nauman (1976); Beauchamp, Pasternak, and Straus (1979); Roth (1979); Simpson, Johnson, and Hammen (1986); Rothstein, Stoller, and Rajan (1988); de Chastonay, Müller, and Tobler (1990); Piotte et al. (1994); Balsamo and Manicardi (1995). Priapulida—Schreiber, Sturenberg, and Storch (1994); Tweedie et al. (1997). Mollusca—Shapiro (1970*a*, 1970*b*); Vanyushin, Tkacheva, and Belozersky (1970); Hinegardner (1974); Bird and Taggart (1980); Martinez-Lage, Gonzalez-Tizon, and Mendez (1994). Annelida—Shapiro (1970*a*, 1970*b*); Conner, Hinegardner, and Bachmann (1972); Cavalier-Smith (1978); Sella et al. (1993). Crustacea—Bachmann and Rheinsmith (1973); Rheinsmith, Hinegardner, and Bachmann (1974); Bird and Taggart (1980); Warner and Bagshaw (1984); Beaton and Hebert (1989); Dufresne and Hebert (1995); Lecher, Defaye, and Noel (1995); Wyngaard et al. (1995). Orthoptera—Wyatt (1951); Wilmore and Brown (1975); Rees, Shaw, and Wilkinson (1978); Sentis et al. (1990); Lopez-Leon, Cabrero, and Camacho (1991, 1995); Sarkar et al. (1992); Suja et al. (1993); Newfeld and Gelbart (1995). Homoptera—Achwal, Ganguly, and Chandra (1984); Scarbrough, Hattman, and Nur (1984); Field et al. (1989); Manicardi et al. (1994, 1995); Finston, Hebert, and Foottit (1995). Coleoptera—Brown et al. (1990); Alvarez-Fuster, Juan, and Petitpierre (1991); Juan and Petitpierre (1991); Petitpierre, Segarra, and Juan (1993); Hunt and Page (1995). Diptera—Shapiro (1970*b*); Jost and Mameli (1972); Manning, Schmid, and Davidson (1975); Crain, Davidson, and Britten (1976); Wells, Royer, and Hollenberg (1976); Adams et al. (1979); Rae and Steele (1979); Samols and Swift (1979); Eastman et al. (1980); Urieli-Shoval et al. (1982); Eick, Fritz, and Doerfler (1983); Rao and Rai (1987); Black and Rai (1988); Petitpierre,

blonka and Lamb (1995, pp. 208–213) went much further. While accepting that the primary role of DNA methylation was in eliminating genomic parasites, they argued that its role in the control of gene expression was a parallel function, already present in primitive unicellular organisms. They suggested that the present distribution of DNA methylation in multicellular animals is correlated primarily with mode of development, particularly with the amount of cell turnover. Animals with short life spans and little cell turnover make less use of the methylation-dependent cell memory system than do those with long life spans and a lot of cell turnover. DNA methylation is mutagenic (Holliday and Grigg 1993), so in animals with little cell turnover, where the DNA methylation EIS is not critical for the maintenance of gene activity patterns during repeated rounds of cell division, natural selection has reduced, and sometimes even eliminated, DNA methylation. According to this hypothesis, increase in genome size is associated with DNA methylation only indirectly, through the effect of genome size on developmental strategies.

In an attempt to evaluate the two hypotheses, we made the first comprehensive survey of existing data on levels of methylation, cell turnover, genome size, and repetitive sequence content in different invertebrate taxa, and determined the level of DNA methylation in three invertebrate species in groups for which data were either not available or scarce.

Results and Discussion

A comprehensive search of the literature for methylation levels, genome sizes, and cell turnover in different invertebrate phyla yielded the data shown in table 1. In addition, we assembled the available information on the percentages of repetitive DNA in invertebrate genomes. The table also includes the new data obtained in the present study using the modified nearest-neighbor analysis (Gruenbaum et al. 1981) for the crustacean *Penaeus semisulcatus,* the annelid *Aporrectodea caliginosa trapezoides,* and the parasitic platyhelminth *Schistosoma mansoni.* The data in table 1 are summarized in table 2, which gives the means and variances of genome size and mean methylation levels for different phylogenetic groups.

The data in table 1 were assembled from literature reports of studies that were made for different purposes but included estimates of DNA content, methylation levels, or cell turnover in adults. Consequently, the amount and quality of the information available for each group is very variable. For example, we do not always have information about genome size, methylation level, and cell turnover for the same species. The amount of cell turnover in adult tissues is not always known in detail. Methylation levels have been determined with methods of varying sensitivity and have been reported in different ways, sometimes only as methylation present or absent, so not all data could be included in the calculations that produced table 2. Data about genome size are much more comprehensive than are data about methylation levels, and the genome size ranges (table 1) as well as the means and variances (table 2) include all available data for a group. However, specific genome sizes are included in table 1 only when methylation data for the same or a very closely related species is also available.

Clearly, the nature of the data allows only limited statistical analysis. However, although when taken in isolation some of the data are not substantial, when they are taken together, a fairly clear and consistent picture emerges. This is the first time that all available data have been used to evaluate two hypotheses about the role of cytosine methylation in invertebrates.

The first hypothesis suggests that methylation in nonvertebrates silences genomic parasites and represses repeated DNA sequences (Bestor 1990; Bird 1995; Yoder, Walsh, and Bestor 1997). Thus, for animals of similar complexities, the extent of DNA methylation should be correlated with genome size (Bestor 1990; Bestor and Tycko 1996), and no correlation with cell turnover is expected. The second hypothesis links DNA methylation with mode of development and amount of cell

Table 1
Footnote Continued

Gatewood, and Schmid (1988); Brown et al. (1990); Marchi and Mezzanotte (1990); Nayak et al. (1991); Warren and Crampton (1991); Besansky and Powell (1992); Hunt and Page (1995). Lepidoptera—Shapiro (1970*b*); Gage (1974); Efstratiadis et al. (1976); Knebel, Lübbert, and Doerfler (1985); Patel and Gopinathan (1987); Taylor et al. (1993). Hymenoptera—Shapiro (1970*b*); Crain, Davidson, and Britten (1976); Rae and Steele (1979); Hunt and Page (1995). Bryozoa—Tweedie et al. (1997). Echinodermata—Chargaff, Lipshitz, and Green (1951); Shapiro (1970*a*); Vanyushin et al. (1973); Graham et al. (1974); Cavalier-Smith (1978); Bird and Taggart (1980); Gruenbaum et al. (1981); Raff et al. (1990); Fronk, Tank, and Langmore (1992); Tweedie et al. (1997). Cephalochordata—Schmidtke, Epplen, and Engel (1979); Holland et al. (1994); Tweedie et al. (1997). Urochordata—Shapiro (1970*a*, 1970*b*); Schmidtke, Epplen, and Engel (1979); Bird and Taggart (1980); Holland et al. (1994).

[a] The reported percentages of repetitive sequences were calculated by different methods of varying accuracy, so in certain cases there may be an overestimate.

[b] Genome size of other species from the same genus.

[c] The low value is only a partial estimate for this species. The high value is a complete estimate for another species of the same genus.

[d] Percent of methylated CpG.

[e] Different methylation levels in male/female and adult/embryo.

[f] No B chromosomes.

[g] With B chromosomes (facultative heterochromatin).

[h] A very low amount of 5-mC found in polytene chromosomes (Eastman et al. 1980).

[i] Methylation not restricted to CpG dinucleotides.

[j] Different estimates for ex vivo (tissue samples) and in vitro (cell culture) samples.

[k] Different estimates for different strains.

Table 2
Averages and Variances of Genome Size and Average Methylation Levels in Different Invertebrate Groups[a]

	Genome Size (pg)		Log Genome Size (log pg × 10^2)			Methylation Level	
	Mean	Variance	Mean	Variance	No. of Species	Mean	No. of Species
Porifera	0.06	~0.00	0.76	0.080	2	3.78	15
Cnidaria	0.53	0.080	1.69	0.059	2	4.60	2
Platyhelminthes[b]	2.00	12.020	2.05	0.160	16	0	6
Platyhelminthes[b]	1.15	0.390	1.98	0.070	15	0	6
Nematoda	0.72	0.920	1.46	0.450	7	0	6
Mollusca	1.80	0.910	2.19	0.054	113	3.43	3
Annelida	1.90	2.070	2.13	0.156	49	8.40	1
Crustacea[c]	4.28	14.200	2.50	0.110	79		
Orthoptera	9.40	12.120	2.92	0.060	27	1.98	2
Homoptera	0.51	0.026	1.69	0.023	34	1.11	4
Coleoptera	0.53	0.024	1.62	0.072	91	0	1
Diptera	0.80	0.230	1.78	0.130	42	0.05	7
	0.41[d]		1.46[d]				
Drosophilidae	0.18		1.25		4		
Culicidae	0.96		1.92		31		
Lepidoptera	0.65	0.094	1.78	0.038	3	0.04	4
Hymenoptera	0.26	0.007	1.49	0.020	5	0	2
Echinodermata	1.05	0.089	2.00	0.012	22	5.19	3
Urochordata	0.18	~0.001	1.25	0.004	2	4.90	1

[a] Based on the data and references in table 1.

[b] The turbellarian flatworm, *Mesostoma ehrenbergii*, has an unusually large genome, of 14.8 pg (Hebert and Beaton 1990). In order not to bias the mean and variance values for the entire flatworm group, both values were calculated twice, either including *M. ehrenbergii* (first entry) or excluding it (second entry).

[c] The average methylation level of crustaceans could not be calculated as the only nonzero value was given in percentage of methylated CpGs and was therefore noncomparable to other values.

[d] Value calculated after averaging first for the different families (Cecidomiidae, Dixidae, Drosophilidae, Muscidae, Chironomidae, Trychodidae, Psychodidae, Sarcophagidae, Culicidae). This was done to prevent overestimation of the Drosophilidae (4 species, see entry) and the Culicidae (31 species, see entry) over the other families (1 species each).

turnover. It assumes that, as well as being a defense mechanism against intragenomic parasites, methylation is an EIS that enables determined and differentiated states to be transmitted in cell lineages through many cell divisions (Jablonka and Lamb 1995, pp. 90–102; Jablonka and Regev 1995). However, since methylation is also a hazard because methylated cytosines are hot spots for mutations (Holliday and Grigg 1993), the extent of methylation in a particular species is the result of an evolutionary compromise between the benefits of methylation for gene regulation, cell memory, and the repression of intragenomic parasites, and the mutational hazards imposed by methylation (Hurst 1995; Jablonka and Lamb 1995, pp. 208–213). In large organisms with high cell turnover, the methylation EIS is indispensable, and its benefits as a reliable cell memory system outweigh its mutational hazards, whereas in small organisms, with little multiplication of determined cells, the mutational hazards of methylation may outweigh its benefits. In general, DNA methylation should be correlated with high cell turnover.

Table 1 and figure 1 show that, contrary to the predictions of the genome defense hypothesis, there is no clear relationship between genome size and the presence or extent of DNA methylation. Some organisms with very small genomes have methylated DNA, while others with comparable or higher amounts of DNA show no detectable DNA methylation. For example, whereas the DNA of sponges (genome size 0.055–0.06 pg) and tunicates (e.g., *Ciona*, with a 0.2-pg genome) is methylated, that of nematodes, with their somewhat larger genomes (0.08–2.5 pg), and *Drosophila melanogaster*, with a genome size of 0.18 pg, is not. Even insects with relatively large genomes, in the range 0.157–3.692 pg, have less than 1% methylation. Some animals with quite large genomes, such as the *Culex* mosquitoes (0.2–1.8 pg), that presumably contain many genomic parasites, have almost no methylation, whereas sea urchins, with a similar genome size range (0.67–1.9 pg), have significant levels of DNA methylation.

Within the Arthropoda the situation is complex, with variable methylation levels in different groups. Again, the level of methylation is not correlated with genome size. Within the Diptera, large-genome organisms such as *Musca* have no detectable methylation, whereas the relatively small genome of the mosquito *Anopheles* is methylated, albeit at a low level. A similar situation is found in crustaceans, where the *Penaeus* 2.4-pg genome is methylated but the *Artemia* (1.5–3.0 pg) genome is not. As table 2 and figure 1 show, there is no overall trend relating genome size and DNA methylation levels either within or between groups. A wide variation in genome size is seen both in methylated groups (e.g., molluscs and annelids), and in groups which seem to be nonmethylated (e.g., dipterans and nematodes). The data thus provide no evidence that variation in genome size within groups is associated with variation in methylation level.

One of the problems with the genome defense hypothesis is that it is difficult to see why organisms that lack or have little methylation do not need to silence genomic parasites. The small, methylation-free *Dro-*

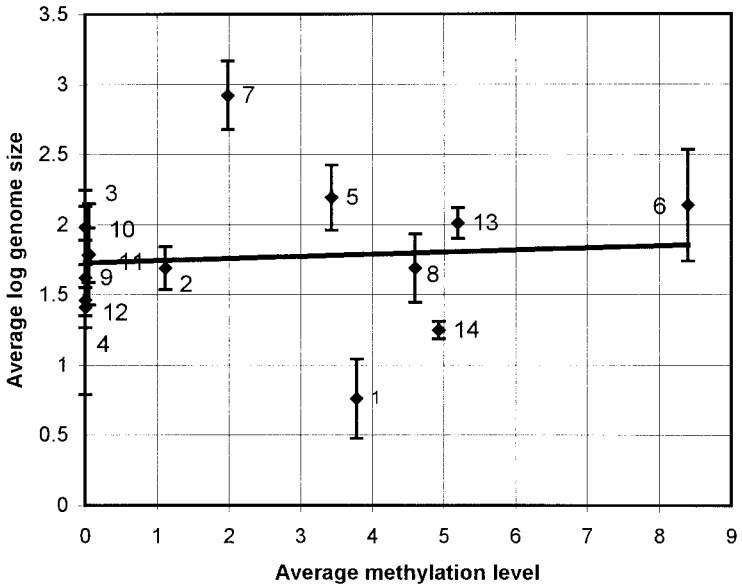

FIG. 1.—Relationship between genome size and methylation levels in invertebrate groups. 1, Porifera; 2, Cnidaria; 3, Platyhelminthes; 4, Nematoda; 5, Mollusca; 6, Annelida; 7, Orthoptera; 8, Homoptera; 9, Coleoptera; 10, Diptera; 11, Lepidoptera; 12, Hymenoptera; 13, Echinodermata; 14, Urochordata. Bars indicate standard error values. The line fitted is the least-squares linear regression line, showing a very poor correlation ($r = 0.081$). A similar regression analysis using the data for individual species also yielded a low correlation coefficient ($r = 0.024$, data not shown).

sophila genome has a large number of transposable elements (10% of the genome) and these are the cause of most mutations (Rubin 1983). If anything, the potential hazard from transposable elements might be enhanced in small genomes; yet, as table 1 and figure 1 show, small genome size is not correlated with either the presence or absence of methylation. The lack of methylation in *Drosophila* and other animals has to be attributed to something other than the superfluity of the antiparasite function of methylation in organisms with small genomes.

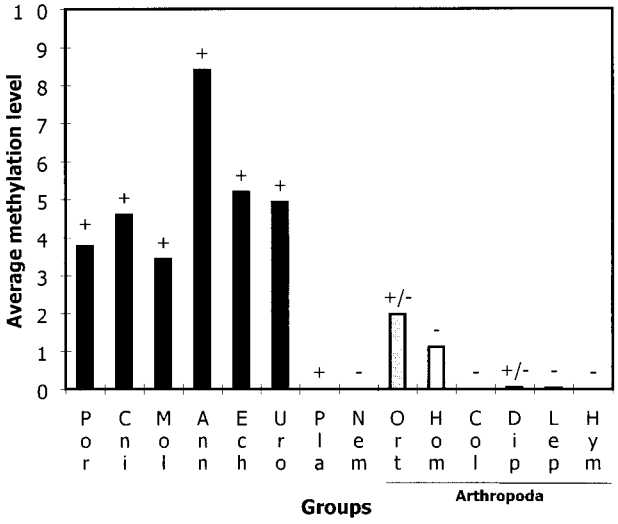

FIG. 2.—Methylation levels and cell turnover in invertebrate groups. Black bars: high cell turnover (+); white bars: little or no cell turnover (−); gray bars: some cell turnover (+/−). Por, Porifera; Cni, Cnidaria; Mol, Mollusca; Ann, Annelida; Ech, Echinodermata; Uro, Urochordata; Pla, Platyhelminthes; Ort, Orthoptera; Hom, Homoptera; Nem, Nematoda; Col, Coleoptera; Dip, Diptera; Lep, Lepidoptera; Hym, Hymenoptera.

Furthermore, as Bestor has stressed (Yoder, Walsh, and Bestor 1997), if the role of DNA methylation is to suppress genomic parasites, there should be a quantitative relationship between the percentage of methylation and the percentage of repetitive sequences. No such correlation can be inferred from the data on repetitive sequences in invertebrate genomes (table 1). For example, in several cases, there is a relatively high percentage of repetitive sequences, but the level of methylation is very low or nondetectable. This is the case in certain mosquitoes (Black and Rai 1988; Nayak et al. 1991; Warren and Crampton 1991), in *Drosophila virilis* (Petitpierre, Gatewood, and Schmid 1988), in *Tribolium* (Brown et al. 1990), in *Musca domestica* (Crain, Davidson, and Britten 1976), in *Bombyx mori* (Gage 1974), and in *Artemia* (Lecher, Defaye, and Noel 1995). In general, there is good correlation between the number of repetitive sequences within a taxon and total genome size (Schmidtke and Epplen 1980; Rao and Rai 1987; Black and Rai 1988). Table 1 and figure 1 show no correlation between the percentage of DNA methylation and genome size within and between groups. Therefore, the strong version of the defense hypothesis, claiming that the role of DNA methylation in nonvertebrates is limited to genome defense, is refuted.

Table 1 and figure 2 show that, as expected from the hypothesis that methylation is important in cell memory, there is a good correlation between DNA methylation and cell turnover in eight out of nine phyla examined. The groups having a significant amount of cell turnover between consecutive meioses include those with regulative development, those able to regenerate, and those

that reproduce asexually by budding, fission, or ameiotic parthenogenesis. DNA methylation has been found in sponges, cnidarians, molluscs, annelids, some crustaceans and some insects, echinoderms, and chordates; all have moderate or high levels of cell turnover. In contrast, nematodes, some crustaceans, and most of the insects studied have low cell turnover and low or undetectable DNA methylation. The insects that do have appreciable levels of 5-methyl cytosine (5-mC) usually also have significant levels of cell turnover in the adult. For example, in the locust *Locusta migratoria,* cells in the midgut and ceca divide during adult life, and there is a low level of DNA methylation.

The new data obtained in the present study agree with the general pattern seen in earlier work. For DNA of the annelid *A. caliginosa trapezoides,* about 13% of CpG sequences were methylated, and for that of the crustacean *P. semisulcatus,* about 4.5% were methylated. The relatively high level of methylation found in annelids is consistent with the developmental hypothesis, since annelids have extensive cell turnover and regenerative abilities. The low level of DNA methylation found in the crustacean *Penaeus* was detected using the very sensitive modified nearest-neighbor analysis. A study of a different crustacean, the crab *Cancer pagurus,* failed to detect DNA methylation (Bird and Taggart 1980). We attribute the difference to the different methods used: Bird and Taggart (1980) used the restriction pattern of the enzymes *Hpa*II and *Msp*I, a method known to be less sensitive than that used in this study. However, it is clear that a methylated genome is not characteristic of all crustaceans, since no methylation was found using methods sensitive enough to detect $<0.01\%$ of total cytosines in the brine shrimp *Artemia* (Warner and Bagshaw 1984). Since crustaceans differ widely in their modes of development, regenerative abilities, and lengths of life, differences between species are consistent with the developmental hypothesis.

We found no evidence of DNA methylation in the parasitic platyhelminth *S. mansoni.* This confirms earlier investigations using the same and different species, other developmental stages, and different techniques (Simpson, Sher, and McCutchan 1982; Cox, Phares, and Schmidt 1990; Sermswan, Mongkolsuk, and Sirisinha 1991). Since the complicated life cycles of flukes include extensive ameiotic multiplication during the immature stages, the result seems inconsistent with the developmental hypothesis. In addition to the parasitic platyhelminthes, two other groups, coccids and aphids (Homoptera), seem to be exceptions to the general rule relating methylation to cell turnover. However, scrutiny of the characteristics of these groups suggest that they are, in fact, exceptions that prove the rule.

Adult coccids have no cell turnover, yet their DNA is methylated. The explanation of this may be that all the species examined have the lecanoid type of sex determination system: maleness is the result of the heterochromatinization during early embryonic development of the paternally derived chromosome set (Sager and Kitchin 1975). The system depends on the maintenance and transmission in the male of the heterochromatic conformation of whole chromosomes. Methylation levels are higher in males than in females (Scarbrough, Hattman, and Nur 1984), suggesting that DNA methylation may be important in the marking and heterochromatinization of the paternal chromosomes. A similar relationship between heterochromatinization and methylation has been found in other invertebrate genomes, including molluscs (Martinez-Lage, Gonzales-Tizon, and Mendez 1994) and Orthoptera (Sentis et al. 1990; Suja et al. 1993). The orthopteran *Gryllotalpa fossor,* which is unique among the nonmammalian organisms in that it shows facultative heterochromatinization of the female somatic X chromosome, has significant CpG methylation, although with no apparent differences between females and males (Sarkar et al. 1992). The correlation between facultative heterochromatinization and methylation in the inactive X chromosome of eutherian female mammals is well known (Holliday 1990), and methylation seems to play an essential role in domain- and chromosome-level regulation of gene activity in mammals. If methylation has been retained in lecanoid coccids because of its role in chromosome silencing in males, it would be interesting to investigate the diaspidid coccids for which sex determination involves the elimination of paternal chromosomes rather than their heterochromatinization. It is possible that chromosome loss, which is an extremely strong cell memory mechanism, eliminates the need for facultative heterochromatinization and hence for methylation.

Adult aphids are extremely small and have no cell turnover, yet their DNA is methylated. Aphids, however, have many cycles of ameiotic parthenogenesis between meioses, and such an extended clonal somatic life requires efficient EISs. It has been suggested that this is why methylation has been retained in this group (Jablonka and Lamb 1995, pp. 208–213). No methylation is expected or found in parthenogens like *Artemia* or *Drosophila mangabeiri*, which have meiotic parthenogenesis (Maynard Smith 1986). It would be interesting to look for DNA methylation in the bdelloid rotifers, which are obligatory ameiotic parthenogens. Although they have no cell turnover, they have indefinite clonal life span, and therefore would be expected to have efficient EISs. The presence of selectable variations in genetically pure clones of bdelloid rotifers suggests that there are indeed efficient EISs in these animals (C. Ricci, personal communication).

The absence of methylation in parasitic flatworms is puzzling, since flukes have life cycles which include extensive phases of asexual multiplication in the preadult stages, and tapeworms form new segments by budding. However, as discussed by Musto et al. (1994), the lack of DNA methylation may be of relatively recent origin. CpG dinucleotides are underrepresented in fluke genomes, whereas TpG and CpA levels are higher than expected. These biases are similar to those found in methylated genomes, for which spontaneous deamination of 5-mC reduces the amount of CpG and increases the amount of TpG and the complementary CpA dinucleotides. Musto et al. (1994) also found that the distribution of dinucleotide biases in fluke genomes is similar

to that typical of methylated genomes: mammalian housekeeping genes have methylation-free islands on their 5′ control regions and, hence, no CpG bias in these regions, and, correspondingly, there was no bias in 5′ flanking regions of the fluke genes studied. This suggests that the recent ancestors of the flukes probably had methylated genomes. If the loss is recent and related to the parasitic mode of life, studies of DNA methylation in free-living platyhelminthes would be informative. Some of these reproduce asexually by budding, and planarians are well known for their ability to regenerate missing parts, so we predict that free-living platyhelminthes will be found to have methylated genomes.

With the exception of the parasitic platyhelminthes, all the existing data, from 8 animal phyla and 80 different species, support the hypothesis that DNA methylation is correlated with the mode of development, usually with the amount of cell turnover between consecutive meioses. In groups with high cell turnover levels, where a reliable long-term cell memory system is essential, the level of DNA methylation is significant, since the advantages outweigh the deleterious mutational effects. In groups for which development is highly mosaic with a small number of cell divisions between consecutive meioses, the benefits of DNA methylation as an EIS are small, and methylation has usually been lost (Jablonka and Lamb 1995, pp. 208–213). In such organisms, the mutational burden of methylation may be particularly high because there would be little opportunity for replication-related repair (Griffin and Karran 1993) or for the purifying somatic selection that would eliminate mutated cells. In addition, if the statistical model of Otto and Walbot (1990) is correct, it may not be possible to establish stable methylation patterns with so few cell generations. Steady-state EISs and chromatin-marking EISs based on protein marks (e.g., Pirrotta 1997) are likely to play a central role in cell memory in small, short-lived animals. These memory systems are stable enough for species with short life spans and limited numbers of cell divisions. A low level of methylation may still be important in developmental regulation, since the control of some genes could still involve methylation. As Holliday (1994) has pointed out, even though the techniques used would have detected 0.01% 5-mC in *C. elegans* (Simpson, Johnson, and Hammen 1986) and 0.003% 5-mC in *Drosophila* (Patel and Gopinathan 1987), there could still be about 3,500 undetected methylated cytosines in *C. elegans* and 2,000 in *Drosophila.* Cytosine methylation may have become restricted to a few essential sites in species with little adult cell turnover, rather than being completely lost. The presence of methyltransferase activity in sea urchins and coccids (sometimes correlated with developmental changes) (Devajyothi and Brahmachari 1989; Tosi et al. 1995) supports the conjecture that low-level methylation may still be important in gene regulation. On the other hand, a low level of methylation in species with a large genome and many genomic parasites is unlikely to be an effective genome-defense mechanism.

Bird (1995) claimed that only in vertebrates is DNA methylation associated with the control of endogenous gene expression. If this is so, it is difficult to see why endogenous genes in urochordates are methylated (Tweedie et al. 1997), and why methylation is associated with the mode of development in invertebrates. Although in this paper we have considered the role of DNA methylation only in multicellular invertebrates, there are sufficient data linking methylation changes to changes in gene expression and development in a variety of organisms to make it seem likely that the role of DNA methylation in controlling gene activity is very ancient, probably preceding the origins of multicellularity. Fungi, protists, and even some prokaryotes show developmental changes that point to a general involvement of DNA methylation in developmental regulation (Jablonka and Lamb 1995, pp. 208–213; Jablonka and Regev 1995). For example, starvation-induced differentiation of the slime mold *Physarum polycephalum* is accompanied by DNA methylation in specific genes associated with differentiation (Fronk and Magiera 1994; Magiera and Fronk 1994), and developmental changes in the level of methylated cytosine have been observed in several species of fungi, among them *Candida albicans* (Russell et al. 1987*b*), *Neurospora crassa* (Russell et al. 1987*a*), *Phymatotrichum omnivorum* (Jupe, Magill, and Magill 1986), and *Phycomyces blakesleeanus* (Antequera et al. 1985). As with invertebrates, the distribution of DNA methylation in lower eukaryotes is extremely variable. In addition to its postulated role in the developmental regulation of gene expression, in certain species methylation is also an important antiparasitic device (Selker 1997). Lachmann and Jablonka (1996) have argued that reliable EISs, such as the DNA methylation EIS, also have an important role in unicellular organisms that live in fluctuating environments. Clearly, unravelling the roles of DNA methylation in lower eukaryotes will be difficult, and requires knowledge of life history strategies, genome size, genomic parasite load, and the level of methylation in different species. However, the evidence just outlined suggests that, at least in some species of fungi, methylation is associated with the regulation of gene activity. Methylation is also involved in gene regulation in plants (Finnegan, Brettell, and Dennis 1993), so this function is taxonomically widespread.

There is some indirect evidence supporting a developmental role for DNA methylation in multicellular invertebrates. In the sea urchin *Strongylocentrotus purpuratus*, the methylation status of gene families and single genes changes throughout development (Fronk, Tank, and Langmore 1992). Changes have been found in the specific activity of methyltransferase during the development of another sea urchin, *Sphaerechinus granularis* (Tosi et al. 1995). In the potato aphid, *Myzus persicae,* loss of insecticide resistance is associated with changes in the methylation of amplified esterase genes (Field et al. 1989). Changes in DNA methylation occur during development in the nucleolar organizer of the B chromosome of the grasshopper *Eyprepocnemis plorans* (Lopez-Leon, Cabrero, and Camacho 1995), and tissue- and stage-specific differences in the 5-mC level have been detected during development of the silkworm moth *Bombyx mori* (Patel and Gopinathan 1987).

Although the data are limited at present, they indicate that DNA methylation is involved in developmental regulation in many nonvertebrates. The wide taxonomic distribution of developmentally correlated changes in methylation suggests that the role of methylation as a regulator of gene expression is an evolutionary old function, which in some groups has also assumed a role as a cell memory system. Unfortunately, the methylated part of invertebrate genomes is not yet well characterized, and studies of patterns of methylation and the way they change will be necessary to determine the particular role played by methylation in each group. The compartmentalization of methylated DNA found in plants and in urochordates (Tweedie et al. 1997) is particularly intriguing. Although compartmentalization is clearly compatible with the developmental function of methylation in plants (Richards 1997) and seems compatible with regulatory function in some invertebrate groups, it is likely that the pattern of DNA methylation is also related to the overall structural organization of the chromosome and genome. It must be remembered that even in vertebrates, for which there is a large body of evidence supporting a role for DNA methylation in gene regulation during development, definitive evidence for a causal relationship is still elusive (Bestor and Tycko 1996; Yoder, Walsh, and Bestor 1997). The identification of DNA sequences and chromatin cues that direct DNA methylation will help to identify the function of methylation in both vertebrates and invertebrates (Bestor and Tycko 1996).

Bird (1995) postulated that the transition from invertebrates to vertebrates was a result of a change in the role of DNA methylation from that of a general repressor of genomic parasites and repetitive sequences to that of a regulator of protein-encoding endogenous genes. Our discussion suggests that the role of DNA methylation in gene control is much older. However, the role of DNA methylation has certainly evolved during evolutionary history, and the relative importance of its different functions may have changed for different groups. It should be stressed that the different roles of DNA methylation are probably not mutually exclusive, and during evolution, methylation may have been recruited for various functions, including genome defense and cell memory.

Our survey of DNA methylation shows that there is a striking correlation between the level of methylated cytosine in DNA and an organism's mode of development. This correlation suggests that DNA methylation is an important EIS in the invertebrate groups in which it has been retained. DNA methylation emerges as a key, although not unique, cell memory mechanism accompanying the evolution of development of complex multicellular organisms. The evolutionary loss, retention, and elaboration of DNA methylation are part of the evolution of development.

Acknowledgments

We would like to thank Aharon Razin and Agnes Yeivin for providing the conditions and helping to perform the experiments. We would also like to thank Drs. Ester Lubsenz, Rivka Hisdai, and Naomi Zack for their help in obtaining tissue and DNA samples and Dr. Emilia Rota for classifying the annelid specimens.

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DAN GRAUR, reviewing editor

Accepted March 24, 1998