Variable patterns of total DNA and rDNA methylation in animals

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Received 5 February 1980

ABSTRACT

Restriction endonucleases were used to determine the degree of methylation at the sequences CCGG and GCGC in a wide range of animal DNAs. Both total DNA methylation and ribosomal DNA methylation were studied. Whole DNA methylation was undetectable in arthropods, fractional in other invertebrate phyla, and high in the vertebrates. Ribosomal DNA was predominantly unmethylated in all animals except fish and amphibia, where it was heavily methylated. We discuss the evolutionary and functional implications of these results, and suggest that the large differences between genome types are the result of evolutionary changes in the relative size of heavily methylated and unmethylated compartments.

INTRODUCTION

5-Methylcytosine (5mC) is present as a rare base in the DNA from many animals (1-4). It is known to arise by direct methylation of C in the DNA polymer following DNA replication (5-8), but its function in the cell remains unknown. Recent interest in eukaryotic DNA methylation has been stimulated by the discovery of prokaryotic restriction/modification systems. On a theoretical level, restriction/modification systems show that methylated bases such as 5mC can determine whether or not a protein interacts productively with its recognition sequence on the DNA (9,10), and therefore raise the possibility that DNA methylation in animals is also involved in switching protein-DNA interactions (11,12). On a practical level, restriction endonucleases are themselves valuable tools for mapping 5mC in DNA. The usefulness of restriction enzymes as probes for 5mC relies on the marked preference of animal DNA methylation for the sequence mCpG and on the inability of certain restriction enzymes to cleave if mCpG occurs within their recognition sequence (10, 15,16). Taking advantage of these properties, it has been shown firstly, that 5mC is not distributed over the DNA at random, but conforms to a pattern (15,17); secondly, that this

pattern can vary in different tissues when specific sequences are examined (18-21); and thirdly, that the pattern of DNA methylation appears to be replicated following S-phase, and is thus transmitted to the progeny cells (11, 12, 22).

These recent experiments have been conducted against a background of uncertainty about variation in DNA methylation within the animal kingdom as a whole. It has been known for some time that levels of 5-methylcytosine in different animal groups are not constant, since base composition analyses showed that in insects the level is low (less than 0.2 moles per cent of bases) compared to other animals (0.5-2.8 moles per cent of bases) (1-4).

More recently this variability has been emphasised by using the isoschizomers Hpa II and Msp I. Hpa II cleaves CCGG (23) but not CmCGG (10), whereas Msp I cleaves both sequences (18), so by comparing Hpa II and Msp I digests of a particular DNA it is possible to assay for methylation of this subset of CpGs (i.e. those preceded by C and followed by G). Experiments of this kind showed that in four species of insect, Hpa II and Msp I digests of total DNA were indistinguishable, suggesting that methylated Hpa II sites are very rare (25), whilst in the mouse more than 70% of Hpa II sites were in the methylated form (24). Between these extremes, the sea urchin Echinus esculentus has one third of its DNA as heavily methylated sequence blocks, and the remaining two thirds not detectably methylated (17).

Variable DNA methylation is also evident at the level of specific DNA sequences. The 18S and 28S ribosomal RNA genes (rDNA) of four insect species (25), and of Echinus (17) are not detectably methylated at Hpa II or Hha I (GCGC) (26) sites whilst chromosomal rDNA in Xenopus laevis is heavily methylated (15,27). Similarly, 5S RNA genes in Echinus are not detectably methylated (17), though the same genes in Xenopus are heavily methylated (28,29).

In an attempt to discover the spectrum of DNA methylated patterns, we have examined DNA from a wide variety of animals using Hpa II/Msp I and other mCpG-sensitive restriction endonucleases. We analysed both total DNA patterns visualised on agarose gels, and rDNA patterns visualised by blotting and hybridisation (30). Our results have allowed us to categorise the wide range of methylation levels amongst animals.

MATERIALS AND METHODS

DNA sources and preparation

Drosophila melanogaster DNA was a gift from D. Finnegan (Department of

Molecular Biology, Edinburgh University); Paracentrotus and Strongylocentrotus DNAs were gifts from K. Gross (present address Roswell Park Memorial Institute, Buffalo); Triturus cristatus DNA was a gift from D. Malcolm (St. Andrew's University); trout, snake, chick and quail DNAs were gifts from K. Jones and I. Purdom (Department of Genetics, Edinburgh University); human placental DNA was a gift from H. Cooke (this laboratory); sheep, pig and rat somatic DNAs were a gift from A. Mitchell (MRC Clinical and Population Cytogenetics Unit, Edinburgh). Calf thymus DNA was obtained from Sigma Chemicals. Marine invertebrates were supplied by the Marine Biology Laboratory, Millport, Scotland. DNA was prepared as described previously (12) except in the case of pig sperm. Diluted boar sperm was a gift from the Meat and Livestock Commission (Selby, North Yorkshire, England). Sperm were recovered by centrifugation, washed in SSC (0.15M NaCl, 0.015M sodium citrate) and resuspended in 10 ml of 7M urea, 2% SDS, 0.34M NaCl, 1mM EDTA, 10mM Tris (pH 8) (40). 10mg of dithiothreitol were added, and the cells allowed to lyse for 15 minutes at room temperature. Phenol (1 volume) was added, followed by chloroform-isoamyl alcohol (1 volume) and the mixture was shaken. The supernatant was re-extracted with phenol-chloroform and precipitated with ethanol. DNA was further purified by RNase and pronase digestion, phenol and chloroform extraction, and finally, ethanol precipitation.

Restriction endonuclease digestions

Restriction endonucleases Hpa II, Hha I, EcoR I and Hind III were prepared according to published procedures by B. Smith (this laboratory). Msp I was obtained from New England Biolabs (USA). Digestion and gel electrophoresis methods have been described previously (17).

Ribosomal DNA hybridization

Gels were blotted and hybridized (30) as described previously. The rDNA probe for hybridization (pXlr 101) was a gift from Ronald Reeder (Hutchinson Institute, Seattle, USA). The plasmid is a hybrid between one Xenopus laevis rDNA repeat unit cleaved at Hind III sites, and plasmid pMB 9.

RESULTS

Variation in Whole DNA Methylation Patterns

DNA samples from each animal were digested separately with Msp I, Hpa II, Hha I, or Hpa II + Hha I and then fractionated on 1.2% agarose gels. Figure 1 shows ethidium bromide staining patterns for the fruit fly (Drosophila melanogaster), the sea urchin (Echinus esculentus), the mouse

Figure 1. Examples of the three types of whole DNA methylation patterns in animals as revealed by Hpa II and Hha I digestion. (a) *Drosophila melanogaster*; (b) *Echinus esculentus*; (c) *Mus musculus*; (d) *Xenopus laevis*. The marker on each 1.2% agarose gel was bacteriophage lambda DNA digested with Eco RI and Hind III. The numbers refer to fragment size in kilobase pairs. Contaminating RNA is visible at the bottom of each track in panel C (*Mus*).

(*Mus musculus*) and the African clawed frog (*Xenopus laevis*). These patterns cover the range of total DNA patterns observed among 25 animal species.

Drosophila DNA (Figure 1a) gave indistinguishable banding patterns after Hpa II or Msp I digestion, implying very low levels of methylation at these sites. Like Hpa II, Hha I digested the DNA to a low average molecular weight, suggesting that Hha I site methylation is also minimal in *Drosophila*. *Echinus* DNA methylation has been discussed in detail elsewhere (17). About

one third of the DNA is relatively resistant to Hpa II and Hha I owing to methylation of these sequences, and the remaining two thirds is not detectably methylated. Mouse DNA appeared markedly more methylated than *Echinus* DNA since it was poorly digested by Hpa II and Hha I compared to Msp I (see also reference 24), though the presence of discrete bands indicated that periodic unmethylated Hpa II and Hha I sites are present in the genome. *Xenopus* DNA also appeared highly resistant to both Hpa II and Hha I, though Msp I digested the DNA normally. We conclude that *Xenopus* DNA and mouse DNA are both methylated to a high degree.

Each of the three methylation patterns in Figure 1 was typical of a group of animals. Thus all the insects studied by others (25,33) and a crab (*Cancer pagurus*) resembled *Drosophila melanogaster* in showing no evidence of methylation, while all the non-arthropod invertebrates (a coelenterate, a mollusc, eight echinoderms and a chordate) conformed to the partially methylated *Echinus* pattern (Figure 2). Similarly, thirteen vertebrates (seven mammals, two birds, a reptile, two amphibians and a fish) all displayed high levels of DNA methylation similar to the mouse and *Xenopus laevis*. Table 1 groups the total DNA results according to the above categories: insect-type (no detectable methylation), echinoderm-type (methylated and unmethylated fractions) and vertebrate-type (heavily methylated), respectively. Categorisation of the various total DNA patterns is independent of which tissue was the source of DNA (see Table 1). For example, mouse DNA was highly resistant to Hpa II and Hha I regardless of whether brain, liver or testis were examined, and *Echinus* DNA gave a similar pattern whether derived from germ cells, embryonic cell or adult

Figure 2. Methylated and unmethylated fractions of the genome in three invertebrate phyla: a coelenterate (sea anemone), a mollusc (mussel) and an echinoderm (sea urchin). Genomes of this type are referred to as "echinoderm-type" in table 1. About 2 μ g of each DNA were digested separately with Hpa II + Hha I (left-hand track of each pair) and Hpa II + Hha I + Msp I (right-hand track). A fraction of each DNA is seen to be resistant to Hpa II + Hha I, but is digested by Msp I.

Hpa II/Hha I Methylation Patterns in Animal DNAs

Methylation pattern	Phylum	Species	Common name	Tissue	rDNA [#]
INSECT-TYPE	ARTHROPODA (INSECTA)	<u>Drosophila melanogaster</u> <u>Drosophila virilis</u> *	fruit-fly fruit-fly	adult adult embryo	≡ ≡ ≡*
	(CRUSTACEA)	<u>Psithyrus sp.</u> * <u>Musca domestica</u> * <u>Sarcophaga bullata</u> * <u>Aedes albopictus</u> ** <u>Cancer pagurus</u>	bee house-fly flesh-fly mosquito edible crab	adult adult adult adult cultured cells mixed	≡ ≡ ≡* ≡* not measured ≡
ECHINODERM-TYPE	COELENTERATA MOLLUSCA ECHINODERMATA	<u>Metridium senile</u> <u>Mytilus edulis</u> <u>Echinus esculentus</u>	sea anemone common mussel sea urchin	whole testis egg sperm embryo intestine tubefoot sperm	≡ ≡ ≡ ≡ ≡ ≡ ≡
		<u>Paracentrotus lividus</u> <u>Strongylocentrotus purpuratus</u> <u>Psammechinus miliaris</u> <u>Asterias rubens</u> <u>Thyone fusus</u> <u>Ophiopholis</u> <u>Ophiothrix fragilis</u> <u>Ciona intestinalis</u>	sea urchin sea urchin sea urchin starfish sea cucumber brittle-star brittle-star sea-squirt	sperm sperm sperm intestine sperm sperm sperm mixed	≡ ≡ ≡ ≡ ≡ ≡ ≡ ≡
CHORDATA (TUNICATA)					
VERTEBRATE-TYPE	(VERTEBRATA) Osteichthyes Amphibia	<u>Salmo trutta</u> <u>Xenopus laevis</u>	trout clawed frog	testis liver brain sperm blood	m+ m+ m+ m+ m+
	Reptilia	<u>Triturus cristatus</u> <u>Elaphe radiata</u>	crested newt Indian cobra (snake)	liver liver	m- (m+?) m- (m+?)
	Aves	<u>Gallus domesticus</u> <u>Coturnix coturnix</u>	chicken quail	liver liver	m- (m+?) m- (m+?)
	Mammalia	<u>Homo sapiens</u> <u>Mus musculus</u> <u>Rattus norvegicus</u> <u>Oryctolagus cuniculus</u> <u>Ovis aries</u> <u>Sus domesticus</u> <u>Bos taurus</u>	man mouse rat rabbit sheep pig calf	placenta liver brain testis liver liver liver sperm thymus	m- (m+?) m- (m+?) m- (m+?) m- (m+?) m- (m+?) m- (m+?) m- (m+?) m- (m+?) m- (m+?)

Table 1. A summary of total DNA and ribosomal DNA methylation data obtained using restriction endonucleases. The "insect-type" genomes resemble Drosophila melanogaster (Figure 1a) in having no detectable Hpa II or Hha I site methylation. "Echinoderm-type" genomes resemble Echinus esculentus (Figures 1b and 2) by displaying both methylated and unmethylated fractions in the total DNA. The "vertebrate-type" genomes resemble heavily methylated mouse and Xenopus laevis genomes (Figures 1c and d).

* data from Rae and Steele, 1979 (25).

** data from Adams et al, 1979 (33).

m- = unmethylated; m+ = methylated (17).

somatic cells (17).

Ribosomal DNA Methylation

The 28S and 18S ribosomal RNA gene sequence (rDNA) has been highly conserved during evolution (34-36), and is therefore an ideal probe for specific sequence methylation in a wide range of animals. Each rDNA repeat unit is tandemly repeated many times at one or more locations in the genome. We have assayed for rDNA methylation at CCGG by comparing the Hpa II and Msp I digestion patterns of sequences complementary to a *Xenopus* rDNA plasmid. Paired Hpa II/Msp I digests of each DNA sample were blotted onto a nitrocellulose filter (30) and hybridised to p³²-labelled plasmid DNA. The results show that in all the invertebrates tested (i.e. insect-type and echinoderm-type), Msp I and Hpa II rDNA digestion patterns are indistinguishable (Figure 3a). Ribosomal DNA from these organisms is also extensively digested by Hha I and Ava I (data not shown), reinforcing the conclusion that rDNA in these invertebrates is primarily unmethylated.

The picture is more complicated among vertebrates. There are two types of pattern, one found in a reptile, two birds and seven mammals, and the other in a fish and two amphibians. Reptiles, birds and mammals gave the complete Msp I pattern in all Hpa II tracks (Figures 3b and c), showing that some rDNA repeat units are not methylated at CCGG. Hha I also generated an apparently complete digestion pattern of low molecular weight fragments (data not shown). Both Hpa II and Hha I, however, gave indications that some sequences complementary to the rDNA probe are methylated. Firstly, the Msp I pattern was invariably more intense than the equivalent Hpa II bands, implying that not all rDNA was susceptible to Hpa II. Secondly, both Hpa II and Hha I digested tracks often displayed some high molecular weight sequences complementary to *Xenopus* rDNA. In addition, our current work (in progress) has provided evidence that a small fraction of rDNA in the mouse is in a heavily methylated form. Nevertheless, the majority of rDNA in reptiles, birds and mammals appears to be unmethylated at Hpa II and Hha I sites. This is also true in germ cells of the pig. Sperm and liver were compared and were found to give identical levels of unmethylated rDNA (data not shown).

In contrast to all other genome types, rDNA from the amphibia and bony fish that we tested was heavily methylated at Hpa II (Figure 3b) and Hha I sites (data not shown). In *Xenopus* this has been known for some time (27) and it has been demonstrated that, at a finer level, undermethylated sites exist within the repeat unit (15). It should be noted that fish and

Figure 3. Variable Hpa II site methylation of animal ribosomal DNA. About 1 μ g of each DNA was digested separately with Msp I (left-hand slot of each pair) and Hpa II (right-hand slot). The DNA was fractionated on 2% agarose gels, transferred onto nitrocellulose filters and hybridised to the *Xenopus* rDNA plasmid pXlr 101. Hpa II - Msp I comparisons of rDNA from (a) a coelenterate (*Metridium senile*), a mollusc (*Mytilus edulis*), an echinoderm (*Echinus esculentus*) and an arthropod (*Drosophila melanogaster*); (b) left panel, a bony fish (*salmo trutta*), two amphibians (*Xenopus laevis* and *Triturus cristatus*); right panel, a reptile (*Elaphe radiata*), two birds (*Gallus domesticus* and *Coturnix coturnix*); (c) mammals (see Table 1). Owing to inefficient transfer of low molecular weight fragments (30), only bands of equal size can be compared. In particular, the high relative intensity of hybridisation near the top of some tracks in Figure 3b is exaggerated by the greater efficiency with which these fragments bind to nitrocellulose filters.

amphibia amplify rDNA in the germ cells (37-39), and in *Xenopus* at least, the amplified rDNA is not methylated (27,15). There are no well documented instances of rDNA amplification in reptiles, birds or mammals.

DISCUSSION

Evolutionary Changes in the Pattern of DNA Methylation

The comparative study described above shows that whole DNA methylation ranges from barely detectable levels in insects, through intermediate levels in non-arthropod invertebrates, to high levels in vertebrates. Among the species chosen, the patterns do not vary continuously between these extremes, but are grouped in three distinguishable types as outlined in Table 1 and Figure 1. Organisms that are closely related in evolution generally exhibit methylation patterns of similar type, so we feel justified in extrapolating from our limited studies to the larger taxonomic groups of which these animals are a part. Figure 4 illustrates the approximate phylogenetic relationship between the animal phyla and sub-phyla studied, and gives the inferred methylation status of each group.

The results do not tell us which methylation pattern is the ancestral one, but there are reasons for believing that the echinoderm-type pattern may have preceded both insect-type and vertebrate-type patterns. Each of the latter genome types has been found in only a single phylum, whereas the echinoderm-type pattern is widely distributed in phyla as distantly related as coelenterates and chordates (Figure 4). It is simpler to assume that this pattern was found in an ancestor common to all these phyla, than to assume that echinoderm-type methylation has arisen independently on several occasions. In the case of vertebrates, there is another good reason for

Figure 4. Accepted phylogenetic relationships between organisms with "vertebrate-type", "echinoderm-type" and "insect-type" methylation patterns. Vertebrates and tunicates are sub-phyla of the phylum chordate.

believing that the present pattern of methylation has evolved from the echinoderm-type pattern. Both echinoderms and tunicate chordates are thought to be closely related to the vertebrate lineage, and both have echinoderm-type methylation. Indeed, tunicates and vertebrates are members of the same phylum.

What is the basis of these differences in methylation? One simple possibility is that the average density of 5mC along each stretch of nuclear DNA has changed in evolution, arthropods acquiring a low density, and vertebrates a high density. A closer examination of organisms with echinoderm-type genomes suggests that this is not the case. In *Echinus*, the best studied example, the genome is divided between unmethylated (m-) and methylated (m+) "compartments", and specific sequences can be assigned to one or the other (17). Sequences belonging to (m-) appear to lack methylation at CpG, while we have argued that in (m+), most CpGs are methylated regardless of their flanking nucleotide sequences. Our evidence suggests that compartmentalisation of this kind also occurs in the other echinoderm-type organisms listed in Table 1. Thus, 5mC is not evenly distributed within these genomes, but is found in large clusters that are separated from one another by unmethylated DNA. Generalising from the echinoderm-type pattern, we suggest that 5mC compartments are also present in vertebrates, with the difference that (m+) now predominates over (m-) compared to the ancestral echinoderm-type pattern. The insect pattern can also be explained in this way by assuming that the (m-) compartment now makes up most of the nuclear DNA and (m+) is so small as to be undetectable.

Our studies of rDNA methylation support the idea that vertebrates contain both (m+) and (m-) compartments. Thus, in reptiles, birds and mammals, most of the rDNA is completely digested by Hpa II, and probably Hha I, although the DNA as a whole is poorly digested by both these enzymes. It appears, then, that unmethylated rDNA belongs to a small (m-) compartment in this group of vertebrates. The situation is different in bony fish and amphibia, where all chromosomal rDNA is (m+). Since rDNA is predominantly unmethylated in all other tested animals, this result suggests that during evolution expansion of the (m+) compartment can occur at the expense of (m-) by methylation of pre-existing (m-) sequences. In this context, it is significant that while we have detected both (m+) and (m-) rDNA among animals, we have not yet seen any sparsely methylated sequences which are intermediate between (m+) and (m-). This is in agreement with the hypothesis that methylation of long sequence tracts does not occur piecemeal, but is an

"all or none" phenomenon (17). Further work is needed to test this point.

At first sight Table 1 suggests that an increasing proportion of (m+) in the genome correlates with increasing genome size, since *Drosophila*, which has a relatively small genome, is poorly methylated compared to the much larger vertebrate genomes. Recent evidence shows that more DNA does not necessarily correlate with more methylation. The mosquito *Aedes* has a large genome, comparable in size with that of mammals, yet there is little detectable 5mC, and *Aedes* DNA appears typically insect-type after Hpa II digestion (32).

Finally, it is necessary to consider recent experiments showing that the pattern of specific sequence methylation can change between tissues of the same organism (19-21). A full discussion of these changes is outside the scope of this paper, except to observe that methylation of all sequences so far examined is at its highest level in sperm DNA. This is consistent with the view that germ cells exhibit the "ground state" of methylation in a species, and all somatic cells acquire their specific methylation patterns by loss of methyl groups relative to germ cell DNA. As a consequence of this, we think it preferable to define sequences as (m+) or (m-) according to their state of methylation in the germ cells, disregarding any subsequent changes in this pattern during differentiation of the soma. The (m+) and (m-) compartments in most echinoderm-type genomes conform to this definition since they have been established in sperm DNA (Table 1). Similarly, unmethylated rDNA in the pig, and probably therefore in other mammals, is (m-) by this criterion.

Functional Implications

The results described in this paper, together with those of Rae and Steele (25) show that methylation of Hpa II and Hha I sites is not an obligatory feature of animal DNA. This is most strikingly apparent when the same family of genes is compared in different species, since the ribosomal genes can be found either heavily methylated or unmethylated. The implication is that both methylated and unmethylated sequences can be transcribed normally. This is not, however, sufficient to discount the existence of a link between DNA methylation and transcription. There are now several systems in which a normally heavily methylated gene is seen to be less methylated in tissues where that gene is expressed (19-21). Also, in the sea urchin, all the genes tested so far - histone genes, ribosomal genes, 5S RNA genes (17) and actin genes (Southern, unpublished results) - are found in the unmethylated compartment of the gen-

one. Finally, despite the high level of methylation observed in Xenopus rDNA, there are, at a finer level, two undermethylated regions in the repeat unit, and it is conceivable that one, or both, of these may be required for transcription. The possibility remains open that "removal" of methyl groups is a pre-requisite for the transcription of any fully methylated sequence.

ACKNOWLEDGEMENTS

We are grateful to Ed Southern, Chris Bostock, Jim Dahlberg and Elsebet Lund for criticism of the manuscript. The work was supported by the Medical Research Council (London).

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