The sequence specificity of vertebrate DNA methylation

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ABSTRACT

The relative quantity of 5-methyl cytosine in vertebrate nuclear DNA shows species and tissue variation. To determine whether this is due to the action of species or cell specific DNA methylases the sequence specificity of the 5-methyl cytosine distribution in the DNA of a range of cells has been partially characterised. The pattern of methylation was found to be remarkably constant and indicates stringent evolutionary conservation of the characteristics of vertebrate DNA methylation.

INTRODUCTION

The DNA of both prokaryotes and eukaryotes has been shown to contain low levels of bases which arise by the enzymatic methylation of cytosine or adenine already incorporated into DNA.^{1,2} In prokaryotes methylation of DNA has been assigned a role in the phenomenon of restriction/modification,³ the function of the only methylated base to appear in the higher eukaryotes, 5-methyl cytosine, is as yet unknown. Current evidence suggests the 5-methyl cytosine (5 MeC) is not connected with maintenance of the stability of vertebrate DNA^{4,5,6} or DNA synthesis^{7,8,9,10}. Several lines of evidence have implicated DNA methylation in the control of cellular differentiation, (a) DNA from different tissues of the same animal has a differing content of 5-methyl cytosine^{11,15} (b) the 5-methyl cytosine content of sea urchin DNA rises during development¹⁶ (c) DNA of cultured hamster BHK-21/C13 cells transformed by polyoma virus contains almost double the quantity of 5 MeC found in normal cells.^{17,18} Rubery & Newton¹⁸ suggest that the increased content of 5-methyl cytosine in transformed BHK-21/C13 cells might be due to the presence of a new virus coded or virus induced

DNA methylase: more generally others^{19,20} have proposed that during the course of cellular differentiation there are present, in the vertebrate cell, a number of DNA methylases able to methylate previously unmethylated sites in DNA.

Bearing in mind the differing sequence specificities of the bacterial DNA methylases³ it might be expected that vertebrate DNA methylases would also show a range of sequence specificities. If this is the case variation of the DNA methylases present in virus transformed cells (or different tissues of one animal) should be rendered detectable by their differing nucleotide sequence specificities. We have examined the distribution of 5 MeC in "transformed" and "non-transformed" hamster and mouse cells and also compared two human cell lines of differing origin in an attempt to reveal the activities of novel DNA methylases in these cells.

MATERIALS AND METHODS

(i) Cells.

Hamster BHK-21/Cl3 cells²¹; Hamster BHK-21/PyY cells²²; Chang liver cells; HeLa cells²³; mouse L929 cells²⁴; mouse SVT2 cells; and a Xenopus laevis liver cell line (originally a gift from Dr. K. Jones, Edinburgh University) were used in this study. All cell lines were maintained as monolayers in Eagles' Minimal Essential Medium to which had been added 10% (v/v) calf serum²⁵. Frequent PPLO checks were carried out and fresh cultures were recovered from liquid nitrogen at two monthly intervals.

(ii) [¹⁴C] methyl labelling of DNA.

The conditions of labelling are based on techniques used to label rRNA²⁶. Cells were seeded into Roux bottles (5×10^6 cells in 50 ml of medium), after incubation overnight the medium was removed and the cell layer washed in warm medium containing no methionine or serum. Fresh medium (50 ml) containing 5% (v/v) freshly thawed dialysed calf serum was then added to the cells along with 125 μ Ci L[methyl-¹⁴C] methionine (5.8 mCi/mmol) and the cells allowed to grow for 48 hours in the presence of the isotope before harvesting.

(iii) [³H] methyl and ³²PO₄ labelling of DNA.

L-[methyl-³H] methionine can be obtained at a higher specific activity (5 Ci/mmol) than L-[methyl]¹⁴C methionine and was therefore used in many of these experiments (up to 75 µCi per Roux bottle), sufficient non-radioactive methionine being added to bring the total methionine concentration up to 50% of the normal level. As ³H was not suitable for autoradiographic detection DNA was labelled in some experiments using a combination of L-[methyl-³H] methionine and ³²PO₄. The procedure adopted was essentially the same as (ii) except that 100 µCi ³²PO₄ (carrier free) was added to the medium at the same time as the radioactive methionine (the phosphate concentration of the medium was not modified).

(iv) Cell Fractionation and DNA purification.

After exposure to the appropriate isotopes, cells were washed once in ice-cold BSS²⁷ and then scraped off the surface of the Roux bottle into 10 ml of ice-cold BSS. Nuclei were prepared using suitable dilutions of reticulocyte standard buffer (RSB) to disrupt the outer cell membrane²⁸. DNA was prepared from nuclei as described elsewhere²⁹.

(v) "Fingerprinting" of DNA.

DNA was depurinated and "fingerprinted" using electrophoresis at pH 3.5 in the 1st dimension and homochromatography (5% homomixC) in the second dimension as described elsewhere³⁰. The use of ³²PO₄ to label DNA as well as L-[methyl-³H] methionine enabled checks to be made for preferential loss of oligonucleotides in either the preparation of the depurination products³¹ or during transfer between the first and second dimension of the "fingerprinting" procedure. No preferential loss of any of the oligonucleotides could be detected.

The use of ³²PO₄ label also allowed the detection of methylated oligonucleotides by autoradiography, in a much shorter time than would be the case if only L-[methyl-C¹⁴] labelled DNA was used.

(vi) Autoradiography.

Kodirex film was used. For [^{14}C] methyl-labelled DNA the exposure time was 3 to 6 weeks, for [^3H] methyl and $^{32}\text{PO}_4$ labelled DNA the exposure time was approximately one week.

(vii) Scintillation Counting.

Spots were excised from the DEAE cellulose thin layer plates after reference to their autoradiographs, the spots were placed in scintillation vials and radioactive material solubilised by addition of 0.5 ml hyamine hydroxide (1 M solution in methanol) and incubation for 15 min at 60°C . Radioactivity was estimated by use of a scintillation counter, allowance being made for 'spillover' between counting channels in the case of ^3H and $^{32}\text{PO}_4$ labelled experiments.

(viii) Calculation of Frequency of Oligonucleotides.

The relative frequency of the oligonucleotides appearing in a fingerprint of $^{32}\text{PO}_4$ labelled DNA can be calculated once allowance has been made for the 3' terminal $^{32}\text{PO}_4$ group. Relative frequencies of pyrimidine isostichs and isomers within isostichs were as expected³²⁻³⁵. (Base compositions were determined by inspection of the relative mobilities of the oligonucleotide in both dimensions.³⁰)

(ix) Calculation of Frequency of Cytosine in each Oligonucleotide.

The relative frequency of occurrence of cytosine in each oligonucleotide may be calculated as follows: $^{32}\text{PO}_4$ labelled dCMP per oligonucleotide = $\frac{N_{\text{cyt}}}{N_{\text{T}}}$

N_{cyt} = No. of cytosine bases (determined from base composition of the oligonucleotide)

N_{T} = Total no. bases (determined from base composition of the oligonucleotide).

Values in the results have been expressed relative to the monopyrimidine as 100 as it is difficult to determine the total amount of $^{32}\text{PO}_4$ label on a fingerprint.

(x) Acid Hydrolysis of DNA.

Hydrolysis of the DNA and chromatography was as

described elsewhere. (see references 10 and 18 respectively).

(xi) Enzymic Digestion of DNA.

The conditions of DNase digestion and electrophoresis have been described³⁶, d5MeCMP and dCMP were resolved, using a solvent of isobutyric acid/NH₃, on Whatman No.1 paper.³⁷

RESULTS

(i) DNA Sequences Methylated in Virus-'transformed' Cells and in non-transformed Cells.

A means of producing a type of DNA 'fingerprint' is provided by chemically depurinating DNA to produce pyrimidine tracts which may then be resolved by base composition and length using a combination of electrophoresis and homochromatography³⁰. The presence of a novel DNA methylase in virus transformed cells might be revealed by differences in the distribution of 5-methyl cytosine in pyrimidine tracts of DNA from the 'normal' (BHK-21/Cl3) and polyoma virus 'transformed' (BHK-21/PyY) cell lines.

DNA labelled with L[methyl-¹⁴C] methionine alone (under such conditions that at least 95% of the [¹⁴C] label appeared in 5-methyl cytosine, Table I) was used in early experiments.

TABLE I
THE DISTRIBUTION OF 5-METHYL CYTOSINE IN
PYRIMIDINE TRACTS OF HAMSTER DNA

<u>OLIGONUCLEOTIDE*</u>	<u>CELL LINE</u>	
	<u>BHK-21/Cl3</u>	<u>BHK-21/PyY</u>
<u>BASE COMPOSITION</u>		
C	100	100
C ₂	23.1(7.0)	32.4(3.1)
C ₃	5.4(1.0)	7.3(4.1)
CT	22.8(5.8)	26.1(5.1)
C ₂ T	11.1(2.3)	14.0(2.5)
C ₃ T	4.6(0.8)	6.2(0.1)
CT ₂	5.4(2.2)	6.7(1.3)
C ₂ T ₂	5.2(1.2)	6.3(1.3)
CT ₃	1.8(0.7)	2.1(0.8)

DNA was labelled with L[methyl-¹⁴C] methionine and 'fingerprinted' as in Materials and Methods. The frequency of occurrence of 5-methyl cytosine in each oligonucleotide has been expressed relative to the amount of 5-methyl cytosine in the monopyrimidine (pCp) fraction as 100. The results are based on 3 fingerprints from a single cell culture in each case, the values in parentheses are standard deviations.

* Pyrimidine oligonucleotides defined by base composition e.g. CT₂ comprises pCpTpTp, pTpCpTp and pTpTpCp (the d, deoxy, prefix has been omitted).

Fig.1(a) shows an autoradiograph of a 'fingerprint' of hamster BHK-21/C13 cell nuclear DNA labelled with L(methyl- ^{14}C) methionine. Two points are noteworthy (i) while the most frequently methylated 'spot' corresponds to the monopyrimidine pCp, a considerable amount of label also appears in other oligonucleotides (ca. 50% of the total) (ii) there is no visible spot corresponding to [^{14}C] labelled pTp, pTpTp or pTpTpTpTp thus further demonstrating the specificity of the labelling procedure.

The areas of the DEAE cellulose thin layers corresponding to spots on the autoradiographs were excised and the amount of [^{14}C] label in each estimated as in Materials and Methods, the results are shown in table I.

Fig.1 Fingerprints of L(methyl- ^{14}C) methionine labelled DNA

Nuclear DNA, labelled with L(methyl- ^{14}C) methionine as described in Materials & Methods was depurinated, and 'fingerprinted' by electrophoresis at pH 3.5 (1st dimension) and homochromatography at 60°C (2nd dimension) as in Materials & Methods. (a) fingerprint of BHK-21 cell DNA; (b) fingerprint of HeLa cell DNA; (c) key to fingerprints illustrating the relationship between the composition of an oligonucleotide and its position on the thin-layer plate. Solid lines join nucleotide spots of similar T content and broken lines join those of similar C content as in Ling²⁰ and Y and B represent the position of coloured dye markers.

DNA labelled with L[methyl- ^{14}C] methionine was also prepared from BHK-21/PyY cell nuclei and fingerprinted in the same manner, the distribution of 5-methyl cytosine is also shown in table I.

The experiments were repeated using DNA labelled with L-[methyl- ^3H] methionine and $^{32}\text{PO}_4$ for the reasons stated in Materials and Methods and also because this second procedure allowed a direct comparison between the distribution of 5-methyl cytosine and cytosine in the same sample of DNA. The relative distributions of 5-methyl cytosine and cytosine are shown in Table II. The results for BHK-21/C13 and BHK-21/PyY DNA again show the distribution of 5-methyl cytosine to be very similar.

A similar set of experiments was performed in two mouse cell lines: L929 cells and the SV40 virus transformed SVT2 cell line. The distribution of 5-methyl cytosine and cytosine in the 'transformed' (SVT2) and 'non-transformed' (L929) cells are shown in Table III.

TABLE II
THE DISTRIBUTION OF 5-METHYL CYTOSINE IN
PYRIMIDINE TRACTS OF HAMSTER CELL DNA

OLIGONUCLEOTIDE BASE COMPOSITION	CELL LINE			
	BHK-21/C13		BHK-21/PyY	
	5-Methyl cytosine	Cytosine	5-Methyl cytosine	Cytosine
C	100	100	100	100
C ₂	28.4(6.0)	49.6(11.0)	31.7(4.9)	45.0(4.6)
C ₃	6.9(3.1)	22.7(7.8)	7.0(2.2)	17.0(2.7)
CT	25.6(3.1)	73.6(6.4)	25.0(9.3)	73.7(4.5)
C ₂ T	13.0(0.8)	39.1(6.2)	13.7(3.2)	47.6(5.1)
C ₃ T	7.0(4.7)	25.9(8.4)	7.3(1.6)	24.8(2.7)
CT ₂	8.4(2.4)	29.2(4.3)	6.6(2.0)	31.6(2.1)
C ₂ T ₂	7.5(3.1)	34.9(9.0)	6.9(1.4)	31.4(5.1)
C ₃ T ₂	2.8(1.8)	20.1(3.3)	3.3(0.7)	17.8(1.7)
CT ₃	2.6(0.4)	18.4(-)	1.6(1.2)	15.3(4.2)
T	1.3(1.3)		0.2(0.2)	

DNA was labelled with L[methyl- ^3H] methionine and $^{32}\text{PO}_4$ and fingerprinted as in Materials and Methods. The frequency of occurrence of 5-methyl cytosine or cytosine in each oligonucleotide has been expressed relative to the amount of 5-methyl cytosine or cytosine in the monopyrimidine fraction as 100. The values in parentheses are standard deviations based on results from 4 cultures of BHK-21/PyY cells and 3 cultures of BHK-21/C13 cells.

TABLE III
THE DISTRIBUTION OF 5-METHYL CYTOSINE IN
PYRIMIDINE TRACTS OF MOUSE CELL DNA

<u>OLIGONUCLEOTIDE</u> <u>BASE</u> <u>COMPOSITION</u>	<u>CELL LINE</u>		<u>SYT2</u>	
	<u>L929</u>			
	<u>5-Methyl</u> <u>cytosine</u>	<u>Cytosine</u>	<u>5-Methyl</u> <u>cytosine</u>	<u>Cytosine</u>
C	100	100	100	100
C ₂	14.8(3.3)	42.4(7.4)	19.2(3.6)	44.3(2.3)
C ₃	2.7(0.6)	12.5(2.1)	4.3(1.7)	14.4(3.3)
CT	16.5(3.0)	61.3(9.5)	21.7(3.7)	67.9(4.1)
C ₂ T	6.5(0.7)	38.0(5.3)	7.8(0.7)	42.5(1.4)
C ₃ T	3.0(0.5)	13.2(3.9)	4.3(1.2)	20.4(1.2)
CT ₂	4.6(1.8)	26.5(5.0)	4.6(0.2)	26.0(1.4)
C ₂ T ₂	3.3(1.0)	24.4(6.4)	3.5(0.4)	30.0(1.3)
C ₃ T ₂	1.9(0.9)	12.5(2.3)	1.6	14.0(1.8)
CT ₃	4.2(0.4)	12.9(1.4)	3.9(0.1)	11.4(1.0)
T	0.6(0.4)		<0.1	

³²P₀ DNA was labelled with L[methyl-³H] methionine and fingerprinted as in Materials and Methods. The frequency of occurrence of 5-methyl cytosine or cytosine in each oligonucleotide has been expressed relative to the amount of 5-methyl cytosine or cytosine in the monopyrimidine fraction as 100. The values in parentheses are standard deviations.

Comparison of the distribution of 5-methyl cytosine and cytosine (Tables II & III) shows the distribution of 5-methyl cytosine to be highly sequence specific (i.e. there are substantial differences in distribution of cytosine and 5-methyl cytosine and that this specific distribution is almost identical in the 'transformed' and 'non-transformed' cells of the same species. The general features of the distribution of 5-methyl cytosine are similar to previous reports^{39,40}. The maintenance of the pattern of methylation in 'transformed' cells is emphasised when one considers that the BHK-21/PyY cells contain at least 70% more 5-methyl cytosine than BHK-21/Cl3 cells,^{17,18} (Table VI), thus no one oligonucleotide in the 'transformed' cell DNA has accommodated the increased level of 5-methyl cytosine as might be expected if a novel DNA methylase of unusual sequence specificity was in operation.

(ii) EVOLUTIONARY CONSERVATION OF THE SEQUENCE SPECIFICITY OF DNA METHYLATION

In view of these observed similarities between the cells

TABLE IV
THE DISTRIBUTION OF 5-METHYL CYTOSINE IN
PYRIMIDINE TRACTS OF HUMAN CELL DNA

OLIGONUCLEOTIDE BASE COMPOSITION	CELL LINE	
	HeLa 5-Methyl cytosine	Chang 5-Methyl cytosine
C	100	100
C ₂	27.6(2.9)	27.7
C ₃	9.3(2.1)	8.2
CT	22.6(3.0)	23.0
C ₂ T	10.2(3.2)	10.0
C ₃ T	6.8(2.6)	6.5
CT ₂	5.3(1.6)	5.6
C ₂ T ₂	6.4(2.1)	8.4
C ₃ T ₂	2.1(0.7)	2.8
CT ₃	2.3(1.9)	1.8
T		

³²P₄ DNA was labelled with L[methyl-³H] methionine and ¹⁴C₄ (or in the case of 2 cultures of HeLa cells L[methyl-¹⁴C]₄ methionine alone) and fingerprinted as in Materials and Methods. The frequency of occurrence of 5-methyl cytosine in each oligonucleotide has been expressed relative to the amount of 5-methyl cytosine in the monopyrimidine as 100. Values in parentheses are standard deviations based on 5 HeLa cell cultures, the data for Chang liver cells are based on one cell culture only.

of two distinct rodent species, the scope of the investigation was extended to include the patterns of DNA methylation in two human cells (HeLa and Chang liver cells) (Table IV). A fingerprint of L[methyl-¹⁴C] methionine labelled HeLa DNA is shown in Figure 1(b), it can be seen that it bears a strong resemblance to that of the analogous fingerprint of BHK-21/C13 DNA. In fact it appears that the distribution of 5-methyl cytosine in three different mammals (man, mouse and hamster) is remarkably similar (Tables I to IV) and suggests that the features of the DNA sequence dictating methylation have been highly conserved. To investigate this suggestion the distribution of 5-methyl cytosine in a distantly related vertebrate i.e. the amphibian, *Xenopus laevis* was determined (Table V).

Tables I to V show the distribution of 5-methyl cytosine in pyrimidine tracts in all the cells examined to be remarkably constant especially in view of the reported variation in the content of 5-methyl cytosine^{10,17,18}. Our own cell stocks were checked to ensure that these had maintained their 5-

TABLE V

THE DISTRIBUTION OF 5-METHYL CYTOSINE IN
PYRIMIDINE TRACTS OF XENOPUS LAEVIS DNA

OLIGONUCLEOTIDE BASE COMPOSITION	RELATIVE DISTRIBUTION OF 5-METHYL CYTOSINE	RELATIVE DISTRIBUTION OF CYTOSINE
C	100	100
C ₂	25(2.5)	41.4(3.5)
C ₃	4.9(0.7)	12.0(1.3)
CT	34.1(2.6)	70.3(5.5)
C ₂ T	11.3(2.4)	36.9(1.2)
C ₃ T	4.8(1.5)	16.6(0.2)
CT ₂	18.2(0.9)	29.2(3.2)
C ₂ T ₂	7.8(2.0)	20.0(2.7)
CT ₃	3.7(0.2)	13.1(1.9)
T	1.2(-)	

Details are as in Tables II to IV, the results are based on fingerprints from 2 separate Xenopus cell cultures.

TABLE VI

5McC Content of DNA in Cultured Cells

Cell Line	<u>5-methyl cytosine x100</u> <u>5-methyl cytosine + cytosine</u>
HeLa	1.3 ± 0.2
L929	2.9 ± 0.4
BHK-21/Cl3	1.0 ± 0.1
BHK-21/PY _y	1.7 ± 0.2
<u>Xenopus</u>	4.1 ± 0.2

DNA was labelled for 48 hours with ³²P₀ and extracted (Materials & Methods) digested to the mononucleotides, the mononucleotides separated by electrophoresis³⁶ and d5MeCMP resolved³⁷ from dCMP by chromatography in isobutyric acid/NH₃.

methyl cytosine complement and that the similarity of fingerprint data was not merely a reflection of a constant level of 5-methyl cytosine in all the cells; the detected content of 5-methyl cytosine in our cell stocks is given in Table VI.

DISCUSSION

One of the prime problems in examining features of DNA methylation labelled with L[methyl-³H] or L[methyl-¹⁴C] methionine is that of achieving specific labelling of 5-methyl

cytosine, particularly as the label is liable to enter thymine via the C₁ carbon pool. The fact that the labelling procedure adopted is highly specific is indicated by (a) base analysis (b) the absence of label corresponding to pTp or pTpTp on fingerprints of L[methyl-¹⁴C] labelled DNA (Figure 1) (c) the lack of ³H label corresponding to pTp or pTpTp in fingerprints of DNA labelled with L[methyl-³H] methionine and ³²PO₄ (Tables II to V) we are thus confident that our results reflect the true distribution of 5-methyl cytosine.

The presence of virus coded or virus induced DNA methylases has been invoked to explain the increased level of 5-methyl cytosine in a polyoma virus transformed hamster cell line¹⁵. The results presented in Tables I to V whilst not conclusively ruling out the presence of a novel DNA methylase in virus transformed cells certainly suggests that the nucleotide sequence specificity of the DNA methylase system may be unaltered.

It is possible that mechanisms not relying on the action of a 'novel' methylase are responsible for the elevated content of 5-methyl cytosine in the transformed hamster cells we have examined. For example a small region of the chromosome which is normally highly methylated could have become preferentially amplified either at 'transformation' or as an outcome of continuous subculturing of these particular cell stocks. Alternatively a pre-existing DNA methylase might have had its normal activity altered by the action of virus coded or virus induced control factors. Finally differences in the 5-methyl cytosine content could simply be due to differing time lags between DNA synthesis and methylation¹⁸.

With regard to the possibility of tissue variation in distribution of sequences containing 5-methyl cytosine the results in Table V show that the distribution of 5-methyl cytosine in pyrimidine tracts of HeLa (a human cervical epithelial cell line) and Chang liver (a human liver fibroblastic line) cells to be identical. However further experiments with primary cell cultures will really be required to clarify this point.

The data in Tables I to V show that there may be some variation in the pattern of DNA methylation from species to species yet this pattern is remarkably constant. The similarity between species is emphasised when one considers that the level of 5-methyl cytosine varies at four fold in the cell lines used (Table VI)^{14,15,38}

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