NUCLEOTIDE CLUSTERS IN DEOXYRIBONUCLEIC ACIDS

XII. THE DISTRIBUTION OF 5-METHYLCYTOSINE IN PYRIMIDINE OLIGONUCLEOTIDES OF MOUSE L-CELL SATELLITE DNA AND MAIN BAND DNA

K. Harbers, B. Harbers and J. H. Spencer

Department of Biochemistry, McGill University
Montreal, Quebec, Canada H3G 1Y6.

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SUMMARY

Mouse L-cell DNA radioactively labeled in the 5-methylcytosine (5-MeC) residue was fractionated into satellite and main band DNA. Satellite DNA was found to contain about four times the molar concentration of 5-MeC than the main band DNA. Based on the known 5-MeC content of total L-cell DNA it was calculated that satellite DNA contains 3.5 - 4.6% 5-MeC. Both DNA fractions were depurinated and the pyrimidine oligonucleotides released separated by ionophoresis-homochromatography. In satellite DNA 5-MeC is distributed non-randomly. About 40% of the total 5-MeC is present in the sequence Pu - 5-MeC - Pu. The remainder occurs in the oligonucleotides CT, CT_3, C_2T_4, C_2T_5 and C_3T_5 only. The distribution of 5-MeC in main band DNA differs from that in satellite DNA indicating that two different fractions of the same nuclear DNA are methylated in different sequences.

INTRODUCTION

5-Methylcytosine (5-MeC) is a minor base found in all animal DNAs so far investigated (1,2). Although the biological significance of this modification is still obscure, it can have profound effects on the physical properties of DNA as demonstrated with synthetic polymers (3). Recently Holliday and Pugh (4) have proposed that enzymatic modification such as methylation of repeated DNA is a possible mechanism by which gene activity is regulated during development. In mouse L-cell DNA most of the 5-MeC is present in the highly repetitive satellite DNA (5) which is located at the centromeric end of nearly every chromosome (6). Antisera to 5-MeC also showed preferential binding to the C-band region of centromeric heterochromatin (7). Sequence studies have shown (8,9) that mouse satellite DNA is composed of a short

repetitive base sequence and a number of minor variants of this sequence. Previous experiments on the distribution of 5-MeC in mouse L-cell DNA did not reveal any feature to distinguish satellite DNA from main band DNA (5).

In this communication we report that in mouse L-cell satellite DNA the distribution of 5-MeC is non-random and differs from that of main band DNA.

MATERIALS AND METHODS

L-[^{3}H-methyl]methionine, specific activity 3-5 Ci/mmole (New England Nuclear) was used to label the methyl group of 5-methylcytosine. L-929 mouse fibroblasts were grown in spinner modified Eagles minimum essential medium (Grand Island Biological Co.) supplemented with 5% fetal calf serum. When the cell concentration reached 0.5 x 10^6 cells/ml, the cells were harvested, washed once in fresh medium and then transferred to a medium containing one-fifth the normal concentration of L-methionine (originally 5 µg/ml), in the presence of 5 µCi/ml L-[^{3}H-methyl]methionine, 10% dialyzed fetal calf serum and 1 mg sodium formate/ml (10,11). The cells were grown for a further 35 h, and then harvested by centrifugation. The DNA was isolated and purified as described previously (12). Main band DNA and satellite DNA were separated by Ag^+/Cs_2SO_4 density gradient centrifugation (13). The specific radioactivity was determined by counting a small aliquot (usually 50 µl) of the DNA solution of known concentration in 10 ml Bray's scintillation fluid (14). Hydrolysis of the DNA by formic acid and separation of the bases released by two-dimensional thin-layer chromatography was by published procedures (11,15). Ultraviolet-absorbing spots and blank areas from adjacent regions of the chromatogram were cut out and counted in 10 ml Bray's solution (14) after incubation with shaking for 1 h at room temperature. Recovery of radioactivity in the bases from the starting radioactivity added to the hydrolysis tubes was better than 85% when corrected for 20% quenching due to the presence of thin-layer chromatography discs in the scintillation fluid.

The depurination products (16) were fractionated by the ionophoresis-homochromatography thin-layer system of Brownlee and Sanger (17), as modified for pyrimidine oligonucleotides by Ling (18). [^{3}H]-labeled oligonucleotides were visualized by fluorography (19), scraped from the thin-layer plate, 10 ml of Bray's solution (14) added and then the radioactivity measured. The base composition of the spots was inferred from the position on the chromatogram as described elsewhere (8,18).

RESULTS

DNA specifically labeled in the 5-methylcytosine residue was obtained by growing mouse L-cells in the presence of [^{3}H-methyl]methionine. To reduce the introduction of non-specific label into bases via the one-carbon pool sodium formate was included in the medium (10). The distribution of radioactivity in the various bases was determined after hydrolysis of the DNA and separation of the bases released by two-dimensional thin-layer chromatography. Fluorography of the chromatogram showed that the radioactivity was present almost exclusively in 5-MeC (Figure 1). Each of the spots on the chromatogram

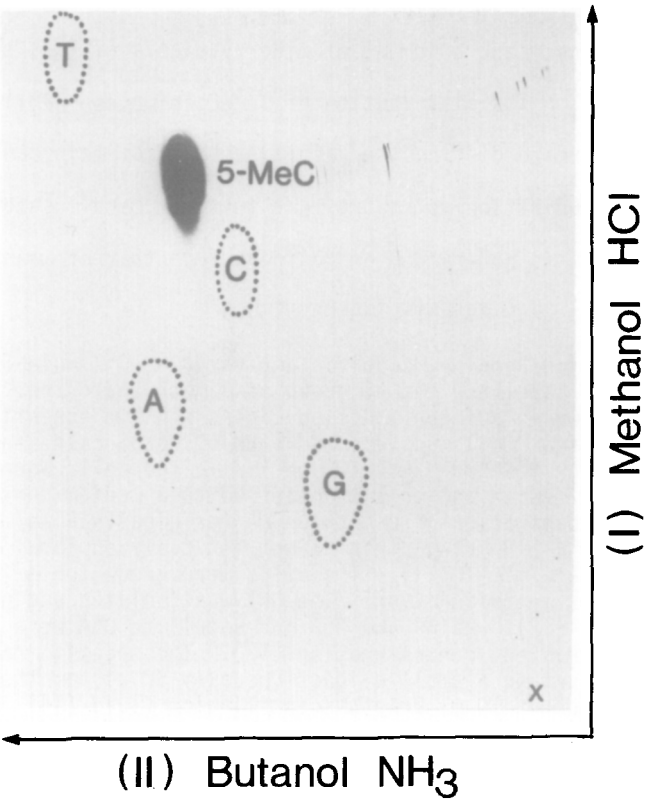

Figure 1. Two-dimensional thin-layer chromatography of bases from mouse L-cell DNA labeled *in vivo* with [^{3}H-methyl]methionine. Chromatography was performed as described by Randerath (15). After identidication of ultraviolet absorbing spots, radioactive bases were visualized by fluorography (19). X indicates origin.

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was cut out and the radioactivity determined. The results from three different DNA preparations are summarized in Table 1. 94-97% of the radioactivity was present in 5-MeC. A very small amount of radioactivity was also incorporated into thymine, cytosine and adenine. The specific radioactivity of unfractionated L-cell DNA from three different preparations was determined to be 350-450 cpm/µg DNA. After fractionation the specific radioactivity of the satellite DNA (1200-1300 cpm/µg DNA) was, in all cases, approximately 4 times higher than that of the main band DNA (300-350 cpm/µg).

Figure 2a shows a radioautogram of the pyrimidine oligonucleotides containing [^{3}H]-labeled 5-MeC residues which are present in a diphenylamine-

TABLE 1

In Vivo Incorporation of Label From L-[methyl-^{3}H] Methionine Into Bases of Mouse L-cell DNA in the Presence of Unlabeled Sodium Formate (1 mg/ml)

	Prep. I		Prep. II		Prep. III	
	cpm*	%	cpm*	%	cpm*	%
G	25	0.1	-		-	
A	170	0.6	525	2.3	210	0.8
C	350	1.2	160	0.7	110	0.4
5-MeC	26,350	97.4	21,480	94.0	25,000	96.5
T	190	0.7	690	3.0	590	2.3
Origin	-		-		-	

*After subtraction of background.

formic acid hydrolysate of satellite DNA. 5-MeC occurs in the mononucleotide fraction and in five other oligonucletoides only whose base compositions are indicated on the chromatogram. Even after long exposure times the autoradiogram showed no additional radioactive spots. Each oligonucleotide was eluted from the chromatogram and the radioactivity determined. The results are summarized in Table 2. The radioactivity in cpm (column III) has not been corrected to 100% recovery because preferential losses of short, cytosine-rich oligonucleotides occur during the transfer of oligonucleotides from the cel-

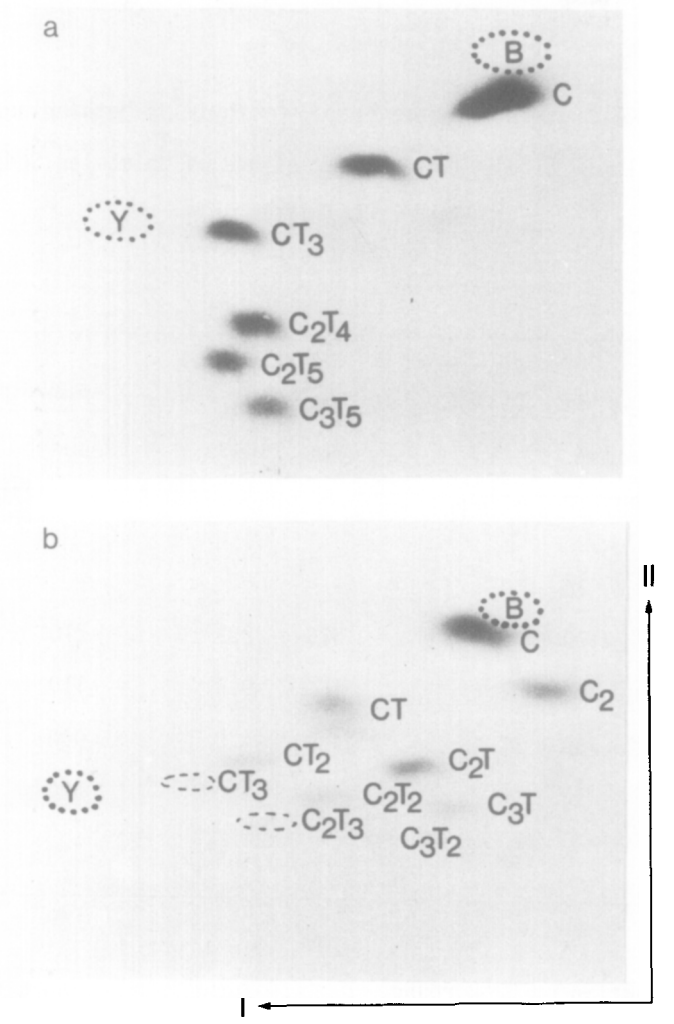

Figure 2. Two-dimensional fraction of pyrimidine oligonucleotides of mouse L-cell satellite DNA (a) and main band DNA (b) [^{3}H]labeled in the 5-MeC residue.

Dimension I. electrophoresis on a cellulose acetate strip in pyrimidine-acetate buffer, pH 3.5.

Dimension II: homochromatography on DEAE-cellulose thin-layer plates at 60°C, eluent 3% partially hydrolyzed yeast RNA containing 7 M urea.

The separated oligonucleotides were visualized by fluorography (19). Y = position of yellow dye marker; B = position of blue dye marker.

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lulose acetate strip to the DEAE-cellulose plate (unpublished observation). The sequences of the oligonucletoides containing 5-MeC given in column II have been determined previously (8). At the present time it is not known which

TABLE 2

Distribution of 5-Methylcytosine in the Pyrimidine Oligonucleotides of Mouse L-Cell Satellite DNA

I Oligonucleotide	II Sequence	III cpm[a]	IV % Radioactivity[b]	V Moles pyrimidine/ 100 g-atoms DNA-P[c]	VI cpm/mole cytosine[d]	VII No.of tracts/ 15,000 base pairs[c]
C	-	1870	40	3.75	500	950
CT	C-T T-C	750	15	4.79	310	625
CT_3	T-T-T-C T-T-C-T	680	13	2.43	1130	205
C_2T_4	T-T-T-T-C-C T-T-T-C-T-C	640	13	4.8	400	300
C_2T_5	T-T-T-T-C-T-C C-T-T-T-T-T-C	460	10	1.1	1480	56
C_3T_5	T-T-T-T-C-C-T-C C-C-T-T-T-T-T-C	410	9	1.79	610	89

[a] Values from one chromatogram

[b] Average values obtained from three different chromatograms and two different DNA preparations

[c] From Harbers and Spencer (11)

[d] The moles of cytosine per fraction were determined from the moles pyrimidine per 100 g-atoms DNA-P (column V) and the base composition (column I). cpm are from column III.

of the cytosine residues are methylated. The cpm per mole cytosine shown in column VI of Table 2 have been calculated in order to allow a ready comparison of the frequencies with which the different oligonucleotides are methylated which is not apparent from cpm or % radioactivity. Since the radioactivity is associated almost exclusively with the methyl group of 5-MeC a high value in column VI indicates a high frequency of methylation.

For comparative reasons the pyrimidine oligonucleotides of mouse main band DNA [^{3}H]-labeled in the 5-MeC residue were also separated by ionophoresis-homochromatography (Figure 2b). It can be seen that most of the radioactivity is present in the mononucleotide fraction and the longest oligonucleotide containing 5-MeC is a pentanucleotide. We can not exclude the possibility that longer oligonucleotides are also methylated in main band DNA and that they do not appear on the radioautogram because of the very low specific radioactivity of the starting material. Figure 2b shows that the distribution of 5-MeC in main band DNA is quite different from that in satellite DNA. 5-MeC is present in several oligonucleotides (e.g. C_2, C_2T, CT_2, C_2T_2) which are not methylated in satellite DNA (Figure 2a).

DISCUSSION

The DNA obtained from L-cells grown in the presence of [^{3}H]methionine and sodium formate was almost exclusively labeled in the 5-MeC residue . The small amount of radioactivity found in thymine may originate from *in vivo* deamination of 5-MeC (20,21). The radioactivity corresponding to adenine may result from oxidation of the methyl groups of methionine to formate and/or CO_2 which was not diluted sufficiently by the presence of unlabeled formate (11)

The specific radioactivity of the satellite DNA was, on average, approximately 4 times higher than that of main band DNA and 3.2 times higher than that of total L-cell DNA. Assuming that no preferential *in vivo* labeling of satellite DNA occurs under the conditions used in our experiments we can conclude that satellite DNA contains about 3.2 times as much 5-MeC as the total DNA. Previously Vanyushin *et al.* (2) had determined the content of 5-MeC in

total mouse DNA to be 1.1%. A higher value (1.45%) was reported by Salomon *et al.* (5). Depending on which value is used we have calculated that mouse satellite DNA contains 3.5% or 4.6% 5-MeC respectively. The lower value (3.5%) is in good agreement with previous estimates of the 5-MeC content of mouse satellite DNA (5,22).

The diphenylamine-formic acid hydrolysate of uniformly [^{32}P]-labeled mouse L-cell satellite DNA can be fractionated into 29 pyrimidine oligonucleotides of different base composition by ionophoresis-homochromatography (8). The results presented here show that only 5 of these oligonucleotides contain 5-MeC, and approximately 40% of the 5-MeC is present in the sequence Pu - 5-MeC - Pu. Each of the oligonucleotides containing 5-MeC are composed of two isomeric sequences (Table 2). Some of the most frequently occurring oligonucleotides in the satellite DNA such as CT_5, CT_4 and C_2T_2 (8) are not methylated. These findings clearly indicate that 5-MeC is distributed non-randomly in satellite DNA. A comparison of the cpm/mole cytosine (Table 2, column VI) indicates the preferential methylation of cytosines in certain oligonucleotides such as C_2T_5 and CT_3 which are not among the most commonly occurring pyrimidine oligonucleotides in mouse satellite DNA (column VII). For example, oligonucleotide C_2T_5 is methylated approximately four times more frequently as C_2T_4 yet there are six tracts of C_2T_4 for every tract of C_2T_5 in satellite DNA (column VII).

In mouse main band DNA most of the 5-MeC is also present in the sequence Pu - 5-MeC - Pu, but methylation of the various pyrimidine oligonucleotides is markedly different than those in satellite DNA, as described in the results. Thus the two fractions of the same nuclear DNA are methylated in different sequences and at different levels.

The biological significance of the high content and specific distribution of 5-MeC in mouse satellite DNA is unknown. Further studies using DNAs from other organisms may indicate whether the features described here for mouse satellite DNA apply also to other highly repetitive DNAs.

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