THE DISTRIBUTION OF MODIFIED AND NON-MODIFIED C–G DOUBLETS IN BHK-21 CELL DNA

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Received 19 April 1978

1. Introduction

Although a distinctive feature of vertebrate DNA is the relative scarcity of the dinucleotide C–G [1,2], analysis of methylation patterns has indicated that 5-methyl cytosine (5MC) residues are most commonly recovered in this dinucleotide [3–5]. Further studies [6] show that the pattern of methylation of pyrimidine tracts in DNA has been stringently conserved through vertebrate evolution.

The present study examines the distribution of 5MC in relatively long DNA sequences of baby hamster kidney fibroblast cells (BHK-21/C13). Our results indicate preferential methylation of (C + G)-rich and rapidly-reannealing BHK-21/C13 DNA, these findings are compared with human and mouse cell DNA.

We have shown that approx. 1% of all cytosines in BHK-21/C13 DNA are methylated [6], whereas nearest neighbour frequency data [1,2,7] indicate that 2.4% of all cytosines occur in the C–G dinucleotide. This implies that many of the C–G dinucleotides in these cells are not in fact methylated. We have been able to detect such non-methylated C–G dinucleotides using the bacterial *Hpa*II restriction endonuclease.

2. Materials and methods

BHK-21/C13 cells were inoculated into Roux bottles (5×10^6/50 ml Eagles medium containing 10% (v/v) calf serum). After 24 h growth at 37°C the medium was removed and replaced with fresh medium containing 2 mCi $^{32}PO_4$ or 50 μCi [6-^{3}H]thymidine and growth continued for 48 h. In some experiments where the cells were to be labelled with L-[*methyl*-^{14}C]-methionine or L-[*methyl*-^{3}H]methionine the cells were grown for 24 h, and the medium replaced with medium containing 10^{-2} M sodium formate, 2×10^{-5} M adenosine and guanosine, and sufficient non-radioactive and radioactive methionine to give a final methionine concentration of 5×10^{-5} M. The levels of isotope used were 125 μCi and L-[*methyl*-^{14}C]-methionine or up to 750 μCi L-[*methyl*-^{3}H]methionine and the medium 5% (v/v) with dialysed calf serum.

DNA was extracted by the method in [8], and where relevant sheared to 1.2×10^5 daltons by the method in [9]. Analysis on actinomycin–CsCl gradients was carried out following the procedure in [10] and the kinetics of renaturation determined following the procedure in [11].

For digestion with *Hpa*II restriction nuclease (Miles Labs) 1 μg DNA was dissolved in 10 mM Tris, pH 7.4, 10 mM $MgCl_2$, 6 mM KCl, 1 mM dithiothreitol, 5 μg gelatin and 1 unit *Hpa*II added (1 unit is the amount of enzyme which is required to digest 1 μg of λ-phage DNA in 1 h). After incubation for 4 h at 37°C the products were subjected to electrophoresis through 1.5% cylindrical gels of agarose (0.6×14 cm), at 10 A/gel for 2 h. The gels were then sliced into 2 mm slices, and after solubilisation in 0.3 ml H_2O_2 at 200°C were assayed for radioactivity.

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3. Results and Discussion

3.1. *Equilibrium density centrifugation*

Equilibrium density centrifugation was used to examine the distribution of modified cytosines in relatively long (2.3–3.5 X 10^6 dalton double-stranded) stretches of DNA. A modification of the basic equilibrium density gradient technique was suggested in [10] where CsCl gradients containing actinomycin were used to isolate sea urchin histone genes. The actinomycin interacts with (C + G)-rich DNA [12] and preferentially alters its density.

The distribution of BHK-21/C13 cell DNA labelled in vivo with L-[*methyl*-^{3}H]methionine and $^{32}PO_4$ is shown in fig.1(a). The distribution of *methyl*-^{3}H radioactivity is biased towards the (C + G)-rich region of the gradient (in these gradients this is on the right hand side of the main band DNA [10]). Preferential methylation of (C + G)-rich DNA in chinese hamster DON cells has been shown [13] using conventional gradients but this necessitated synchronisation of the cell cultures.

Further experiments showed HeLa cell DNA fig.1(b) to be similar to BHK-21/C13 DNA in the bias of methylation to (C + G)-rich DNA. Mouse L-929 DNA, on the other hand, showed a bias of 5MC towards the (A + T)-rich fraction of the gradient fig.1(c). While the reasons for these differences are unclear there would appear to be some degree of species specificity in 5MC distribution in the genome as a whole, perhaps reflecting methylation of the various DNA satellites shown to exist in many species [14].

3.2. *Reassociation kinetics of DNA*

To examine further the sequence distribution of modified cytosine residues, sheared BHK-21/C13 DNA labelled in vivo with [^{3}H]thymidine and L-[*methyl*-^{14}C]methionine was denatured and its reassociation kinetics followed. From fig.2 it is clear that the DNA sequences that reanneal rapidly contain about twice the level of 5MC that occurs in non-repetive sequences. Experiments carried out with mouse DNA (L929) and HeLa cell DNA give similar data, thus by this criterion there is no obvious species difference. The significance of such 5MC-rich regions in the genome is not understood. However studies in this laboratory indicate that regions of DNA in hamster cell chromatin which are particularly sensitive to DNase I digestion and which may be related to transcription are also rich in 5MC [15].

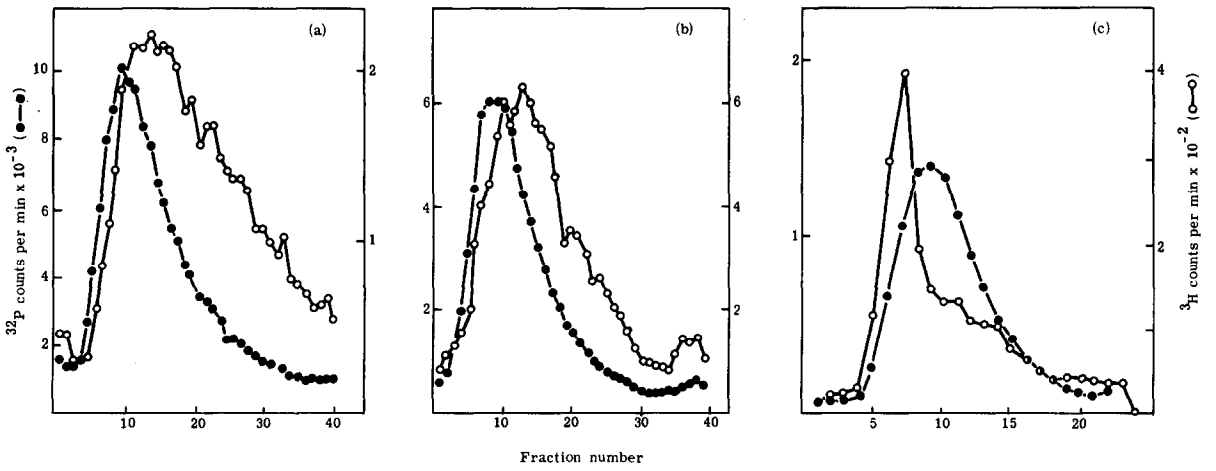

Fig.1. Analysis of $^{32}PO_4$ and L-[*methyl*-^{3}H]methionine labelled DNAs on actinomycin–CsCl gradients. CsCl (refractive index 1.3900 at 3°C) 5 ml in 0.05 M sodium borate, pH 9.0, containing 200 μg actinomycin D and containing 5–15 μg labelled DNA (2.3–3.5 X 10^6 dalton) (see section 2) was centrifuged to equilibrium at 35 000 rev./min in a Beckman Ti50 rotor for 72 h at 4°C. Fractions were collected dropwise onto Whatman 3MM discs. These were washed in ice-cold 5% trichloroacetic acid, dried and the radioactivity determined after solubilisation with hyamine hydroxide. (○——○) *methyl*-^{3}H derived cpm; (●——●) ^{32}P radioactivity, cpm. Density increases from left to right. (a) BHK-21 cell DNA; (b) HeLa cell DNA; (c) L-929 cell DNA.

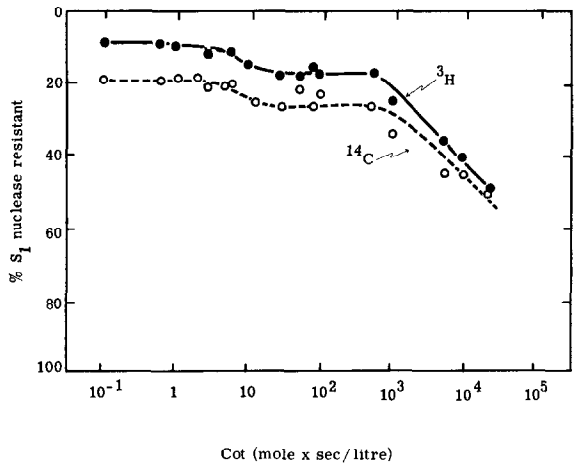

Fig.2. Reassociation kinetics of *methyl*-^{14}C and ^{3}H-labelled BHK-21 cell DNA. BHK-cell DNAs were labelled, extracted and sheared as in section 2. The kinetics of reassociation were followed using the procedure in [17]. % ^{3}H-labelled DNA that becomes resistant to S_1-nuclease digestion (●——●); % *methyl*-^{14}C-labelled DNA that becomes resistant to S_1-nuclease digestion (o– – –o).

3.3. *Restriction of DNA by* HpaII

The genomic distribution of non-methylated C–G dinucleotides may be probed using the bacterial restriction endonuclease *Hpa*II. This nuclease cleaves unmodified double stranded DNA as follows [16]

C – C – G – G

G – G – C – C

Methylation of the 3′-cytosine in the cytosine doublet prevents restriction cleavage [17]. Samples of $^{32}PO_4$-labelled BHK-21 DNA were digested with *Hpa*II and the digestion products electrophoresed on 1.5% agarose gels (fig.3). The digestions were assumed to be complete as no further degradation was apparent when the digestion time was increased from 4–16 h (under the same conditions samples of SV40 DNA containing both form I and form II molecules were completely digested to the linear form III).

In DNA of random base composition each possible tetranucleotide should occur once every 256 base pairs. However taking into account the low C–G

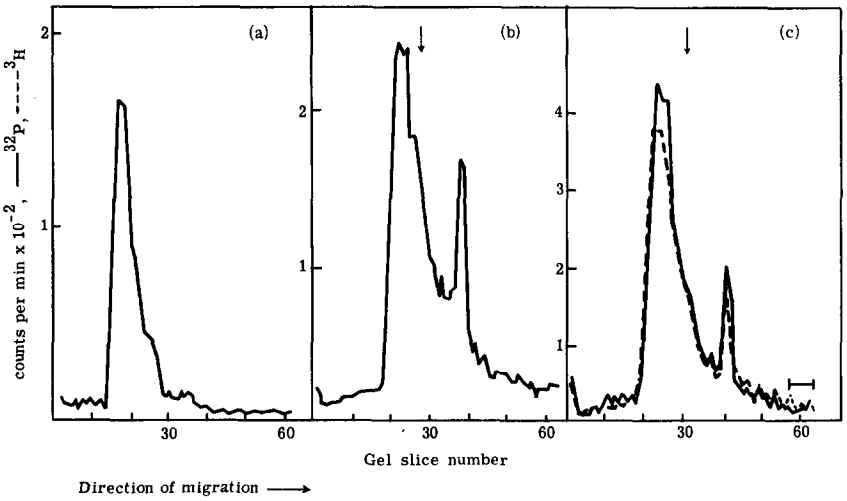

Fig.3. Agarose–gel electrophoresis of BHK-cell DNA after digestion with *Hpa*II restriction nuclease. (a) Gel profile of undigested BHK-21 cell nuclear DNA labelled with $^{32}PO_4$ (the profiles from BHK-21 strain C13 and strain PyY were identical). (b) Gel profile of *Hpa*II-digested BHK-21/C13 DNA labelled with $^{32}PO_4$. (c) Gel profile of *Hpa*II-digested BHK-21/PyY DNA labelled both with $^{32}PO_4$ and with L-[*methyl*-^{3}H]methionine. Conditions of digestion and electrophoresis through 1.5% agarose were as described in section 2. The 'bar' line indicates the position reached by marker bromophenol blue under the conditions used. (↓) indicates position of SV40 form III molecular weight marker.

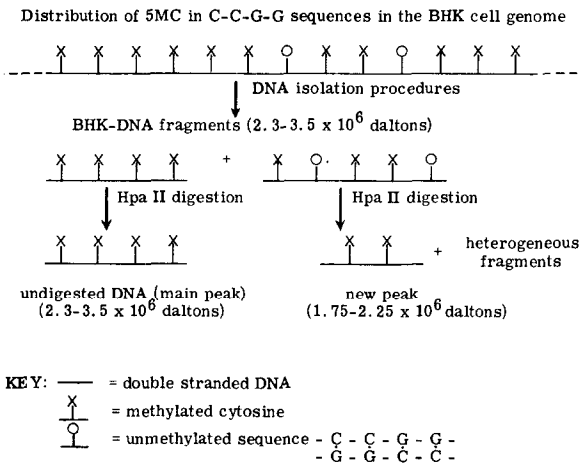

Fig.4. A schematic diagram indicating the possible distribution of unmethylated CpG dinucleotides in sequences sensitive to cleavage by *Hpa*II.

dinucleotide frequency [1,2] and the 40% (G + C) base content of vertebrate DNA, the tetranucleotide C–C–G–G should occur approximately once every 256 X 4 X 1.25 base pairs. Thus cleavage of total unmodified vertebrate DNA should produce fragments of average double-stranded length 8×10^5 dalton. Examination of the gel profiles in fig.3 shows that *Hpa*II does digest a proportion of the BHK-21/C13 DNA indicating that there are indeed non-methylated C–G dinucleotides in this DNA. However if non-methylated and thus cleavable *Hpa*II sites were distributed randomly the digestion should have produced DNA fragments of heterogeneous molecular weight. This is clearly not the case as the digestion results not only in the production of some heterogeneous DNA but also a new fairly discrete size class of DNA moving faster than the main band. (By comparison with the mobility of undigested DNA and linear SV40 DNA this is about $1.75–2.25 \times 10^6$ dalton.) This finding implies that many of the nonmethylated *Hpa*II cleavage sites are separated in a highly specific fashion in the BHK genome.

A digestion of BHK-21/PyY cell DNA double-labelled with $^{32}PO_4$ and L-[*methyl*-^{3}H] methionine resulted in a distribution of $^{32}PO_4$ which was qualitatively similar to that of the BHK-21/C13 DNA. Examination of the distribution of the *methyl*-^{3}H label shows this to closely parallel that of the $^{32}PO_4$ (fig.3(c)). Restriction of the DNA is thus likely to have occurred as shown schematically in fig.4.

However, autoradiographic data [18] suggest that DNA from mouse L-cells contains regions 8–20 μm long which may be unmethylated. If the sequence C–C–G–G is randomly distributed throughout these unmethylated regions then DNA fragments produced by *Hpa*II restriction enzyme should be unmethylated. The fact that the fragments of BHK-21/PyY cell DNA are apparently methylated to the normal extent (fig.3C) suggests that they are not related to the long unmethylated regions proposed in [18].

The existence of specific unmethylated C–G doublets in mammalian DNA implies rigorous control of the operation of the DNA methylation system. In this context it is noteworthy that the DNA of SV40 virus, despite having 54 C–G dinucleotides amongst its 10 490 nucleotides [19] does not serve as a substrate for mammalian DNA methylase [20].

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