Lack of 5-methylcytosine in *Dictyostelium discoideum* DNA

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We find no evidence for the presence of 5-methylcytosine in the DNA of *Dictyostelium discoideum*. Methylation was absent from CCGG sites in repetitive DNA and in DNA from the actin multigene family. Nor was 5-methylcytosine detected in total DNA when base composition was determined by means of h.p.l.c.

INTRODUCTION

Modern systematists recognize five kingdoms of living things, comprising nearly 100 phyla (Margulis & Schwartz, 1988). Diversity among the phyla extends to the molecular level. Genome size, organization and modification each reflect that diversity. 5-Methylcytosine is a modified base that is present in the DNA of many organisms. In the kingdom Animalia, for example, representative chordates and echinoderms (Bird, 1980) possess 5-methylcytosine contents ranging from 2 to 7% of total cytosine. On the other hand, certain nematodes (Simpson *et al.*, 1986) and insects (Urieli-Shoval *et al.*, 1982) appear to be devoid of this minor base. Most species within the fungal kingdom either lack 5-methylcytosine or have very low contents of this modified base (reviewed in Magill & Magill, 1990). Relatively little is known about the presence of 5-methylcytosine in the 27 phyla comprising the kingdom Protocista. The DNA of the acellular slime mould *Physarum polycephalum* (a protocist in the phylum Myxomycota) is known to possess 5-methylcytosine (Evans & Evans, 1970; Whittaker & Hardman, 1980). Although the cellular slime moulds are classified in the same kingdom, they are only distantly related to the acellular slime moulds, and have been classified in a separate phylum, Acrasiumycota. In the present paper we show that, unlike the situation in *Physarum polycephalum*, 5-methylcytosine cannot be detected in the acrasiumycote *Dictyostelium discoideum*.

EXPERIMENTAL

High-molecular-mass DNA from vegetative axenically grown cells of *Dictyostelium discoideum* strain AX3, axenically grown plasmodia of *Physarum polycephalum* strain M₃C or from BALB/c mouse spleen was prepared as described in Blin & Stafford (1976). End-labelling, gel electrophoresis and autoradiography were performed as described in Smith & Thomas (1981). Blots were prepared by the alkaline method with Zeta-probe membranes (Reed & Mann, 1985). The actin gene family was probed with plasmid pcDdActinB1 (McKeown *et al.*, 1978) labelled by means of the random priming method (Feinberg & Vogelstein, 1983). *Hpa*II and *Msp*I were from New England Biolabs, Beverly, MA, U.S.A. The completeness of restriction digestion was monitored by the method described in Smith & Thomas (1981) and Tolberg & Smith (1984).

The procedure described by Palmgren *et al.* (1990) was used without modification for the determination of 5-methylcytosine contents by means of h.p.l.c. DNA was digested with nuclease P₁ and bacterial alkaline phosphatase and separated on a μ Bondapak C₁₈ column (Waters Associates, Milford, MA, U.S.A.). Nucleosides were detected at 285 nm with a Waters Associates 484 Tunable Absorbance Detector. Peak areas were calculated

with the aid of a Waters Associates 745 Data Module. Authentic nucleoside standards were used to determine retention times, and serial dilution of standards was used to establish the detection limit for 5-methylcytosine. As little as 1 ng of input 5-methylcytosine was reproducibly detected with the method in the present study.

RESULTS

Restriction analysis

The restriction endonucleases *Hpa*II and *Msp*I both recognize the DNA sequence CCGG, but differ in their sensitivity to cytosine methylation (McClelland & Nelson, 1988). Unmethylated DNA is cleaved by both enzymes. When either of the two cytosine residues at a CCGG site is methylated at C-5, cleavage by *Hpa*II is blocked. However, only methylation of the external cytosine residue blocks cleavage by *Msp*I. Given these specificities, identical patterns of DNA cleavage by both enzymes can only be interpreted as a complete absence of cytosine methylation at the CCGG sites examined. Differences in cleavage pattern would indicate methylation of internal cytosine residues whereas the absence of cleavage would indicate methylation at external cytosine residues.

Two methods were employed to detect differences between *Hpa*II and *Msp*I cleavage patterns. In the first method, genomic DNA was digested with *Bam*HI and end-labelled by filling in the uneven ends of the restriction fragments with DNA polymerase I in a reaction mixture containing [³²P]dGTP (Smith & Thomas, 1981). When the end-labelled *Bam*HI digest was separated by agarose-gel electrophoresis, autoradiographs of dried gels revealed a pattern of several prominent high-molecular-mass bands above a background of several hundred bands of lower intensity (result not shown). Portions of the end-labelled digest were then re-digested with either *Hpa*II or *Msp*I, and the cleavage products were separated electrophoretically. Gels were dried and ³²P was detected by autoradiography.

Representative results are depicted in Fig. 1. Numerous prominent bands were detected after digestion with *Hpa*II or *Msp*I, indicating that extensive cleavage had occurred. Moreover, no differences in banding pattern were detected when *Hpa*II and *Msp*I digests were compared. Identical extents of cleavage by both enzymes showed that the central cytosine residues in the cleaved CCGG sites were not methylated. In contrast, control BALB/c mouse DNA, which is known to be methylated at CG sites (Bird, 1980), was cleaved more extensively by *Msp*I than it was by *Hpa*II (Fig. 1a).

The end-labelling technique allows the detection of highly repeated sequences as bands that are darker than the complex background of low-copy-number bands. In the *Dictyostelium*

Fig. 1. Identical patterns of cleavage by restriction enzymes *HpaII* and *MspI* in repetitive DNA of *Dictyostelium*

High-molecular-mass DNA was digested to completion with *BamHI* and end-labelled with [32 P]dGTP. The end-labelled digest was cleaved with either *HpaII* or *MspI*. (a) Digests of BALB/c mouse (*Mus musculus*) DNA and *Dictyostelium discoideum* DNA were separated by electrophoresis through a 1.2% agarose gel. Mouse DNA serves as a control to show differential cleavage by *HpaII* and *MspI* for DNA known to be methylated at CG sites. (b) Digests of *Dictyostelium* DNA were separated by electrophoresis through a 0.6% agarose gel. The pattern detected by autoradiography of dried gels is shown. Size markers are from bacteriophage λ DNA digested with *EcoRI* and *BamHI*.

pattern, many of the most intense bands arise from extra-chromosomal ribosomal DNA or mitochondrial DNA. The many fainter bands of Fig. 1 also show identical profiles for the two enzymes, but these low-copy-number DNAs cannot be unequivocally analysed by this method. In order to inspect representative chromosomal genes for methylation at CCGG sites, Southern blots were prepared from DNA digested with *HpaII* or *MspI*.

Fig. 2 depicts the results obtained when such blots were probed with DNA homologous to the 15–20-member (Kimmel & Firtel, 1982) actin gene family. More than 20 bands were discernible, identical in DNA digested with *HpaII* or *MspI*. As with the end-labelling method, the identical cleavage patterns of the two lanes of Fig. 2 imply that none of the 20–40 CCGG sites examined was methylated at C-5 of either cytosine residue (McClelland & Nelson, 1988).

H.p.l.c. analysis

5-Methylcytosine was also undetectable by h.p.l.c. analysis. A representative chromatogram from *Dictyostelium* DNA is given in Fig. 3(a). *Dictyostelium* DNA has an unusually low G+C content (22% G+C; Fasman, 1976), and this base composition was confirmed by the chromatographic results obtained here. 5-Methylcytosine was easily detected in DNA from *Physarum polycephalum* (Fig. 3b), where it was found to be 3.6 mol% of total cytosine, in agreement with earlier reports (Evans & Evans,

Fig. 2. Identical patterns of cleavage by restriction enzymes *HpaII* and *MspI* in the actin gene family of *Dictyostelium*

High-molecular-mass DNA was cleaved with either *HpaII* or *MspI* and separated by electrophoresis through a 1.2% agarose gel. The DNA was transferred to Zeta-probe membranes and probed with DNA homologous to the actin gene family that had been labelled with 32 P by random priming. DNA homologous to the probe was detected in Southern blots by autoradiography. Size markers are bacteriophage λ DNA digested with *HindIII* and bacteriophage ϕ X174 DNA digested with *HaeIII*.

1970). Three unidentified chromatographic peaks (numbered 1, 2 and 3 in Fig. 3) were noted in *Dictyostelium* DNA chromatographs. A mixture of *Dictyostelium* DNA and *Physarum* DNA showed that 5-methylcytosine from *Physarum* DNA did not co-chromatograph with any of the unknown peaks seen in *Dictyostelium* DNA digests.

Since *Dictyostelium* DNA has a low G+C content, the absolute amount of cytosine present in chromatographs is low. In order to search for low contents of 5-methylcytosine, experiments were performed in which nucleosides from as much as 8 μ g of total DNA were chromatographed in a single run. 5-Methylcytosine was still not observed when as much as 580 ng of cytosine was detected in chromatographs. With a detection limit of 1 ng for 5-methylcytosine established for the method, the upper limit for 5-methylcytosine in the genome of *Dictyostelium* is less than 0.2 mol%.

DISCUSSION

The data above show that cytosine methylation is absent from the *Dictyostelium* CCGG (*HpaII*/*MspI*) sites inspected here. These data are consistent with a published report showing that *HpaII* [another methylation-sensitive restriction enzyme (McClelland & Nelson, 1988)] cleaves *Dictyostelium* DNA extensively (McKeown *et al.*, 1978). It is, of course, conceivable that some cytosine residues in *Dictyostelium* DNA are methylated either at CCGG sites not examined or at cytosine residues within other sequences. However, failure to detect methylation with *HpaII* and *MspI* restriction enzymes (Rae & Steele, 1979; Smith & Thomas, 1981; Simpson *et al.*, 1986) has implied, in other organisms, a complete lack of detectable methylation at cytosine

Fig. 3. Chromatographic analysis of DNA from *Dictyostelium* and *Physarum*

DNA was digested with nuclease P₁ and bacterial alkaline phosphatase. The resulting nucleoside mixture was resolved on a μ Bondapack C₁₈ column by the method described by Palmgren *et al.* (1990). Absorbance was monitored at 285 nm. (a) *Dictyostelium* DNA (1 μ g); (b) *Physarum* DNA (1 μ g); (c) *Dictyostelium* DNA (1 μ g) mixed with *Physarum* DNA (0.35 μ g).

(Urieli-Shoval *et al.*, 1982; Simpson *et al.*, 1986). The h.p.l.c. data support this by showing that 5-methylcytosine can be no more than 0.2 mol% of cytosine in *Dictyostelium* DNA.

In eukaryotes that contain 5-methylcytosine, CG is the most frequently modified dinucleotide pair (Bird, 1980). Nearest-neighbour analysis has shown that methylation of CG sites is associated with an under-representation of this dinucleotide pair compared with the GC pair. These dinucleotides are, however, present at equal frequencies in *Dictyostelium discoideum* DNA (Skalka *et al.*, 1966). This is again consistent with a general absence of CG methylation in the organism.

We infer from these data that *Dictyostelium* resembles *Drosophila* and *Caenorhabditis* in its lack of methylation of cytosine residues in CCGG and in other sequence contexts. Such methylation is not required for developmentally regulated gene expression (Smith, 1991).

The absence of detectable cytosine methylation in *Dictyostelium discoideum* should be contrasted with the easily detected methylation in *Physarum polycephalum* (Whittaker & Hardman, 1980). It would be of interest to examine methylation in other prototists, in part to consider the evolutionary relationships between them and higher organisms.

This work was supported by grants from The Council for Tobacco Research (1571B), the March of Dimes (1-1130), the National Institutes of Health (GM38350-02) and the National Science Foundation (DCB8911049). We thank David Baker for his help with Southern blotting and h.p.l.c.

REFERENCES

- Bird, A. P. (1980) *Nucleic Acids Res.* **8**, 1499–1503
- Blin, N. & Stafford, D. W. (1976) *Nucleic Acids Res.* **3**, 2303–2308
- Evans, H. H. & Evans, T. E. (1970) *J. Biol. Chem.* **245**, 6436–6441
- Fasman, G. D. (1976) in *Handbook of Biochemistry and Molecular Biology* (Fasman, G. D., ed.), pp. 236–240, CRC Press, Cleveland
- Feinberg, A. P. & Vogelstein, B. (1983) *Anal. Biochem.* **132**, 6–13
- Kimmel, A. R. & Firtel, R. A. (1982) in *The Development of Dictyostelium discoideum* (Loomis, W. F., ed.), pp. 233–324, Academic Press, New York
- Magill, J. M. & Magill, C. W. (1989) *Dev. Genet. (N.Y.)* **10**, 63–69
- Margulis, L. & Schwartz, K. V. (1988) *Five Kingdoms: An Illustrated Guide to the Phyla of Life on Earth*, pp. 75–149, W. H. Freeman, New York
- McClelland, M. & Nelson, M. (1988) *Gene* **74**, 291–304
- McKeown, M., Taylor, W. C., Kindle, K. L. & Firtel, R. A. (1978) *Cell* **15**, 789–800
- Palmgren, G., Mattsson, O. & Okkels, F. T. (1990) *Biochim. Biophys. Acta* **1049**, 293–297
- Rae, P. M. M. & Steele, R. E. (1979) *Nucleic Acids Res.* **6**, 2987–2995
- Reed, K. C. & Mann, D. A. (1985) *Nucleic Acids Res.* **13**, 7207–7221
- Simpson, V. J., Johnson, T. E. & Hammen, R. F. (1986) *Nucleic Acids Res.* **14**, 6711–6719
- Skalka, A., Fowler, A. V. & Hurwitz, J. (1966) *J. Biol. Chem.* **241**, 588–596
- Smith, S. S. (1991) *Mol. Carcinog.* **4**, 91–92
- Smith, S. S. & Thomas, Jr., C. A. (1981) *Gene* **13**, 395–408
- Tolberg, M. E. & Smith, S. S. (1984) *FEBS Lett.* **176**, 250–254
- Urieli-Shoval, S., Gruenbaum, Y., Sedat, J. & Razin, A. (1982) *FEBS Lett.* **146**, 148–152
- Whittaker, P. A. & Hardman, N. (1980) *Biochem. J.* **191**, 859–862

Received 4 April 1991; accepted 24 April 1991