Methylation of nuclear DNA in *Physarum polycephalum*

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The restriction endonucleases *HpaII* and *HhaI*, whose action is inhibited by the presence of methylated base analogues at the recognition sequences in the DNA substrate, were used to investigate the distribution of 5-methylcytosine in nuclear DNA from *Physarum polycephalum*. *Physarum* DNA is digested into two fractions by these enzymes: a low-molecular-weight (M[−]) compartment comprising 80% of the DNA, and a high-molecular-weight (M⁺) compartment containing 20% of the DNA. The DNA fraction showing resistance to digestion by restriction endonuclease *HpaII* is cleaved by its isoschizomer *MspI*, indicating that methylated endonuclease-*HpaII*-specific sites are present in M⁺ DNA. Additional properties of sequences in the M⁺ compartment were investigated.

5-Methylcytosine is the major modified base present in the DNA of eukaryotic organisms (Vanyushin *et al.*, 1970). It arises by methylation of cytosine residues at the polynucleotide level (Scarano *et al.*, 1965; Burdon & Adams, 1969; Sneider & Potter, 1969; Kappler, 1970). Although the function of this modified base in DNA is not known, there have been many speculations, including the idea that it may serve a role in the control and genetic expression (Riggs, 1975; Holliday & Pugh, 1975) and cell differentiation (Jones & Taylor, 1980).

5-Methylcytosine is found almost exclusively in the dinucleotide CpG (Daskocil & Sorm, 1962; Grippo *et al.*, 1968). Thus it is possible to locate 5-methylcytosine by using restriction endonucleases with recognition sites in DNA which include CpG, and which fail to cleave the DNA if the cytosine is methylated (Mann & Smith, 1978). This approach has been made more powerful by the discovery of restriction-enzyme isoschizomers with the same sequence specificity, but which differ in their action on methylated DNA. The most useful of such isoschizomer pairs are endonucleases *HpaII* and *MspI* (Waalwijk & Flavell, 1978; Singer *et al.*, 1979), both of which recognize the sequence CpCpGpGp, but only enzyme *MspI* cleaves the methylated analogue CpmCpGpGp. Hence the enzymes can be used as probes for the distribution of 5-methylcytosine in DNA by direct comparison of the restriction patterns generated by each nuclease.

Endonuclease *HpaII*, and another restriction endonuclease *HhaI*, which recognizes the sequence GpCpGpCp and is also sensitive to methylation of

cytosine residues in CpG doublets, have been used to determine the degree of DNA methylation in a wide range of animal species (Bird *et al.*, 1979; Bird & Taggart, 1980). DNA methylation is undetectable in arthropods, partial in other invertebrate phyla and high in vertebrates. In *Echinus*, the best-studied example, the genome can be divided into an unmethylated (M[−]) and methylated (M⁺) compartment, M⁺ DNA being composed of long segments containing several endonuclease-*HpaII* restriction sites, all of which are methylated. Specific sequence components have been ascribed to one or other of the compartments (Bird *et al.*, 1979).

Lower eukaryotic organisms such as the slime moulds are useful model systems for studies of chromatin structure and phenomena related to genetic expression and differentiation. Hence it was considered pertinent to examine the distribution of 5-methylcytosine in the genome of *Physarum polycephalum*, which is known to contain significant amounts of 5-methylcytosine (Evans & Evans, 1970; Evans *et al.*, 1973).

In the present paper we describe experiments in which endonucleases *HpaII* and *MspI* have been used to probe the sites of methylation in *Physarum* nuclear DNA. Similar to the observations in *Echinus* and other organisms with an echinoderm-type pattern of DNA methylation, we find evidence for an M⁺ and an M[−] compartment in *Physarum* DNA. In contrast with these previous studies, however, a significant fraction of the DNA is comparatively resistant to the action of both endonucleases *HpaII* and *MspI*. Possible explanations for this result are discussed. The evidence presented lends support to

the idea that the echinoderm-type pattern of DNA-sequence methylation may have preceded both the insect- and vertebrate-type patterns.

Materials and methods

Culture methods and isolation of nuclear DNA

Physarum polycephalum strain LU648 × LU688 (Cooke & Dee, 1975) was used in this work. Microplasmodia were grown as described previously (Hardman *et al.*, 1979). DNA was labelled in microplasmodia by growth in the presence of 1 μ Ci of [methyl-³H]thymidine/ml of culture, as described previously (Hardman & Jack, 1977). Nuclei were isolated by the method of Mohberg & Rusch (1971) as modified by Hardman & Jack (1978). DNA was isolated from purified nuclei by lysis and proteinase K treatment, followed by isopycnic centrifugation as described previously (Hardman & Jack, 1978). Density-gradient fractions containing nuclear DNA were pooled and dialysed against 20 mM-NaCl/1 mM-EDTA/10 mM-Tris/HCl, pH 8.0.

Restriction nucleases

Enzymes *MspI* and *HhaI* were obtained from CP Laboratories, P.O. Box 22, Bishops Stortford, Herts., U.K., and enzymes *AluI*, *HaeIII*, *HindIII*, *HpaIII* and *TaqI* were from Boehringer Corp. (London), Bell Lane, Lewes, East Sussex, U.K. Enzymes were used under conditions recommended by the supplier.

Agarose-gel electrophoresis

Agarose gels were electrophoresed at 60 mA for 3 h in buffer consisting of 5 mM-sodium acetate, 1 mM-EDTA and 40 mM-Tris/HCl, pH 7.4. Gels were stained after electrophoresis with 1 μ g of ethidium bromide/ml in electrophoresis buffer, and viewed under short-wave u.v. light. Gels were photographed with Kodak Tri-X film through an orange filter.

Densitometric analysis of endonuclease-*HpaII* and -*MspI* digests of *Physarum* DNA was performed by scanning photographic negatives of gels with a Joyce-Loebl microdensitometer.

Sucrose-density-gradient centrifugation

Preparative 5–20% (w/v) sucrose gradients (5 ml) in 50 mM-NaCl/1 mM-EDTA/10 mM-Tris/HCl, pH 7.4, were formed in cellulose nitrate tubes. DNA (10 μ g) in 0.1 ml of restriction buffer was layered on to each gradient and centrifuged at 40 000 rev./min for 90 min at 20°C in the SW50.1 rotor in a Beckman L5-50 preparative ultracentrifuge. Appropriate gradient fractions were pooled and the DNA was precipitated by the addition of 2 vol. of 95% (v/v) ethanol.

Results and discussion

Physarum nuclear DNA was digested to termination with the restriction endonucleases *HpaII*, *MspI* and *HhaI*, and the products of digestion were analysed on agarose gels. The results are shown in Fig. 1. Compartmentation of the DNA into high- and low-molecular-weight fractions can be seen clearly in digests obtained with endonucleases *HpaII* and *HhaI* (lanes 5, 9 and 4, 8 respectively, Fig. 1). These will be referred to as M+ and M– fractions respectively, by analogy with the results obtained in other systems by Bird *et al.* (1980). Double digestion using both enzymes increases the compartmentation by decreasing the average fragment size of the M– fraction, while sparing the high-molecular-weight M+ DNA (lanes 3 and 7, Fig. 1). Endonuclease *MspI* significantly decreases the fragment size of the M+ enzyme-*HpaII*-resistant material, thus demonstrating that this fraction results, at least partly, from the presence of methylated *HpaII*-specific sites in *Physarum* nuclear DNA (lanes 2 and 6). Interestingly, the presence in the M+ fraction of repetitive sequences which contain regularly spaced methylated *HpaII*-specific sites is indicated by the generation of discrete bands of DNA in endonuclease-*MspI* digests that are either absent from endonuclease-*HpaII* digests, or present in undetectably small amounts (compare lanes 2 and 6 with 5 and 9; Fig. 1). The most prominent of these repetitive DNA bands correspond to fragments about 5000, 7000 and 10000 nucleotide pairs in length. These results are in general agreement with previous observations in other systems indicating that highly repetitive DNA is heavily methylated (Gautier *et al.*, 1977; Browne *et al.*, 1978).

In addition to the experiments illustrated, control experiments were conducted to verify that the results were not due to trivial factors. Parallel digests were carried out which included bacteriophage- λ DNA to monitor completeness of digestion by each enzyme. Various treatments used in the preparation of the *Physarum* DNA, and the addition of excess restriction endonucleases, did not affect the differences in the digestion patterns shown in Fig. 1. It was also verified that, under the conditions used, both endonucleases *MspI* and *HpaII* gave identical digestion patterns with bacteriophage- λ DNA, thus eliminating the possibility of the presence of a contaminating restriction or modification activity in the enzyme preparations.

The M+ fraction amounts to about 20% of the total nuclear DNA complement in *Physarum*, as determined from the fluorescence intensities of ethidium bromide-stained gels, and consists of DNA segments ranging from about 20 000 to 50 000 base-pairs in length. This is considerably longer than the average size of DNA in the M– fraction

Fig. 1. Separation of *Physarum* DNA fragments by agarose-gel electrophoresis after digestion with restriction endonucleases

DNA was digested to completion with restriction endonucleases and subjected to electrophoresis on agarose slab gels as described in the text, with reference to restriction fragments of DNA from bacteriophages λ and PM2. Electrophoresed in separate lanes of the same gel. The same amount of DNA ($3\mu\text{g}$) was loaded into each slot in the gel. After electrophoresis, gels were stained with ethidium bromide and photographed under u.v. light. (a) 1% agarose gel; (b) 0.5% agarose gel. Lanes: 1, mixture of DNA from phages λ and PM2 limit-digested with endonuclease *Hind*III; 2 and 6, *Physarum* DNA digested with endonuclease *Msp*I; 3 and 7, *Physarum* DNA digested with endonucleases *Hpa*II plus *Hha*I; 4 and 8, *Physarum* DNA digested with endonuclease *Hha*I; 5 and 9, *Physarum* DNA digested with endonuclease *Hpa*II; 10, undigested *Physarum* DNA.

(300–2000 base-pairs). These results suggest that 5-methylcytosine is distributed non-randomly in *Physarum* DNA, methylated *Hpa*II- and *Hha*I-specific sites being clustered together in regions of DNA that are separated from each other by unmethylated segments. However, analysis of the fragment sizes obtained on digestion of DNA with endonuclease *Msp*I (Fig. 2) shows that, although enzyme *Msp*I indeed decreases the average size of the M+ compartment, it does not cleave the DNA to an extent equivalent to that found for the M- fraction. This may be an indication that *Physarum* DNA might contain two types of modified loci: DNA segments containing consecutively methylated *Hpa*II- and *Hha*I-specific sites, and additional sites that are resistant to cleavage by both enzymes *Hpa*II and *Msp*I. The latter could result from further modification of a number of the *Hpa*II-specific sites in M+ DNA, rendering them refractory to cutting by either enzyme. For example, the doubly methylated sequence mCpmCpGpGp is found in human

DNA and has been shown to be resistant to cleavage by both endonucleases *Hpa*II and *Msp*I (Van der Ploeg & Flavell, 1980). Alternatively, *Hpa*II-specific sites could occur much less frequently in M+ DNA sequences than in M- DNA, perhaps owing to the presence of tandemly repeated sequences that are devoid of *Hpa*II-specific sites. We have been able to detect several repetitive sequence components in the *Physarum* genome by the generation of different families of sharp DNA bands, ranging in size from 100 to 5000 base residues, in agarose gels of restriction fragments obtained by digestion of total *Physarum* DNA with endonucleases *Alu*I, *Hae*III and *Taq*I (A. C. Robinson, P. A. Whittaker & N. Hardman, unpublished work). The amount of DNA estimated to be present in these repetitive DNA bands, however, is far too small to provide a satisfactory explanation of the refractory nature of M+ DNA to digestion with enzymes *Hpa*II and *Msp*I, based on the assumption that tandem repeats peculiar to M+

Fig. 2. Size distribution of *Physarum* DNA digested with restriction endonucleases

DNA was restricted and electrophoresed on 0.5% agarose gels similar to that illustrated in Fig. 1. Gels were photographed, and the negatives scanned with a microdensitometer. The numbers ($10^{-3} \times$ base-pairs) refer to the size standards of bacteriophage- λ DNA digested with enzymes *EcoRI* or *HindIII* described in Fig. 1. ----, Undigested *Physarum* DNA; —, DNA digested with endonuclease *HpaII*; ····, DNA digested with endonuclease *MspI*.

DNA dominate the compartment and are devoid of *HpaII*-specific sites. Further experiments are required, however, to confirm this result.

The results reported here for *Physarum*, normally regarded as a lower eukaryotic organism, would tend to support the idea that the type of DNA-methylation pattern observed in *Echinus*, and which is observed in phyla as distantly related as the coelenterates and chordates, could well have preceded both the insect-type and vertebrate-type patterns, as suggested by Bird & Taggart (1980). The findings have been illuminated further by determining the distribution of sequences in the M+ and M- fractions, which show homology with cloned repetitive *Physarum* DNA sequences. This work confirms and extends the study reported here (A. McLachlan, P. A. Whittaker & N. Hardman, unpublished work). It is noteworthy that similar experiments using cloned *Physarum* rDNA (ribosomal DNA) sequences (Reilly *et al.*, 1980) demonstrate that *Physarum* rDNA *in vivo* contains several *HpaII*-specific sites, none of which is methylated. Hence these sequences can be considered to reside in the M- compartment, as defined here.

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