# METHYLATION IN *PHYSARUM* DNA

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Received 4 June 1980

## 1. Introduction

The biological function of methylated bases in eukaryotic DNA is not known. An attractive hypothesis suggests a correlation between gene activity and state of methylation [1–3]. The major modified base in eukaryotes is 5-methylcytosine (MeC) which is primarily found in the doublet CpG [4,5]. Changes in the relative amount of MeC occur during the development of sea urchin [6] and tissue differences are known in several mammals [4]. The pattern of modified bases in chicken globin DNA [7], ovalbumin DNA [8], rabbit globin DNA [9], sea urchin rDNA [10] and *Xenopus* ribosomal DNA (rDNA) [11] have been partially determined. The DNA of *Physarum polycephalaum* is known to contain significant amounts of MeC [12,13]. Furthermore, the nuclei of this organism contain ~200 linear, extrachromosomal DNA molecules that are 60 kilobases (kb) long. Each molecule is composed of two inverted sequences, ~30 kb long, both of which contain one copy of the ribosomal RNA genes [14,15]. These genes are thought to be vigorously transcribed in vivo, while other regions of this molecule, such as the satellite-like spacers, are presumably not transcribed at all. In this report, we present evidence that this extrachromosomal DNA, called rDNA here, is broken by *Hpa*II and *Msp*I in an identical manner, suggesting that most of the CCGG restriction sites are free of methylation. Chromosomal DNA, by contrast, shows significant differences. Equivalent experiments with *Dpn*I, *Mbo*I and *Sau*3A, which are sensitive to methyladenine at GATC, indicate that this site is not methylated in either DNA.

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## 2. Materials and methods

Ribosomal and chromosomal DNA from *Physarum*, strain M3cVIII, was isolated by the method in [16]. In brief, plasmodia were homogenized, nuclei and nucleoli isolated by differential centrifugation, followed by DNA extraction using SDS and proteinase K. Density gradient centrifugation with CsCl separated rDNA from chromosomal DNA. Mouse DNA was prepared from NIH-3T3 cells by standard procedures [17].

Restriction enzymes were used under conditions described by manufacturers, Bethesda Res. Labs (*Hpa*II, *Mbo*I, *Taq*I, *Dpn*I, *Sau*-3A, *Hae*III, *Hin*dIII, *Eco*RI), and New England Biolabs (*Msp*I). *Hin*dIII was prepared by an unpublished method of J. G. R. and S. S. Smith. *Msp*I was a gift of Joel Gottesfeld.

Agarose electrophoresis (low EEO agarose, Sigma Chemical Co.) was in 40 mM Tris (pH 7.9), 5 mM Na-acetate, 1 mM EDTA. Gels were run, stained with ethidium bromide and photographed as in [18].

## 3. Results and discussion

The availability of restriction endonucleases whose action is affected by the presence of a methyl group on a base within the nuclease recognition sequence, has opened new ways to explore sequence-specific methylation. Table 1 lists the recognition sequences and cleavage sites of some restriction endonucleases that are useful in determining the state of methylation at a specific position in the chromosome. For example, one approach is to treat a sample of a DNA with either *Hpa*II or *Msp*I, both of which recognize CCGG. However, cleavage by *Hpa*II is blocked completely if this sequence contains MeC, while

Table 1
Effect of methylation on restriction enzyme activity

| Enzyme | Site | Modified site | Ref. |
|---|---|---|---|
| *Hpa*II | C↓CGG | C*CGG | [19,20,31] |
| *Msp*I | C↓CGG | C↓*CGG | [19,20,32] |
| *Taq*I | T↓CGA | T*CGA | [21] |
| *Dpn*I | GATCX | GA*TC↓X | [22] |
| *Mbo*I | X↓GATC | GA*TC | [23] |
| *Sau*-3A | X↓GATC | X↓GA*TC | [23] |

This table shows the recognition and cleavage sites for some restriction endonucleases that are useful for determining stage of methylation. The arrow indicates cleavage; no arrow indicates that no cleavage will occur. Methyl group is indicated by an asterix

*Msp*I is unaffected. Thus, as shown in fig.1, both mouse and *Physarum* chromosomal DNA are broken less frequently by *Hpa*II than by *Msp*I, even in the presence of an excess of nuclease. This suggests that many CCGG sequences are blocked by methylation. When these enzymes are applied to the purified rDNA, an identical pattern of ~20 bands is seen (fig.2). Because of the palindromic nature of this molecule, even the faintest bands must correspond to 2 segments/molecule; yet, judging by the fluorescent intensity, many bands contain 5–10-times more DNA than the faintest ones. These probably arise from numerous repeating sequences (R. B., unpublished). Thus, one may conclude that ⩾100 CCGG sequences within the rDNA are unmethylated. This stands in contrast with the chromosomal DNA where ⩾10–20% of the DNA survives *Hpa*II in a relatively high molecular weight condition. This estimate roughly agrees with the observation that in total *Physarum* DNA 1 cytosine in 20 is methylated [12,13]. If all these MeCs were in CpG, as the majority are in mammalian DNAs [4,5], and if 1 C in 4 were in a CpG doublet, then 1 CpG in 5 (20%) would be methylated.

Fig.1B shows that the portion of chromosomal DNA, which survives *Hpa*II, is found in relatively high molecular weight fragments. This suggests that methylated CCGG sequences are clustered and not evenly distributed throughout the chromosomal DNA. In comparison, we would expect ~20% of the CCGG sequences in rDNA to be methylated and unavailable to *Hpa*II. This is clearly not the case and suggests that this extrachromosomal rDNA is somehow protected from the methylation that occurs on the chromosomal DNA.

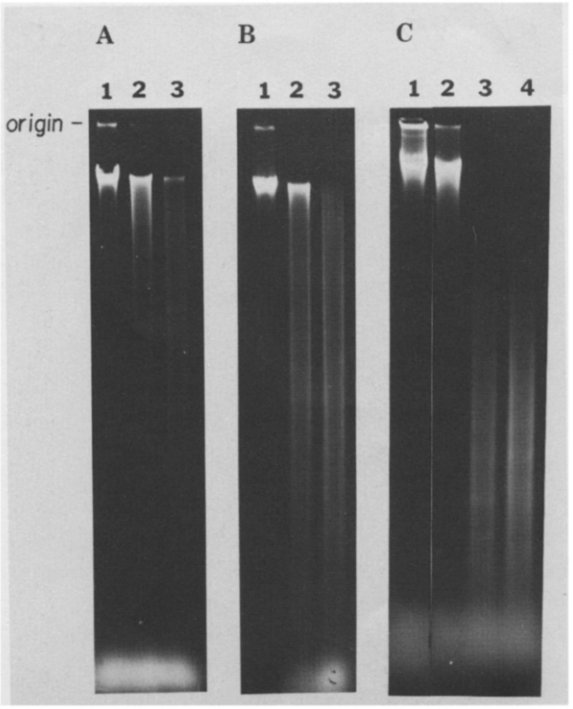


Fig.1. Restriction patterns of total mouse and *Physarum* chromosomal DNA electrophoresed on 0.8% agarose gels. (A) Shows mouse DNA: unrestricted (1); restricted with *Hpa*II (2); restricted with *Msp*I (3). (B) Shows *Physarum* chromosomal DNA: unrestricted (1); restricted with *Hpa*II (2); restricted with *Msp*I (3). (C) Shows *Physarum* chromosomal DNA: unrestricted (1); restricted with *Dpn*I (2); restricted with *Mbo*I (3); restricted with *Sau*-3A (4). Each channel contained 6 μg DNA. Electrophoresis, staining, and photography was performed as in [18]. Terminal digestion was verified by incubating ~10% of the reaction mix with 0.5 μg pBR322. The appearance of expected bands confirmed that terminal digestion occurred.

A second approach to this problem is to compare the restriction patterns of a specific segment derived from the extrachromosomal DNA (the natural DNA) with the same segment after cloning in *E. coli*. In this case, one assumes that growth in *E. coli* (particularly in strains that are mutationally deficient in ability to modify DNA) results in a DNA that is free of modification at CpG. This is illustrated in fig.2B where the natural 2.7 kb *Eco*RI segment that contains a portion of the 26 S rRNA gene [24] is compared with its cloned counterpart. As can be seen, both *Hpa*II and *Taq*I produce identical restriction patterns with both DNAs. This means that all the CCGG and

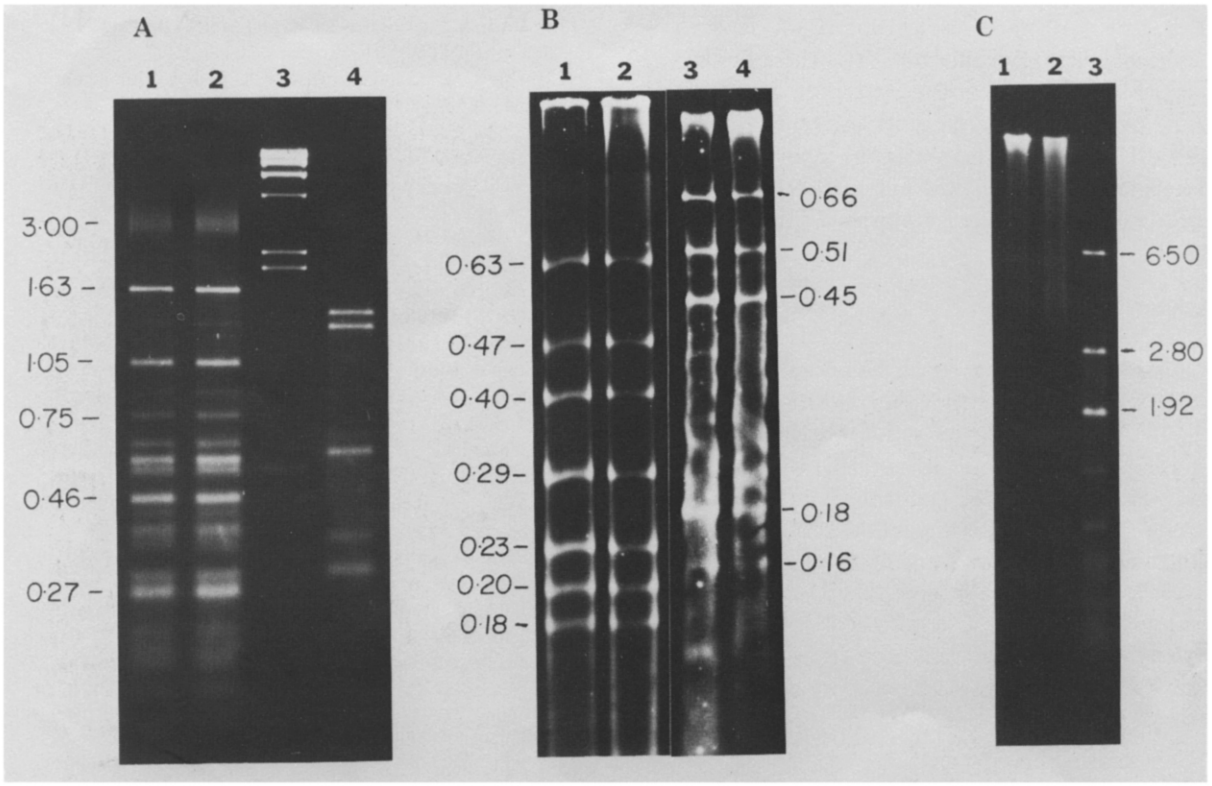


Fig.2. Restriction patterns of *Physarum* ribosomal DNA. (A) ~4 μg rDNA was terminally digested with *Hpa*II (1) and *Msp*I (2), run on a 2.5% agarose gel, stained and photographed; (3) contains λ/*Hin*dIII markers (23.6, 9.64, 6.64, 4.34, 2.26, 1.98, and 0.56 kb [25] and (4) contains pBR322/*Taq*I (1.44; 1.31, 0.62, 0.37, 0.32 and 0.31 kb [26]). The lengths of some rDNA segments are shown to the left.

(B) Shows the restriction pattern of a cloned rDNA segment and its natural counterpart. A plasmid containing a 2.7 kb *Eco*RI segment that has homology to a portion of the 26 S RNA [24] was amplified in *E. coli*, purified, restricted with *Eco*RI and electrophoresed on 0.8% agarose. The 2.7 kb DNAs were recovered by dissolving the agarose in Na-perchlorate [27] and chromatography on hydroxyapatite. These DNAs were broken with either *Hpa*II or *Taq*I, and the uneven ends filled in with d[$\alpha^{32}$P]-GTP using *E. coli* polymerase I (Boehringer). After electrophoresis on 5% acrylamide gels (acrylamide:bis-acrylamide, 25:1) with pBR322/*Msp*I and pBR322/*Hae*III, the gels were stained, photographed and autoradiographed. (1,3) Contain the cloned segment broken with *Hpa*II and *Taq*I, respectively; (2,4) contain the natural segment broken with these same nucleases. The calculated lengths of the *Hpa*II segments are noted on the left, the *Taq*I segments on the right.

(C) Shows the restriction patterns of rDNA on 0.8% agarose gels; (1) no enzyme; (2) *Dpn*I; (3) *Mbo*I. Separate experiments showed that *Dpn*I is active on pBR322 DNA (grown on $r^-m^-$*E. coli* where all GATC sequences are methylated [28]) and that the conditions of digestion would have resulted in a terminal pattern if sites were available. The lengths of some prominent *Mbo*I fragments are noted.

TCGA sequences in both the natural and cloned segment are equivalent, and therefore, unmethylated.

A third approach is illustrated with *Dpn*I which *requires* a GA(me)TC sequence in order to cleave the DNA. As seen in fig.1C and fig.2C, this enzyme fails to cleave the rDNA or chromosomal DNA, although GATC sequences do exist as indicated by the cleavage observed with *Mbo*I.

## 4. Conclusions

The extrachromosomal DNA (rDNA) of *Physarum polycephalaum* appears to have unmethylated CCGG sequences, while these sequences are significantly methylated in the chromosomal DNA. The rDNA has no detectable methylation at CCGG or TCGA within an *Eco*RI segment covering a portion of the 26 S

RNA gene. Both chromosomal and rDNAs have no detectable methyladenine at GATC. These findings parallel the observation that extrachromosomal rDNA from *Xenopus* oocytes is significantly undermethylated [10], and contrasts with the report that dinoflagellate DNA is highly methylated [29]. A comparison of many species has appeared [30].

## Acknowledgements

This work was supported by American Cancer Society Postdoctoral Fellowship, California Division (J-457-01) to G. R., Swiss National Science Foundation (no. 3.312.78) to R. B., and NIH GM 25531 and NSF PCM 78-16282 grants to C. A. T. We thank Dr Steven Smith for his unpublished method for end-labelling restriction segments.

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