DNA methylation during differentiation of a lower eukaryote, *Physarum polycephalum*

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Starvation-induced differentiation of the slime mould *Physarum polycephalum* is accompanied by continuous methylation of DNA. No stable changes in the overall level of DNA methylation are evident, but a gene known to be transcribed specifically during differentiation is subject to increased methylation.

Inhibitors of DNA methylation preclude differentiation of *P. polycephalum*, although they are only marginally inhibitory to normal growth. Taken together these results indicate that methylation of DNA is involved in differentiation of this lower eukaryote.

INTRODUCTION

Cytosine methylation is a common modification of nuclear DNA in vertebrates and vascular plants. Ample evidence indicates the role of DNA methylation in regulating gene activity in differentiation in these organisms (reviewed in [1,2]). Usually methylation of a region within or flanking a gene correlates with its transcriptional inactivity, but the opposite relationship has also been observed [3]. Methylated-DNA-binding proteins [4] are a likely go-between linking DNA methylation and transcriptional activity.

Elucidation of the mechanisms regulating methylation of individual nucleotide sequences and of the influence of DNA methylation on transcription would be greatly advanced if less complex model organisms, amenable to genetic analysis and manipulation, were available to study these processes. Unfortunately, the best characterized lower eukaryotes, i.e. yeasts, *Dictyostelium discoideum*, *Caenorhabditis elegans*, *Drosophila melanogaster*, all lack detectable methylation in their DNA [5]. Among the less-thoroughly investigated 'simple' organisms the slime mould *Physarum polycephalum* stands out as a very promising candidate for studies of DNA methylation. Its level of DNA methylation is similar to that of vertebrates [6] and a preliminary study has suggested that DNA methylation is a necessary step in its sporulation [7]. Several inducible transitions among a number of life-forms (e.g. diploid and haploid, uni- and multinucleate, metabolically active and dormant) offer a wide range of differentiation programmes to study [8]. Recent progress in transient [9] and stable [10] transformation of *P. polycephalum* opens it up for genetic manipulations.

In this paper we sought evidence for the involvement of DNA methylation in starvation-induced formation of vegetative spores (spherulation) in *P. polycephalum*. This is probably the best biochemically characterized transition in *P. polycephalum*. Numerous genes expressed selectively during spherulation have been cloned [11,12], making it possible to investigate relationships between DNA methylation and expression of individual genes.

EXPERIMENTAL

Spherulation

P. polycephalum strain M₃CIV was grown in semi-defined liquid medium (growth medium) and spherulation was induced by

transferring 48-h-old cultures into salts-only medium (starvation medium) [8]. The time of transfer into starvation medium will be further referred to as time zero. Mature spherules were operationally defined as structures capable of germinating after being subjected to a 48 h period of desiccation over anhydrous CaCl₂ at 4 °C.

Isolation of DNA

DNA was prepared by Proteinase K digestion of SDS-lysed nuclei [13] followed by phenol–chloroform and chloroform extractions and RNAase A digestion. Spherules were disrupted by grinding in a porcelain mortar in liquid nitrogen. The final step of KI density gradient centrifugation [14] was used to free the DNA from contaminating slime.

Pulse-labelling with [methyl-³H]methionine

To 3 ml of culture in starvation medium [methyl-³H]methionine (Amersham; 83 Ci/mmol) was added to achieve a concentration of 5 µCi/ml and incubation was continued for 1 h. DNA was purified as above and digested to completion with DNAase I. Radioactivity and A₂₆₀ of the 0.2 M perchloric acid-soluble supernatant were measured and the intensity of DNA methylation was expressed as counts/min per A₂₆₀ unit.

H.p.l.c. analysis

DNA was hydrolysed in 98% formic acid and analysed by h.p.l.c. as in [15] on a 5 µm C₁₈ 4.6 mm × 250 mm column with 45 mm precolumn (Beckman). All chemicals were of the highest quality available from Merck. Detection was at 280 nm and individual bases were identified by comparing their retention times with those of authentic standards (Sigma). For calculation of mol% of methylated cytosine [(mol of 5-mC):(mol of 5-mC + mol of cytosine)] the peak areas of cytosine were multiplied by 1.04 to compensate for different molar absorption coefficients of the two bases.

Southern hybridization

DNA was digested for 2 h with 4 units of restriction endonuclease per µg of DNA and electrophoresed at 5 µg per lane in 2%

Abbreviations used: AzaC, 5-azacytidine; 5-mC, 5-methylcytosine.

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agarose. *Hae*III-digested Φ X-174 DNA was run as size markers. Gels were electroblotted on to Zeta-Probe membrane (Bio-Rad). cDNA inserts cut out from LAV2-1, 2-2, 2-4, 6-1, 6-3 and 6-4 plasmids [11,12] were used individually as hybridization probes. Probes, supplemented with 10 pg of Φ X-174 DNA to visualize the size markers, were labelled with 32 P using the Megaprime system (Amersham). Hybridization and washes were at high stringency.

RESULTS

The influence of inhibitors of DNA methylation on spherulation

After being transferred to starvation medium *P. polycephalum* cultures undergo a massive metabolic transition resulting in the formation of dormant spherules [8]. First spherules appear after approx. 48 h in starvation medium and by 72 h the transition is completed. To check whether methylation of DNA is a necessary element of this transition we added 5-azacytidine (AzaC), a potent inhibitor of DNA methylation [16], to the starvation medium at time zero and assayed for the presence of mature spherules after 72 h incubation. It was found that AzaC at 50 μ M precluded the formation of spherules, with lower concentrations being ineffective. Increasing the time the cultures spent in starvation medium supplemented with AzaC up to 168 h did not produce spherules either, thus indicating that spherulation was inhibited completely rather than being only slowed down.

To ensure that this effect was not caused by the overall toxicity of the inhibitor we checked the influence of AzaC on growth of *P. polycephalum* in growth medium and on the viability of cultures in starvation medium. The growth rate of the cultures, as measured by the increase in total protein, in the presence of AzaC was approx. 75 % of that in normal growth medium (results not shown). Cultures incubated in starvation medium plus AzaC for 72 h resumed active growth immediately upon transfer into normal growth medium. These results prove that the inhibition of spherulation was not caused by non-specific toxicity of AzaC.

We then checked the influence on spherulation of AzaC present at different periods of the process. The results are shown in Table 1.

Consistent with the mechanism of action of AzaC [16] its continuous presence during the initial 6 h of spherulation is sufficient to inhibit the process. Shorter incubation with AzaC is

not effective, which probably reflects the need for sufficient amounts of the drug to be incorporated into DNA to inhibit the DNA methyltransferase. The inhibitory action of AzaC added later in spherulation indicates that DNA methylation necessary for spherule formation takes place after 24 h into spherulation; this does not exclude the possibility that DNA methylation at earlier periods is also important.

AzaC had no influence on germination of spherules produced in its absence (results not shown), which suggests that methylation of DNA is required for the formation of spherules rather than for their subsequent germination. However, one cannot exclude the possibility that AzaC had no effect on spherule germination due to its inefficient uptake.

To ensure that the action of AzaC was indeed effected through the inhibition of DNA methylation we used two other drugs known to inhibit methylation of DNA: 5-aza-2'-deoxycytidine and L-ethionine. These compounds at 12.5 μ M and 200 μ M respectively also inhibited the formation of spherules.

DNA methylation in spherulating *P. polycephalum*

Results described above suggested that methylation of DNA takes place even after 24 h in starvation medium. To estimate the intensity of DNA methylation during spherulation cultures of *P. polycephalum* were pulse-labelled with [*methyl*- 3 H]methionine at different times after the induction of spherulation and the incorporation of 3 H into DNA was determined. Results (not shown) indicated that the average intensity of DNA methylation remains virtually unchanged from time zero through 30 h into spherulation, with values obtained for individual measurements at any particular time-point differing by as much as 50 %. Measurements were not performed at later stages as the formation of thick-walled spherules was likely to interfere with the uptake of the label.

We next determined the level of DNA methylation by h.p.l.c. analysis of the percentage of methylated cytosine (5-mC) in DNA preparations from cultures at different times of spherulation. Results are given in Table 2.

The apparently small increase in the mean level of DNA methylation during spherulation is statistically not significant. Nevertheless, a substantial difference is evident in the ranges of values measured for individual time-points. While in the actively growing cultures (0 h in starvation medium) individual DNA preparations all had similar contents of 5-mC (range 5.1–5.4 %), for DNAs from spherulating cultures the ranges were 4.0–8.7 %, 4.1–6.9 % and 3.9–8.5 % for cultures at 24 h, 48 h and 72 h respectively. These results suggest that transient changes in the

Table 1 The influence of AzaC on spherulation

Cultures were transferred into starvation medium at time zero. AzaC was added at time points indicated and incubation in the presence of AzaC was continued as shown. Next, cultures were transferred into fresh starvation medium without AzaC and were incubated further to give the total time of 72 h. Spherule formation was assayed at 48 h and 72 h. Symbols denote: +, spherules formed after 48 h; +/–, spherules formed only after 72 h; –, no spherules formed.

Time in the presence of AzaC (h)	Spherule formation
0–2	+
0–4	+ / –
2–6	+ / –
4–6	+
0–6	–
12–24	–
24–48	–

Table 2 The content of 5-methylcytosine (5-mC) in DNA from cultures at different times of spherulation

Mean $\pm$ S.D. of mol % 5-mC: (C + 5-mC). Numbers of independent DNA preparations analysed for each time-point are given in parentheses.

Time in starvation medium (h)	5-mC (%)
0	5.25 $\pm$ 0.14 (6)
18	5.13 $\pm$ 0.44 (4)
24	5.47 $\pm$ 1.67 (6)
36	5.45 $\pm$ 0.15 (3)
48	5.46 $\pm$ 0.89 (8)
72	5.76 $\pm$ 1.78 (5)

Figure 1 Methylation of spherulin-4 gene during spherulation of *P. polycephalum*

DNA from actively growing cultures (lanes 1), from cultures after 24 h in starvation medium (lanes 2), from mature spherules (lanes 3) and from germinating spherules (lanes 4) was digested with restriction endonuclease isoschimer pairs [*HpaII* and *MspI* in (a), *MboI* and *Sau3AI* in (b)], Southern-blotted and hybridized with LAV6-1 cDNA. Sizes of bands are given in base pairs.

rate of DNA methylation and/or demethylation take place in spherulating cultures, leading to changes in the overall level of DNA methylation in individual DNA preparations.

Methylation of individual DNA sequences

Results obtained up to this point constitute a strong indication that methylation of DNA is an important element of spherulation. To investigate the methylation status of defined nucleotide sequences we analysed several spherulation-specific genes by Southern blotting of genomic DNA digested with pairs of methylation-sensitive/methylation-insensitive restriction endonuclease isoschimers. The pairs used were *MspI*/*HpaII* recognizing the sequence CCGG, with *MspI* inhibited by methylation of the outer C and indifferent to methylation of the inner C and *HpaII* inhibited by methylation of either C, and *MboI*/*Sau3AI* recognizing the sequence GATC with *MboI* indifferent to and *Sau3AI* inhibited by cytosine methylation. DNA from actively growing cultures, from cultures after 24 h in starvation medium, from mature spherules and from germinating spherules was probed with six spherulation-specific cDNA probes (see the Experimental section); five of them gave hybridization patterns identical for both isoschimers in each pair and for all DNAs tested (results not shown), indicating no methylation of CCGG and GATC sequences in these genes.

LAV6-1 (spherulin 4) cDNA [12] gave a strikingly different picture (Figure 1). In DNAs from cultures starved for 24 h and from mature spherules several hybridizing bands appear in addition to those present in DNA from actively growing cultures and from germinating spherules, which indicates changes in methylation of this gene. To estimate the reproducibility of this phenomenon additional independent DNA preparations were analysed as above (results not shown). All DNAs from actively growing cultures gave hybridization patterns identical with those in lanes 1 in Figure 1. Of seven preparations from spherules, two gave patterns identical with those from actively growing cultures, while five gave more complex patterns, qualitatively similar to those in lanes 3 in Figure 1. This indicates that changes in methylation of the spherulin 4 gene are indeed associated with the formation of spherules but are probably transient.

Alternative explanations of the observed changes in the hybridization patterns were ruled out based on the results of

control experiments. Digesting the DNA with doubled amounts of restriction endonucleases did not lead to any changes in hybridization patterns (results not shown), thus ruling out incomplete digestion as the source of additional bands. Upon progressively higher stringency washes of the hybridization membranes all bands were weakening in parallel (results not shown), thus indicating that all the signal comes from true homologous hybridization rather than from non-specific association of the probe with, for instance, repetitive sequences.

DISCUSSION

Our studies with inhibitors of DNA methylation indicated that for *P. polycephalum* to differentiate into spherules methylation of DNA must take place at least during the second half of spherulation (after 24 h in starvation medium). Two classes of inhibitors were used: (i) nucleoside analogues, which upon incorporation into DNA selectively and irreversibly inhibit the DNA methyltransferase, and (ii) L-ethionine, a competitive inhibitor of all S-adenosylmethionine-dependent methyltransferases. Since these two classes of inhibitors operate by distinct mechanisms their influence on spherulation is likely to be caused via the feature they have in common, i.e. by inhibition of DNA methylation. Consistent with the observation that adding the inhibitors of DNA methylation after 24 h of starvation prohibits spherule formation is the finding that methylation of DNA proceeds at a roughly constant rate for at least the first 30 h of spherulation. We have also shown that inhibiting methylation of DNA does not lead to significant overall growth retardation of *P. polycephalum*, thus indicating the specific role of DNA methylation in spherulation. Similar conclusions were reached before [7] concerning the participation of DNA methylation in another differentiation programme of *P. polycephalum*, i.e. formation of meiotic spores, although no proof was offered for the specificity of the effect. Recent studies using transgenic mice indicate that DNA methylation is dispensable for cell growth and divisions but is required at a certain stage of embryonic development [17].

Inhibitors of DNA methylation alter the development of another 'lower' eukaryote, a sea urchin [18]; subsequent studies indicated that indeed during its development individual genes undergo differential methylation [19] and that artificially increasing the level of DNA methylation alters the expression pattern of developmentally regulated genes [20].

In another species of *Physarum*, *P. flavicomum*, formation of dormant cysts was found to be accompanied by an approximately fourfold decrease in the content of 5-mC [15]. In contrast, no statistically significant changes in the average level of DNA methylation could be observed in spherulating cultures of *P. polycephalum*. However, pronounced differences in the content of 5-mC among individual DNA preparations from spherulating cultures and spherules indicate that transient changes in DNA methylation do take place. These changes could take the form of waves of demethylation and subsequent *de novo* methylation resulting in altered patterns of methylation of individual nucleotide sequences.

The latter supposition is supported by the observation of changes in the methylation pattern of a spherulation-specific gene, spherulin 4. Based on the specificity of the restriction nucleases used for the experiment we suggest the following interpretation of the hybridization patterns shown in Figure 1. In the genomic sequence of the spherulin 4 gene several CCGG and GATC sequences are present which, when unmethylated (as in actively growing cultures), will be cut by appropriate restriction endonucleases, giving rise to a number of DNA fragments some

of them shorter than approx. 100–150 bp. Such fragments would be lost from Southern blots. During spherulation, methylation of cytosines in some of these sites renders them refractive to cutting, thus giving rise to longer fragments appearing as additional bands of 320 and 450 bp in *MspI*- and *HpaII*-cut DNA or as the 1600 bp band in *Sau3AI*-cut DNA. Since the genomic organization of the gene is unknown positions of these differentially methylated sites cannot be inferred. Methylation of these sites is not permanent in mature spherules as shown by some of the DNA preparations from spherules having hybridization patterns indistinguishable from that of the actively growing cultures. As spherulin 4 mRNA is detectable only in late spherulating cultures and mature spherules [12] methylation of the gene apparently accompanies its transcriptional activation. Similar correlation has been observed for some genes in higher vertebrates [3], albeit the inverse relationship holds true much more frequently [1].

A study published when this work was in progress failed to detect any changes in the methylation pattern of several genes during differentiation of *P. polycephalum* [21]. The most likely explanation of this discrepancy is the fact that not all developmentally regulated genes undergo differential methylation detectable by the method used in the study in question as well as in this work; for five other genes studied we also failed to detect any changes in their methylation.

This report is among the very few cases to date of the involvement of DNA methylation in differentiation of a lower eukaryote; the detection of changes in methylation of a spherulation-specific gene suggests that *P. polycephalum* should be considered as a new, easy to manipulate model with which to study the participation of DNA methylation in differentiation.

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