Developmentally Regulated DNA Methylation in

Dictyostelium discoideum†

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DNA methylation is linked to various aspects of epigenetic regulation, including silencing of gene expression, organization of chromatin structure, and cellular differentiation of eukaryotes. Previous studies failed to detect 5-methylcytosine in Dictyostelium genomic DNA, but the recent sequencing of the Dictyostelium genome revealed a candidate DNA methyltransferase gene (dnmA). The genome sequence also uncovered an unusual distribution of potential methylation sites, CpG islands, throughout the genome. DnmA belongs to the Dnmt2 subfamily and contains all the catalytic motifs necessary for cytosine methyltransferases. Dnm2 activity is typically weak in Drosophila melanogaster, mouse, and human cells and the gene function in these systems is unknown. We have investigated the methylation status of Dictyostelium genomic DNA with antibodies raised against 5-methylcytosine and detected low levels of the modified nucleotide. We also found that DNA methylation increased during development. We searched the genome for potential methylation sites and found them in retrotransposable elements and in several other genes. Using Southern blot analysis with methylation-sensitive and -insensitive restriction endonucleases, we found that the DIRS retrotransponson and the guaB gene were indeed methylated. We then mutated the dnmA gene and found that DNA methylation was reduced to about 50% of the wild-type level. The mutant cells exhibited morphological defects in late development, indicating that DNA methylation has a regulatory role in Dictyostelium development. Our findings establish a role for a Dnmt2 methyltransferase in eukaryotic development.

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ylation is developmentally regulated and that deletion of the *dnmA* gene results in reduced methylation and in developmental defects. We found that CpG dinucleotides have a unique distribution in the *Dictyostelium* genome and that 5mC residues are found around some of the DIRS transposable elements and in the *guaB* gene.

**MATERIALS AND METHODS**

Growth, development, and generation of mutants. Wild-type *Dictyostelium* strain AX4 (19) and the *dnmA* mutant were grown in HL-5 liquid medium (41) and developed as described previously (36). The *dnmA* knockout strain was generated in AX4 by substituting a 1.2-kb fragment of the *Dictyostelium* *dnmA* gene (nucleotides 80 to 1292 relative to the first ATG) with a 4.4-kb plasmid containing the blasticidin resistance gene (1). Transformants were generated by homologous recombination, selected as described previously (29), and verified by Southern blot analysis and by PCR across the homologous recombination junctions. Two independently derived strains were constructed which had identical phenotypes.

Purification of genomic DNA and dot blot analysis. Genomic DNA was purified using three methods. The CTAB method (47) was used with minor modifications. Nuclei were lysed in 100 mM EDTA and 5% sodium lauryl sarcosyl at 55°C for 20 min. Genomic DNA was isolated in CTAB solution (1% CTAB, 0.7 M NaCl, 10 mM EDTA, 50 mM Tris-HCl [pH 8.0], 0.5% polyvinylpyrrolidone) at 65°C for 5 min and extracted with chloroform and phenol-chloroform and purified by ethanol precipitation. For the sodium dodecyl sulfate (SDS)-proteinase K digestion, nuclei were lysed in 1% SDS and 0.2 mg/ml proteinase K for 60 min at 60°C. Genomic DNA was extracted with phenol and phenol-chloroform and purified by ethanol precipitation. For extraction with plant DNAzol (Invitrogen), nuclei from vegetative cells or whole cells from developing cultures were treated according to the manufacturer’s recommended protocol. Genomic DNA from all three methods was treated with 100 μg/ml RNase A (Sigma) for 1 h at 37°C. The different methods produced essentially identical results, supporting the notion that the signals observed were not an artifact of the purification method. PCR fragments of the thymidine kinase gene *thyB* (dictyBase identification no. DDB0191436) were used as positive or negative controls for dot blots. To generate a fully methylated positive control fragment, 5-methyl-dCTP (Roche Applied Science) was used instead of dCTP in the PCR. These DNA fragments were used as standards to quantify the amount of 5mC in the genomic DNA samples (see Fig. S1 in the supplemental material).

Detection and quantification of 5mC in genomic DNA were performed by dot blot analysis with an antibody against 5mC. This sensitive immunological method has been used by others to detect 5mC in *Entamoeba histolytica* (10) and in liver tumors (42) and is especially suitable for detection and quantification of small amounts and small changes in 5mC levels. Genomic DNA (1 to 25 μg) and PCR fragments were denatured with 0.4 N NaOH at 100°C for 10 min, neutralized with ammonium acetate (pH 7.0), and blotted on a nitrocellulose membrane (Schleicher & Schuell). Dot blots were incubated with a 1:750 dilution of anti-5-methylcytosine antibody (Megabase Research). Signals were detected by chemiluminescence with the Western-Star system (Applied Biosystems) according to the manufacturer’s recommended protocol. Antibodies were then removed by incubation with 62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 100 mM 2-mercaptoethanol at 50°C for 30 min, and the blots were hybridized with a radiolabeled DNA probe for DIRS-1 (37) and the control probe * thyB*.

**RESULTS**

Evidence for general methylation of *Dictyostelium* genomic DNA. Previous reports suggested that *Dictyostelium* genomic DNA was not methylated (40), but recent analysis of the *Dictyostelium* genome suggested otherwise (9). To test whether *Dictyostelium* DNA contains methylated cytosines, we extracted genomic DNA from cells during growth and during development. The DNA was denatured and blotted onto a nitrocellulose membrane. The presence of 5mC was monitored by incubation with a specific antibody followed by chemiluminescence detection. Figure 1A shows that *Dictyostelium* genomic DNA does contain 5mC and that the level of methylation was higher in developed cells than in vegetative cells. As controls, we used PCR fragments amplified from the *thyB* gene with dCTP (negative control) or with 5-methyl-dCTP (positive control). The antibody did not react with the negative control and reacted with the positive control in a dose-dependent manner, indicating high specificity and linearity with the amount of 5mC (see Fig. S1 in the supplemental material). A quantitative analysis comparing the methylation signal of genomic DNA to a dilution series of the positive control suggests that as many as 3 in 10,000 (0.03%) cytosine residues are methylated in vegetative *Dictyostelium* cells. The methylation level increased to 0.14% of the cytosine residues in developed cells. We treated the genomic DNA with RNase and with proteinase K and confirmed the absence of protein and RNA by electrophoresis and by spectrophotometry (data not shown). We conclude that the signal we observed was indeed due to methylation of cytosine in DNA.

The dot blots were stripped and hybridized with a probe against the DIRS-1 repetitive element to normalize the signal to the amount of DNA bound to the membrane. Normalized values of the 5mC signal show that basal levels of DNA methylation occur in vegetative cells and are increased about five-fold by the end of development (Fig. 1B). We therefore con-
clude that *Dictyostelium* genomic DNA is methylated and that methylation is developmentally regulated.

**Unusual distribution of CpG islands in the *Dictyostelium* genome.** DNA methylation often occurs on cytosines in the dinucleotide CpG. One of the reasons to suspect that *Dictyostelium* DNA was indeed methylated was the observation that the dinucleotide CpG was underrepresented in the genome (9). In other organisms, methylation of CpG has a profound regulatory role that is associated with the CpG islands (7). CpG islands have been reported around RTEs in other genomes, and the function of methylation at RTEs is a defense mechanism against the expression of these parasitic elements (5, 46). Therefore, we tested whether they were also overrepresented near RTEs in the *Dictyostelium* genome. Figure 2A shows the distribution of RTEs and CpG islands along the 6 chromosomes and several unassigned contigs in the *Dictyostelium* genome. We observed that the CpG islands are not distributed evenly throughout the genome and that they are more frequently found around RTEs (Fig. 2A). The most striking correlation is found at the centromeric end of chromosomes 1 and 6 and at the opposite end of chromosome 2 (*Dictyostelium* chromosomes are telocentric and the map places the centromeres on the left). We also searched for CpG islands using a model that accounts for the bimodal distribution of C+G in coding and noncoding regions, and the results were essentially identical to the ones shown (data not shown).

Surveys of other genomes have indicated that the dinucleotide CpG has a unique distribution around the translational start site (3, 38). To test whether that was also true in *Dictyostelium*, we have aligned all the predicted genes in the genome at the translational start and the translational end sites of their respective open reading frames (ORFs). We then calculated the frequency of CpG dinucleotides from all genes and plotted it for a region of 20 kb, centered on the respective borders of the ORF (Fig. 2B and Fig. 2C). We found that the regions 5’ of the ORF start site (Fig. 2B) and 3’ of the ORF end site (Fig. 2C) are significantly deprived of CpGs.

Due to the unique nucleotide composition of the genome, *Dictyostelium* ORFs have significantly higher G+C contents than their flanking and intergenic regions (9). Plotting the average G+C content around the ORF borders revealed the expected drop 5’ of the start site, followed by a sharp increase 3’ of the start site (Fig. 2B). The end of the ORF exhibited the expected opposite trend with a mild increase 5’, a sharp decline immediately 3’, and a gradual increase back to the average level following the ORF end (Fig. 2C). We therefore tested the ratio between the observed number of CpG dinucleotides and the number expected from the G+C content. We found that the observed/expected ratio increased sharply immediately 5’ of the ORF start site, sharply decreased immediately 3’, and gradually returned to average further 3’ of the start site (Fig. 2B). At the end of the ORF, the CpG ratio gradually decreased 5’ to the end, sharply increased at the end, and then sharply decreased back to the average level 3’ to the end of the ORF (Fig. 2C). We tested the distribution of all other dinucleotides but found none that behaved like CpG (data not shown). Finally, we performed the same analysis on about 3,700 genes that were known to be expressed from microarray data (43). The results were essentially identical to those shown for the entire predicted ORFeome (data not shown). These results support the notion that CpG dinucleotides have a special role around the borders of open reading frames.

**Evidence for selective DNA methylation in the *Dictyostelium* genome.** The correlation between RTEs and CpG islands (Fig. 2A) prompted us to test whether DNA methylation could be observed specifically in RTEs. We digested genomic DNA from growing and developing cells with restriction endonucleases that are sensitive or resistant to the presence of 5mC and separated the restriction fragments by gel electrophoresis. Examination of the banding pattern after ethidium bromide staining revealed that the methylation-sensitive and the methylation-insensitive enzyme reactions were carried out with the same efficiency (Fig. 3A). We therefore conclude that the reactions were carried out to completion. The DNA was then subjected to Southern blot analysis with a probe against one of the most abundant RTEs, DIRS-1 (6, 9). The data in Fig. 3B show the expected multitude of bands but also show that one of the bands was methylated. Comparing the pattern obtained with the 5mC-sensitive endonucleases in lane 3 with the pattern obtained with the 5mC-resistant endonucleases in lane 4, it is clear that at least one type of DIRS RTEs was protected from digestion by the methylation-sensitive endonuclease. This finding was best observed at 16 h of development (Fig. 3B). It is also likely that most of the DIRS RTEs were not methylated.

**FIG. 1. DNA methylation is developmentally regulated.** Genomic DNA was prepared from cells at different developmental stages and dot blotted on nitrocellulose membranes in the indicated amounts. (A) DNA methylation was detected by reacting the membranes with an antibody directed against 5-methylcytosine (α5mC), and the total amount of DNA was estimated by hybridization with a radioactive probe against DIRS-1 (DIRS). (B) The antibody and hybridization signals were quantified, and methylation levels were normalized to the amounts of DNA and plotted as a function of developmental time. The plot indicates that DNA methylation is increased during *Dictyostelium* development. Results are the averages and standard deviations from 3 replications.
on CpG, since their Southern blot patterns were indistinguishable by this method.

The computational analysis of CpG island distribution (Fig. 2A) revealed several genes that were good candidates for DNA methylation. We selected one of these genes, *guaB*, for further analysis because it carries convenient restriction sites within CpG islands. The Southern blot described above was stripped and reprobed with the *guaB* probe (Fig. 3C). We observed a band at 1.6 kb that was digested efficiently into 1.0-kb and 0.6-kb bands by the methylation-insensitive endonucleases (Fig. 3C, lanes 2 and 4) but was partially protected from the methylation-sensitive endonucleases (Fig. 3C, lanes 1 and 3). We speculate that the partial protection was observed because DNA methylation occurred only in some of the cells.

To further test the notion that *Dictyostelium* DNA methylation is rather sparse, we performed quantitative analysis of DNA methylation at the *guaB* locus. The *guaB* locus contains 3 putative methylation sites that can be detected by differential restriction endonuclease digestion (Fig. 4A). We designed PCR primer pairs that flank these sites as well as one pair to amplify a control fragment near the 5′/H11032 border of the ORF. Genomic DNA was digested with the appropriate restriction endonucleases and purified, and quantitative PCRs were performed and normalized (Fig. 4B). We found that site 1 was not methylated, since both types of endonuclease digestion eliminated the ability to generate a PCR product. Both site 2 and site 3 were protected from digestion with 5mC-sensitive endonucleases but not protected from the 5mC-insensitive endonuclease. We therefore conclude that the sites within the *guaB* ORF are methylated, whereas the site 5′ of the ORF is not. The data also support the observation that DNA methylation is developmentally regulated because the phenomenon was more pronounced in DNA from developing cells than in DNA from vegetative cells (Fig. 4B).

The *dnmA* gene encodes a DNA methyltransferase. The *Dictyostelium* genome project predicted the presence of a single
copy of dnmA, a DNA methyltransferase gene of the Dnmt2 family (30). To test that prediction, we generated a knockout strain by replacing most of the gene with a blasticidin resistance cassette. We verified the gene disruption by Southern blot analysis (Fig. 5) and by PCR across the relevant junctions (data not shown). Using most of the dnmA gene as a probe (Fig. 5, probe 1), we observed a single band in each of the lanes containing wild-type DNA, verifying the observation that the gene is present as a single copy. These single bands were absent in the lanes containing mutant DNA, indicating that the sequences were deleted from the genome. Probing with the plasmid used to replace the dnmA gene (Fig. 5, probe 2), we found no signal in the lanes containing wild-type DNA and the expected sized single bands in lanes containing the mutant DNA, indicating that a clean replacement has occurred. We then tested the level of DNA methylation in growing (0 h) and in developing (24 h) mutant cells with the anti-5mC antibody (Fig. 6). The data indicate that deletion of dnmA resulted in a decrease in the level of DNA methylation in developing cells. We therefore conclude that dnmA is a bona fide DNA methyltransferase whose activity can account for about 50% of the 5mC in the genome. We also postulate that another activity, encoded by a yet unidentified gene in the genome, must be responsible for the remaining methylation.

To further test the target specificity of DnmA, we compared the guaB methylation patterns in the mutant and in the wild type. Using the quantitative PCR method described in the legend to Fig. 4, we found that site 2 was completely unmethylated in the mutant but protected from the 5mC-insensitive endonuclease in the wild type (Fig. 7). The guaB expression levels were not affected by the dnmA mutation (data not shown).

We conclude that dnmA is responsible for nearly 50% of the 5mC in the genome and that its activity is rather selective, but we cannot link it to gene expression.

dnmA activity is essential for proper culmination. Gross morphological aberrations are a good measure of developmental function in Dictyostelium. We therefore developed the dnmA knockout mutant side by side with wild-type cells and compared their morphologies. The mutant and wild-type morphological and temporal progression through development were essentially identical for the first 20 h of development (data not shown). During culmination however, the dnmA knockout mutant exhibited subtle but reproducible defects. Many of the mutant sori were fragmented along the stalk, and they were clearer than the wild-type sori (Fig. 8). Clear sori sometimes indicate inefficient sporulation or precocious germination, and we indeed found that the mutant formed fewer spores than the wild type (data not shown). However, the sporulation phenotype was less penetrant and less reproducible than the sorus fragmentation phenotype. Overall, it is clear that dnmA is essential for proper terminal development, consistent with the temporal increase in DNA methylation during development.

DISCUSSION

DNA methylation is found in both animals and plants (11), indicating that it has evolved before the separation of the two kingdoms. Dictyostelium has evolved from the evolutionary line leading to animals, after the separation from plants (24), so the
reported lack of DNA methylation was peculiar (40). Our findings indicate that Dictyostelium is not an exception to the evolution of DNA methylation in that it has at least two mechanisms of DNA methylation. One of the mechanisms depends on the dnmA gene, and the other remains to be identified. DNA methylation was also reported in Candida albicans, which is not known to have any putative Dnmt methyltransferases in its genome (34).

The function of the Dmnt2 family of DNA methyltransferases is somewhat enigmatic. In most organisms, these enzymes have little or no activity, and mutations in the respective genes have almost no consequences (20, 28, 45). DNA methylation in Dictyostelium is fairly rare, in that only 0.03 to 0.14% of the total cytosines are methylated. The fraction of methylated cytosines is higher in other organisms with about 0.1% in Drosophila melanogaster, 2 to 10% in mammals, and more than 30% in some plants (2, 14). In addition, the G+C content in the Dictyostelium genome is very low (22.43%), so there is just one methylated cytosine per 7 to 30 kb in the genome. This low level of DNA methylation is consistent with the weak activity of Dmnt2 enzymes. Nevertheless, mutating dnmA resulted in a subtle yet obvious developmental defect such as fragmentation of the sori midway along the stalk. The positioning of the sorus to the top of the stalk is likely to have a selective advantage in

FIG. 5. Physical maps of the dnmA locus. (A) Restriction maps of the dnmA locus in the wild-type strain (AX4) and in the dnmA knockout strain (KO). Probe 1 consists of the dnmA gene, and probe 2 consists of the plasmid DNA used to replace the gene in the knockout mutant. Open boxes and arrowhead indicate the dnmA exons. Restriction sites are indicated as follows: EV, EcoRV; EI, EcoRI; CI, ClaI. (B) Genomic DNA from the wild-type strain (AX4) and from the dnmA knockout strain (KO) was digested as indicated and subjected to Southern blot analysis with probes 1 and 2.

FIG. 6. Reduced global DNA methylation in the dnmA knockout strain. Genomic DNA was extracted from vegetative (0 h) and developed (24 h) cells of the wild-type (AX4) and dnmA knockout (KO) strains. DNA methylation was detected by dot blotting with the anti-5mC antibody as described in the legend to Fig. 1. (A) DNA methylation signal intensity during growth (black bar) and development (white bar) are the averages of results from two independent replications of each of two independent knockout strains (KO) and the averages of results from three independent replications of the wild-type strain, showing a reduction in the developmentally regulated methylation. (B) Representative dot blot data comparing the dnmA knockout strain (KO) and the wild-type strain (AX4) signals in growth and development.

FIG. 7. dnmA is responsible for selective DNA methylation at the guaB locus. Genomic DNA was prepared from developed wild-type (AX4) and dnmA knockout (KO) cells. The DNA was digested with a methylation sensitive (S, AvaI) or a methylation-insensitive (IS, BsoBI) endonuclease and analyzed by PCR using the primer set indicated in the legend to Fig. 4. PCR products were resolved on a 1% agarose gel containing ethidium bromide and visualized by fluorescence under UV light. Photographs are shown, and control PCR amplification products are shown below the experimental lanes.
spore dispersal. Therefore, a mutant lacking \textit{dnmA} would probably have a competitive disadvantage against wild-type cells. These findings provide a demonstration of function for a Dnmt2 DNA methyltransferase. Another recent example is the finding that \textit{Drosophila} Dnmt2 might be involved in longevity and aging (22).

\textit{Dictyostelium} DnmA contains all 10 conserved motifs, including the methyl donor (S-adenosyl-L-methionine) binding motif and the active site, and an invariant polypeptide in the target-recognizing domain, TRD, which is the putative DNA recognition site (21). The latter domain is not found in other Dnmt family members, so it is thought that Dnmt2 recognizes a specific kind of target through this TRD domain (8). In this paper, we found that the \textit{Dictyostelium} DnmA functions as one of the DNA methyltransferase and methylates its targets in a selective manner. In \textit{D. melanogaster}, most methylation was seen at CpT, and only a small fraction of the 5mC was detected in CpG dinucleotides (25). Overexpression of Dmnt2 in \textit{D. melanogaster} increased the methylation levels of cytosine in the non symmetrical CpT and CpA dinucleotides (20). It is therefore conceivable that the cytosines in CpT and CpA are also recognized by the \textit{Dictyostelium} DnmA.

The low level of DNA methylation and the developmental regulation explain why previous studies have failed to find it (40). A random choice of probes for Southern blot analysis would have to be very lucky to reveal a methylated gene, and physical methods like HPLC analysis are limited to fairly high proportions of 5mC. We have tried to analyze \textit{Dictyostelium} DNA by HPLC, followed by mass spectrometry, but found that the proportion of 5mC in the genome was below the limit of detection of the method (data not shown). We were fortunate that the antibody we used is highly sensitive and highly specific to 5mC in DNA. We also had the advantage of knowing the genome sequence as a guide in searching for good probes for Southern blot analysis.

DNA methylation in \textit{Dictyostelium} is developmentally regulated, and the highest degree of methylation is observed in cells at the end of development. It is tempting to speculate that the timing of highest methylation and the late morphological phenotype of the \textit{dnmA} mutant are causally correlated. It is possible that the methylation of a small subset of genes is required for regulation of late gene expression and that disruption of the process leads to developmental defects. Another possibility is that the correlation is indirect. Terminal morphology is the result of proper execution of all developmental pathways. Therefore, we cannot rule out the possibility that the \textit{dnmA} knockout phenotype is the result of subtle defects in processes that are executed early in development and are manifested only later on.

The unusual distribution of CpG islands in the \textit{Dictyostelium} genome is also quite interesting. The association of CpG islands with RTEs in other organisms is usually attributed to the silencing effects of DNA methylation (5, 46). It is reasonable to hypothesize that silencing RTE transcription by DNA methylation may have a selective advantage in maintaining genome stability and reducing the metabolic burden associated with expressing the RTE genes. This assertion may have to be reexamined in light of our findings. In \textit{Dictyostelium}, CpG islands are also associated with RTEs, but most of them are probably not methylated because we could not detect methylation around most DIRS1 RTEs. It is therefore possible that the CpG islands have a role that is independent of their methylation in addition to their proven, methylation-dependent role in the regulation of chromatin structure and gene expression. In that context, CpG dinucleotides have an unusual distribution around the borders of ORFs. It is conceivable that most of them are not methylated in \textit{Dictyostelium}, so we propose that CpGs and CpG islands have a role that is independent of methylation. What that role might be is currently a matter of speculation, but the fact is that the unusual distribution is also found in other organisms (3, 38).

Finally, we did not find evidence for the role of DNA methylation in the regulation of gene expression in \textit{Dictyostelium}, but we only examined one gene. A more comprehensive study might reveal whether DNA methylation is important for gene regulation and which genes and pathways are regulated by that mechanism.

In an independent study, Nellen and coworkers have detected 5mC DNA methylation of CpA and CpT dinucleotides. In their hands, deletion of \textit{dnmA} did not result in morphological defects, but their parental strain (AX2) was different from the one we used (AX4). These researchers found that DNA methylation was necessary for transcriptional silencing of the Skipper RTE and showed evidence for the correlation of DNA methylation and RNA interference (19a). Together, our data establish that \textit{Dictyostelium} cells utilize DNA methylation for regulation of gene expression and of development.

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