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Silencing of retrotransposons in *Dictyostelium* by DNA methylation and RNAi

Markus Kuhlmann, Branimira E. Borisova, Markus Kaller, Pontus Larsson[1], Dirk Stach[2], Jianbo Na[3], Ludwig Eichinger[3], Frank Lyko[2], Victor Ambros[4], Fredrik Söderbom[5], Christian Hammann and Wolfgang Nellen*

Abt. Genetik, Universität Kassel, Heinrich-Plett-Str. 40, 34132 Kassel, Germany, [1]Department of Cell and Molecular Biology, Biomedical Center, Uppsala University, Box 596, S-751 24 Uppsala, Sweden, [2]Arbeitsgruppe Epigenetik, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany, [3]Institut fuer Biochemie I, Medizinische Einrichtungen der Universitaet zu Koeln, Joseph-Stelzmann-Str. 52, 50931 Koeln, Germany, [4]Department of Genetics, Dartmouth Medical School, Hanover, NH 03755, USA and [5]Department of Molecular Biology, Biomedical Center, Swedish University of Agricultural Sciences, Box 590, S-75124 Uppsala, Sweden

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ABSTRACT

We have identified a DNA methyltransferase of the Dnmt2 family in *Dictyostelium* that was denominated DnmA. Expression of the dnmA gene is downregulated during the developmental cycle. Overall DNA methylation in *Dictyostelium* is ~0.2% of the cytosine residues, which indicates its restriction to a limited set of genomic loci. Bisulfite sequencing of specific sites revealed that DnmA is responsible for methylation of mostly asymmetric C-residues in the retrotransposons DIRS-1 and Skipper. Disruption of the gene resulted in a loss of methylation and in increased transcription and mobilization of Skipper. Skipper transcription was also upregulated in strains that had genes encoding components of the RNA interference pathway disrupted. In contrast, DIRS-1 expression was not affected by a loss of DnmA but was strongly increased in strains that had the RNA-directed RNA polymerase gene rrpC disrupted. A large number of siRNAs were found that corresponded to the DIRS-1 sequence, suggesting concerted regulation of DIRS-1 expression by RNAi and DNA modification. No siRNAs corresponding to the standard Skipper element were found. The data show that DNA methylation plays a crucial role in epigenetic gene silencing in *Dictyostelium* but that different, partially overlapping mechanisms control transposon silencing.

INTRODUCTION

DNA methylation plays a crucial role in epigenetic gene regulation in many organisms (1). In most eukaryotes, cytosines in the symmetric CpG or CpNpG context are methylated (2) and methylation is propagated by maintenance methyltransferases (3,4) on the newly synthesized DNA strand shortly after replication. In addition, other methyltransferases may place new marks on DNA by *de novo* methylation (5–8), including the modification of asymmetric sites (9). Methylated cytosines may recruit specific chromatin components and initiate heterochromatin formation by histone deacetylation and methylation (10–12). On the other hand, modified histones may recruit DNA methyltransferases and mediate C-methylation (13,14). Though DNA methylation is found in organisms as diverse as animals, plants and fungi, it appears to be absent in *Schizosaccharomyces pombe* (15,16), *Caenorhabditis elegans* and other organisms where epigenetic silencing is mediated by histone modification alone.

It was first detected in tobacco that RNA can direct the methylation of homologous sequences in the genome (17,18). In plants and yeast (10) silencing of chromatin was found to be mediated by small RNAs that originate from the RNA interference pathway. RdDM (RNA-directed DNA methylation) is thus another branch of regulation by small

*To whom correspondence should be addressed. Tel: +49 561 8044805; Fax: +49 561 8044800; Email: nellen@uni-kassel.de

The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

RNAs. Recently, several models have been presented as to how the RNA guides for DNA modification may be generated and which components of the cellular machinery are involved (19–21). Targets for epigenetic chromatin silencing are frequently transposons and retrotransposons that are a source for naturally occurring siRNAs (22,23). Transposons are reactivated (22) and may be mobilized when components of the RNAi pathway are disrupted (24).

The presence of a functional RNAi machinery (25), the cloning of numerous siRNA-like molecules from *Dictyostelium* containing sequences of retrotransposons (F. Söderbom and V. Ambros, manuscript in preparation) and the suggestion that pericentric heterochromatin contained clustered retrotransposons (26) raised the question whether RNA-mediated DNA modification may be involved in epigenetic gene regulation in *Dictyostelium*. Similar to the situation in *Drosophila*, previous work had suggested that *Dictyostelium* DNA was not methylated (27). The discovery of a methyltransferase gene of the Dnmt2 family in the *Drosophila* genome and the subsequent detection of low levels of cytosine methylation (12) prompted us to reinvestigate this issue in *Dictyostelium*. In the recently completed genome of the social amoeba (26), we found a Dnmt2-like gene that had also been independently detected by Ponger *et al.* (28).

Our data show that *Dictyostelium* contains a functional DNA methyltransferase enzyme that methylates specific C-residues in the retrotransposons DIRS-1 and Skipper. Skipper silencing depends on both DNA methylation and a functional RNAi machinery.

MATERIALS AND METHODS

Strains

All experiments were carried out with Ax2, strain 214 and derivatives. The following transgenic strains were used: dnmA$^-$ (25), rrpA$^-$ (25), rrpB$^-$ (25), rrpC$^-$ (25), hcpA$^-$ (M. Kaller, U. Euteneuer and W. Nellen, manuscript submitted), helF$^-$ (B. Popova, M. Kuhlmann and W. Nellen, submitted) drnA$^-$ (B. Popova, B.E. Borisova, M. Kuhlmann, C. Hammann and W. Nellen), actin 15::dnmA-myc and actin 15::dnmA-gfp.

Growth and development of *Dictyostelium* cultures

Dictyostelium Ax2 cells and transformants were grown in association with *Klebsiella aerogenes* on SM agar plates, in suspension culture or plates in HL5 medium. *Dictyostelium* cells were developed in phosphate buffer suspension culture as described previously (29).

For growth and development cycles, cells were plated out on SM agar in association with *K.aerogenes* and allowed to grow and develop. Spores were harvested after 7 days and used to inoculate a fresh plate.

Generation of the dnmA knock-out strain

For amplification of the genomic 5′ and 3′ parts of the dnmA gene the following primer sets were used. 5′ part: forward, (Eco31I) GGTCTCATGGAACAATTGAGAGTATTAGAA and reverse, (BamHI)GATCCGCTTAACCAAATTGTTGTGGTG; 3′ part: forward, (Eco31I) GGTCTCGATTTTCAGTTACAATGAATTGGT and reverse, (BamHI) 5′ GGATCCTTTTGTATTGCAAAAAGAAATGG. The PCR products of 782 bp (5′ part) and 1100 bp (3′ part) were inserted into the plasmid pGEM T-easy. The plasmid was digested at the central BamHI site to introduce the 1343 bp BS^R cassette (30). The resulting plasmid was cut with Eco31I and the ~3200 bp dnmA fragment with the inserted BS^R cassette was isolated. The fragment was introduced into *Dictyostelium* cells by electroporation as described previously (31,32).

Clonal isolates were obtained by plating ~500 cells on an SM plate with *K.aerogenes*. Cells were picked from clones into Costar plates containing HL5 medium with the appropriate selective drug. After several days, cells were transferred to suspension culture.

DnmA expression constructs

The coding sequence of the dnmA gene was amplified by PCR using the primers forward: ATTGAATTCAAAATGGAACAATTGAGAGTATTAG and reverse: AGGAAGCTTATTGGATCCTTTTTTTCCTTCTTTTTCCTTTTG.

The product was cloned via EcoRI and BamHI into pDdGFP to generate a C-terminal GFP fusion. For expression of the Myc-tagged protein, the same PCR product was cloned via EcoRI and HindIII into pDEX_RH. Two complementary oligonucleotides encoding the Myc-tag were inserted into the BamHI and HindIII sites to obtain a C-terminal Myc fusion.

RNA isolation and northern blotting

Total cellular RNA was prepared as detailed in (33). For northern blots, 20 μg of total cellular RNA were separated on a 1.8% agarose gel containing 20 mM guanidiniumthiocyanate and blotted to a nylon membrane (porablot Nyamp, Macherey & Nagel). Pre-hybridization and hybridization were carried out as described previously (34). Radioactively labelled PCR fragments were generated by incorporation of [α-^{32}P]dATP with Klenow-fragment^{exo-} (MBI, Fermentas) as described previously (35).

PCR-primers for fragment amplification used for random-prime Klenow-labelling. DnmA 801 bp (+183 bp intron): Forward, TTAATGAGTCCACCATGTCAAC; reverse, CCTGTACCTTCAATAAATTTTCC; DIRS-1-RT-LTR 726 bp: forward, GTATGCCCTGTTCGCCACCTTGC; reverse, AACATTTATTTATTTGAATTTCCC; Skipper GAG 400 bp: forward, TGAAGCTAAAACCATTGACGC; reverse, CTAATTGAACTTCAGCAGTACC; eriA 660 bp: forward, GGATCCGATGTCCACAACAACAACATC; reverse, GGATCCTTTACTGATTTCATTGTTGAAAC.

RT–PCR

RNA was isolated from wild-type cells and mutants and treated with RNase free DNase (MBI, Fermentas). Equal amounts of total RNA were used for reverse transcription with oligo(dT) primers. PCRs for dnmA were carried out on cDNA using the following primers: forward, TTAATGAGTCCACCATGTCAAC; reverse, CCTGTACCTTCAATAAATTTTCC.

For the thioredoxin control: forward, GAACGAGCTCCATGGCCAATAGAGTAATTCATG; reverse, CGCGGATCCTTATTTGTTTGCTTCTAGAGTACTTC.

Small RNA libraries

Sequences for siRNAs were retrieved from two small RNA libraries (~18–25 nt long) that were constructed according to Lee and Ambros (36) and Lau *et al*. (37). Details on these libraries will be published elsewhere (F. Söderbom and V. Ambros, manuscript in preparation).

DNA isolation and Southern blotting

A total of 1×10^8 *Dictyostelium* cells were harvested from HL5 medium, washed in ice-cold phosphate buffer (100 mM, pH 6.7) and collected at 540 *g* for 10 min at 4°C. The cell pellet was lysed in 50 ml nuclear-lysis-buffer (50 mM HEPES, pH 7.5, 40 mM $MgCl_2$, 20 mM KCl, 5% saccarose and 1% NP-40). Nuclei were lysed in 5 ml of 0.7% SDS and 100 µl (25 mg/ml) proteinase K for 60 min at 65°C. The lysate was extracted twice with phenol/chloroform and the genomic DNA was precipitated with 1/10 volume of 3 M sodium acetate and 2.5 vol of ethanol. The pellet was washed twice with 70% ethanol, air-dried and dissolved in an appropriate volume of ultra-pure water.

Aliquots containing 10 µg of DNA were digested with the restriction enzymes indicated, separated on 1% agarose gels, blotted to nylon membrane and hybridized with PCR fragments labelled with [α-^{32}P]dATP by random priming. Primers for PCR amplification are listed as control primers in 'bisulfite sequencing' and in 'northern blotting'.

Calculation of retrotransposon copy numbers

Total nuclear DNA was digested with SacI, and ~10 µg were loaded on a 1% agarose gel containing ethidium bromide. After electrophoresis, the gel was photographed with an EASY documentation system, blotted on nylon membrane and hybridized with a [^{32}P]labelled random primed probe of the DIRS-1 right LTR and part of the RT sequence. After exposure to a phosphoimager screen and scanning of the autoradiogramm, the blot was stripped and hybridized with a [^{32}P]labelled random primed probe of the Skipper gag sequence. The blot was exposed, scanned and rehybridized with a [^{32}P]labelled random primed probe of the single copy eriA gene, encoding a homologue of the *C.elegans* ERI-1 gene (38). Using the TINA program, pixels were counted for ethidium bromide staining (two defined sections of the stained gel) and PSL (photo-stimulated light units) for the major bands of the retrotransposons and for the single copy gene fragment. Counts for the quantitation standards were averaged for each lane; PSL of the main DIRS-1 and Skipper bands were divided by the averaged quantitation standard and set to 1 for fresh wild-type cells. The results did not change significantly when only the eriA or only the ethidium staining were used for quantitation.

Bisulfite sequencing

Bisulfite sequencing was essentially carried out according to (39). Briefly, 5 µg of genomic, RNase-treated DNA were denatured with 1/10 vol of 3 M NaOH for 20 min at RT and 3 min at 100°C in a total volume of 60 µl. Aliquots containing 600 µl of freshly prepared bisulfite solution (3.1 M $Na_2S_2O_5$, 5 mM hydroquinone, pH 5) were added and the samples were overlayed with mineral oil. Reactions were carried out for 3 h at 55°C; they were then briefly heated (1 min) to 95°C and incubated for an additional 12–16 h at 55°C. DNA was purified with Geneclean® or glass milk and dissolved in 100 µl H_2O. Desulfonation was carried out for 20 min at 37°C by addition of 11 µl of 3 M NaOH. The converted DNA was precipitated with 35 µl ammoniumacetate and 3 vol of 100% ethanol, washed with 70% ethanol and dissolved in 20 µl H_2O; 2–4 µl were used for PCRs with control primers (complementary to non-converted DNA) and bisulfite primers (complementary to converted DNA).

Primers for DIRS-1 LTR PCR. Control PCR: forward, ATCAAATTGTTTTAGTTTTTAGTG; reverse, AACATTTATTTATTTGAATTTCCC. Bisulfite PCR: forward, ATCAAATTATTTTAATTTTTAATA; reverse, AATATTTATTTATTTGAATTTTTT.

Primers for Skipper RT. Control PCR: forward, CTGTTACCTTAGTGAAGATGGG; reverse, GGGCATCTATTGTCTTATGACATGG. Bisulfite PCR: forward, AAATCTTACATATATTATCAATAAA; reverse, AATAATTGAGTAGTATGTTGGGT.

Primers for Skipper LTR: Control PCR: forward, GTTAGAGACTCAAAACTAAATTA; reverse, TGTAAAAGTCACTCACACTAATC. Bisulfite PCR: forward, ACTCAAAACTAAATTAATTTAAAATTAA; reverse, ATGAAGGATAGAAAGAGTGAAAGAT.

PCRs were carried out on bisulfite treated and on untreated DNA with both sets of primers. Experiments were continued when only the appropriate primers yielded products on the different DNA samples. PCR products from cloned DIRS-1 LTR and Skipper RT fragments were also subjected to bisulfite sequencing to confirm that all cytosine residues could be converted.

In some experiments, DNA for the retrotransposon fragments was enriched by restriction digestion, gel electrophoresis and preparation of the appropriate size fragment for bisulfite treatment. The results were, however, not significantly improved in comparison with those obtained from bisulfite treatment of total DNA. PCR fragments were purified by MicroSpin™S-400HR columns before sequencing (MWG Biotech).

Fluorescence microscopy

Cells were fixed at −20°C, with methanol for 20 min, washed three times in 1× PBS and stained with DAPI (1 mg/ml, diluted 1:15,000 in 1× PBS).

Cells expressing the DnmA-c-Myc fusion protein were blocked with PBG buffer (1× PBS containing 3% BSA and 0.045% cold water fish gelatin; Sigma) for 1 h at 37°C. Primary mAb 9E10 (directed against the c-Myc epitope) was added in a 1:50 dilution for 3 hours. Cells were washed three times with 1× PBS, then the secondary TRITC coupled polyclonal antibody (Dianova, Hamburg) was added at a 1:1000 dilution in PBG for 1 h.

Microscopic analysis was carried out with a Leica DM IRB inverted fluorescence microscope. For image acquisition a Leica DC 350F digital camera and IM50 software were used. Images were processed in Adobe Photoshop.

Capillary electrophoresis

For DNA methylation analysis, genomic DNA was isolated from wild-type (Ax2) and dnmA knock-out cells. DNA

samples were hydrolysed, derivatized and analysed by capillary electrophoresis as described previously (40).

Microarray

RNA from Ax2 and the mutant cells was isolated with the Qiagen RNA kit. Aliquots containing 20 µg each were precipitated together with the appropriate spike mixture (controls). The RT–PCR for labelling reactions was carried out with oligo(dT) primers. cDNA was labeled with a Cy3 or Cy5 labelling kit (FairPlay^R Stratagene). Dye-coupled cDNA was purified and ethanol precipitated.

Corning ultra GAPS arrays containing probes for 450 defined genes, 5400 non-redundant expressed sequence tags (ESTs), and positive and negative controls were prehybridized and subsequently hybridized with the dye coupled cDNA preparations (41). After washing, arrays were scanned by Scan-Array^R 4000XL and analysed by Scan Array Express Version 2.1 (Perkin Elmer Life Sciences, Wellesley, USA). Furthermore, the R project for Statistical Computing (http://www.r-project.org) and Significance Analysis of Microarrays (SAM) Version 1.21 (http://www-stat.stanford.edu/~tibs/SAM/) were used.

Southwestern blots

DNA was isolated from wild-type cells and from the dnmA gene disruption mutant, digested with various restriction enzymes and separated on a 1% agarose gel. The gel was blotted and the a nylon membrane was incubated with an antibody directed against 5-methyl cytosine (Eurogentec, Seraing, Belgium) at 4°C overnight. Further processing was carried out as described previously for western blotting (42) using an alkaline phosphatase coupled secondary antibody. Similar experiments were carried out using slot-blotting instead of Southern blotting. Purified DNA was denatured in 0.4 M NaOH, 10 mM EDTA, heated to 100°C for 10 min., neutralized by addition of an equal volume of 2 M ammoniumacetate, pH 7 and applied to a 6× SSC saturated nitrocellulose membrane in a slot-blot-apparatus. After washing with 2× SSC, DNA was UV-crosslinked to the filter, incubated with the anti 5-methyl cytosine antibody and processed as described above.

Accession numbers

dnmA: DictyBase DDB0231095, NCBI: XM_631863 eriA: DictyBase DDB0231961; hcpA: DictyBase DDB0220646; rrpC: DictyBase DDB0216193; rrpA: DictyBase DDB0191515; rrpB: DictyBase DDB0220017.

RESULTS

Dictyostelium contains an expressed DNA methyl transferase of the Dnmt2 type

Global analysis of genomic DNA from *Dictyostelium* by capillary electrophoresis revealed a faint signal corresponding to ~0.2% cytosine methylation (Figure 3B). This indicated that the *Dictyostelium* genome contains a DNA methyltransferase gene that catalyses DNA methylation in a restricted sequence context. Accordingly, we detected in the *Dictyostelium* genome database a single gene with high similarity to human Dnmt2 (Figure 1) and denominated it dnmA. The predicted gene product of 379 amino acids and 44.1 kDa displayed 41% identity with the human enzyme. All 10 characteristic motifs of DNA methyltransferases (43) could be identified. Additional genes with similarity to DNA methyltransferases of the Dnmt1 or Dnmt3a/b type were not detected by blast searches in the *Dictyostelium* genome.

RT–PCR showed that dnmA RNA expression was high in vegetative cells and decreased during development (Figure 2A). N-terminal fusions with GFP or a c-Myc-tag were predominantly found in the nucleus (Figure 2B), thus

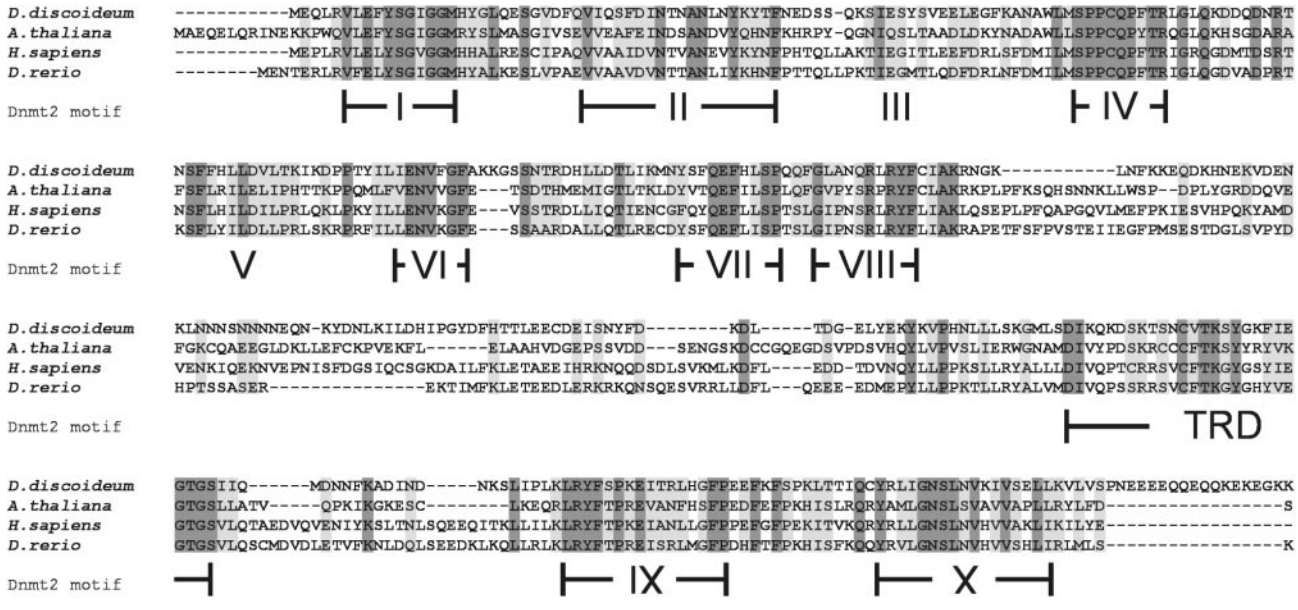


Figure 1. Amino acid sequence alignment of Dnmt2 homologues from *D.discoideum*, *Arabidopsis thaliana*, *Homo sapiens* and *Danio rerio*. The 10 characteristic Dnmt2 motifs (44) are indicated by roman numbers. TRD, target recognition domain.

confirming the *in silico* predicted nuclear localization by PSORTII (44) and LOCtree (45).

We generated a knock-out strain of the dnmA gene by homologous recombination. The gene disruption was confirmed by PCR, Southern blot (data not shown) and RT–PCR (Figure 3A). No obvious phenotypic alterations were observed during growth or in development.

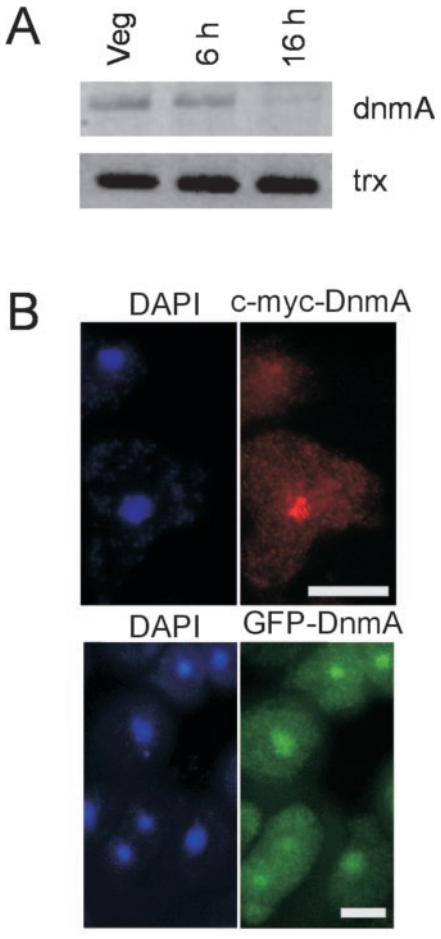


Figure 2. Expression of dnmA. (**A**) Semi-quantitative RT–PCR was performed on total mRNA isolated from vegetatively grown cells (veg) and from cells after 6 and 16 h of development on filters. Expression of the thioredoxin (trx) gene family is shown as a comparison. (**B**) Expression of DnmA-c-Myc fusion protein in *Dictyostelium* visualized by staining with mAb 9E10 and a secondary TRITC coupled polyclonal antibody (top right). The DnmA-GFP fusion protein (bottom right) showed the same localization. DNA was stained with DAPI to localize the nucleus (left). Bars are 8 μm.

The transposons DIRS-1 and Skipper are methylated by DnmA at specific, mostly asymmetric C-residues

By using global methods for DNA methylation analysis, such as capillary electrophoresis and Southwestern blots with an antibody directed against 5-methyl cytosine, we were unable to obtain conclusive evidence for a loss of DNA methylation in the mutant strain, presumably, because both methods were operating close to their detection limit (Figure 3B and data not shown). For this reason, we focused our experiments on gene-specific methylation analysis. Based on observations in plants that RNAi could mediate transcriptional silencing by DNA methylation (46), we first investigated one of the discoidin gene loci after silencing by an RNAi construct (25). Bilsulfite sequencing revealed complete conversion of C-residues and no evidence for DNA methylation (data not shown). This was not unexpected since RNAi-mediated DNA methylation is predominantly found in promoter regions (18). The RNAi construct was, however, directed against the coding sequence. Attempts to achieve silencing with a hairpin construct in order to generate siRNAs directed against the promoter region failed (data not shown).

In order to identify specific targets for DNA methylation, we analysed RNA derived from the dnmA knock out and from wild-type cells by microarray. Setting a threshold of at least 2-fold difference, 7 genes were found to be overexpressed in the KO strain and 12 were underexpressed. Among the upregulated genes, the ESTs SLD246 and SLE355 contained sequences encoding the gag and RT genes, respectively, of the retrotransposon Skipper (47). We concentrated initially on the analysis of Skipper, as this was of special interest since transposable elements are frequently targets for methylation (22). Interestingly, the ESTs SSL485 containing non-coding Skipper sequences and SLC703 at the very end of the RT/integrase sequence were not overexpressed in the mutant. Skipper has 20 copies in the *Dictyostelium* genome and consists of direct long terminal repeats and three open reading frames (ORFs) encoding GAG (matrix protein), PRO (protease) and RT (reverse transcriptase), POL (polymerase) and IN (invertase) (47). Skipper is expressed at very low levels during growth and development. Bisufite sequencing of a 294 bp region in the RT gene bottom strand showed that 12 out of 21 C-residues were methylated. Methylation was

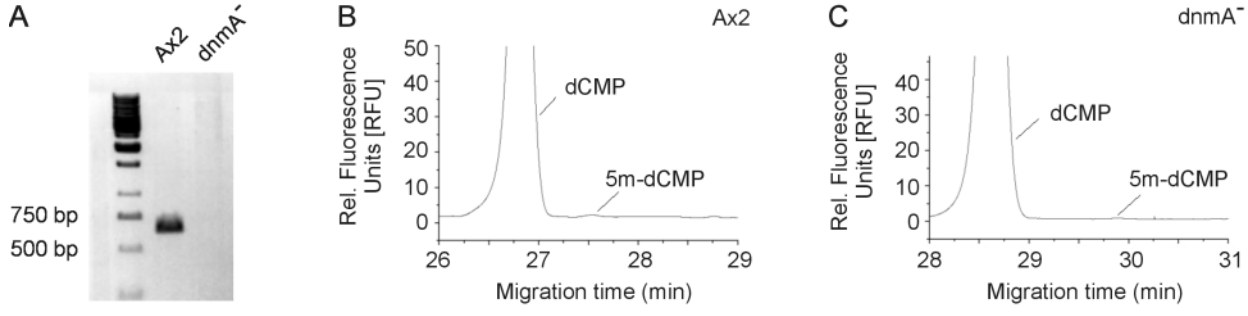
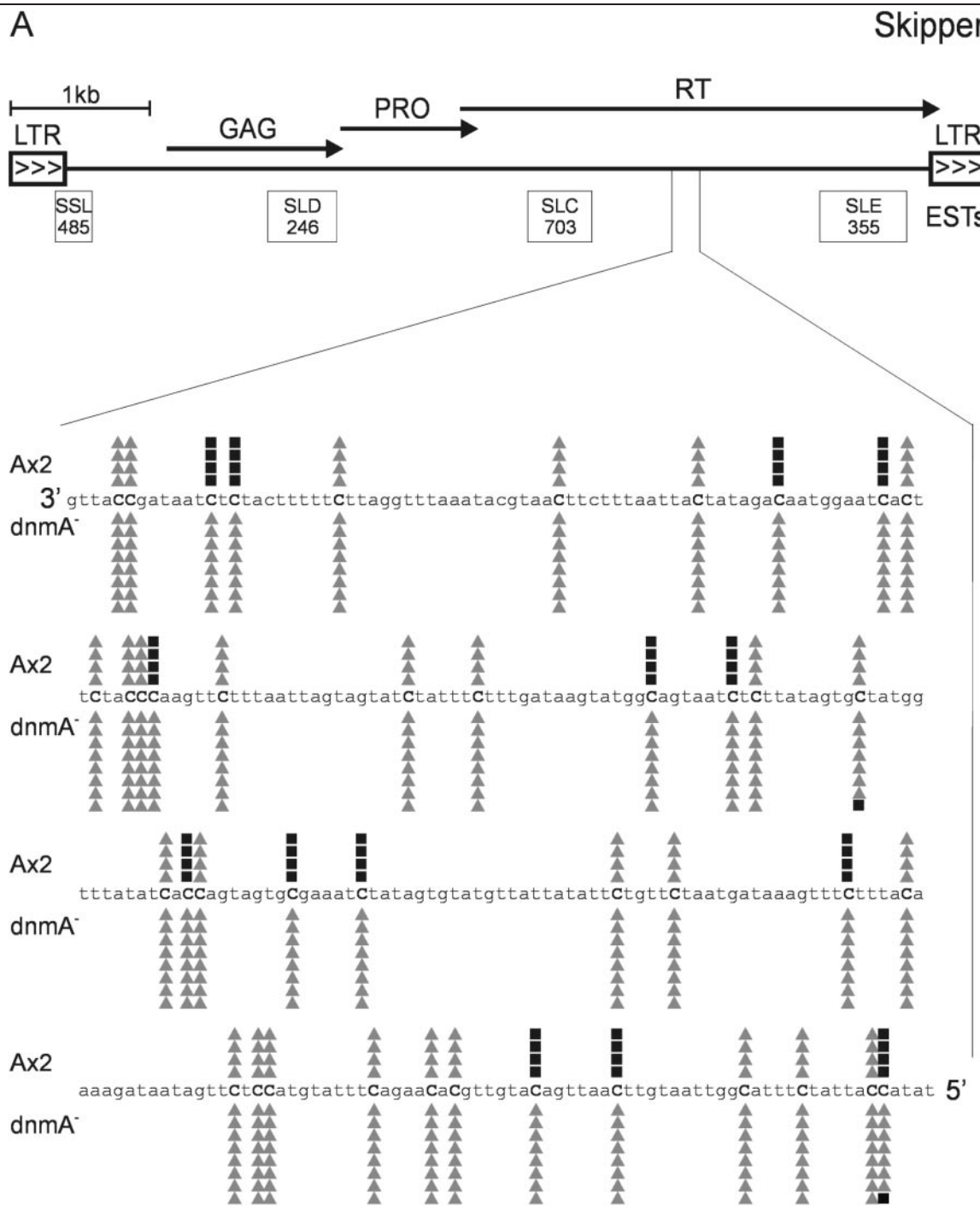


Figure 3. (**A**) Semi-quantitative RT–PCR on dnmA gene transcripts from wild-type Ax2 cells and the dnmA disruption mutant. Capillary electrophoresis on DNA from wild-type cells (**B**) and the dnmA disruption strain. (**C**) The position of 5-methyl cytosine is indicated.

mostly at asymmetric sites and could not be correlated with any obvious sequence context (Figure 4A). By bisulfite sequencing of the RT fragment derived from the dnmA knock-out strain no methylated C-residues (with the exception of two that are likely to be artifacts, see below) could be detected. This indicated that methylation within this sequence depended on DnmA.

Bisulfite sequencing of 214 bp of the Skipper LTR bottom strand revealed no methylation of the 12 C-residues within this sequence (Figure 4B).

We then analysed the retrotransposon DIRS-1 that was not covered by the microarray. DIRS-1 occurs in 40 complete and ~200 incomplete copies in the genome. It consists of inverted long terminal repeats and three overlapping open reading frames encoding protein1, reverse transcriptase and a recombinase. In addition, a heat-shock-induced transcript in antisense orientation termed E1 is derived from the right LTR of the locus. Transcription of DIRS-1 is developmentally regulated and expression increases substantially after the onset of starvation (48–50).



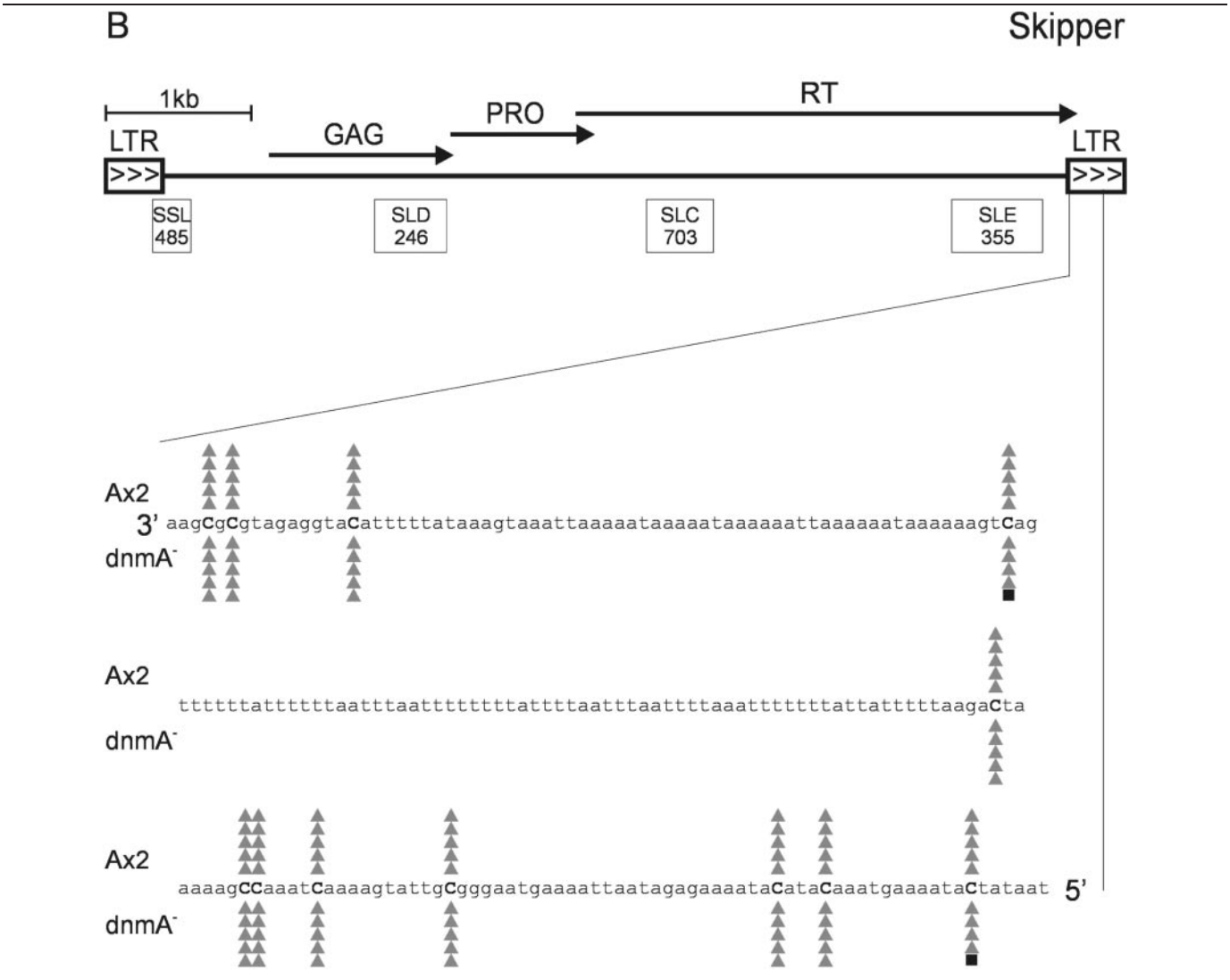
We sequenced 128 bp of the right LTR (bottom strand) and found that 7 out of 23 C-residues in this region were consistently methylated (Figure 4C). Similar to the results from Skipper, methylation sites were mostly asymmetric and no obvious sequence context could be correlated with methylation.

Using DNA derived from the knock-out strain, no DNA methylation (with the exception of four that are likely to be artifacts, see below) could be detected, thus indicating that DnmA was responsible for all C modification of DIRS-1, as we had seen for Skipper.

Overall, eight non-converted C-residues were found by bisulfite sequencing in the dnmA mutant strain (two in Skipper RT, two in Skipper LTR and four in DIRS-1 LTR). This is a very small proportion of the unambiguously sequenced C-residues and we assume that this is due to an incomplete bisulfite reaction, or to PCR or sequencing errors. Importantly, most of these cytosine residues were never found to be methylated in the wild type and thus likely represent an *in vitro* artifact. However, we cannot exclude that an additional minor, non-conventional methyltransferase is active in the knock-out strain.

Expression of Skipper and DIRS-1 in dnmA knock-out cells and other mutants

Skipper as well as DIRS-1 are barely expressed in cells growing in axenic suspension culture. We were interested to see whether the loss of C-methylation had an influence on the abundance of transcripts from both transposons as suggested for Skipper by the microarray. We examined RNA in the mutant and in the wild type after multiple passages through the differentiation cycle, assuming that transposon mobilization may be activated during development. Cells were grown on a lawn of *K.aerogenes* until they had developed mature fruiting bodies; spores were harvested and used to inoculate a new plate to begin the next cycle. After 0 and 16 cycles, spores were transferred to axenic medium and cells were grown up to prepare RNA. After 16 cycles, mutant cells were severely compromised in growth on axenic medium in one experiment (generation time of ~25 h) but not in a second experiment over 16 cycles. This could be due to retrotransposon activation that may affect growth in one but not the other population. Wild-type cells displayed the normal generation time of 8 h over at least 20 passages in two independent experiments. Northern



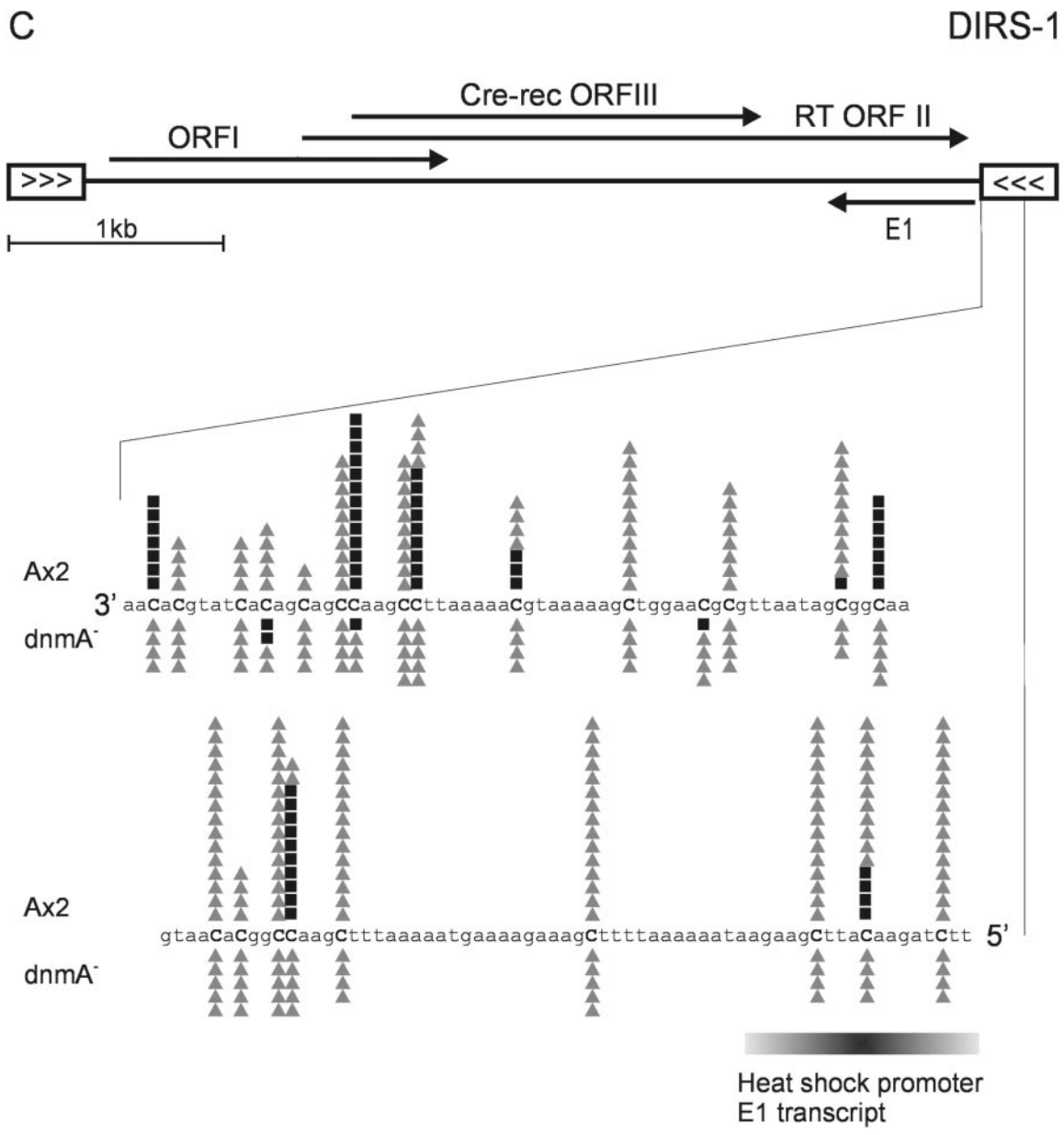


Figure 4. (**A**) Bisulfite sequencing of 294 bp in the Skipper RT gene (bottom strand). The top part shows the organization of the retrotransposon with GAG, PRO and RT genes and LTRs. ESTs that were included in the microarray are indicated below. (**B**) Bisulfite sequencing of 214 bp in the Skipper LTR region (bottom strand). (**C**) Bisulfite sequencing of 128 bp in the right LTR of DIRS-1 (bottom strand). The top part shows the organization of the retrotransposon with the overlapping reading frames ORF I, III and II, LTRs and the E1 antisense transcript. Grey triangles indicate unmethylated C-residues; black squares methylated C-residues. Symbols above the sequence refer to DNA from Ax2 wild-type cells; symbols below the sequence refer to DNA from the dnmA$^-$ strain. No symbol is shown when the sequence could not be unambiguously determined.

blot analysis revealed that DIRS-1 expression during growth was unaffected in the mutant while Skipper expression was significantly upregulated and further increased after 16 rounds of development (Figure 5A). Interestingly, Skipper expression was also stronger in wild-type cells after 16 rounds of growth and development even though no significant effect on the doubling time was detected. This suggested that the frequently observed genome instability of laboratory strains after prolonged growth could be due to transposon activation.

In *S.pombe* siRNAs are involved in chromatin remodelling (10,51) and in plants(18), *de novo* DNA methylation is mediated by small RNAs (18). We therefore examined if Skipper and DIRS-1 expression was impaired in mutants affecting the RNAi pathway. As shown in Figure 5B, Skipper expression was not only upregulated in the dnmA knock-out but also in strains where one of the three RdRP genes (25) was disrupted. A minor degree of overexpression was also observed in dicer A (drnA) and the heterochromatin protein gene hcpA (M. Kaller, U. Euteneuer and W. Nellen, manuscript submitted) knock-outs. In contrast, DIRS-1 expression was only affected in the rrpC mutant. The data showed that components of the RNAi pathway are involved in suppression of both retrotransposons but that DIRS-1 and Skipper are regulated by distinct mechanisms.

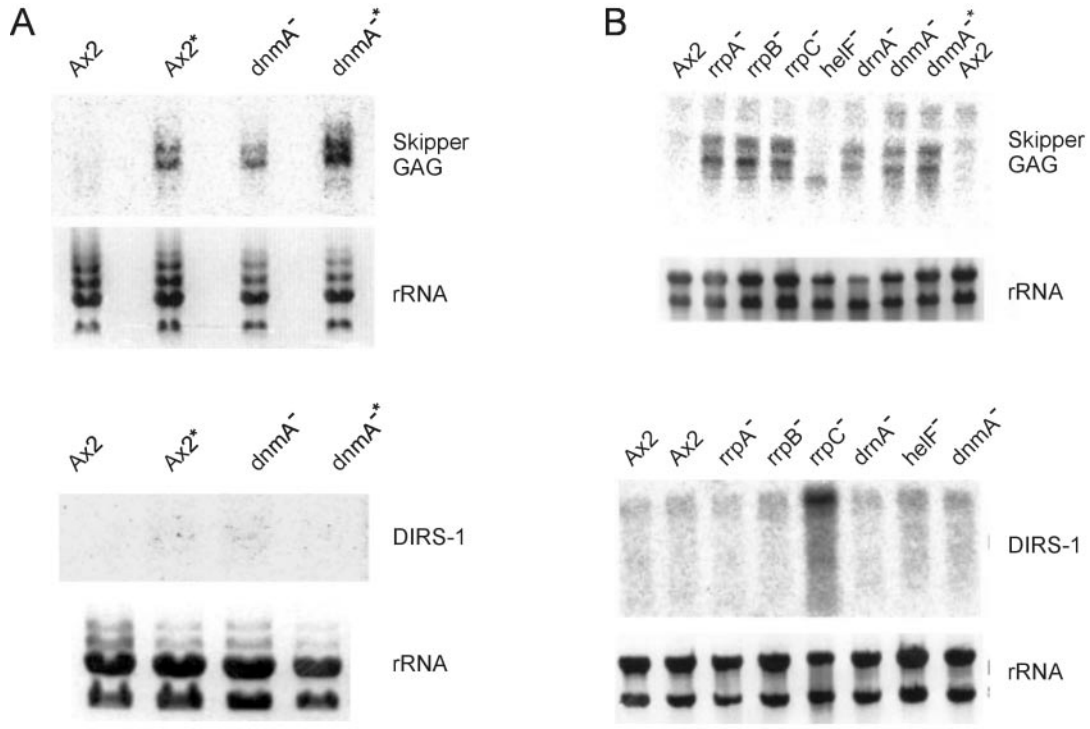


Figure 5. (**A**) Northern blots with specific Skipper (top) and DIRS-1 (bottom) probes on RNA isolated from wild-type Ax2 and dnmtA$^-$ cells after a short period of growth in axenic medium and after 16 passages through the developmental cycle (indicated by asterisk). Ethidium bromide staining is shown for quantitative comparison. (**B**) Northern blot with a Skipper (top) and a DIRS-1 probe (bottom) on RNA isolated from axenically growing cells of different mutants. Ax2: wild-type control, rrpA$^-$, rrpB$^-$, rrpC$^-$: knock outs of the RNA-directed RNA polymerase genes rrpA, B and C, respectively, helF$^-$: knock out of the gene encoding the putative RNA helicase HelF, drnA: knock out of the gene encoding the putative dicer homologue A, dnmA$^-$: knock out of the gene encoding the DNA methyltransferase A, dnmA^{-*}: knock out of the gene encoding dnmA, RNA from cells after 16 rounds of the developmental cycle.

siRNAs cover the entire DIRS-1 sequence

The observation that components of the RNAi pathway were involved in the silencing of the retrotransposons prompted us to search for siRNAs complementary to Skipper and DIRS-1. Out of more than 9000 small RNAs represented in two small RNA libraries, ~600 were complementary to either the bottom or the top strand of DIRS-1 (Figure 6, for details see Supplementary Figure 1S). Even though the libraries are probably not complete, DIRS-1 siRNAs are clearly overrepresented because the retrotransposon contributes ~3.2% to the total *Dictyostelium* genome (26) but more than 7.2% of the siRNAs contain DIRS-1 sequences. In contrast, ~1% of the genome are Skipper sequences but no siRNAs corresponding to the complete element were found.

Mobilization of Skipper

To examine whether the increase of Skipper transcription had an effect on retrotransposon mobility, DNA was isolated from cell populations grown continuously over 0 and 16 passages of the life cycle. DNA was digested with SacI that generates a close to full-length fragment of 7 kb for Skipper and more than 4.5 kb for DIRS-1 from all complete copies. Southern analysis was carried out using specific Skipper and DIRS-1 fragments as probes. Hybridization signals were adjusted by comparison with ethidium bromide staining in two regions of the gel and by hybridization to a single copy gene (eriA) on the same filter. The relative copy number was calculated for both transposable elements. Figure 7A shows the Southern blot for wild-type and dnmA$^-$ cells after 0 and 16 cycles of growth and development, and Figure 7B shows the change in relative copy number of the retrotransposons. No difference was detected for DIRS-1 while Skipper had already increased by 40% in mutant cells after selection of the transformants. A further increase was observed after 16 passages of growth and development to a total of 70% above the wild-type value. Wild-type cells were largely unaffected by multiple growth and development cycles and no increase of copy number was observed for either transposon.

DISCUSSION

Until now, *Dictyostelium* DNA was considered not to be modified by cytosine methylation (27). With the completion of the genome sequencing project (26), a gene, dnmA, highly homologous to the methyltransferases of the Dnmt2 family, was detected. We show here that the dnmA gene product is responsible for methylation of C-residues in the retrotransposons Skipper and DIRS-1. As might be expected, a disruption of the methyltransferase gene results in transcriptional activation of Skipper and, probably as a consequence, in mobilization of the retrotransposon. Surprisingly, DIRS-1 expression is not affected by a loss of DNA methylation. We assume that the different organization and the different strategies of transposition of the elements may cause this discrepancy: While DIRS-1 has inverted terminal repeats that contain methylated C-residues, Skipper has direct terminal repeats that are not

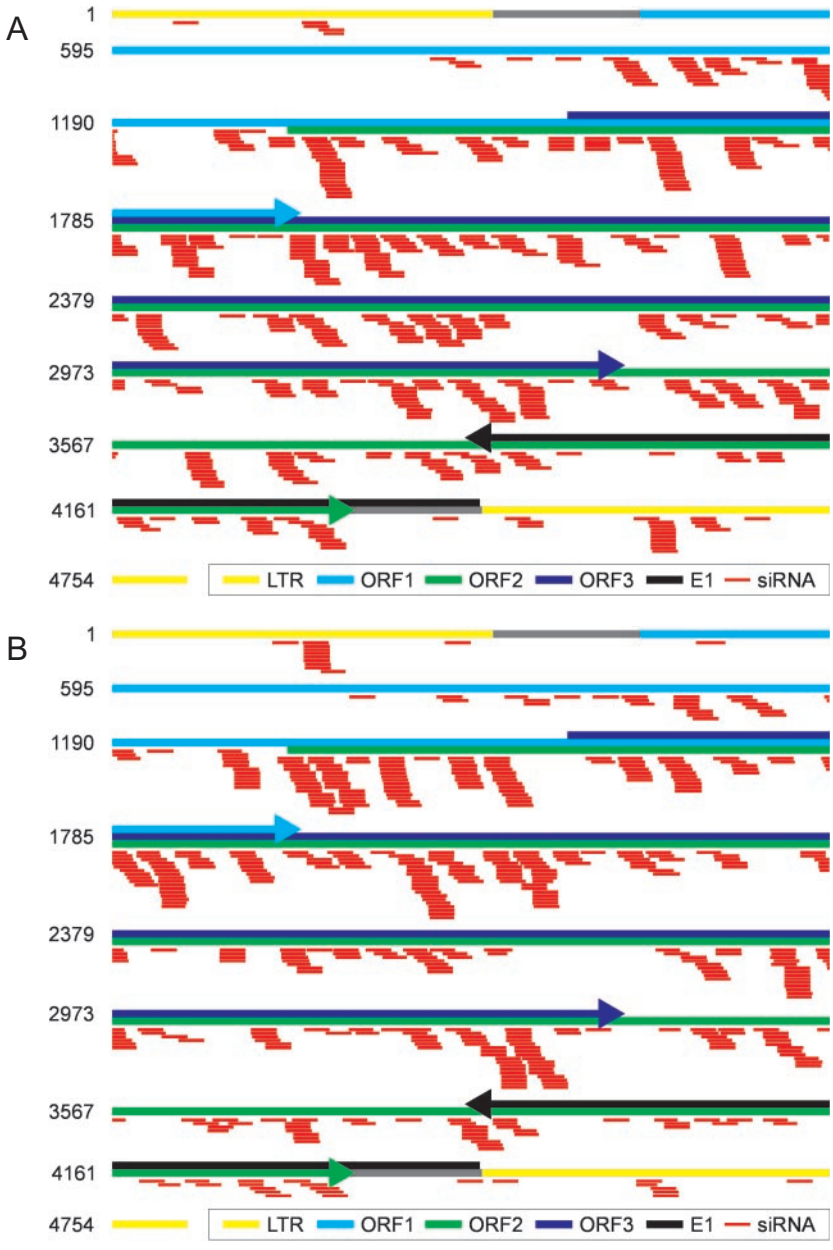


Figure 6. RNAs derived from the small RNA library are aligned on the schematic depiction of DIRS-1: sense strand (**A**) and antisense strand (**B**). The inverted repeats are marked in yellow. The ORFs I, II and III and the E1 antisense transcript are depicted as colored arrows. siRNAs are shown as red lines. Most of the small RNAs are in the range of 21 nt; some longer molecules have been retrieved and are also shown. Small RNA alignment in respect to the DIRS-1 sequence is shown in Supplementary Figure 1S.

methylated. Furthermore, there are many incomplete copies of DIRS-1 in the genome and the element frequently transposes into its own copies while Skipper has almost exclusively complete and separate copies in the genome. DIRS-1 appears to constitute centromeres in *Dictyostelium* and is also clustered in the vicinity of the telomeres while Skipper is more spread out over the genome (50). Though these characteristics argue for mechanistic differences in transposition, they cannot functionally explain the susceptibility of Skipper but not DIRS-1 expression to DNA methylation. It is noteworthy that the promoter for Skipper transcription is probably located in the LTR and promoter methylation usually causes transcriptional silencing. In the case of Skipper, however, coding sequences rather than the LTR are methylated. We therefore propose that DNA methylation causes chromatin remodelling over the entire retroelement and thus blocks accessibility for the transcription machinery. The component(s) mediating chromatin remodelling remain elusive since a search for methyl-C binding proteins in the *Dictyostelium* database did not reveal any candidate gene containing significant similarity to methyl binding domains. This may not be surprising since conventional MBDs bind to the symmetric CpG motif in a certain sequence context (52) and most methylated C-residues are in a non-symmetric context in *Dictyostelium*. In contrast, *Drosophila* contains a putative methyl-C binding protein MBD2/3 that associates with specific regions in the DNA

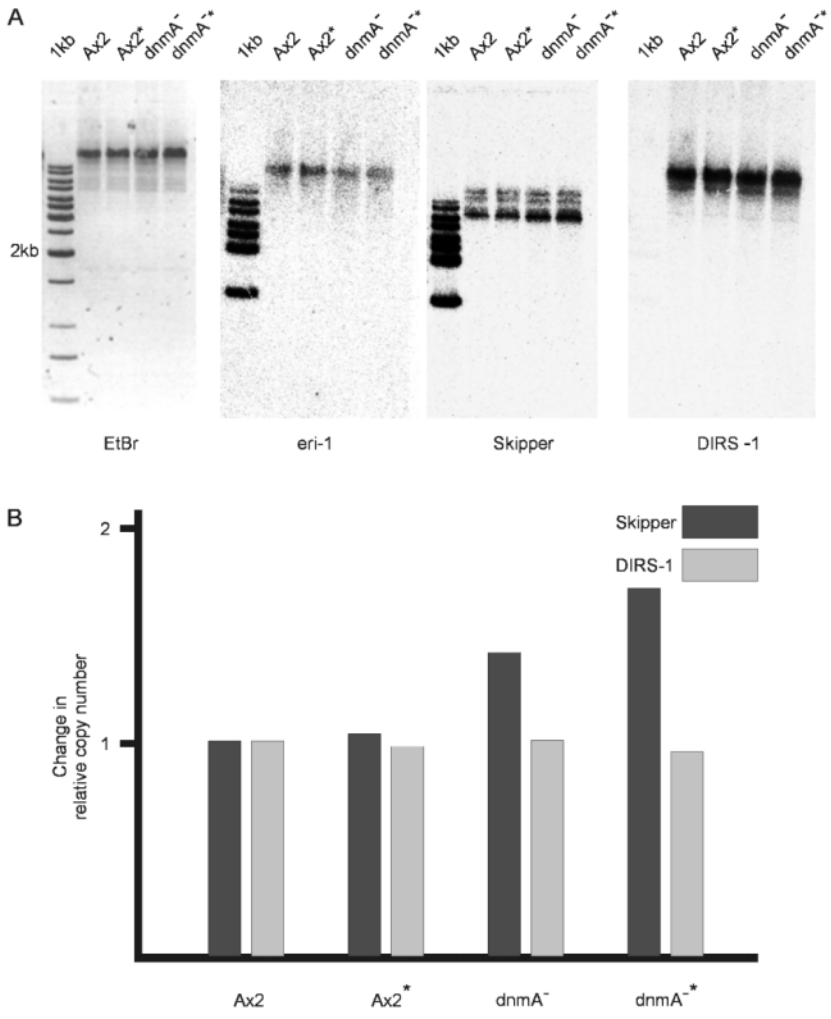


Figure 7. (**A**) Southern blot with Skipper and DIRS-1 probes on wild-type Ax2 and dnmA$^-$ mutant cells after 0 and 16 rounds (indicated by asterisk) of the developmental cycle. From left: ethidium bromide staining, hybridization with an eriA probe, hybridization with a Skipper probe, hybridization with a DIRS-1 probe. (**B**) Relative copy numbers were calculated in respect to ethidium bromide staining and to hybridization to the single copy gene eriA (see Materials and Methods).

(53) but it has not been shown to bind to the methylated, asymmetric C-residues.

In yeast, siRNAs are required to recruit heterochromatin components to specific sites in the genome (10) and in plants DNA methylation is targeted by small RNAs (18). We therefore investigated whether siRNAs complementary to retrotransposon sequences could be found. Again, completely different results were obtained for the two retroelements: while no siRNAs corresponding to the complete Skipper element could be detected, more than 600 different molecules were found for DIRS-1. Since siRNAs covered essentially the entire DIRS-1 sequence, all methylation sites in the short segment that we sequenced by the bisulfite method had a corresponding siRNA. However, not all siRNAs covered C-residues that were methylated. Since methylation sites are mostly asymmetric, cytosine modification has to occur *de novo* as would be predicted for a Dnmt2-like enzyme (54). Our observations strongly suggest that siRNAs are involved in DNA methylation. However, their role in the regulation of DNA methylation cannot be determined at this point. In fact, it has been shown in *Neurospora* that *de novo* DNA methylation is independent of RNAi (55).

With the detection of siRNAs for DIRS-1, it was obvious to examine if components of the RNAi machinery were involved in silencing of the retrotransposons. The results were unexpected since Skipper silencing was strongly dependent on functional RdRPs and the dicer A gene even though no siRNAs had been found. In contrast, DIRS-1 expression was induced only when the RdRPC gene (rrpC) was knocked out. In a previous study (25), we had shown that rrpC was not required for post-transcriptional gene silencing by RNAi. However, rrpC is necessary for PTGS mediated by antisense RNA, indicating differences between gene silencing by single stranded (antisense) and double-stranded (RNAi) RNA molecules (H. Martens and W. Nellen, unpublished data). It is tempting to correlate this with the fact that the antisense transcript E1 is transcribed from the right DIRS-1 LTR. The observation that a knock out of the *Dictyostelium* Dicer homologue drnA had no effect on DIRS-1 expression could be explained by the activity of the second Dicer gene drnB. However, since Skipper was clearly upregulated in a drnA knock-out strain, this provides evidence that the two dicer genes are not completely redundant.

In a transposition active transcript of DIRS-1, the inverted repeats can form a double strand of more than 300 bp and the

formation of a 1.7 kb double strand with the antisense transcript E1 (49,56) is feasible. However, siRNAs are also found in regions that do not form putative double strands. They may therefore originate from RdRP copies of the primary transcript and would thus constitute secondary siRNAs (57). Alternatively, an antisense strand could be generated by an RNA polymerase IV-like activity that has recently been detected in *Arabidopsis* (19,21). However, a polymerase IV-like enzyme has not yet been identified in *Dictyostelium*. Though we do not know whether the abundant siRNAs that we found for DIRS-1 are functionally involved in silencing, it is still surprising that most knock outs of RNAi components (except for rrpC) did not show an effect on DIRS-1 activity. Possibly, DNA methylation followed by chromatin remodelling as proposed above and antisense regulation provide redundant pathways for DIRS-1 silencing and both alone are sufficient. In contrast, Skipper, responded to the gene knock outs as if it was silenced by the RNAi machinery. Though no Skipper siRNAs were found, they could still be generated but escape detection due to rapid turnover or low abundance.

Various mechanisms to silence transposons and retrotransposons in one organism have been observed (19,22). In *Arabidopsis*, different classes of transposable elements are regulated by different, though overlapping mechanisms that require DNA methylation, histone modification and the RNAi system. Remarkably, different components contribute to various degrees to silencing of specific transposable elements. In addition, transcriptional and post-transcriptional mechanisms contribute simultaneously to the silencing machinery.

In an independent approach, Shaulsky and co-workers detected low levels of DnmA dependent DNA methylation in Dictyostelium (M. Katoh, T. Curk, Q. Xu, B. Zupan, A. Kuspa, G. Shaulsky, in press). Using methylation sensitive restriction enzymes, they identified methylated CpG sites in DIRS-1 and in the guaB gene.

Taken together, our data establish Dictyostelium as a model system to study mechanisms of epigenetic gene regulation by DNA methylation and RNA interference. Both mechanisms appear to overlap in function to silence the retrotransposons DIRS-1 and Skipper but further experiments are required to elucidate how the two pathways interact.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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