DNA Methylierung in *Dictyostelium discoideum*

Inaugural-Dissertation

zur

Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften (Dr. rer. nat.)

im Fachbereich Naturwissenschaften

der Universität Kassel

vorgelegt von

Branimira Emilova Borisova - Todorova

Kassel, September 2006

First supervisor:	Prof. Dr. Wolfgang Nellen
Second supervisor:	Prof. Dr. Friedrich W. Herberg
Date of defence:	20.10.2006
Ph.D. committee:	Prof. Dr. Wolfgang Nellen
	Prof. Dr. Friedrich W. Herberg
	Prof. Dr. Markus Maniak
	Prof. Dr. Mireille A. Schäfer

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Zusammenfassung

Dnmt2 DNA Methyltransferasen stellen eine hoch konservierte Proteinfamilie mit enigmatischer Funktion dar. Ziel dieser Arbeit war es, DnmA, die Dnmt2 Methyltransferase aus *Dictyostelium discoideum* zu charakterisieren und weiterhin ihre Beteiligung an DNA Methylierung und transkriptioneller Genstilllegung zu untersuchen.

Das Genom der sozialen Amöbe *Dictyostelium* kodiert DnmA als die einzige DNA Methyltransferase. Das Enzym enthält alle zehn charakteristischen DNA Methyltransferase Motive in seiner katalytischen Domäne. Mittels RT-PCR konnte gezeigt werden, dass die DnmA mRNA im vegetativen Wachstum exprimiert und während der Entwicklung herabreguliert wird. Untersuchungen mittels Fluoreszenz-Mikroskopie zeigten, dass DnmA-myc und DnmA-GFP Fusionen hauptsächlich im Kern lokalisieren.

Die Funktion von DnmA blieb zunächst unklar, jedoch zeigten spätere Experimente, dass das Enzym eine aktive DNA Methyltransferase ist, die für die gesamte DNA (Cytosin) Methylierung in *Dictyostelium* verantwortlich ist.

Weder in Gel-Retardations Untersuchungen noch durch das Yeast Two-hybrid System konnten Anhaltspunkte zur Funktionalität von DnmA gewonnen werden. Jedoch gab der immunologische Nachweis der Methylierungsmarkierung mittels eines anti-5mC Antikörpers einen ersten Hinweis darauf, dass die DNA von *Dictyostelium* methyliert ist. Zugabe von 5-Aza-Cytidine als demethylierendem Agens zum Dictyostelium Medium und anschließende *in vitro* Inkubation der aus diesen Zellen gewonnenen DNA mit rekombinanter DnmA zeigte weiterhin, dass das Enzym etwas besser an diese DNA bindet.

Zur weiteren Untersuchung des Proteins wurde ein Gen Knock-out von dnmA generiert. Das Gen wurde erfolgreich durch homologe Rekombination unterbrochen, der Knock-out Stamm zeigte jedoch keinen offensichtlichen Phänotyp unter normalen Laborbedingungen.

Um Zielsequenzen für die DNA Methylierung zu identifizieren, wurde eine Microarray Analyse durchgeführt. Unter Benutzung eines Grenzwerts von mindestens 1.5facher Veränderung in der Stärke der Genexpression, wurden mehrere dem entsprechende Gene im Knock-out Stamm für weitere Untersuchungen ausgewählt. Unter der hochregulierten Genen waren ESTs, die die gag und RT Gene des Retrotransposons *skipper* repräsentieren, und Northern Blot Analysen bestätigte die Hochregulierung von *skipper* im DnmA Knock-out Stamm.

Bisufitbehandlung und Sequenzierung spezifischer DNA Bereiche von *skipper* zeigte, dass DnmA für die Methylierung hauptsächlich asymmetrischer Cytosine verantwortlich ist. Außer für *skipper* wurde dies später auch für das Retrotransposon *DIRS-1*, das nicht im Microarray vorhanden war, gezeigt.

Weiterhin war die *skipper* Transkription auch in Stämmen hochreguliert, in denen Gene unterbrochen waren, die für Komponenten im RNA Interferenz Mechanismus kodieren. Im Gegensatz dazu war die *DIRS-1* Expression nicht durch den Verlust von DnmA verändert, aber stark erhöht in einem Stamm, in dem das RNA abhängige RNA Polymerase rrpC Gen unterbrochen war.

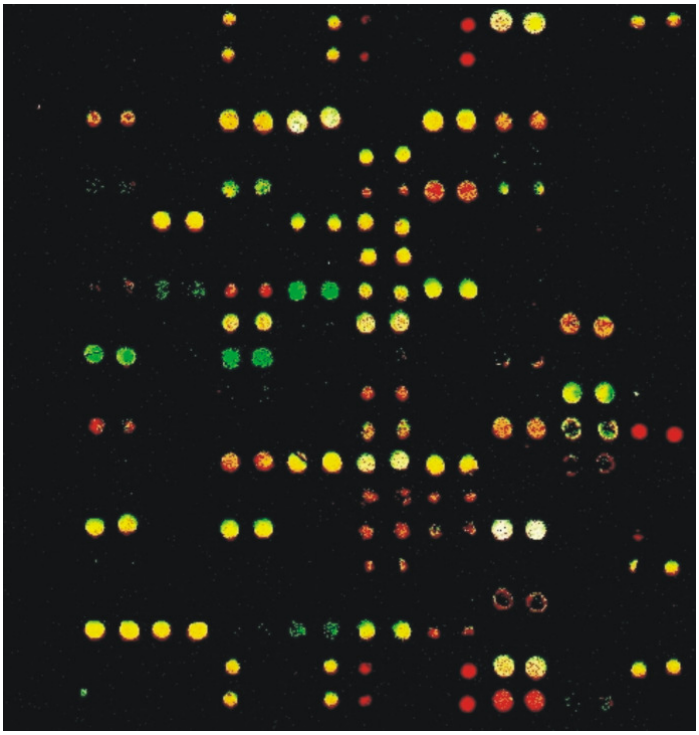
Stämme, die aus normalen Ax2 Wildtyp und DnmA Knock-out Zellen dadurch generiert wurden, dass sie 16 Runden der Entwicklung durchlaufen hatten, wurden auf Transposon Aktivität untersucht. Dabei zeigten Northern Blot Analysen eine Aktivierung der *skipper* Expression, aber nicht der von *DIRS-1*.

Eine große Anzahl an siRNAs, die sich mit der *DIRS-1* sequence decken, wurde gefunden, was auf eine konzertierte Regulation der *DIRS-1* Expression durch RNAi and DNA Methylierung hindeutet. Im Gegensatz dazu wurden keine siRNAs gefunden, die sich mit dem vollständigen *skipper* Element decken.

Die Daten zeigen, dass DNA Methylierung eine entscheidende Rolle in der epigenetischen Genregulation in *Dictyostelium* spielt und dass verschiedene, teilweise überlappende Mechanismen die Transposon Stilllegung für *skipper* and *DIRS-1* kontrollieren.

Um den Mechanismus zu analysieren, durch den das Protein bestimmte Gene im *Dictyostelium* Genom angreift, wurden weitere Gene, die im DnmA Knock-out Stamm hochreguliert waren, durch Bisulfitsequenzierung analysiert. Die ausgewählten Gene sind in anderen Arten an der Multidrug response beteiligt, ihre Funktion in *Dictyostelium* ist allerdings unbekannt. Die Bisulfitdaten zeigten, dass zwei dieser Gene an asymmetrischen Cytosinen im Wildtyp methyliert waren, aber nicht in DnmA Knock-out Zellen. Dies deutet darauf hin, dass DNA Methylierung in *Dictyostelium* nicht nur in der Stilllegung von Transposons involviert ist, sondern auch in der Regulation der Transkription spezifischer Gene.

DNA methylation in *Dictyostelium discoideum*



Branimira Emilova Borisova - Todorova

Department of Genetics

Kassel University

Cooperation and contributions

Some of the constructs used in this work were contributed by Markus Kaller, as indicated throughout.

Some of the Northern blots and bisulfite sequencing reactions were done in cooperation with Dr. Markus Kuhlmann.

Microarray analyses were done with the kind cooperation of the members of the Institute of Biochemistry I at the University of Cologne.

Published material

Parts of this thesis have been published in the following paper:

Kuhlmann M*, Borisova BE*, Kaller M, Larsson P, Stach D, Na J, Eichinger L, Lyko F, Ambros V, Söderbom F, Hammann C, Nellen W.

Silencing of retrotransposons in Dictyostelium by DNA methylation and RNAi. NAR, 2005 Vol.33, 6405-6417

* The first two authors equally contributed to this work.

Supervision

First supervisor: Prof. Dr. Wolfgang Nellen

Department of Genetics, University of Kassel, Germany

Second supervisor: Prof. Dr. Friedrich W. Herberg

Department of Biochemistry, University of Kassel, Germany

Acknowledgements

My thanks go to:

Prof. Dr. Wolfgang Nellen for giving me the possibility to join his group, be my supervisor and for his support all of the time of my PhD.

Dr. Christian Hammann in whose associated group I have done this work, and I appreciate his great help and willingness to share his knowledge at any time with me.

Prof. Dr. Friedrich W. Herberg for accepting to be my second supervisor.

Prof. Dr. Markus Maniak for reading and making comments on the manuscript of this work, and discussing problems during my PhD time.

Dr. Ludwig Eichinger for the possibility to make the microarray nalyses in his laboratory and for his kind agreement to serve as a reference in my future applications.

Jianbo Na for introducing me to the microarray analysis.

Prof. Dr. Victor Ambros and Dr. Fredrik Söderbom for their kind collaboration on the siRNA library.

Harald Rühling for his support with microscopy.

Iris Müller for performing the phagocytosis analysis in this work.

The Genetics department for the nice working atmosphere and support.

The Cell Biology department for most of the antibodies used in this work and for the nice disscusions.

The Botany department for giving me the possibility to learn and perform sequence analysis.

The Developmental Biology department for providing positive controls used in the yeast two-hybrid system.

The late Prof. Dr. Martin Tabler for his great help and support.

The Otto-Braun Foundation for the stipend during the first two years of my PhD time.

The DFG project number 5538804 and the Europian project FOSRAK number 5538805, by which I was supported for the rest of the PhD time.

My husband for his help in the technical part of this thesis.

My parents for their support throughout the years.

Summary

DNA methyltransferases of type Dnmt2 are a highly conserved protein family with enigmatic function. The aim of this work was to characterize DnmA, the Dnmt2 methyltransferase in *Dictyostelium discoideum*, and further to investigate its implication in DNA methylation and transcriptional gene silencing.

The genome of the social amoeba *Dictyostelium* encodes DnmA as the sole DNA methyltransferase. The enzyme bears all ten characteristic DNA methyltransferase motifs in its catalytic domain. The DnmA mRNA was found by RT-PCR to be expressed during vegetative growth and down regulated during development. Investigations using fluorescence microscopy showed that both DnmA-myc and DnmA-GFP fusions predominantly localised to the nucleus.

The function of DnmA remained initially unclear, but later experiment revealed that the enzyme is an active DNA methyltransferase responsible for all DNA (cytosine) methylation in *Dictyostelium*.

Neither in gel retardation assays, nor by the yeast two hybrid system, clues on the functionality of DnmA could be obtained. However, immunological detection of the methylation mark with an α - 5mC antibody gave initial evidence that the DNA of *Dictyostelium* was methylated. Furthermore, addition of 5-aza-cytidine as demethylating agent to the *Dictyostelium* medium and subsequent *in vitro* incubation of the DNA isolated from these cells with recombinant DnmA showed that the enzyme binds slightly better to this target DNA.

In order to investigate further the function of the protein, a gene knock-out for dnmA was generated. The gene was successfully disrupted by homologous recombination, the knock-out strain, however, did not show any obvious phenotype under normal laboratory conditions.

To identify specific target sequences for DNA methylation, a microarray analysis was carried out. Setting a threshold of at least 1.5 fold for differences in the strength of gene expression, several such genes in the knock-out strain were chosen for further investigation. Among the up-regulated genes were the ESTs representing the gag and the RT genes respectively of the retrotransposon *skipper*. In addition Northern blot analysis confirmed the up-regulation of *skipper* in the DnmA knock-out strain.

Bisufite treatment and sequencing of specific DNA stretches from *skipper* revealed that DnmA is responsible for methylation of mostly asymmetric cytosines. Together with *skipper, DIRS-1* retrotransposon was found later also to be methylated but was not present on the microarray.

Furthermore, *skipper* transcription was also up-regulated in strains that had genes disrupted encoding components of the RNA interference pathway. In contrast, *DIRS-1* expression was not affected by a loss of DnmA but was strongly increased in the strain that had the RNA directed RNA polymerase gene rrpC disrupted.

Strains generated by propagating the usual wild type Ax2 and the DnmA knock-out cells over 16 rounds in development were analyzed for transposon activity. Northern blot analysis revealed activation for *skippe*r expression, but not for *DIRS-1*.

A large number of siRNAs were found to be correspondent to the *DIRS-1* sequence, suggesting concerted regulation of *DIRS-1* expression by RNAi and DNA methylation. In contrast, no siRNAs corresponding to the standard *skipper* element were found.

The data show that DNA methylation plays a crucial role in epigenetic gene regulation in *Dictyostelium* and that different, partially overlapping mechanisms control transposon silencing for *skipper* and *DIRS-1*.

To elucidate the mechanism of targeting the protein to particular genes in the *Dictyostelium* genome, some more genes which were up-regulated in the DnmA knock-out strain were analyzed by bisulfite sequencing. The chosen genes are involved in the multidrug response in other species, but their function in *Dictyostelium* is uncertain. Bisulfite data showed that two of these genes were methylated at asymmetrical C-residues in the wild type, but not in DnmA knock-out cells. This suggested that DNA methylation in *Dictyostelium* is involved not only in transposon regulation but also in transcriptional silencing of specific genes.

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1 Abbreviations

Amp	ampicillin
AP	alkaline phosphatase
APS	ammonium persulphate
ATP	adenosine triphosphate
as	antisense
5-aza-cytidine	5-aza-2'-deoxycytidine
BCIP	5-Bromo-4-Chloro-3-Indolylphosphate
bp	base pairs
BS	blasticidin
BS^R	Blasticidin resistance cassette
cAMP	5'-3' cyclic adenosine monophosphate
cDNA	complementary DNA
DEPC	diethylpirocarbonate
DAPI	4',6-Diamidino-2-phenylindole
DNA	deoxyribonucleic acid
DMF	dimethyl formamide
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
dNTP	deoxyribonucleotide triphosphate
ds	double stranded
EDTA	ethylenediaminetetraacetic acid
EMSA	electrophoretic mobility shift assay
Fig	figure
G418	Geniticin
GFP	green fluorescent protein
h	hour
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
IPTG	isopropyl-beta-D-thiogalactopyranoside
kb	kilo base pairs
kDa	kilo dalton

ko	knock-out
l	liter
M	molar
5mC	Methyl cytosine antibody
mA	milliampere
NBT	nitro blue tetrazolium chloride
NP40	Nonident®P40
nt	nucleotide
OD	optical density
P	phosphate
PAA	polyacrylamide
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMSF	phenylmethylsulfonylfluorid
PNK	polynucleotide kinase
PTGS	posttranscriptional gene silencing
RdRP	RNA-directed RNA Polymerase
RT-PCR	Reverse transcription –polymerase chain reaction
RNA	ribonucleic acid
RNAi	RNA interference
rNTP	ribonucleotide
rpm	rounds per minute
RT	room temperature
SAM	S-adenosyl methyonine
SDS	sodium dodecyl sulphate
siRNA	Small interfering ribonucleic acids
ss	single stranded
TBE	Tris-Borat-EDTA
TBq	terabequerel
TEMED	N'N'N'N'-Tetramethylethylendiamin
Tris	Tris(hydroxymethyl)aminomethane
u	unit
UTP	uridine triphosphate

UV	ultraviolet
V	volt
W	watt

2 Introduction

2.1 Concept of epigenetics

Epigenetic modifications of the genome involve DNA (cytosine-5) methylation and chromatin, and therefore produce alterations in gene expression without any differences in DNA sequence (Wolffe and Matzke 1999). With reference to some features of epigenetics, euchromatin (open structured chromatin) contains mainly actively transcribed genes and in contrast, heterochromatin is a transcriptional inactive and displays a compacted structure. At the molecular level, DNA methyltransferases, methyl-CpG binding proteins, chromatin remodeling factors, transcription factors and chromosomal proteins cooperate together to make the system working (Fig. 2.1). Additionally, chromosomal structures such as centromeres, kinetochores and telomeres are influenced by epigenetics even though they are not necessarily connected directly to gene function.

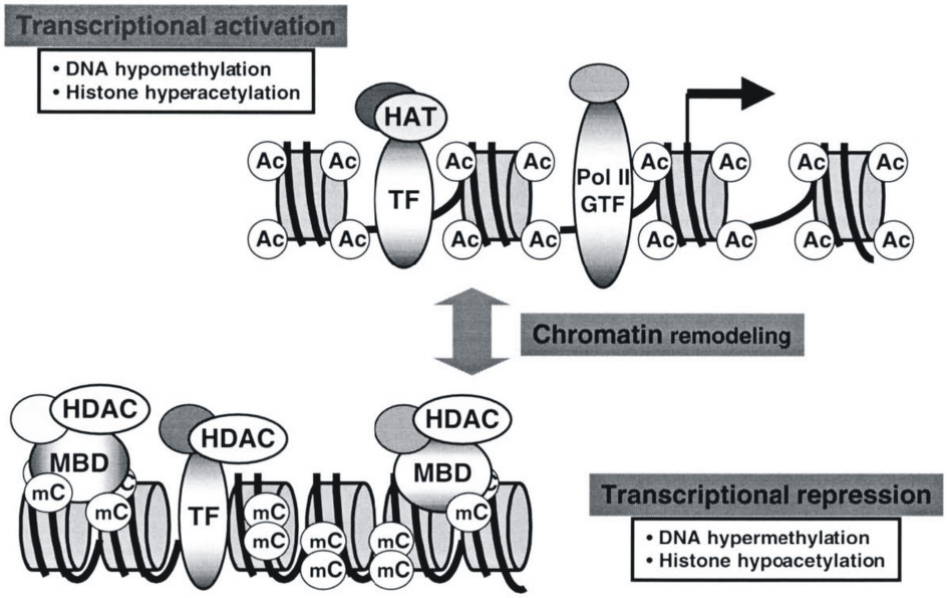

Fig. 2.1 Representation of the molecular level of epigenetics (adapted from (Nakao 2001). Active transcription (top): HAT-histone acetyltransferase, Ac-acetilated histone N-tails, TF-transcription factor, Pol II-DNA polymerase II; Repressed transcription (bottom): HDAC-histone deacetylase, MBD-methyl-binding protein, mC-methylated C5 DNA, TF-transcription factor.

2.2 DNA methylation systems

2.2.1 DNA methylation

In 1925, it has been shown that bacterial DNA contains methylated cytosine (Johnson and Coghill 1925). More than a half century ago, the discovery of 5-methylcytosine was done in calf thymus DNA using paper chromatography (Hochkiss 1948), before the structure and function of DNA as genetic material was known. After the genetic code was solved by (Nirenberg and Matthei 1961), modern molecular biology was directed into beginning of research in DNA methylation. Biological DNA methylation can result in N 6-methyladenine, N 4-methylcytosine, and 5-methylcytosine in prokaryotes but it is restricted to 5-methylcytosine in vertebrates. DNA methylation in vertebrates is a process, by which a methyl group is added to the C5 of cytosines in 5'-CpG-3' dinucleotides as well as to the C5 of cytosines in 3'-CpG-5', in a reaction catalyzed by a special group of enzymes called DNA methyltransferases (Fig. 2.2), (Liu et al. 2003).

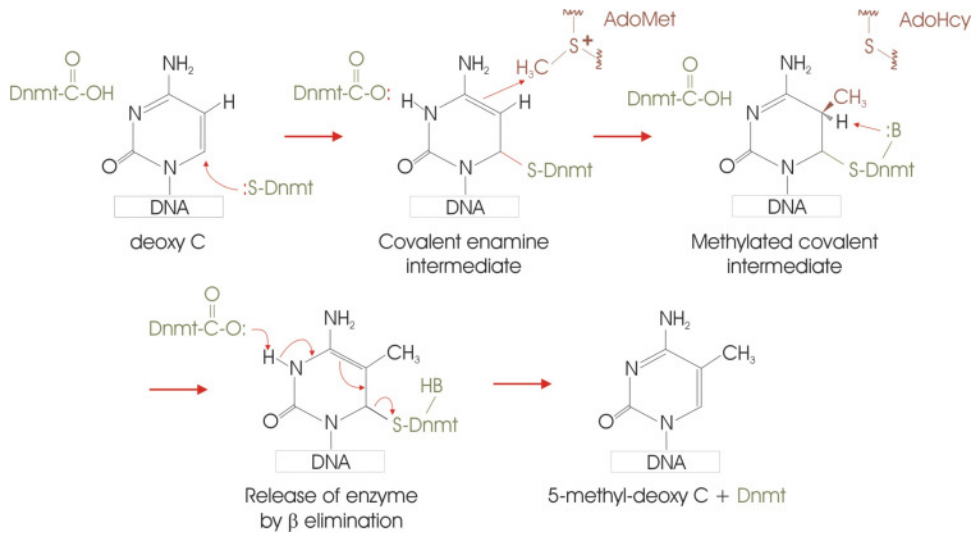

Fig. 2.2 The catalytic mechanism of DNA methylation, where the reaction proceeds with a cysteine SH group from the active site of the enzyme making a nucleophilic attack at position C6 of the target cytosine resulting in covalent complex intermediate between the enzyme and DNA. The attacking cysteine residue is located in the conserved motif IV (PCQ motif) of all known DNA (cytosine-5) methyltransferases. The enzyme facilitates nucleolytic attack on the C6 by transient protonation of the cytosine ring at the endocyclic nitrogen, N3, creating cytosine 4,5 enamine, which in turn attacks the sulphonium linked methyl group of S-Adenosyl-L-methionine. Following methyltransfer, abstraction of a proton from C5 allows reformation of the 5,6 double bond. The enzyme is finaly released by β-elimination. During this process, the cytosine residue is flipped out of the DNA double helix in a localized extrahelical configuration.

Hemi-methylation refers to the situation when only one of the two cytosine residues in a double strand is methylated. This situation occurs transiently after each round of DNA synthesis (Fig. 2.3).

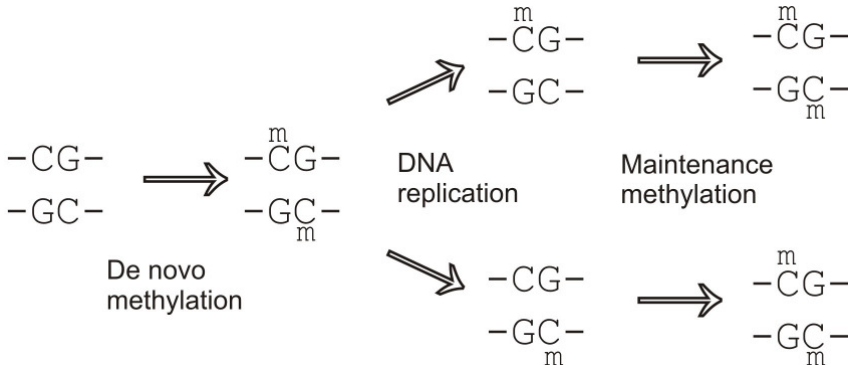

Fig. 2.3 Schematically representation of the two types of DNA (cytosine-5)-methyltransferase activities, before and after DNA replication. DNA methylation is indicated by the letter *m*.

Approximately 60-90% of all CpG sequences in vertebrate genomes are methylated, while unmethylated CpG dinucleotides are mainly clustered in CpG rich sequences termed CpG islands, in promoter regions (Ng and Bird 1999). In plants, DNA methylation occurs in the sequence context of both CpG and CpNpG. Cytosine methylation outside of the sequence context CpG is variable and with different impact on the biological function (Kunert et al. 2003; Kuhlmann* and Borisova* et al. 2005)

For a long time DNA methylation was ignored, since popular model developmental systems *Schizosaccharomyces pombe*, *Caenorhabditis elegans* (Hall et al. 2002; Volpe et al. 2002)together with *Drosophila melanogaster* and *Dictyostelium discoideum* (Smith and Ratner 1991) did not appear to have detectable DNA methylation. The research in that field was boosted when it was shown that a functional methylation machinery is essential for mammalian embryonic development (Li et al. 1992; Okano et al. 1999), and also plays an important role in tumourogenesis and other diseases (Jones and and Baylin 2002).

2.2.2 DNA methyltransferases

Almost a quarter of a century after the discovery of 5-methylcytosine, the first human DNA methyltransferase was purified and characterized (Roy and Weissbach 1975). Mammalian DNA methyltransferases are subdivided based on sequence homology into three families: Dnmt1, Dnmt2 and Dnmt3 (Fig. 2.4, (Colot and and Rossignol 1999). Because of their substrate

preference for hemimethylated CpG dinucleotides (Gruenbaum et al. 1982; Bestor and Ingram 1983), Dnmt1 enzymes are generally regarded as maintenance metyltransferases.

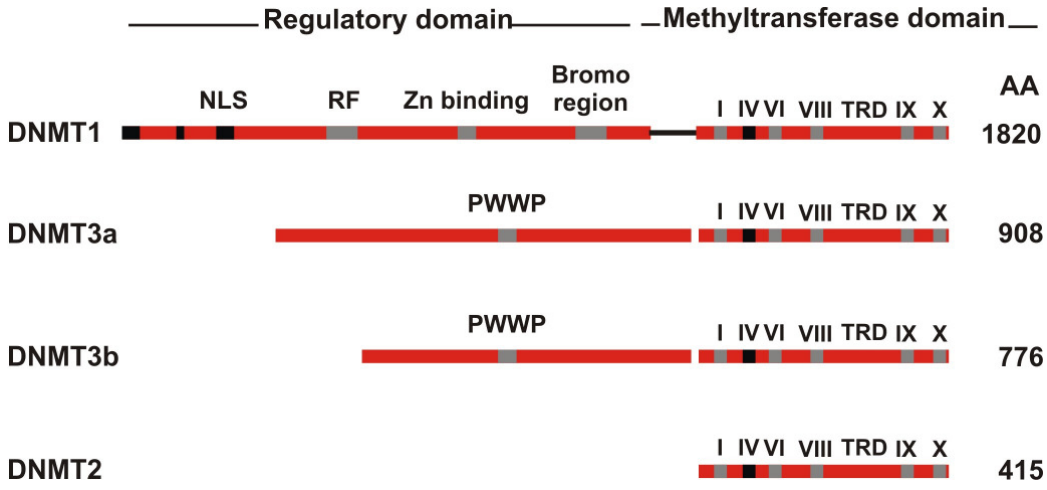

Fig. 2.4 Functional organization of mammalian DNA (cytosine-5) methyltransferases. Functionally mapped regions of the full-length human DNMT1 are illustrated along with the carboxy-terminal catalytic domain. Nuclear localization signal (NLS) (194-213), replication fork (RF) targeting peptide (320-567), and Zn binding region (652-670), and polybromo-1 homology regions are represented. The PWWP is a characteristic domain for both DNMT3a and DNMT3b, which is involved in DNA repair and regulation of transcription. The conserved motifs responsible for the catalytical function of all DNA methyltransferases are indicated on the top of the methyltransferase domain. Of these motifs I-III form the co-factor (AdoMed) binding site; IV (containing the Pro-Cys di-peptide), VI, XIII and X form the catalytic pocket and motifs V and VII are involved in preservation of the common fold of MTases that generate 5mC. TRD: target recognition domain, which is a variable region and recognized as a separate domain. On the right side, the sizes in amino acids (AA) are marked for each of the human DNA-methyltransferase proteins.

They display 7-20 fold more activity on hemimethylated DNA as compared with unmethylated substrates *in vitro* (Pradhan et al. 1999). It has been suggested that the function of these enzymes might be the copying of cytosine methylation patterns from the parental DNA strand to the newly synthesized strand during or shortly after replication (see Fig. 2.3). Deletion of Dnmt1 alone led to embryonic lethality in mice (Okano et al. 1999). More recently, complexes immunoprecipitated with anti-MeCP2 antibody (methyl-CpG-binding protein) were shown to have preferences to hemimethylated DNA. This suggested that Dnmt1 could associate with MeCP2 in order to perform maintenance methylation *in vivo* (Kimura and Shiota 2003). Dnmt1 has several isoforms, including a slice variant known as Dnmt1b (Bonfils et al. 2000) and an

oocyte specific isoform lacking the first 118 amino acids (Dnmt1o) from the N-terminus of the somatic form (Mertineit et al. 1998). In addition to its enzymatic activities, Dnmt1 was reported to repress transcription in a methylation-independent manner, in cooperation with histone deacetylases (HDACs) (Robertson et al. 2000).

The function of the second family of DNA methyltransferases, Dnmt2, has been enigmatic for a long time. The protein has all 10 conserved sequence motifs, including the AdoMet binding pocket formed by motifs I-III and the catalytic Pro-Cys di-peptide in motif IV shown to be essential for the catalytic function. This enzyme lacks the regulatory N-terminal region that is found in the Dnmt1 and Dnmt3 family of enzymes (Fig. 2.4). Targeted deletion of Dnmt2 in embryonic stem cells did not affect methylation, indicating that Dnmt2 is not essential for DNA methylation in development (Okano et al. 1998). It even has been suggested that Dnmt2 proteins might not function as DNA methyltransferases at all (Dong et al. 2001; Goll et al. 2006). However, more recent data indicated weak DNA methyltransferase activity of Dnmt2 in mouse and human cells (Liu et al. 2003). In addition, it has been shown that the observed DNA methylation in *Drosophila* is due to the function of a Dnmt2 homolog (Kunert et al. 2003).

The role of the third family of animal DNA methyltransferases is defined by their distinct preference for unmethylated DNA (Okano et al. 1998). Dnmt3a and Dnmt3b function as *de novo* DNA methyltransferases (Hsieh 1999; Lyko et al. 1999; Okano et al. 1999) and are considered to be important for the establishment of DNA methylation patterns during embryogenesis and also involved in altered DNA methylation in tumourogenesis. The architecture of Dnmt3 enzyme family is similar to Dnmt1 with a large amino terminal regulatory region attached to the catalytic domain (Fig. 2.4). Dnmt3a co-localize with heterochromatin protein (HP1) and methyl-CpG-binding protein (Bachman et al. 2001). Both Dnmt3a and Dnmt3b colocalize on the heterochromatic region of the chromosomes. They both have the characteristic PWWP domain involved in DNA repair and regulation of transcription. Murine null mutants for Dnmt3a and/or Dnmt3b are not viable, similar to Dnmt1null animals, which confirm an essential function also for Dnmt3 enzymes. It has been demonstrated that Dnmt3 can also be associated with HDAC1, leading to methylation independent gene silencing similar to Dnmt1 (Bachman et al. 2001).

There is a growing list of proteins that share homology with the conserved motifs of DNA (cytosine-5) methyltransferases without transmethylase activity. Such an example is the Dnmt3L methyltransferase, which contains motifs I, IV and VI and participates in maternal genomic imprints (Bourc'his et al. 2001). Dnmt3L was shown to interact with Dnmt3a and Dnmt3b and to colocalize with these proteins in the nucleus. Because of lacking critical amino acids residues in

motif I, active PC site in motif IV and all the motifs VII-X, not surprisingly no catalytic function was observed for Dnmt3L.

2.2.3 Methyl-CpG binding proteins

The most common epigenetic modification of vertebrate genomes is CpG methylation, which is primarily associated with transcriptional repression (Bird and Wolffe 1999). In addition to methylated DNA, methyl CpG binding proteins are required to inhibit transcription by mediating the interactions between DNA methylation, histone deacetylation, and chromatin components. MeCP2, MBD1, MBD2, MBD3 and MBD4 constitute a family of vertebrate proteins that share the methyl-CpG-binding domain (MBD). The MBD consists of about 70 residues, possesses a unique α / β -sandwich structure with characteristic loops and is able to bind single methylated CpG pair as a monomer (Nan et al. 1993).

The fact that the MBD recognizes symmetrically methylated CpG dinucleotide suggested that dimerization may be required for the binding of MeCP2 (Nan et al. 1993). Unlike DNMTs, which interact with the DNA substrate transiently, methyl-CpG binding proteins form stable associates with methylated DNA. Binding of these proteins to methylated promoter DNA causes transcriptional repression and assembly of inactive chromatin (Keshet et al. 1986).

2.2.4 DNA demethylases

How DNA gets de-methylated and what enzymes are involved still remains uncertain (Wolffe et al. 1999), in a contrast to histone demethylation, which recently has been shown to be demethylated by family of JmjC domain containing proteins (Tsukada et al. 2006). There are two possible mechanisms for removing a methyl group from methylated DNA. One is passive whereby methylation is not maintained during DNA replication, and the other is an active mechanism catalyzed by alternative DNA demethylase(s). Although MBD4 was reported to have demethylase activities (Bird and Wolffe 1999), this result has not been reproduced. Demethylation by glycosilases removes nucleotide. Active demethylation has to occur upon reprogramming of fertilized mammalian eggs.

2.3 Chromatin remodeling and assembly factors

Chromatin is a nucleoprotein complex, which consists of histone proteins and DNA wrapped around it, in a structure called: “beads on the string”. Chromatin remodeling represents a change

of the nucleosome assembly in a way that lead to heterochromatin formation, recruiting different chromatin remodeling factors (Lusser and Kadonaga 2003). All remodeling complexes are ATP-dependent, especially the SWI/SNF and ISWI families, which were initially found in yeast and *Drosophila* (Workman and Kingston 1998; Kingston and Narlikar 1999). The diversity of these large multimolecular structures among species is immense. In addition the nomenclature is quite complex: CAF1, HuCHRAC, hSWI/SNF, RSF, SIN3, Mi2-NuRD. Each of these complexes is built up of several different proteins and is regulating different processes. Some of them mainly activate gene transcription (hSWI/SNF), some are related to chromatin assembly during DNA replication (CAF1), and others like the Mi2-NuRD together with SIN3 are implicated in repression of gene transcription (Knopfler and Eisenman 1999)

Transcription, DNA replication, repair and recombination are dynamically carried out at the chromatin level. As mentioned previously, the nucleosome is the fundamental unit of chromatin and consists of core histones bound to DNA. The concept of the "histone code" describes combinations of N-terminal modifications on histones, including acetylation, methylation, phosphorylation, ubiquitination and their influence on gene expression, DNA replication and chromatin-dependant processes (Strahl and Allis 2000).

In mammals acetylation of the N-terminal histone tails usually represents transcriptionally active chromatin and is mediated by transcriptional co-activators called histone acetyltransferases (HAT) (Marmorstein and Roth 2001). Phosphorylation at serine 10 of histone H3 is important for chromosome condensation in mitosis and for an initial response to mitogens, and it has been suggested that this phosphorylation induces acetylation of neighboring lysine residues by histone acetylases. Conversely, histone deacetylases (HDACs) are known to contribute to form transcriptional co-repressor complexes (Knopfler and Eisenman 1999). In addition it has been postulated that the MBD3-MBD2 interactions recruits the Mi2-NuRD complex to methylated DNA. Thus, DNA methylation and histone deacetylation are cooperatively involved in transcriptional repression.

There are six residues on the histones H3 and H4 known to be methylated: K4, K9, K27, K36 and K79 of histone H3, and K20 of histone H4. While histone acetylation generally correlates with transcriptional activation, histone methylation can either mark transcriptionally active or inactive chromatin, depending on the lysine residue, which is methylated. Recently, H3-specific methylase (HMTase) was identified as a product of the su(var)3-9 gene in *Drosophila*, and its homologues clr4 in *S. pombe* and suv39h1 and –h2 in humans (Martin and Zhang. 2005). The H3 methylation at lysine 9 generates a binding site for heterochromatin-associated protein HP1

(Jenuwein 2001). In contrast, H3 methylated at lysine 4 by an H3-K4 methyltransferase is specific to the euchromatin regions.

2.4 HP1 – DNA methylation connection

Heterochromatin Protein 1 (HP1) was the first non-histone heterochromatin component to be discovered in *Drosophila* 20 years ago in polytene chromosomes (James and Elgin. 1986) Since then, a large number of genetic and biochemical experimental approaches have elucidated its function in heterochromatin and made it one of the best characterised chromatin proteins to date (Hiragami and Festenstein. 2005).

Genetic studies in different organisms have shown that H3K9 methylation acts upstream of DNA methylation. Consequently, loss of the H3K9-HMTases DIM5 in *N. crassa*, the KRYPTONITE methyltransferase in *Arabidopsis* or the murine Suv39h1/2 causes loss of DNA methylation (Tamaru and Selker 2001). Furthermore, HP1 is essential for DNA methylation in *N. crassa* (Freitag et al. 2004). In numerous cases of transcriptional inactivation, it has been shown that establishment of repressive histone modification patterns chronologically precedes the establishment of DNA methylation, which is therefore regarded as a "final" lock to switch off transcription (Bachman et al. 2003; Feldman et al. 2006).

2.5 RNA interference, DNA methylation and heterochromatin assembly

2.5.1 RNA-directed DNA methylation

RNA interference was introduced by Fire (Fire et al. 1998) as a conserved double-stranded RNA triggered mechanism that degrades homologous mRNA. The canonical effector molecules of the RNAi machinery are small interfering RNAs (siRNAs). These siRNAs are generated from dsRNA precursors (derived from different origins and varying in length) by the action of Dicer, a member of the RNaseIII family of ribonucleases, which processes the dsRNA into short RNA duplexes of 21 to 28 nucleotides in length. The siRNAs are loaded into different effector complexes, such as RISC (RNA-induced silencing complex), or serve as primers for an RNA-dependent RNA polymerase (RdRP), which synthesizes new dsRNA from the targeted complementary RNA-template. On the other hand, the RISC complex is guided to homologous mRNAs, which are cleaved by the endonucleolytic activity of Argonaute-2, a RISC component. RNAi has been identified as an evolutionarily conserved pathway used by many organisms to

shut down expression of retrotransposons, but also to regulate endogenous genes. RNAi-like mechanisms have previously been shown to control transposon activity or transcription of tandem transgene arrays in various organisms (Matzke et al. 2000; Sijen and Plasterk. 2003; Svoboda et al. 2004).

Since RNA can also base pair with DNA; it may guide genome modifications and induce silencing at the transcriptional level. The idea that short RNAs can target epigenetic alterations, such as DNA (cytosine-5) methylation and histone modifications was demonstrated for the first time in plants (Wassenegger et al. 1994). The so called RNA-directed DNA methylation (RdDM) was observed in viroid infected plants where DNA with sequence identity to silenced RNA is *de novo* methylated at its cytosine residues. This methylation is not only at canonical CpG sites but also at cytosines in CpNpG and asymmetric sequence contexts. Primarily the methylation was limited to the region of RNA-DNA sequence identity but subsequently there was also limited spreading of methylation into adjacent DNA sequences observed (Wassenegger 2000; Aufsatz et al. 2002).

2.5.2 RNAi-dependent heterochromatin assembly

Recent work on *S. pombe* has revealed an extensive role for components of the RNAi machinery in transcriptional gene silencing and chromatin modifications that occur in the absence of detectable DNA methylation. This introduced on the stage RNAi proteins such as Dicer, Argonaute and RNA-dependent RNA polymerase, which together with histone methyltransferase Clr4, were responsible for establishing the heterochromatin at *S. pombe* centromeres (Volpe et al. 2002) and the silent mating type locus (Hall et al. 2002). It was found recently, that siRNAs are incorporated into the RITS complex (RNA induced transcriptional silencing), the nuclear counterpart of RISC and subsequently guided to chromatin. This complex is composed of the chromodomain protein Chp1, Tas3 (a protein of unknown function) and Ago1, the sole argonaute protein in *S. pombe*. RITS binds to all known heterochromatic loci in *S. pombe*, e.g. centromeres, telomeres and the silent mating type locus (Noma et al. 2004), indicating a general function in heterochromatin formation.

In contrast to RdDM, which is largely restricted to the region of RNA-DNA sequence identity, RNAi-dependent heterochromatin can spread several kilobases from the RNA-targeted nucleation site in a manner that depends on Swi6, the *S. pombe* ortholog of heterochromatin protein 1 (Hall et al. 2002; Volpe et al. 2002; Schramke and Allshire 2003). In addition

retrotransposon regulation via components of the RNAi machinery could suggest an evolutionary role of RNAi as a host defence mechanism.

2.6 Concept of epigenetics-validation in *Dictyostelium discoideum*

The first description for *Dictyostelium* was given by Oskar Brefeld in 1869 (Brefeld 1869). He named the species *Dictyostelium* from *Dicty* – net like and *stelium* – tower, because the aggregation territories he observed looked like nets and the fruiting bodies like towers. The discovery of *Dictyostelium discoideum* was done in 1935 by Kenneth Raper and since then it has become the type species to study various cell biological processes such as signal transduction and cellular differentiation.

The life cycle of *Dictyostelium* is divided in two: a vegetative part where the cells multiply by mitotic division and a developmental part where upon starvation, unicellular amoebae start to form multi-cellular aggregates, which differentiates into two main cell types: spore and stalk cells.

Recently, the genome of *Dictyostelium discoideum* has been completely sequenced and assembled (Eichinger et al. 2005). This clarified the chromosome organization, with some small exceptions like limited information for centromer and telomer structures.

Dictyostelium became a "model organism" for epigenetics studies in the recent years because several components of the protein machinery required for gene silencing in higher eukaryotes were also identified in the *Dictyostelium* genome. DNA methyltransferase (Kuhlmann* and Borisova* et al. 2005; Ponger 2005; Katoh et al. 2006), histone deacetylases (HDACs) and histone acetyl transferases (Puta pers. communication), ATP-dependent chromatin remodelling enzymes, chromo-domain proteins (including three isoforms of HP1) (Kaller et al. 2006), bromodomain proteins, histone methyltransferases (including a su(var) 3-9 homolog (Essid 2004) and putative histone demethylase (Földesi pers. communication) have been identified and are currently being characterized.

In addition proteins required or influencing RNAi interference have been identified and their function was established (Martens et al. 2002; Kuhlmann* and Borisova* et al. 2005; Popova et al. 2006). Some of these proteins occur in large families for example nine members of putative histone deacetylases, or five members of putative Argonaute proteins in comparison to a single DNA methyltransferase. Until recently, evidence for DNA methylation in the *Dictyostelium* genome was missing (Smith and Ratner 1991), but in 2005/2006 *Dictyostelium* DNA was found to be modified by cytosine methylation. Shortly before the genome was sequenced it was found that

only one gene encoding a putative Dnmt2 DNA methyltransferase is present in the *Dictyostelium* genome (Kaller 2002).

Global analysis of genomic DNA from *Dictyostelium* by capillary electrophoresis revealed a faint signal corresponding to ~0.2% cytosine methylation which suggested that methylation is strongly restricted to defined foci rather than global (Kuhlmann* and Borisova* et al. 2005).

All *Dictyostelium* species contain several types of transposable elements which are relics of events in the earlier history of the species (Geier et al. 1996; Leng et al. 1998). Mobile genetic elements can be divided into retrotransposons with identical long terminal repeats (LTR) or into non-LTR elements, which are often defined as "longer-interspersed nuclear-elements" (LINE). Members of a LTR retroelements in *Dictyostelium* are *DIRS-1* and *skipper*.

DIRS-1 is a 4.7 kb LTR - repetitive element that is present in about 40 copies and approximately 200 incomplete copies in the genome. *DIRS-1* sequences were found near the telomeres of the chromosomes. It consists of inverted long terminal repeats of 330 bp 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 (Rosen et al. 1983; Zuker et al. 1984; Glockner et al. 2001). The organization of *DIRS-1* retrotransposon is shown schematically on Fig. 2.5.

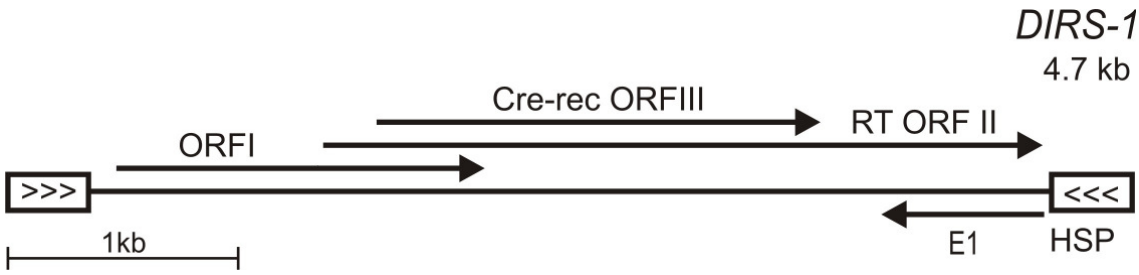

Fig. 2.5 Schema of 4.7 kb *DIRS-1* retroelement. ORF I is encoding protein 1, ORF II for reverse transcriptase and ORF III for recombinase. In addition, HSP is a heat-shock promoter for heat shock transcript in antisense orientation, termed E1. The two arrowed boxes are representing the LTRs (long terminal repeats).

Skipper is 7 kb LTR - repetitative element which has 20 copies in the *Dictyostelium* genome and consists of direct long terminal repeats and three open reading frames encoding GAG (matrix protein), PRO (protease) and RT (reverse transcriptase), POL (polymerase) and IN (invertase) (Leng et al. 1998).

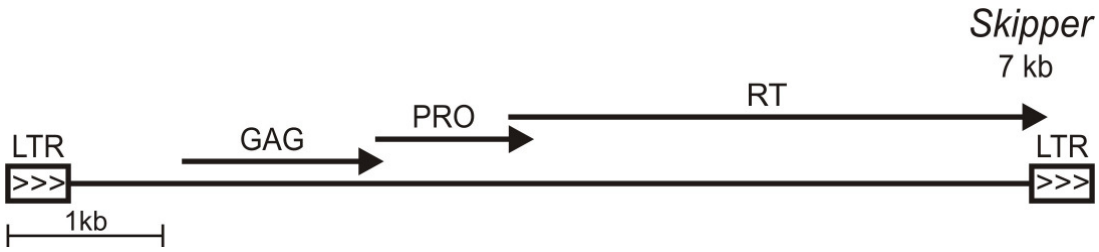

Fig. 2.6 Schema of 7 kb *skipper* retroelement. Three open reading frames: GAG encodes retroviral nucleocapsid domain, PRO encodes protease and POL encodes the RT (reverse transcriptase), and IN (invertase). Arrowed boxes indicate LTRs which in this case are direct repeats.

Skipper is expressed at very low levels during growth and development. Both retrotransposons *skipper* and *DIRS-1* do not integrate near tRNA genes in contrast to non-LTR retrotransposons like TREs (Glockner et al. 2001). More common integration for *skipper* is next to the *DIRS-1* element.

To suppress transposition events other organisms have developed mechanisms of defence. One of them is RNAi like mechanism shown to be involved in such a regulation (Matzke et al. 2000; Sijen and Plasterk. 2003). The question how the transposition events are governed in the *Dictyostelium* genome and what is the mechanism responsible for that was still open.

2.7 Aim of this work

To explore DNA methylation and its function in *Dictyostelium*, the DnmA protein was investigated as the only one member of the DNA methylation machinery present in *Dictyostelium* genome. Using this protein, which is highly conserved in various organisms, should provide information for how the DNA methylation machinery is working in *Dictyostelium* and also provide a comparison to other organisms where different DNA methyltransferases are known to be functional. Having only one DNA methyltransferase belonging to the family of DNMT2 methyltransferases was a challenge to work with it since, it was not even clear by that time, if these enzymes were genuine methyltransferases.

The function of Dnmt2 proteins was enigmatic. Similar to the situation in *Drosophila*, previous work had suggested that *Dictyostelium* DNA was not methylated (Smith and Ratner 1991). The discovery of a methyltransferase gene of the Dnmt2 family in the *Drosophila* genome and the subsequent detection of low levels of cytosine methylation (Lyko et al. 2000) prompted us to reinvestigate this issue in *Dictyostelium* in this work. Further more not only establishing the

function of the enzyme, but also defining the potential targets for methylation was one of the aims here. Our findings reported here that Dnmt2 in *Dictyostelium* (DnmA) methylated asymmetric C-residues, which were also confirmed in *Drosophila* mainly at CpA/T sites.

3 Chemicals and reagents

3.1 Chemicals

acrylamide/bis-acrylamide (30%, 40%)	Roth, Karlsruhe
agar-agar	Euler, Frankfurt am Main
agarose	Sigma, Taufkirchen
ammonium peroxodisulfate (APS)	Merck, Darmstadt
ammonium sulphate	Roth, Karlsruhe
bacto-peptone	Difco, Augsburg
bacto-tryptone	Difco, Augsburg
ß-mercaptoethanol	Fluka, Deisenhofen
boric acid	Roth, Karlsruhe
Bradford solution	Bio-Rad
bromphenolblue	Fluka, Deisenhofen
BSA	Roth, Karlsruhe
calcium chloride (CaCl2)	Roth, Karlsruhe
Coomassie Brilliant Blue G-250	Serva, Heidelberg
dATP	MBI Fermentas, St. Leon-Rot
dCTP	MBI Fermentas, St. Leon-Rot
dGTP	MBI Fermentas, St. Leon-Rot
dTTP	MBI Fermentas, St. Leon-Rot
DAPI	Roth, Karlsruhe
DMSO	Sigma, Taufkirchen
DTT	Roth, Karlsruhe
EDTA	Roth, Karlsruhe
ethanol 99.8%	Roth, Karlsruhe
ethidium bromide	Fluka, Deisenhofen
formaldehyde 37%	Riedel-de-Haen, Seelze
formamide	Roth, Karlsruhe
gelvatol	Fisher Scientific, Nidderau
glycerol, 86%	Roth, Karlsruhe
glycine	Roth, Karlsruhe

guanidine thiocyanate	Roth, Karlsruhe
HEPES	Roth, Karlsruhe
hydroquinone	Sigma-Aldrich, Germany
IPTG	Roth, Karlsruhe
imidazol	Roth, Karlsruhe
isopropanol	Roth, Karlsruhe
liquid nitrogen	Messer Griesheim, Krefeld
lithium chloride (LiCl)	Roth, Karlsruhe
Long Ranger 50% 830611 (250ml)	Biozym FMC
magnesium sulphate (Mg_2SO_4)	Roth, Karlsruhe
methanol	Roth, Karlsruhe
methylene blue	Roth, Karlsruhe
milk powder	TSI, Zeven
Ni-Sepharose™	Amersham, Freiburg
phenol	Roth, Karlsruhe
phenol/chloroform	Roth, Karlsruhe
potassium acetate (KAc)	Riedel-de-Haen, Seelze
potassium chloride (KCl)	Roth, Karlsruhe
potassium hydrogenphosphate (KH_2PO_4)	Fluka, Deisenhofen
PMSF (phenylmethylsulfonylfluoride)	Roth, Karlsruhe
rATP	MBI Fermentas, St. Leon-Rot
rCTP	MBI Fermentas, St. Leon-Rot
rGTP	MBI Fermentas, St. Leon-Rot
rUTP	MBI Fermentas, St. Leon-Rot
sucrose	Roth, Karlsruhe
SDS -sodium dodecyl (lauryl) sulfate	Roth, Karlsruhe
sephadex (G25, G50)	Fluka, Deisenhofen
sodium acetate (NaAc)	Fluka, Deisenhofen
sodium azide (NaN_3)	Merck, Darmstadt
sodium carbonate ($NaCO_3$)	Roth, Karlsruhe
sodium chloride (NaCl)	Fluke, Deisenhofen
sodium citrate	Roth, Karlsruhe
sodium dihydrogenphosphate (NaH_2PO_4)	Fluka, Deisenhofen
disodium hydrogenphosphate (Na_2HPO_4)	Fluka, Deisenhofen
sodium hydrogensulfite	Sigma-Audrich, Germany

Stop/Loading buffer 79448	Amersham Biosciences
TEMED	Roth, Karlsruhe
Tris	Roth, Karlsruhe
Triton-X-100	Roth, Karlsruhe
Tween 20	Roth, Karlsruhe
urea	Roth, Karlsruhe
X-gal	Fermentas, St. Leon-Rot
xylene cyanol FF	Fluka, Deisenhofen
Yeast-extract	Oxoid, England
Yeast-nitrogen base	DIFCO, Augsburg

3.2 Radioactive materials

[α-^{32}P**]** dATP (110 TBq/mmol)	Hartmann Analytic, Braunschweig
[γ-^{32}P**]** ATP (110 TBq/mmol)	Hartmann Analytic, Braunschweig

3.3 Antibiotics

ampicillin	Roth, Karlsruhe
amphotericin	PAA, Cölbe
blasticidin	MP Biomedicals, Eschwege
geneticin (G418)	PAA, Cölbe
penicillin/streptomycin	PAA, Cölbe
kanamycin	Sigma, Deisenhofen
chloramphenicol	Sigma, Deisenhofen
tetracyclin	Serva, Heidelberg

3.4 Antibodies

coronin antibody (176-3-6)	University of Kassel, Cell Biology Dept
discoidinI antibody (80-52-13)	University of Kassel, Cell Biology Dept
c-myc antibody (9-E-10)	University of Kassel, Cell Biology Dept
His-tag antibody (232-470-5)	University of Kassel, Cell Biology Dept
GFP antibody (264-449-2)	University of Kassel, Cell Biology Dept

IgG, goat-anti-mouse, alkaline phosphatase-coupled	Dianova, Hamburg
IgG, goat-anti-rabbit, alkaline phosphatase-coupled	Dianova, Hamburg

3.5 Enzymes and kits

DNase I, RNase free	MBI Fermentas, St. Leon-Rot
Klenow DNA polymerase	MBI Fermentas, St. Leon-Rot
proteinase K	Boehringer Mannheim, Mannheim
restriction endonucleases	MBI Fermentas, St. Leon-Rot, Gibco BRL, Eggenstein New England Biolabs Boehringer Mannheim, Mannheim
reverse transcriptase (Mu-MLV)	MBI Fermentas, St. Leon-Rot
RNase A	Merck Biosciences, Bad Soden
RNase-inhibitor (RNasin)	MBI Fermentas, St. Leon-Rot
RNeasy®Mini kit	Qiagen, Germany
shrimp alkaline phosphatase (SAP)	USB
SP6 RNA-polymerase	MBI Fermentas, St. Leon-Rot
T4 DNA-ligase	MBI Fermentas, St. Leon-Rot
T4- polynucleotide kinase	MBI Fermentas, St. Leon-Rot
T7 RNA-polymerase	Dept. of Genetics, Uni-Kassel MBI Fermentas, St. Leon-Rot
Taq-DNA-polymerase	Dept. of Genetics, Uni-Kassel MBI Fermentas, St. Leon-Rot
NucleoSpin® ExtractII	Macherey-Nagel, Düren
NucleoBond® PC 100	Macherey-Nagel, Düren
pGEM -T-easy cloning kit	Promega, USA
Thermosequenase RPN 2438 (100 reactions)	Amersham Biosciences

3.6 Molecular weight markers

Protein marker (Roti-Mark prestained)	Roth, Karlsruhe
1 kb DNA- ladder	MBI Fermentas, St. Leon-Rot
100 bp DNA- ladder	MBI Fermentas, St. Leon-Rot

3.7 Buffers and solutions

Binding buffer DnmA-DNA (A)	20 mM Tris/HCl pH 8.0 1 mM EDTA 25 mM NaCl 1 mM DTT
Binding buffer DnmA-DNA (B)	Buffer A + 10 mM ATP
Binding buffer DnmA-DNA (C)	Buffer A + 10 mM SAM
Binding buffer DnmA-siRNas	40 mM HEPES pH 7.3 110 mM KOAc 6 mM MgOAc 250 mM Sucrose 1 mM DTT 0.1% NP40 20 units RNasin
Binding buffer HcpA/HcpB-DNA	11 mM Tris/HCl pH 7.4 5 mM $MgCl_2$
Binding buffer HcpA/HcpB-RNA	20 mM HEPES pH 7.6 100 mM KCl 2 mM EDTA 0.01% NP40
Blocking solution	5% milk pulver in 1 x PBS

BCIP solution	50 mg/ml BCIP in DMF
buffer A (lysis buffer)	10 mM Tris/HCl pH 8.0 300 mM NaCl 10 mM imidazole
buffer B (washing buffer 1)	10 mM Tris/HCl pH 8.0 300 mM NaCl 20 mM imidazole
buffer B (washing buffer 2)	10 mM Tris/HCl pH 8.0 300 mM NaCl 50 mM imidazole
buffer C (elution buffer)	10 mM Tris/HCl pH 8.0 300 mM NaCl 250 mM imidazole
buffer D (dialysis buffer)	10 mM Tris/HCl pH 8.0 100 mM KCl 1 mM EDTA 10% Glycerol
Coomassie solution	10% acetic acid 0.1% Coomassie Brilliant Blue (CBB) G250
Coomassie destainer	5 % methanol 7% acetic acid
Comassie solution (improved protocol)	20 ml 85 % H_3PO_4 60 g ammonium sulfate 1 g Coomassie Brillant Blue G-250 ad 1000 ml H_2O destaining with H_2O

DAPI solution	1 mg/ml DAPI in MP-Water
Denhard buffer(100x)	1 g Ficoll 400 1 g polyvinylpyrolidon 1 g BSA ad 50 ml water
DEPC water	0.1% diethylpyrocarbonate in H_2O, incubated overnight, autoclaved
DNA loading buffer	30% glycerol 50 mM EDTA 20 mM Tris/HCl, pH 8.0 0.1% bromphenolblue 0.1% xylene cyanol
EP buffer pH 6.1	10 mM Na_2HPO_4, 50 mM sucrose
Gel solution (Long run 3.7% 66 cm sequencing gel)	25.2 g Urea 4.5 ml Long Ranger 50% 6.0 ml Long Run 10 x TBE buffer 40 µl TEMED 400 µl APS *ad* 60 ml water
2 x HBS, pH 7.05	4 g NaCl 0.18 g KCl 0.05 g NaH_2PO_4 2.5 g HEPES 0.5 g Glucose *ad* 250 ml H_2O

Hybridization solution (Northern and Southern blots)	5 x SSC 120 mM KPi 50% Formamid 5 x Denhard buffer 1% SDS
Kpi (pH 6.5)	56.5 ml 1M KH_2PO_4 43.5 ml 1M K_2HPO_4
2 x Laemmli buffer	62.5 mM Tris HCl (pH 6.8) 2% glycerol 2% SDS 5% ß-mercaptoethanol 0.001% bromphenolblue
9 x Laemmli buffer	3.3 ml glycerol 1.5 ml β-mercaptoethanol 0.69 g SDS 0.228 g Tris ad 10 ml H_2O adjust pH 6.8 0.3 mg bromphenolblue
Native loading dye	70% glycerol 0.1xTBE 0.1% Bromphenolblue
NBT solution	75 mg/ml NBT in 70% DMF
10 x NCP buffer	12.1 g Tris/HCl pH 8.0 87 g NaCl 5 g Tween® 20 *ad* 1000 ml H_2O

Nuclear lysis buffer	50 mM HEPES pH 7.5 40 mM $MgCl_2$ 20 mM KCl 5% Sucrose 1% NP 40
OLB-Mix	200 mM Tris/HCl, pH 7.5 25 mM $MgCl_2$ 10 mM ß-mercaptoethanol 1 M HEPES pH 6.6 13.5 U A_{260} oligos-hexamers (MBI) 0.25 mM dCTP, dGTP, dTTP
10 x PBS buffer	160.12 g NaCl 15.3 g Na_2HPO_4 (2 H_2O) 4.02 g KCl 382 g KH_2PO_4 *ad* 1000 ml H_2O, autoclaved
Phosphate buffer, pH 6.7	56.5 ml 1 M NaH_2PO_4 43.5 ml 1 M Na_2H PO_4
PMSF (100 x)	0.1742 g PMSF in10 ml isopropanol Store in dark.
SDS- lysis buffer	0.7% SDS 25 mg/ml proteinase K, dissolved in TE pH 8.0
SDS PAGE-5 x Running Buffer	151 g Tris 72 g Glycin *ad* 1000 ml with H_2O

SDS PAGE-lower buffer	1.5 M Tris pH 8.8 0.4% SDS *ad* 1000 ml with H_2O
SDS PAGE-upper buffer	0.5 M Tris pH 6.8 0.4% SDS *ad* 1000 ml with H_2O
Semi-dry blot buffer	5.8 g Tris 2.92 g Glycin 0.38 g SDS 200 ml Methanol/Ethanol *ad* 1000 ml H_2O
Solution D (incomplete)	4 mM GTC 25 mM sodium citrate 0.5% sarcosyl To prepare a complete solution D, prior use 360 µl β-mercaptoethanol were added to 50 ml solution (0.1 M).
Solution I (for plasmid preparation)	25 mM Tris-HCl, (pH 7.4) 10 mM EDTA 15% sucrose
Solution II (for plasmid preparation)	200 mM NaOH 1% SDS
Solution III (for plasmid preparation)	3 M sodium acetate, (pH 4.7) 20 x SSC 3 M NaCl 0.3 M sodium citrate
TE buffer (pH 7.4 or 8.0)	10 mM Tris-HCl 1 mM EDTA

10 x TBE Long run buffer pH 8.3	1340 mM Tris-base 450 mM Boric acid 25 mM EDTA
10 x Transcription buffer (Przybilski, R. Diploma thesis 2005)	400 mM Tris/HCl pH 8.0 200 mM $MgCl_2$ 20 mM Spermidine 0.1% Triton X-100
Yeast cracking buffer-stock	8 M Urea 5% (w/v) SDS 40 mM Tris/HCl pH 6.8 0.1 M EDTA 0.4 mg/ml bromophenol blue
Yeast cracking buffer-complete	For 1.3 ml: 1 ml (recipe above) 10 µl β-mercaptoethanol 70 µl protease inhibitor 50 µl PMSF (100x)

3.8 SDS PAGE

	12% resolving gel	Stacking gel
Acrylamide/bis-acrylamide 30% / 0.8%	4.4 ml	450 µl
Lower Buffer	2.64 ml	
Upper Buffer		1 ml
H_2O	3.4 ml	2.5 ml
250 mM EDTA	43.2 µl	16 µl
TEMED	6 µl	4 µl
20% APS	120 µl	60 µl

3.9 Devices

autoclave	Zirbus, Bad Grund
Bio Imaging Analyzer	Raytest, Straubenhardt
BAS cassette 2025	Raytest, Straubenhardt
Biological Workstation (FPLC)	Biorad, München
cell counter (Coulter Counter ZM)	Coulter Electronics, Krefeld
centrifuges:	
- Avanti™ 30	Beckmann, München
- Centrifuge 5417 C	Eppendorf, Hamburg
- Rotina 48R	Hettich, Tuttlingen
- ultracentrifuge L3-50	Beckman, München
DNA sequencer long reader 4200	LI-COR Global Edition IR^2
E.A.S.Y. gel documenting system	Herolab, Wiesloch
elektroporator (Gene PulserII®)	Biorad, München
elektrophoresis chambers:	
- agarose gels	mechanical workshop, Uni Kassel
- protein gels (SE 250)	Hoefer Pharmacia, SF, USA
- PAA gels	mechanical workshop, Uni Kassel
Fluorescent microscope (Leica DM IRB)	Leica, Wetzlar
Geiger counter (Mini-Monitor)	Mini-Instruments, GB
gel casting chambers	mechanical workshop, Uni Kassel
gel dryer	Bachofer, Reutlingen
heating block	electronic workshop, U. of Kassel
hybridization oven	Bachofer, Reutlingen
imager: Fuji X Bas 1500	Raytest, Straubenhardt
laminar flow hood	Nunc, Wiesbaden
magnetic stirring plate	Bachofer, Reutlingen
microscope	Zeiss, Jena
microwave oven	Aldi, Essen
PCR-Mastercycler personal	Eppendorf, Hamburg
pH-Meter 320	Bachofer, Reutlingen
pipetboy (Accu-Jet®)	Brand, Wertheim
pipettes (20µl, 200 µl, 1000 µl)	Gilson, Langenfeld
Photometer (Ultrospec® 2000)	Pharmacia Biotech, Freiburg

power supplies:	
-Power Pac 3000	Bio-Rad, Canada
-EPS	Pharmacia, Freiburg
semidry blotting apparatus	von Kreuz, Reiskirchen
Speed Vac concentrator	Savant, USA
ultra-sonicator UP 200S	Dr. Hielscher GmbH, Stansdorf
UV- table	Bachofer, Reutlingen
Vortex Genie	Bender Hohbein AG, Germany

3.10 Consumables

3MM paper	Whatman, Göttingen
Becher glasses	Schott, Mainz
Costar-plates	Schütt, Göttingen
Cryo-tubes	Nunc, Wiesbaden
disposable pipette tips	Sarstedt, Nürnbrecht
EP-cuvettes (Gene Pulser® 0,4 cm)	Biorad, München
Falcon-tubes (15 ml, 50 ml)	Sarstedt, Nürnbrecht
glass pipettes	Hirschmann, Germany
Hybond™ nylon membranes (NX, N+)	Amersham
HisTrap®, Ni-NTA columns	Pharmacia, Freiburg
injection needles	B.Braun, Melsungen
injection syringes	B.Braun, Melsungen
nitrocellulose membrane (porablot™ NCP)	Macherey-Nagel, Düren
parafilm	Schütt, Göttingen
petri-dishes	Sarstedt, Nümbrecht
PCR-tubes	Sarstedt, Nürnbrecht
sterile-filter (0.22 μm, 0.45 μm pores)	Millipore, Eschborn
scalpels	C.Bruno Bayha GmbH, Tuttlingen
tubes (1,5 ml; 2 ml)	Sarstedt, Nürnbrecht

3.11 *Dictyostelium* medium

AX-medium (pH 6.7)	18 g glucose 14.3 g bacto-peptone 7.15 g yeast extract 0.616 g Na_2HPO_4 x 2 H_2O 0.486 g KH_2PO_4 ad 1000 ml H_2O
DD20-medium, pH 6.5	20 g protease-peptone 8 g glucose 7 g yeast extract 0.47 g Na_2HPO_4 x 2 H_2O 0.33 g NaH_2PO_4 x 7 H_2O ad 1000 ml H_2O
MES-HL5-medium, pH 7.1	10 g Glucose 10 g Protease-Peptone 5 g Hefeextract 1.3 g MOPS ad 1000 ml H_2O
G_0- medium	as AX-medium + 50 µg/ml ampicillin + 10 U/ml penicillin + 10 µg/ml streptomycin + 0.25 µg/ml amphotericin
G10 medium	as G_0-medium + 10 µg/ml geneticin
B10 medium	as G_0-medium + 10 µg/ml blasticidin

Soerensen- phosphate buffer (pH 8.7)	2 mM Na_2HPO_4 15 mM KH_2PO_4
SM agar plates	15 g bacto-agar 10 g peptone 10 g glucose 1 g yeast-extract 1 g $MgSO_4$ 2.2 g KH_2PO_4 1 g K_2HPO_4 ad 1000 ml with H_2O, autoclaved; 10 ml 20% maltose, 10 ml $MgSO_4$ 20 ml/Petri dish

3.12 Bacteria medium

LB medium, pH 7.0	10 g Bacto-Tryptone 5 g yeast-extract 5 g NaCl ad 1000 ml H_2O
LB-agar	LB-medium + 13 g/l Agar-Agar
LB_{amp} plates	as LB-agar, + 50 µg/ml ampicillin

3.13 Yeast medium

YPD – medium:	20 g peptone 10 g yeast extract ad 1l water, adjust pH 5.8 autoclave, cool down to 55°C add 50 ml of a sterile 40% glucose solution For YPD-plates plus 18 g of agar.
SC – yeast medium	1.7 g bacto-yeast nitrogen base, DIFCO (without amino acids) 5g ammonium sulphate add 100 ml 10 x amino acid solution 850 ml water adjusted pH to 5.8 autoclave, after cool down add 50 ml 40% Dextrose solution (sterile) For SC- plates plus 20 g agar.
Amino acid solution (10 x)	200 mg Histidine 300 mg Lysine 200 mg Tryptophane 1 g Leucine 200 mg Adenine 200 mg Uracil For selective media the appropriate amino acids were omitted.

3.14 *Dictyostelium discoideum*

Dictyostelium discoideum	AX2, strain 214

3.15 Bacterial strains

Escherichia coli - DH5α™	Invitrogen, Karlsruhe
Escherichia coli - BL21(DE3)pLysS	Promega, Mannheim
Escherichia coli - Tuner™(DE3)	Novagene, Madison, USA
Escherichia coli Tuner™(DE3) pLacI	Novagene, Madison, USA
Klebsiella aerogenes	(Williams and Newell 1976)

3.16 Yeast strain

Saccharomyces cerevisiae – Y190	Clontech, (Flick and Johnston 1990; Harper et al. 1993)

3.17 Primers

Bsr G1 5’	CGCTACTTCTACTAATTCTAGA
dnmA 5’ outer	GATATAGATTATTTGGGTAATTTAATAATG
dnmAf RT	TTAATGAGTCCACCATGTCAAC
dnmArev RT	CCTGTACCTTCAATAAATTTTCC
MT1	TTAATGAGTCCACCATGTCAAC
MT2	CCTGTACCTTCAATAAATTTTCC
Trx for	GAACGAGCTCCATGGCCAATAGAGTAATTCATG
Trx rev	CGCGGATCCTTATTTGTTTGCTTCTAGAGTACTTC
eriA 5’	GGATCCGATGTCCACAACAACAACATC
eriA 3’	GGATCCTTTACTGATTTCATTGTTGAAAC
pACT2seq/PCR 263	GAGATGGTGCACGATGC
pACT2seq/PCR 262	CGCGTTTGGAATCACTAC
DIRS-LTRrev	AACATTTATTTATTTGAATTTCCC
DIRS-2F	GTATGCCCTGTTCGCCACCTTGC

Skipper RT for	CTGTTACCTTAGTGAAGATGGG
Skipper RT rev	GGGCATCTATTGTCTTATGACATGG
Skipper LTR for	GTTAGAGACTCAAAACTAAATTA
Skipper LTR rev	TGTAAAAGTCACTCACACTAATC
Skipper GAG for	TGAAGCTAAAACCATTGACGC
Skipper GAG rev	CTAATTGAACTTCAGCAGTACC
GuaBf	AATGGATACAGTTACAGAACAT
GuaBr	GAGTTTCTTATTTTCATGGTCT
MvpB for new	TTAAGTGCCAAAGAGGAATTTG
MvpB rev new	AGATAATTTGAGTGAGAGACGA
Tel A for new	TATGTCTAAAGGGTCACTCAA
TelA rev new	CTACGATTGAAGGAACATGTT
ABC 21 for	GATATGTCAACTCCATTCATTAG
ABC 21 rev	GTTCCTCTAACATATCTCTATAC

3.18 Bisulfite primers

bi-DIRS LTR for	ATCAAATTATTTTAATTTTTAATA
bi-DIRS LTR rev	AATATTTATTTATTTGAATTTTTT
bi-Skipper LTR for	ACTCAAAACTAAATTAATTTAAAATTAA
bi-Skipper LTR rev	ATGAAGGATAGAAAGAGTGAAAGAT
bi-SkipperGAG for	CCATTAAAACTAAAACCATTA
bi-SkipperGAG rev	TATTATTATTATTTAAAATTGATTTTAG
bi-SkipperRT for	AAATCTTACATATATTATCAATAAA
bi-SkipperRT rev	AATAATTGAGTAGTATGTTGGGT
bi-guaB for	TCTCTAATATTCCAATTACAA
bi-guaB rev	ATTGGGAAATTTTTATTTTTTTAT

bi-mvpB for	ATCTTACAACTAACACTTTAAA
bi-mvpB rev	GTAATTTTAATGGTTAATTGAAT
bi-mvpB fpouter	AACTCACAACATATTAATTTTAAAA
bi-mvpB rpinner	TAGTTAGAGGAAAAATATCTGAA
bi-mvpB rpouter	ATATGAATTATAGTAGAAGTTTG
bi-Telf	ATCATACTCAAAATATTCTTCA
bi-Telr	TTAGTTAGAATTGTTAATAAATT

3.19 Plasmids

3.19.1 Constructs of various sources

pet15b	Novagene, USA
pGEM T-easy	Promega, Mannheim
pDEX-RH-c-myc	(Faix et al. 1992)
pDd-GFP	(Hanakam et al. 1996)
pGBKT7-53	Clontech
pGADT7-RecT	Clontech
pACT2	Clontech
pGBKT7	Clontech

3.19.2 Constructs created in this department

pDex-dnmA-myc	(Kaller 2006)
pDd-dnmA-GFP	(Kaller 2002)
pGEM-T-easy-DnmA ko	(Kaller 2002)
pACT2-cDNA-library	Adam Kuspa (Baylor College of Medicine, Houston, Texas)
pGBKT7-dnmA	Dr. Christian Hammann (results chapter)

4 Methods

4.1 Isolation of nucleic acids

4.1.1 Isolation of plasmid DNA from *E. coli*

4.1.1.1 Plasmid mini-preparation (Birnboim and Doly 1979)

Bacterial culture inoculated in 1.5 ml LB medium was grown over night at 37°C and used to prepare plasmid DNA by the alkaline lysis method (Birnboim and Doly 1979). *E. coli* cells were collected by centrifugation at 3500 rpm for 5 min and resuspended in 100 µl Solution I by strong vortexing. After adding 200 µl Solution II, the cells were lysed at RT for 5 min, and then neutralized with 150 µl Solution III. After 10 min incubation on ice, the samples were centrifuged at 14000 rpm for 15 min and the supernatant was collected and precipitated with 0.8 ml pure ethanol. The pellet was washed with 70% ethanol, dried and dissolved in 20 µl water.

4.1.1.2 Plasmid maxi-preparation

For the preparation of bigger amounts plasmid DNA 50-100 ml *E. coli* cultures were used. The mini-preparation method was up-scaled respectively, except the last step where the DNA is dissolved in 100 µl water. All plasmids were prepared with the Nucleobond™ midi-columns Macherey&Nagel kit, used according to the manual of the manufacturer.

4.1.2 Isolation of nucleic acids from *Dictyostelium discoideum*

4.1.2.1 Genomic DNA preparation from *Dictyostelium discoideum* - fast mini preparation (Barth et al. 1998)

Dictyostelium cells, grown on Costar 24-well plates (5 x 10^6cells), were collected by centrifugation at 4000 rpm for 5 min The cells were resuspended in 300 µl TES buffer (10 mM Tris/HCl, 1 mM EDTA, 0.7% SDS) and 30 µg Proteinase K (in TE buffer) was added, followed by incubation at 45°C for one hour. The genomic DNA was extracted with phenol/chloroform and precipitated with ethanol. The genomic DNA, prepared by this method, was used for PCR experiments.

4.1.2.2 Genomic DNA preparation from *Dictyostelium discoideum* - maxi preparation

1-2 x 10^8 cells were collected from axenic culture with high cell density (approximately 5 x 10^6 cells/ml) by centrifugation at 1700 rpm for 10 min at 4°C, washed once with ice-cold

phosphate buffer and resuspended in 50 ml nuclear lysis buffer. The cells were lysed by addition of NP40 to a final concentration of 1%. The nuclear fraction was obtained by centrifugation at 4000 rpm for 15 min. The nuclear pellet was carefully resuspended in 5 ml SDS lysis buffer and incubated with 100 µl Proteinase K solution (25 mg/ml in H_2O) at 60°C for 2 hours. The genomic DNA was extracted twice with phenol/chloroform (1:1 vol/vol) and precipitated by adding 1/10 vol. 3M sodium acetate and 2 vol. pure ethanol. The DNA precipitate was washed with 70% ethanol, dried and carefully resuspended in 100 – 150 µl water.

4.1.2.3 Genomic DNA preparation from *Dictyostelium discoideum* in development

Approximately 5 x 10^7 *Dictyostelium* cells were pelleted at 1700 rpm, for 10 min, washed once with phosphate buffer, resuspended and placed on a black nitrocellulose filter (d=5 cm) At the different time points in development, cells were harvested by scratching the filters, disaggregated by vortexing in 10 ml phosphate buffer, and centrifuged for 10 min at 2000 rpm. After discarding the supernatant, cell pellet was placed in a safety Eppendorf tube with two graphite pearls. Eppendorf tubes were stored in liquid nitrogen and placed in carbon-ball mill machine (Retsh MM300) with pre-cooled holder for destroying the spores by intensive shaking. The spore masses were re-suspended in 500 µl of SDS lysis buffer and incubated with15 µl Proteinase K solution (25 mg/ml in H_2O) at 60°C for 1 hour. The genomic DNA was recovered by phenol/chloroform (1:1 vol/vol) and precipitated by adding 1/10 vol. 3M sodium acetate and 2 vol. pure ethanol. The DNA precipitate was washed with 70% ethanol, dried and carefully resuspended in 100 – 150 µl

4.1.2.4 Isolation of total RNA from *Dictyostelium discoideum* (Maniak et al. 1989)

1–3 x 10^7 cells were pelleted at 1700 rpm for 10 min at 4°C and lysed in 500 µl solution D. After adding 50 µl of 3 M sodium acetate (Aravin et al. 2004) and 500 µl phenol/chloroform, the sample was vortexed and incubated on ice for 20 min, followed by centrifugation for 15 min at 14000 rpm. The upper phase was collected and precipitated by adding 1 vol. isopropanol. The RNA was then pelleted, washed with 70% ethanol, dried in the speed-vac and dissolved in 100 µl DEPC water or formamide.

4.2 Molecular biological methods

4.2.1 Polymerase chain reaction (PCR)

The polymerase chain reaction (Mullis et al. 1986) was used to amplify DNA *in vitro*. The following general protocol was used for amplification on plasmid and genomic DNA templates:

100 ng	template DNA
20 pmol	each primer
5 µl	dNTP mix (2mM each)
5 µl	10xPCR buffer (100 mM Tris/HCl pH 8;
	0.1% Triton X-100; 50 mM KCl, 10-25 mM $MgCl_2$)
1 µl	Taq polymerase
ad 50 µl H_2O	

The following typical cycling protocol was used, however modified with respect to the annealing temperature, the elongation time and/or the elongation temperature depending on the primer pair used and on the length of the PCR fragment.

1. step	95° 3’
2. step (30 cycles)	95° 30” denaturation
	50° 30” annealing of primers
	72° 30” polymerization (elongation)
3. step	72° 5’final elongation

4.2.2 Temperature gradient polymerase chain reaction

For difficult templates a temperature gradient was run to define the proper annealing temperature.

1. step	95° 3'	95° 3'
2. step(30 cycles)	95° 30"	95° 30"
	45° 30"	55° 30"
	R = 3.0°	R = 3.0°
	G 5.0°	G 5.0°
	72° 30"	72° 30"
3. step	72° 5"	72° 5"

4.2.3 RT-PCR (Reverse transcription – PCR)

The RT-PCR method was used to produce a cDNA from RNA as a template, using reverse transcriptase, an RNA-dependant DNA polymerase. As a primer for the first strand synthesis, an Oligo (dT) primer or a sequence specific primer was used.

In order to prevent DNA contaminations RNA was precipitated with 1 vol. 8 M LiCl and 2.5 vol. ethanol for 30 min at –20°C and then centrifuged at 14,000 rpm for 30 min, washed in 70% ethanol, dried and dissolved in DEPC water. Alternatively, the RNA sample was treated with DNaseI (RNase-free) for 30 min at 37°C.

Reaction mixture for DNase treatment

10 µl	RNA in DEPC water (~ 5 µg)
2 µl	10 x DNase I buffer
2 µl	DNase I (RNase free) (4 U)
1 µl	RNasin (10 U)

ad 20 µl H_2O

Reaction mixture for cDNA synthesis

2 µl	total RNA (~1-2 µg)
2 µl	Oligo(dT) primer or sequence specific primer (5 pmol/µl)

ad 11 µl DEPC H_2O

For denaturing of the RNA, the reaction mixture was incubated for 5 min at 70°C and then immediately placed on ice. Then 4 µl 5 x RT buffer (MBI Fermentas), 5 µl dNTPs (each 2 mM) and 1 µl RNasin (10 U) were added. The reaction was incubated for 5 min at 42°C, allowing the primer to anneal. After the addition of 1 µl (10 – 20 U) M-MuLV reverse transcriptase the mixture was incubated for 60 min at 42°C for the first strand synthesis. The reaction was stopped by heat inactivation at 70°C for 10 min. 2 µl were used for a standard PCR.

4.2.4 Gelelectrophoresis of nucleic acid samples

Generally, DNA fragments were separated on 0.8 – 1.8% agarose gels. The corresponding amount of agarose was dissolved in 1 x TBE buffer, melted in a microwave, then ethidium bromide was added to a final concentration 0.5 µg/ml and the gel was poured into a horizontal gel-forming chamber. The gels were run using 1 x TBE buffer and were documented using UV light (256 nm) and the E.A.S.Y. system.

Total RNA was separated on 1.8 – 2% denaturing GTC agarose gel. The agarose was melted in 50 ml 1 x TBE buffer using a microwave and after cooling to 60°C 1 ml of 1 M guanidium thiocyanate was added. The RNA was mixed with 1.5 - 2 volumes of denaturing RNA loading buffer (95% Formamide, 2 mM EDTA), heated for 5 min at 65°C and put immediately on ice. The gels were run in the cold room at 90 - 100 V.

4.2.5 Capillary electrophoresis

For DNA methylation analysis, genomic DNA was isolated from wild type and dnmA knock-out cells. DNA samples were hydrolysed, derivatized and analyzed by capillary electrophoresis as described (Feinberg and Vogelstein 1983).

4.2.6 Gel elution of DNA fragments

For elution of DNA fragment from agarose gels, the desired band was cut under UV light (366 nm). The DNA was purified using Nucleospin™ (Macherey & Nagel) purification kit according to the manual of the manufacturer.

Alternatively, the "home" method was used. A small piece of Whatman paper was cut and pressed against the walls of a 1.5 ml Eppendorf tube with the upper side of an yellow tip. The tip was cut shorter, a hole was made at the bottom of the tube and the small "column" was placed in another Eppendorf tube. The Whatman paper was wetted with 100 µl 1 x TBE buffer and centrifuged for 1 min at 14,000 rpm. After discarding the flow-through, the agarose band was cut into small pieces, placed around the yellow tip and centrifuged for 1 min at 14,000 rpm. The DNA containing flow-through was precipitated, washed with 70% ethanol and diluted in H_2O.

4.2.7 Isotachophoresis

One other method for elution of the nucleic acids from polyacrylamide and agarose gels with high recovery is the isotachophoresis (Hammann and Tabler 1999).

20 g of Sephadex G-50 (Pharmacia), pre-equlibrated with 40 mM Tris/HCl pH 8.3 (running buffer), was embedded in a poly-prep chromatography column (Bio-Rad) and washed with running buffer. After washing, a membrane closed and buffer filled valve (see below) was attached to the column. Then it was placed into a beaker that contained running buffer such that the dialysis membrane-closed outlet of the valve is hanging into the buffer. A platinum electrode is placed into the beaker. The gel piece is placed horizontally on the top of the column and covered with 100 mM ε – amino-caproic acid, an amphoteric ion that has in aqueous solution a slightly negative net charge and migrates in the electric field slowly (terminating electrolyte), while the , in Cl^- ions serve as the leading electrolyte. The upper part of the column was then closed with a lid containing a second electrode. Upon applied voltage (20 V/cm), the nucleic acid becomes eluted from the gel piece and due to the different migration paces of the leading and the terminating electrolyte, the nucleic acid, which has an intermediate velocity, is concentrated at the boundary between the two ionic environments. The position of the migrating boundary can be visualised during the electrophoresis by supplementing the terminating electrolyte at the start with traces of phenol red, which co-migrates with the nucleic acid. When the boundary has reached the lower part of the column which is typically in about 2 h, the electrical field is switched off, and the nucleic acid is collected in a small collecting tube. If desired, the nucleic

acid can be purified further by a phenol/chloroform extraction, and concentrated by subsequent NaOAc/ethanol precipitation.

Preparation of the LUER three-way-valve. A 2 cm piece of a conventional dialysis membrane (2 cm flat width) was cut. The tube was opened, delivering a piece of membrane of about 2 cm x 4cm. The membrane was washed with the same buffer as the column, and placed around the lower part of the three-way-valve, and attached to it by tight silicon tubing such that the outlet is sealed by the dialysis membrane.

4.2.8 Bisulfite method

Bisulfite treatment was done according to (Curradi et al. 2002).

Generally, 5 µg of genomic, RNase-treated DNA were denatured with 1/10 volume of 3 M NaOH for 20 min at RT and 3 min at 100°C in a total volume of 60 µl. 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 overlaid 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 done for 20 min at 37°C by addition of 11 µl 3 M NaOH. The converted DNA was purified with Geneclean® or glass milk and dissolved in 40 µl H_2O, 2 – 4 µl were used for PCR-reactions with control primers (complementary to non-converted DNA) and bisulfite primers (complementary to converted DNA). Experiments were continued when only the appropriate primers yielded products on the different DNA samples. In most of the cases temperature gradient PCR was used.

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 to those obtained from bisulfite treatment of total DNA. PCR fragments were purified by MicroSpin^{TM}S-400HR columns before sequencing in (MWG Biotech Company - Germany).

In the latest experiments Qiagen bisulfite kit was used, and the treatment was done according to the manual of the manufacturer.

4.2.9 Sequencing

Sequencing was performed according to Dideoxy termination method, also known as Sanger method (Sanger and Coulson 1975).

Thermo Sequenase sequencing kit (Amersham) was used for the purposes of DNA sequencing. The use of Thermo Sequenase DNA polymerase, a thermo-stable, exonuclease-free enzyme, improves the efficiency of incorporating ddNTPs, which results in a sequencing data with very uniform signal intensities and peak heights. In addition fluorescent dye-labelled primers derived using cyanine dyes with an absorption maximum at either 700 nm for Cy5 or 580 nm for Cy3 were used for amplification.

For performing the sequencing reactions the following protocol was used:

All PCR products were cloned in pGEM T-easy vector, and plasmid DNA preparation was done accordingly to the plasmid-maxi preparation as described. Typically, 100 ng DNA per kb template was used, and 2 pmol of the dye- labelled M13 forward and reverse primers. When sequencing was done on pACT2 vector (prey vector for yeast two hybrid system), special primers (see materials) were used but the same labeling system.

Plasmid DNA preparation was done according to the plasmid-preparation in *S. cerevisiae* part in this chapter, and subsequently transformed and multiplied in *E. coli* before sequencing.

DNA/Primer Premix:

x µl	DNA
x µl	Primer1 (2 pmol)
x µl	Primer2 (2 pmol)
ad 21 µl	H_2O

1.5 µl of the A, C, G, T reagents were placed into the wells of a microtiter plate or sequencing tubes. An aliquot 4.5 µl of the DNA/primer premix was added to each tube. The cycling reaction was performed according to the following protocol:

SEQ 55:

1. step	95°	2’
2.step (25 cycles)	95°	45”
	55°	30”
	70°	1’
3. step	hold at 12°C	

After cycling add 6 µl of the stop solution, denature at 70°C for 2 min and load 1 µl on the gel.

The LI-COR system used for sequencing offers a high flexibility in the choice of glass plates, gel types and running conditions. For long runs, 66 cm gels were used.

For assembling the gel both plates were rinsed twice with water and ethanol and dried with a white paper tissue. The spacers were placed between the glass plates and fixed in the sandwich with the rails. The gel solution was done as described. Degassing and filtering of the mixture was done to improve the resolution capacity of the gel. Degassing was performed by an exicator. Filtration was carried out while pouring the gel using a filter on top of the syringe. The gel sandwich was placed in a horizontal position, lift it at the top and the mixed gel-solution was applied through a syringe. The polymerisation was carried out for at least 1.5 h and prevented from drying. As a running buffer 1 x TBE was used.

Data Collection of BaseImagIR software was used with programmed configuration files for each gel type and glass plate size (for details, see chapter „electrophoresis“ of the LI-COR sequencing manual). Further more, sequencing data was evaluated using LI-COR e-Seq V2.0, and LI-COR Align V2.0 programs, as well as Molecular Biology Online Tools for blast and analysis.

4.2.10 Microarray analysis

The RNA from Ax2 and mutant cells was isolated with Qiagen RNA kit (RNeasy®Mini kit). Aliquots containing 20 µg RNA 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 labelled with Cy3 or Cy5 labelling kit (FairPlayR 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 positives and negative controls were pre-hybridized and subsequently hybridized with the dye coupled cDNA preparation (Farbrother et al. 2005). After washing, arrays were scanned by Scan-Array® 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.

4.2.11 Calculation of retrotransposon copy numbers

Total nuclear DNA was digested with SacI and approximately 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 (Kennedy et al. 2004). 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 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 eri-A or only the ethidium staining were used for quantitation.

4.2.12 Preparation of competent *E. coli* cells

In order to transform bacterial cells with nucleic acids it is necessary to render the bacteria partially permeable for nucleic acids, thus making them competent. This can be achieved by several methods, like electroporation or physico-chemical ($CaCl_2$- method) creation of holes in the bacterial cell wall. Here the $CaCl_2$-method (Dagert and Ehrlich 1979) was used.

5 ml of LB medium was inoculated with the appropriate strain and incubated overnight at 37°C. 50 – 100 ml fresh LB medium was inoculated with 1 ml overnight culture. Cells were incubated at 37°C by vigorous shaking until OD reached OD_{600nm}=0.4. Then, they were collected by centrifugation in a Falcon tube (4000 rpm, 10 min, 4°C). The cell pellet was suspended very

carefully in 50 ml (50 mM $CaCl_2$ sterile and cold), and incubated for 30 min on ice. Centrifugation was performed (4000 rpm, 10 min, 4°C), and cells were resuspended in 18 ml (50 mM $CaCl_2$ sterile and cold) plus 2.7 ml 100% Glycerol. Aliquots of 200 or 400 µl were prepared in precooled Eppendorf tubes, and stored at -80°C.

4.2.13 Transformation of competent *E. coli* cells

One aliquot (200 µl) of competent *E.coli* was thawed on ice and a ligation mixture (20 µl) or 1 µl plasmid DNA was added by gently stirring the cells while pipetting. After 40 min incubation on ice, a heat shock was done for 90 sec. at 42°C and the cells were put on ice for 10 min. Then 1 ml LB medium was added and the cells were incubated for 30 min at 37°C. After 5 min centrifugation at 3500 rpm, the cell pellet was resuspended in ~200 µl LB medium, plated on LB_{Amp} agar plate and incubated overnight at 37°C.

4.3 Preparation of radioactively labeled probes

4.3.1 Klenow random labelling

The method was used for labelling PCR fragments. The DNA template together with the OLB-mix and the Klenow buffer was denatured by heating at 95°C for 5 min followed by cooling step on ice for 5 min and the Klenow fragment^{exo-} and [α^{32}P] – dATP were added.

Reaction mixture

5 µg	template DNA
3 µl	OLB-mix
2µl	10 x Klenow buffer
3 µl	[α^{32}P] - dATP
1µl	Klenow fragment^{exo-}

ad 20 µl H_2O

The reaction was incubated at 37°C for 1 h. The free nucleotides were separated by centrifugation through a Sephadex G50 spinning column. The purified radioactive probe was then denatured by heating at 90°C for 5 min, and used for hybridization.

4.3.2 End labelling with T4 Polynucleotide Kinase (PNK)

End labelling of the 5' termini of DNA was done using T4 Polynucleotide Kinase and [γ ^{32}P] – ATP.

10 pmol	DNA/RNA/synthetic DNA oligo
2 µl	10 x reaction buffer A (MBI)
3 – 5 µl	[γ ^{32}P] - ATP
10 U	T4 PNK
ad 20 µl	H_2O

The reaction was incubated at 37°C for 30 min, extracted with an equal volume of phenol/chloroform, and annealed to non-labeled oligo by adding 10 pmol of it, heated up to 60°C, and cooled down to room temperature. Then the double stranded DNA is formed and processed over a Sephadex G-50 spin column and precipitated with ethanol. In case of using dsDNA/RNA, was performed directly after labelling.

4.3.3 *In vitro* transcription

In vitro transcription was used to generate radio-labeled or non-labeled RNA sequences that were used as probes for hybridization, antisense RNA, substrates for studies of RNA-protein interactions, etc. As templates different vectors were used (for example pGEM 7Z, pGEM T-Easy), which possess promoter sequences for T7 or SP6 Polymerases. The plasmid DNA was cut with an appropriate restriction enzyme (run-off transcription), separated on an agarose gel and gel eluted. Alternative, when PCR fragments were used as templates for *in vitro* transcription, T7 or SP6 promoter sequence were included in the primers.

Reaction mixture

1–5 µg	template DNA
5 µl	10 x transcription buffer (see below)
5 µl	rNTP mix (5 mM ATP, CTP, GTP, 1 mM UTP)
3-5 µl	[α^{32}P] - UTP
1 µl	RNase inhibitor RNasin
1-2 µl	T7 /SP6 Polymerase

ad 50 µl H_2O

4.4 Hybridization techniques

4.4.1 Southern blot analysis (Southern 1975)

This method enables the identification of a specific genomic DNA fragment, digested with restriction enzymes and hybridized to a radioactively labelled DNA probe. 10 µg of genomic DNA were digested with one or several restriction enzymes and the DNA fragments were separated on an agarose gel. The gel was photographed with a ruler for calculation of the length of the DNA fragments in comparison with a DNA marker after the hybridization. The DNA was denatured by soaking the gel in denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 30 min, followed by neutralization for 30 min in neutralization solution (1.5 M NaCl, 0.5 M Tris/Cl, pH 7.0). The capillary transfer of the DNA on a nylon membrane was conducted overnight using 20 x SSC in the buffer reservoir and paper towels placed on top of the membrane. The membrane was washed in 2 x SSC and water, dried and cross-linked using a UV light - crosslinker (256 nm, 0.125 J/cm^2). The membrane was placed in a hybridization tube and incubated in hybridization solution at 48°C (pre-hybridization) for at least one hour. Hybridization using radioactively labelled DNA probes was performed overnight at the same temperature. The membrane was then washed with 2 x SSC, 0.1% SDS for 30 min; subsequently washed two times with 0.2 x SSC, 0.1% SDS for 15 min (stringency washes). After that the membrane was exposed on an imaging plate for analysis in a Fuji X Bas 1500 bio-imaging analyzer.

4.4.2 Northern blot analysis

Total RNA (~10 µg) was separated by gel-electrophoresis in denaturing (GTC) agarose at 90 V in the cold room. The gel was photographed under UV light and the capillary transfer of RNA on a nylon membrane and the hybridization of the membrane were conducted in the same way as in the Southern blot. The hybridization was performed overnight at 50 - 58°C for an RNA radioactive probe and at 42 - 48°C for a radioactive DNA probe.

4.4.3 SDS polyacrylamide gelelectrophoresis (SDS-PAGE) of protein samples (Laemmli 1970)

Protein samples were separated with the help of discontinuous SDS polyacrylamide gelelectrophoresis (SDS-PAGE). Depending on the molecular mass of the proteins, different resolving gels were used (7 - 12%).

Protein samples were supplemented with 2 x Laemmli buffer, heat-denatured for 5 min at 95°C and then separated on SDS-polyacrylamide gels.

The gels were prepared using glass plates and gel-casting chambers. Electrophoresis was carried out in 1 x running buffer at 20 – 40 mA. The gels were electro-blotted or stained with Coomassie dye.

4.4.4 Western blotting (electroblotting, semidry-blotting)

Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes using a semi-dry blotting system. The transfer was performed at 2 mA/cm^2 membrane (max. 40 V) for 1 h in transfer buffer.

4.4.5 Colony blotting

Colony blotting can be used to examine the expression of *Dictyostelium* proteins in different developmental stages. *Dictyostelium* colonies, grown on a KA plate, were transferred on a nitrocellulose membrane. The membrane was placed on a cold metal plate, incubated at -80°C for 20 min and washed 3 times with 1 x NCP buffer to remove the bacterial cells. The proteins were denatured by boiling the membrane for 1 - 2 minutes in 5% SDS solution in a microwave.

4.4.6 DNA Slot Blot Analysis

This alkaline blotting method was done as follows:

The DNA (concentration varied from 0.1 – 1 µg) was denatured prior application to the nitrocellulose membrane, with NaOH and EDTA solution to final concentrations of 0.4 M NaOH, 10 mM EDTA. The samples were heated to 100°C for 10 min to insure complete denaturation. Then, the DNA was neutralised by addition of equal volume of cold 2 M ammonium acetate, pH 7.0, to the target DNA solution.

Before applying the DNA, the membrane was pre-wetted in 6 x SSC buffer. Next, the membrane was assembled in the Bio-Dot apparatus and after applying the vacuum all the screws were retightened so that the apparatus was held together during blotting. The wells were washed with 500 µl TE buffer, and the probes were applied in a volume of 100 µl.

After the sample was filtered, 500 µl 2 x SSC buffer was added. The apparatus was disassembled, the membrane removed and rinsed in 2 x SSC. The membrane was air dried and UV irradiated. The next steps were done according to the western blot procedure, using 5mC as a primary antibody for detection of methylated cytosines.

4.5 Immunodetection

The Western blots or colony blots were immersed in blocking buffer (1 x PBS or 1 x NCP + 5% milk), then incubated with the primary antibody at a proper dilution. The blots were washed 3 x 5 min each with 1 x PBS (or 1 x NCP) and incubated with an alkaline phosphatase (AP) conjugated secondary antibody directed against the primary antibody (e.g. goat anti-mouse, goat anti-rabbit IgG). Antigens were identified by chromogenic visualization in BCIP substrate solution (0.2 mg/ml in 0.1 M Na_2CO_3).

4.6 Protein quantification using Bradford assay

Protein concentration was determined using the Bradford assay (Bradford 1976). 5 to 10 µl protein samples were diluted in 800 µl water and 200 µl Bradford solution (Bio-Rad) was added. The absorption was measured photo-metrically at 595 nm. BSA was used as a standard (1 –

20 μg/ml) and the concentration of the protein sample was determined according to the standard calibration curve.

4.7 Overexpression and purification of His-tagged recombinant proteins

As an expression system, the plasmid pET15b and the bacterial strain *E. coli* BL21 (DE3) were chosen. The gene of interest was cloned after the strong bacteriophage T7 promoter, since the transcription from this promoter is highly selective after transformation of the vector into an expression host, containing a chromosomal copy of the T7 RNA Polymerase gene under the control of lacUV5 promoter. The expression was induced by addition of IPTG to the culture.

The expression vector was transformed in BL21 competent *E. coli* cells. One clone was inoculated in 5 – 10 ml LB-Amp medium overnight. On the next day, different amounts of shaking cultures were inoculated (50 – 100 ml) with the pre-culture. The cells were induced at an OD_{600}=0.4 – 0.5 with 1 mM IPTG usually for 2 hours. Shaking cultures were incubated at 30°C after induction. The cells were harvested by centrifugation at 4000 rpm for 15 min, resuspended in buffer A (1/10 from the initial volume of the culture), and sonicated3 x 20 s with 10 s breaks in between the sonifing rounds. The crude extract was centrifuged for 15 min at 10,000 rpm and the pellet and the supernatant were examined for the presence of the recombinant protein by Western blotting. The supernatant was processed further for purification of the recombinant protein on a Ni-NTA column (Ni Sepharose 6 Fast Flow, Amersham). Usually 1 ml Ni-Sepharose (for ~ 300 ml bacterial culture) was equilibrated with buffer A. The lysate was loaded into a column. The column was washed with 10 ml washing buffer B and the protein was eluted two times with 0.5 ml elution buffer C. The flow-through, washing and elution fractions were collected for analysis by SDS-PAGE. The protein was finally dialyzed overnight at 4°C against buffer D.

4.8 Electrophoretic Mobility Shift Assay (EMSA)

Proteins were incubated with the RNA/DNA substrates in gel-shift binding buffer for 30 min at RT in a total volume of 20 μl. Usually 0.2 pmol RNA/DNA substrate were used per reaction (~1000 c.p.m.). After addition of native loading dye, the reactions were immediately loaded onto a pre-run non-denaturing 5% polyacrylamide gel (5% PAA, 5% glycerol, and 1 x TBE), 1 x TBE as a running buffer. Electrophoresis was performed at 150 V for 2 hours. The gels were sealed in nylon sheets and the products were detected by autoradiography.

4.9 Cell biological methods

4.9.1 *Dictyostelium* axenic cell growth

Dictyostelium discoideum AX2 strain and the derived transformants were grown in Go or selection media. Shaking cultures were grown from 1 – 5 x 10^4 cells/ml, shaken at 150 rpm at 22°C and harvested at indicated cell densities.

4.9.2 Sub-cloning of *Dictyostelium* on SM plates

KA suspension was prepared by washing one KA plate with 5 ml phosphate buffer. To obtain single clones of *Dictyostelium*, around 50 - 200 cells were resuspended in 100 µl freshly prepared KA suspension and plated on SM plates. Plates were grown at 22°C for 3 days until colony plaques appeared on the bacterial lawn. Single clones were picked with tooth picks and grown on 24-well Costar plates.

4.9.3 Development of *Dictyostelium* on nitrocellulose filters

Approximately 5 x 10^7 *Dictyostelium* cells were pelleted at 1700 rpm, washed once with phosphate buffer, resuspended and placed on a black nitrocellulose filter (d=5 cm), which had been boiled beforehand for 5 min in water. The nitrocellulose filter had been placed over two layers of Whatman 3MM paper, wetted with phosphate buffer. The development was done at 22°C in a closed chamber to prevent the drying of the filters. After 16 hours the development was checked optically and the aggregates were harvested by scratching the filters with a scalpel. The cells were disaggregated by vortexing in 10 ml phosphate buffer and the cells were processed further for isolation of total RNA or DNA according to the protocols.

4.9.4 5–aza-cytidine treatment of *Dictyostelium*

The cytosine analog 5-azacytidine (5-AzaC) is a demethylating agent, when incorporated into DNA causes extensive demethylation of 5-methylcytosine. This is due to a covalent binding of DNA methyltransferase to 5-AzaC in DNA and subsequent reduction of the enzyme activity.(Santi et al. 1984)

Series of experiments were done with different amounts of the drug and different types of cell cultures, *in vivo* and *in vitro*. DnmA-cmyc overexpressing cells were cultivated as shaking cultures in duplicate for the treatment with 5-AzaC, and non-treated cells as a control. The concentration of 5-AzaC was in a range of 0.1 - 1 µg/ml cells. The cultures were measured every 6 – 8 hours (one duplication time for *Dictyostelium*) for any changes in growth rate. A comparison between treated and non-treated cultures was done, and in parallel aliquots for protein and DNA samples were collected for detection of the protein on western blot and respectively methylation signals on slot blot assays with 5mC antibody. In addition *in vitro* experiment using recombinant DnmA expressed in *E. coli* (BL21 strain) was carried out. Genomic DNA isolated from wild type *Dictyostelium* treated with the drug and non-treated as a control were immobilized on nitrocellulose membrane, and incubated with recombinant protein His-DnmA for 2 hours. After washing the protein out, the membranes were incubated with α-his antibody for detection of the bound protein to the target DNA.

4.9.5 Transformation of *Dictyostelium* using electroporation

2 x 10^7 cells, grown to a density of 1 x 10^6 were collected, washed once with ice-cold phosphate buffer, once with EP buffer and then resuspended in 800 µl EP buffer. 15 – 20 µg DNA was added and the cells were incubated on ice for 10 min. Electroporation was performed at 1 kV, 25 µF in a 0.4 cm electroporation cuvette (the time constant between 3 and 4 ms). The cells were plated on a Petri dish, mixed with 2 drops (8 µl each) of 0.1 M $CaCl_2$ and 0.1 M $MgCl_2$ and incubated at RT for 15 min. Then 10 ml DD20 medium were added for overnight incubation. On the next day, the medium was replaced by the appropriate selection medium. The cells were kept under selection until transformants were obtained.

4.9.6 Classical transformation of *Dictyostelium discoideum* (Nellen and Firtel 1985; Nellen and Saur 1988)

15 ml *Dictyostelium* cells, grown to a density of approx. 1 x 10^6 were poured in a petri dish. After 30 min the cells have settled down on the bottom and the medium was changed with 10 ml MES-HL5. Simultaneously, the DNA sample was prepared: 20 µg DNA were diluted in 600 µl 1 x HBS buffer and 38 µl 2 M $CaCl_2$ were added drop-wise to the solution under vigorous mixing. The DNA precipitated as micro-crystals during the following 25 min incubation. The medium from the petri dish was removed and the DNA solution was distributed drop-wise over the cells. After 20 min incubation, 10 ml MES-HL5 medium were added and the transformation

reaction was incubated for 3 h at 22°C. Then the medium was changed with 2 ml 18% glycerine in 1 x HBS. After 5 min, the glycerine solution was removed carefully and 10 ml MES-HL5 medium was added. On the next day, the medium was changed with a selection medium, containing the appropriate antibiotic. For selection of resistant clones, the medium was changed every 2 – 3 days.

4.10 Fluorescence Microscopy

Cells were fixed at -20°C in methanol for 20 min, washed three times with 1 x PBS and stained with DAPI (1 mg/ml, diluted 1:15000 in 1 x PBS). Cells expressing the c-myc-dnmA fusion were blocked with PBG buffer (1 x PBS containing 3% BSA and 0.045% cold water fish gelatin; Sigma) for 1 hour 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 x PBS, then the secondary TRITC coupled polyclonal antibody (Dianova, Hamburg) was added at a 1:1000 dilution in PBG for 1 hour.

Microscopic analysis was done 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 AdobePhotoshop.

4.11 *Saccharomyces cerevisiae* methods and techniques

4.11.1 Transformation of *Saccharomyces cerevisiae*

The yeast strain used for transformation was *Saccharomyces cerevisiae* Y190 by Clontech. For further growing and transforming, they were slowly defrosted, plated with sterile inoculation loop on YPD medium and incubated at 30°C until colonies have grown. Yeast transformation was done according to the fast protocol by (Gietz and Woods 1994). A sterile loop was used to transfer the yeast colony from an agar plate into 1ml of Millipore-water. Cells were pelleted for 5 sec at high speed in a table centrifuge. The supernatant was discarded and the pellet re-suspended in 1 ml 100 mM LiAc, and the suspension incubated for 5 min at 30°C. Cells were pelleted again for 5 sec at maximal speed in a table centrifuge and the supernatant discarded. The pellet was treated in a following way:

240µl PEG (50% w/v)

36 µl LiAc (1M)

10 µl ssDNA (10 mg/ml); Preparation see below

5 µl plasmid-DNA (100 ng-5 µg)

60 µl Millipore water

The mixture was then vortexed for 1 min and incubated for 20 min at 42°C. The mix was pelleted again for 10 sec at maximal speed, and the pellet was re-suspended in 150 µl Millipore water by careful pipetting. Then, cells were plated out using the appropriate selection medium.

4.11.2 Preparation of high-molecular ssCarrier-DNA

High-molecular DNA from salmon testes (ICN, Eschwege) was diluted with TE buffer (pH 8) to a final concentration of 10mg/ml and stirred overnight at 4°C. The next day, the DNA was sheared by ultra sound sonification (2 x 20 sec at about 75% power) and controlled on a 0.8% Agarose gel (1 x TBE). Subsequently, DNA was extracted first with Tris-buffered phenol, then with Phenol/Chloroform (1:1) and finally with plain chloroform. In the end the DNA was precipitated by addition of 1/10 Vol. 3M NaAc (pH 5.2) and 2.5 Vol Ethanol (abs.), and centrifuged for 30 min at 13,000 rpm. The pellet was suspended in TE buffer to a final concentration of 10 mg/ml. The suspension was aliquoted in 1.5 ml Eppendorf tubes and boiled for 2 - 3min in the microwave to melt the DNA into single strands. The resulting ssDNA was frozen immediately at -20°C.

4.11.3 Protein preparation from *Saccharomyces cerevisiae*

For each transformed yeast strain to be checked in a Western blot, 5 ml overnight culture was prepared in selective medium. For inoculation, single yeast colonies not older than 4 days were used. As a negative control untransformed yeast colonies were used. The overnight culture was vortexed for 1 min and a 50 ml aliquot was inoculated with the entire overnight culture. A shaking culture was incubated at 30°C until OD_{600}=0.4. The culture was quickly chilled on ice and immediately placed in a pre-chilled rotor and centrifuged at 1000 g for 5 min. The supernatant was discarded and the cell pellet re-suspended in 50 ml ice-cold water. The pellet was recovered by centrifugation at 1000 g for 5 min, immediately frozen and stored at -80°C.

The pellets were thawed quickly in the pre-warmed cracking buffer (100 µl per 7.5 OD_{600} units of cells), and incubated at 60°C for 2 min. The cell suspension was transferred into 1.5 ml eppendorf tube containing 80 µl of glass beads per 7.5 OD_{600} units of cells. The sample was heated at 70°C for 10 min. Vigorous vortex was performed for 1 min and centrifugation at 14,000rpm for 5 min. The supernatant was transferred in new eppendorf tube and placed on ice. Protein samples were heat-denatured for 5 min at 95°C and then separated on SDS-polyacrylamide gels.

4.11.4 Plasmid isolation from *Saccharomyces cerevisiae* (Hoffman and Winston 1987)

A sterile culture tube with 2 ml YPD-liquid medium was inoculated with a plasmid-carrying yeast colony and incubated overnight in a shaker at 30°C until the stationary phase was reached. 1.5 ml of the culture were transferred into a 1.5 ml Eppendorf tube and pelleted for 5 sec at max. speed at room temperature. The supernatant was discarded and the cells pelleted by vortexing. The sediment was re-suspended in 200 µl breaking buffer, mixed with about 200 µl Glass beads (diameter between 450 and 520 µ, acid washed) and 200 µl PCIA (phenol/chloroform/iso-amyl alcohol) and then vortexed for 2 min. The suspension was centrifuged for about 5 min at max. speed at room temperature. The plasmid containing aqueous supernatant was used for transformation of *E.coli*.

4.11.5 Genomic DNA preparation from *Saccharomyces cerevisiae*

5 ml yeast (Y190) culture was inoculated in YPD medium overnight. A culture of 20 ml was inoculated with the overnight culture to starting density OD_{600}=0.2. The cells were grown to the density of OD_{600}=0.45, collected and centrifuged at 4000 rpm for 10 min. The pellet was re-suspended in 600 µl of 1 M sorbitol, 100 mM EDTA, 0.1 M β-mercaptoethanol, and 200 units of Lyticase enzyme (SIGMA). Cell lysis was performed at 30°C for 30 min, and the culture was centrifuged at 10,000rpm for 10 min. The finalization of the genomic DNA preparation was performed according to the manual of the Qiagen kit for extraction of genomic DNA (DNeasy® Tissue kit).

5 Results

5.1 Identifying a *Dictyostelium* homolog of DNMT2

Using the sequence of the human DNMT2 (DNA methyltransferase type2) and the BLASTN algorithm (Altschul et al. 1997), only one gene encoding a putative DNMT2 was found to be present in the *Dictyostelium* genome. The gene was denominated dnmA (Kuhlmann* and Borisova* et al. 2005), and further sequence analysis showed that the DnmA protein has all characteristic DNA methyltransferase motifs (Dong et al. 2001). Some of these motifs I-III form the co-factor binding site; IV (containing the PC site), VI, XIII and X form the catalytic pocket and motifs; V and VII are involved in preservation of the common fold of MTases that generate 5mC. The predicted protein of 379 amino acids and 44.1 kD displays 41% identity with the human enzyme. The inferred protein sequence contained all ten diagnostic motifs in canonical order shown on Fig. 5.1

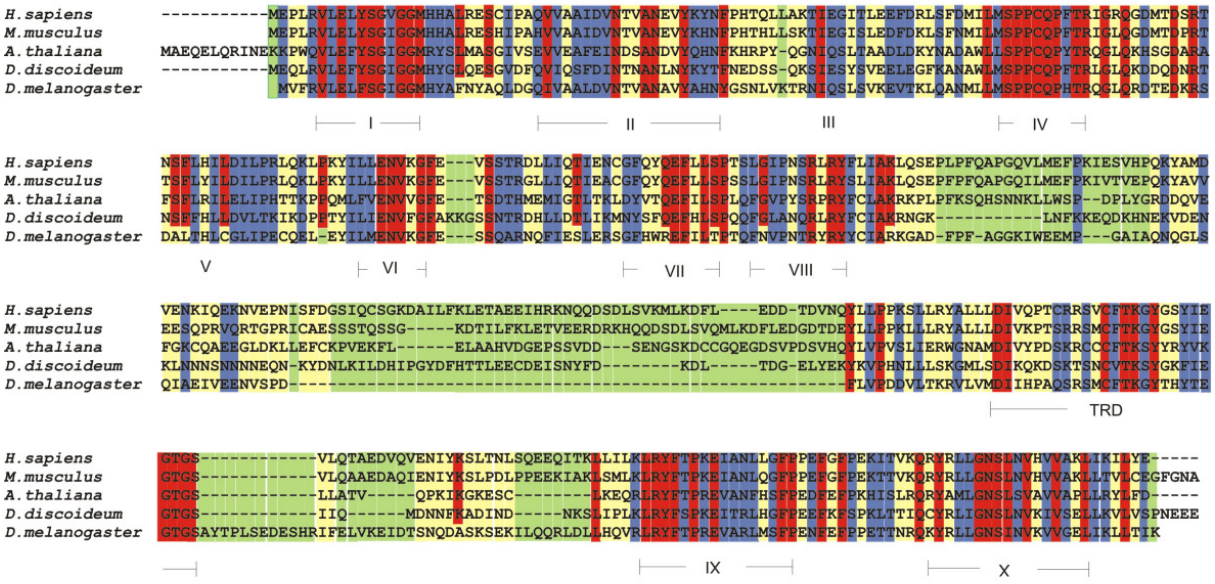

Fig. 5.1 Amino acid sequence alignment of Dnmt2 homologues: *H. sapiens*, *M. musculus*, *A. thaliana, D. discoideum,* and *D. melanogaster*. The ten characteristic Dnmt2 motifs are indicated by roman numbers, TRD: target recognition domain, which is a variable region and recognized as a separate domain. Identical amino acids are indicated in red, similar amino acids in blue, nonconserved regions in yellow, and insertions or delitions in green.

RT-PCR analysis showed that dnmA was expressed in axenically grown cells stronger than in development, where already after 6 hours expression was slightly decreased (Fig. 5.2). After 16 hours of development, expression was barely visible.

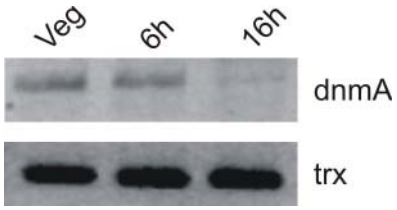

Fig. 5.2 Expression of dnmA. RT-PCR analysis performed on total mRNA of vegetatively (veg.) grown cells and after 6 and 16h in development. Expression of the thioredoxin (trx) gene family was used as control. The PCR was performed at 28 cycles.

5.2 Cellular localization of DnmA

To study the nuclear localization of DnmA in *Dictyostelium* two fusion proteins were created, a C-terminal myc fusion (Kaller 2006) and a C-terminal GFP fusion (Kaller 2002). Fluorescence microscopy revealed that both, DnmA-myc and DnmA-GFP fusions were predominantly found in the nucleus of fixed cells (Fig. 5.3). The distribution of the protein appeared not to be homogeneous in the nuclei, this is most pronounced with the α-myc antibody.

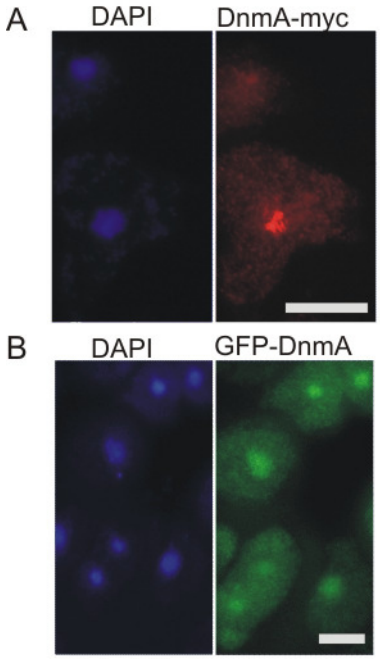

Fig. 5.3 (A) Expression of the DnmA-myc fusion protein in *Dictyostelium* visualized by staining with mAb 9E10 and a secondary TRITC coupled polyclonal antibody (right picture). (B) The DnmA-GFP fusion protein (right picture) showed the same localization. DNA was stained with DAPI to localize the nucleus (left). Bars are 8 μm.

The expression of DnmA-myc and DnmA-GFP could also be demonstrated by western blotting with α-myc and α-GFP antibodies (Fig. 5.4)

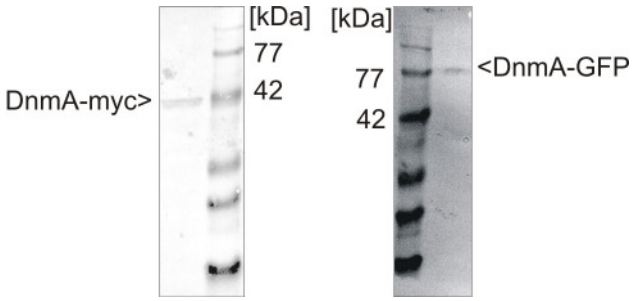

Fig. 5.4 Western blot analysis of cell extracts from DnmA-myc (left) and DnmA-GFP (right) expressing *Dictyostelium* cell lines. The expected sizes for DnmA-myc and DnmA-GFP fusions were 44 kDa and 70 kDa respectively.

To examine the localization of DnmA during mitosis, DnmA-GFP was analyzed in different mitotic stages in asynchronously grown *Dictyostelium* cells by fluorescence microscopy (Fig. 5.5). Mitoses were identified using an antibody directed against DdCP224, a *Dictyostelium* XMAP215 homologue, that stains the centrosome and the mitotic spindle (Graf et al. 2000; Graf et al. 2003).

Significant changes in the localization of DnmA-GFP were not observed in anaphase so no further experiments were done for other mitotic phases.

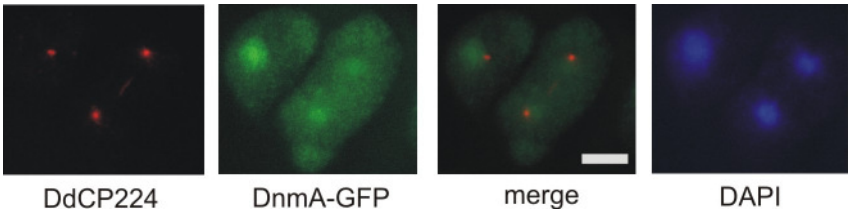

Fig. 5.5 Localization of DnmA-GFP during anaphase. The centrosomes and the mitotic spindles were stained with a α-DdCP224 antibody, and DNA with DAPI. The merged figure shows the distribution of DnmA-GFP in the anaphase. Bar: 8 µm.

5.3 Expression and purification of recombinant DnmA

For analysis of DnmA *in vitro*, the coding sequence of the gene was expressed in *E. coli* BL21 (DE3). For this purpose the cDNA of DnmA was cloned in pet 15b (Novagen) as an expression vector, bearing a 6xHis-tag near the multiple cloning site. The resulting construct denominated as pet 15b-DnmA is shown on Fig. 5.6 (Kaller 2002)

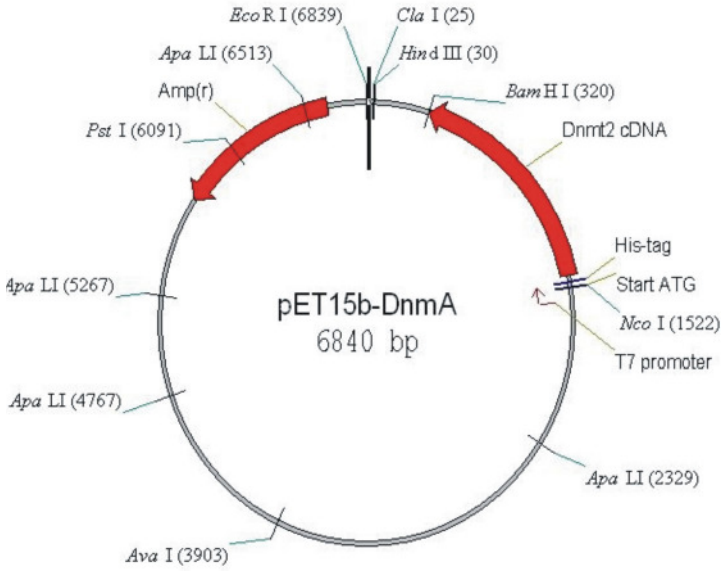

Fig. 5.6 Expression construct pet15b-DnmA. The His-tag is fused N-terminally to the coding sequence.

Extracts from *E. coli* expressing the His-DnmA fusion protein were prepared after induction with 1 mM IPTG for 2 hours together with a non-induced sample as negative control. Subsequently, samples were loaded on SDS-PAGE (Fig. 5.7). Overexpression resulted in a protein with the expected molecular mass of 44 kDa.

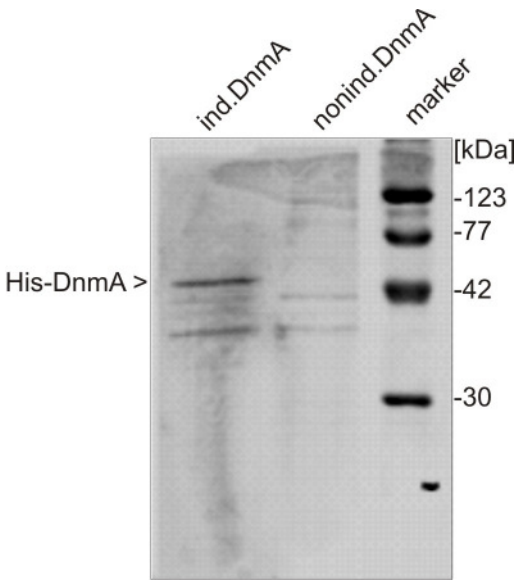

Fig. 5.7 Coomassie staining showing overexpression of His-DnmA in BL21 (DE3) strain. Prestained protein marker (Roti-Mark, Roth) was used to determine the size. The line with induced His-DnmA shows expression of the protein (marked band) after 2 hours of induction with 1mM IPTG.

Preparative amounts of culture (50–100ml) were induced and lysed by sonification. The fusion protein was purified with a Ni-sepharose resin under native conditions, and purification was monitored by separating aliquots of the fractions on SDS-PAGE and subsequent Coomassie staining (Fig. 5.8)

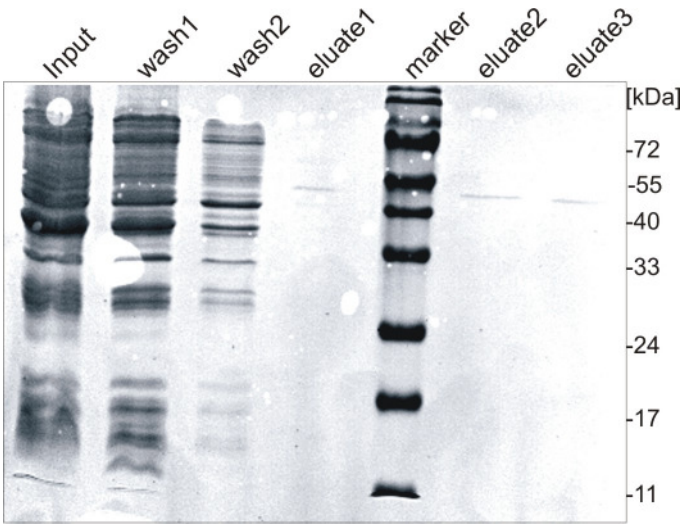

Fig. 5.8 Comassie staining to monitor purification of His-DnmA by affinity chromatography with Ni-sepharose. Wash 1 was done in the presence of 20 mM imidazole and wash 2 with 50 mM. Aliquots of 50µl out of 500µl from each elution step were mixed with 5µl 9 x Laemmli buffer and boiled. 20 µl were loaded on the gel. Eluate1 was done with 250 mM imidazole, eluate 2 with 300 mM, and eluate 3 with 300 mM plus 1 mM EDTA for stripping the column. Prestained marker (Fermentas) was used.

To ensure that the amount of purified protein was high enough to perform binding assays, obtained fractions (eluate 1, 2 and 3) were pooled and enriched for the protein using centricon® concentrator (10 kDa). The resulting protein preparation was 3 fold concentrated and used for all gel shift assays.

5.4 Electrophoretic Mobility Shift Assay

Electrophoretic mobility shift assay (EMSA) or also called gel retardation assay itself is one of the most powerful methods for analysis of nucleic acid - protein interactions (Revzin 1987). Radio-labelled DNA or RNA and protein are mixed together and the solution is subjected to native electrophoresis through polyacrylamide gel. The final result is visualized by autoradiography.

5.4.1 EMSA with recombinant DnmA

To identify potential targets for DNA methylation in the *Dictyostelium* genome, gel shift assay was performed. For this purpose, DNA oligonucleotides were designed that contained DNA methylation target sequences determined for the human DNMT1 and DNMT3 enzymes.

Table 5-1: Oligonucleotides used in the gel shift assays

Direction for the given oligonucleotides is 5'→3'	**Pairs**	**Methylation sensitive enzymes**
GAAATACCAGGATATAACCAGGTTAGAC GTCTAACCTGGTTATATCCTGGTATTTC	Pair 1 CCAGG	Bme 1309 I
GGAAATACAGATATAACAGTTAGAGCCC GGGCTCTAACTGTTATATCTGTATTTCC	Pair 2 CAG	none
GAAAATACCGGATATAACCGGATTAGAC GTCTAATCCGGTTATATCCGGTATTTTC	Pair 3 CCGG	Hpa II

The oligonucleotides were 28 nucleotides in length. Sequences were designed such that each duplex had the same CG content. The specific parts in every duplex formed later on by annealing (heating to 60°C and cooling down to room temperature) are potential symmetrical methylation sites. All oligonucleotides were ran on and recovered from agarose gel using isotachophoresis method (see material and methods). DNA was end-labelled with T4 Polynucleotide Kinase and [γ^{32}P] – ATP, and incubated under different conditions with equal amounts of the crude cell extract for 30 min at room temperature. Since a crude cell extract was used no protein concentration was determined. The difference in the conditions was determined by addition of the donor for the methyl group, S-adenosyl-L-methyonine (SAM) or ATP as a cofactor. The 5% native gels were ran, developed and results detected by autoradiography (Fig. 5.9). In the experiment with pair 2 DNA a slightly different DNA banding pattern was observed but this could not be reproduced in later experiments.

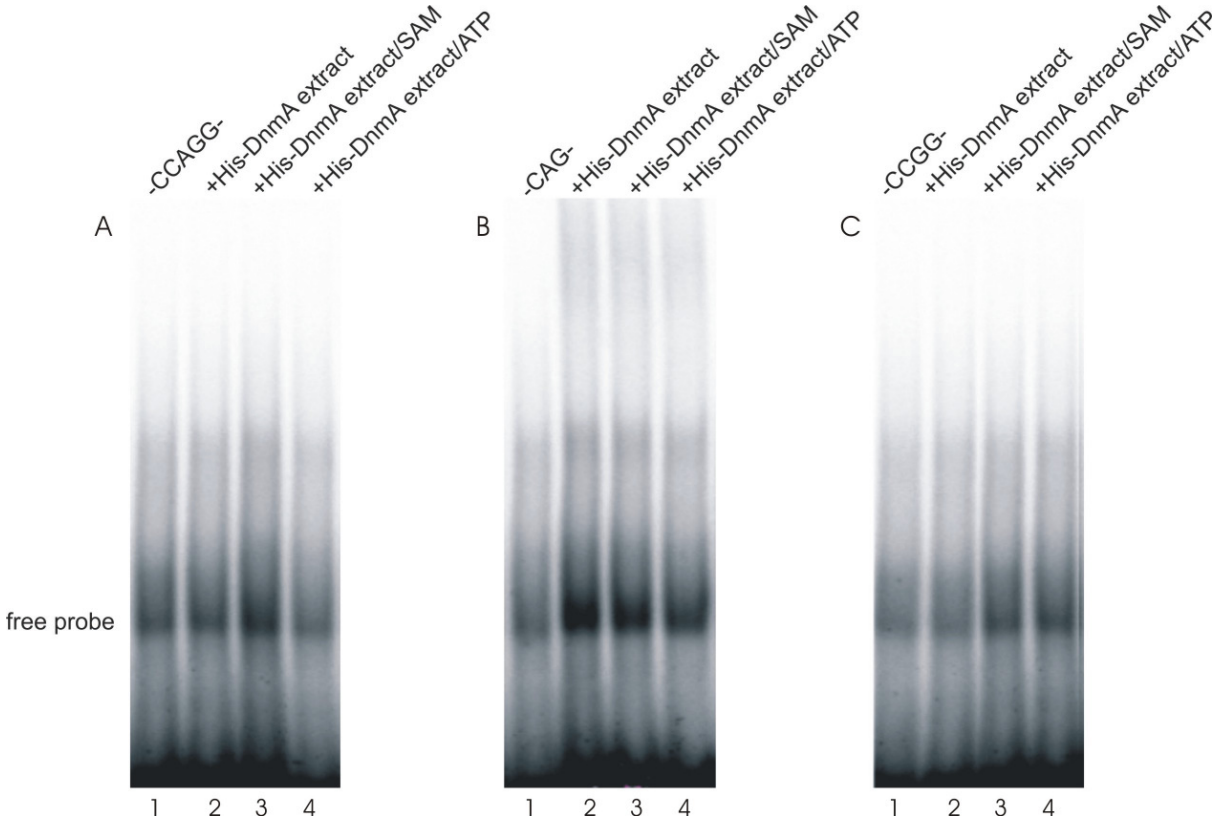

Fig. 5.9 EMSA with DnmA and different DNA substrates. (A) DNA duplex is CCAGG motif (pair 1), (B) DNA duplex is CAG motif (pair 2) (C) DNA duplex is CCGG motif (pair 3), and in all pictures lanes 2-4 contain the appropriate DNA duplex incubated with a His-DnmA extract. Samples in lanes 3 and 4 contain additionally 10 mM SAM or 10 mM ATP respectively.

Different conditions have been used and those described above were determined to work best in respect to running performance. In contrast to observations made for the human DNMT2 (Dong et al. 2001), binding was not observed with either of the DNA duplexes. Since this experiment was done with *E. coli* crude protein extract, there are different possible reasons to explain the failure of detecting a complex: the recombinant protein could be present in too small amounts, essential modifications could be missing, the folding of the protein could be incorrect or essential co-factors that are required for binding could be missing. To investigate the activity of the enzyme by a different means, a restriction digest was done with methylation sensitive enzymes. The duplexes contained sites, which were recognizable by restriction enzymes sensitive to methylation. Exception was pair 2 DNA, where the chosen sequence was not recognizable by any of the restriction enzymes.

The experiment was prompted by the idea that the DNA might be methylated, but that the DNA-enzyme complex was not stably formed. "Touch and go" scenario. However, using

methylation sensitive enzymes, no difference was observed in the pattern of the free probe incubated with the restriction enzyme, and the probe incubated with His-DnmA (crude extract), 10 mM ATP, 10 mM SAM for 30 minutes and subsequently digested in the same way (data not shown). After this experiment was concluded that the potential targets for the enzyme were not methylated either because of missing components in the system, small protein amount as meant before, or the chosen sequences were not of interest for DnmA.

5.4.2 EMSA with purified recombinant DnmA

To further elucidate the DnmA function *in vitro*, several trials were performed with purified protein. Not only DNA, but also RNA was used as a potential target. It has been shown previously that in plants de novo DNA methylation is mediated by small RNAs (Mette et al. 2000) This was based on the assumption that siRNA molecules could provide the specificity to DnmA, as to which regions of the genome to methylate. Commercially synthesized siRNAs derived from the discoidin coding sequence were designed (Popova et al. 2006) according to the Reynolds criteria (Reynolds et al. 2004) and used as substrates for mobility shift assays. siRNAs were end labelled as well as DNA oligos and used in the assay. Every sample contained 5 µl of the substrate DNA or RNA and was incubated for 30 min. at room temperature with the purified His-DnmA as explained in the legend of Fig. 5.10. The buffer conditions were as specified for DNA or RNA respectively as described in material and methods.

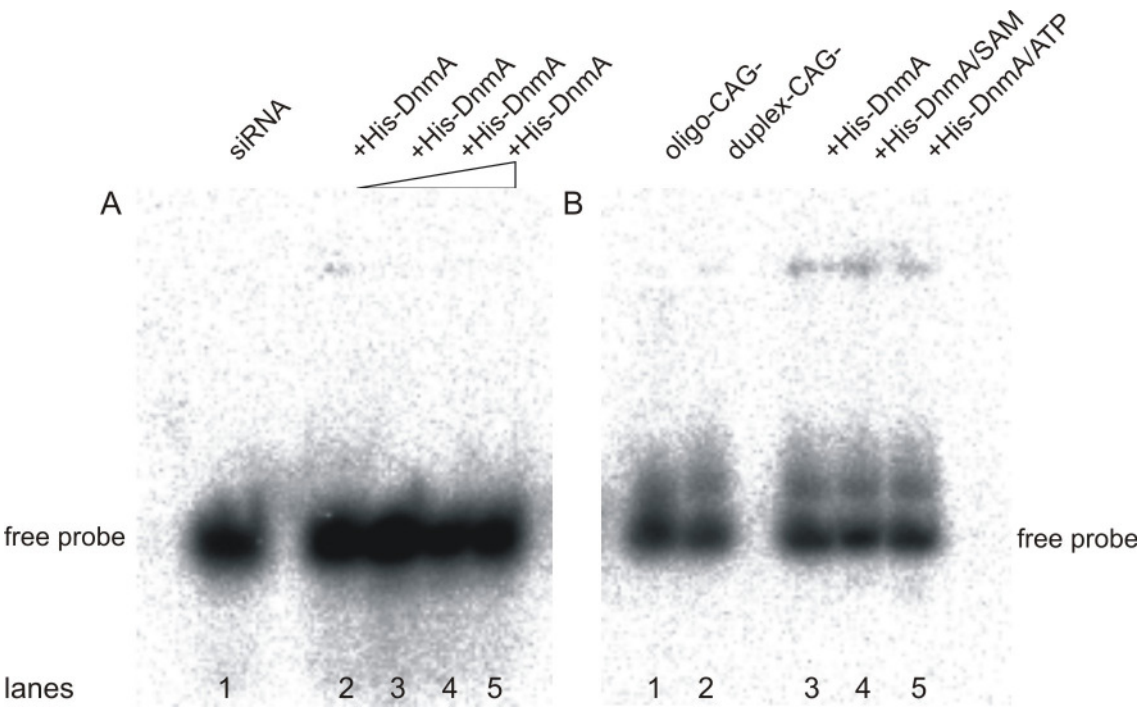

Fig. 5.10 (A) EMSA with purified His-DnmA and siRNAs as a substrate; (B) EMSA with purified His-DnmA and the CAG motif containing DNA duplex (pair 2). In picture (A) lane 1 is the free 21 mere siRNA. The bar on the top represents increasing protein amounts of 5 µl (lanes 2 and 3), 10 µl (lane 4) and 15 µl (lane 5). Since no shift was observed, precise protein amounts were not determined. In picture (B) lane 1 is free DNA oligo, lane 2 is the free DNA duplex, lane 3 -5 the duplex incubated with 10 µl His-DnmA and in addition as follows in lane 4 plus 10 mM SAM, lane 5 plus 10 mM ATP.

As seen in Fig. 5.10 (A) there is no RNP complex formed by DnmA and siRNAs *in vitro,* which could be due either to loss of ability to interact with RNA, which would be in agreement with what had been shown for the mammalian DNMT2 (Jeffery and Nakielny 2004). In the case of the DNA-Protein complex formation, using purified protein did not change the negative result already shown in Fig. 5.9. No complex formation was observed, using the same conditions and co-factors as before. This could suggest for the requirement of more components in an active multi-protein complex to recruit DnmA to its target, or the requirement of post-translational modifications on DnmA.

5.5 Identifying potential protein partners of DnmA by using the Yeast Two-Hybrid System

The yeast Two-Hybrid System (Y2H) is a method, by which direct protein-protein interaction can be easily detected *in vivo*.

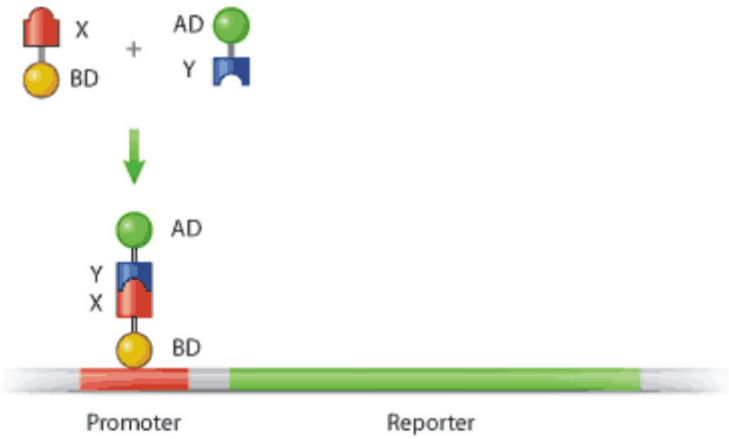

Fig. 5.11 The principle of yeast two-hybrid system. The two-hybrid system by Clontech uses the transcriptional factor GAL4 from bakers yeast. The GAL4 protein has in principle two functional domains, a DNA binding domain (BD) interacting with a specific upstream activator sequence of the DNA and positions the transcription factor in the vicinity of a transcriptional unit. The second domain of the GAL4-transcription factor, the activator domain (AD), is responsible for the contact with the basal transcription apparatus and activates transcription. If the two domains are brought into physical contact, activation of transcription is observed.

The fusion proteins were created in the following way: the protein of interest (X), in our case DnmA was constructed to have the DNA binding domain attached to its N-terminus, and its potential binding partner (Y) in our case cDNA library, which was fused to an activation domain. If protein of interest (X) interacted with protein (Y), the binding of these two would form an intact and functional transcriptional activator. This activator then would transcribe a reporter gene, whose protein product can be detected and measured.

To identify whether *Dictyostelium* (DnmA) formed a complex with other *Dictyostelium* proteins *in vivo*, the coding sequence of DnmA was cloned via non-directional cloning in the pGEM -T-easy vector (Promega). Subsequently, the fragment was cut out with Nde I, and cloned in the Nde I linearized and dephosphorylated pGBKT7 ("bait" vector). Colonies with the right insert were checked for the orientation using T7 and MT2 primers which resulted in a PCR product of the

right size (907 bp) (construct done by Dr. Ch. Hammann). As an additional feature the construct has a C-terminal myc tag for detection.

The pACT2 vector (Clontech) was used as "prey" vector with a cDNA-library from vegetatively grown *Dictyostelium* as an N-terminal fusion to the GAL4 DNA-activating domain. The cDNA-library was done as a phage library by the laboratory of Adam Kuspa (Baylor College of Medicine, Houston, Texas). The conversion of the phage library to a plasmid form was done by transformation in the special *Cre E. coli* strain BNN132 as described (Brodegger, Diploma thesis 1998).

The maps of the two vectors are schematically shown in Fig. 5.12

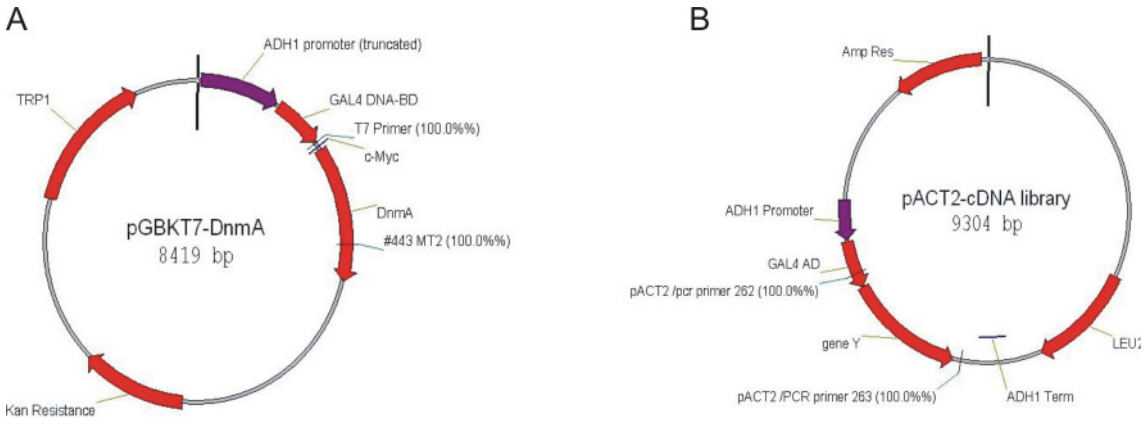

Fig. 5.12 (A) Vector pGBKT7-DnmA ("bait"), (B) Vector pACT2-cDNA Library ("prey")

To establish the Y2H system, two subsequent transformations were done. The yeast strain Y190 used in these experiments is deficient for the synthesis of several amino acids (trp1-901, leu2-3, his3-200), which allowed selection of transformants. The his3 reporter gene in yeast strain Y190 is under the control of the GAL1 UAS (upstream activating sequences) and a minimal promoter containing both his3 TATA boxes:TR, which is regulated, and TC, which is constitutive (Flick and Johnston 1990). Only in case of positive two-hybrid interactions, the expression of the his3 gene is highly increased and selection on His deficient medium is possible. Strain Y190 was transformed first with pGBKT7-DnmA vector, which bears the DnmA-fused GAL4 DNA binding domain and separately the coding sequence for TRP1. Transformants were selected on SC-agar plates without the amino acid tryptophane for 3-4 days at 30°C. Protein samples were loaded on SDS-PAGE (Fig. 5.13)

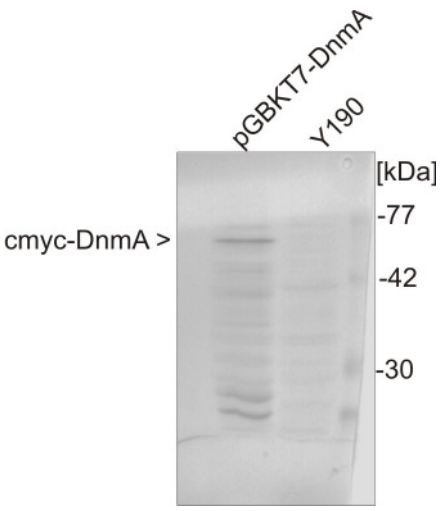

Fig. 5.13 Western blot using an α-myc antibody on total protein extract from a yeast colony expressing Gal4-BD-cmyc-DnmA with an expected size of 64kDa, and Y190 protein extract as a negative control.

A colony was chosen and used for the second transformation with pACT2-cDNA library, having the GAL4 activating domain. The double transformants were selected on SD-agar plate (trp^-, leu^-, his^-) again for 3-4 days at 30°C. In general the entire selection was done as follows (Table 5-2).

Table 5-2 Selection for transformants respectively grown on proper medium

Selection / Type of transformant	Trp^-	Leu^-	$Trp^-/Leu^-/His^-$
Single"bait" transormant	+	-	-
Single"prey" transformant	-	+	+
Double transformant	+	+	+

For further screening of colonies contained DnmA a β-galactosidase assays was performed.

5.5.1 β-galactosidase assay ("colony-lift" filter assay)

If binding occurred between the "bait" and the "prey" proteins, transcriptional activity would be restored for the normal GAL4 activity. The reporter gene for the Gal4 system was LacZ gene, an *E. coli* gene, which was inserted in the yeast DNA immediately after the Gal4 promoter, so that if binding occurred, LacZ is produced and blue colonies occurred upon adding the substrate X-Gal.

The grown and selected colonies were plated on new agar plates for additional growth and were transferred onto filters for the "colony-lift" assay. The selection was done according to blue-white screening, and colonies that turned blue up to 24 hours after the substrate (X-Gal) was added, were selected for further investigations.

Fig. 5.14 β-galactosidase assay on double transformants

Positive controls were used co-transformants containing pGBKT7-53, which encodes murine p53 protein, and pGADT7-RecT, which encodes SV40 large T antigen (Clontech).

For additional confirmation of true positives, cells were grown on his^- agar plates in combination with 3- Amino-1,2,4-triazol (3AT), a drug which can serve as an inhibitor for the yeast HIS3-gene product. This is necessary, because the strain Y190 has significant level of constitutive leaky expression due to the TC TATA box for his3. In case of using 3AT, this constitutive expression was reduced, so the chance for none specific interacting proteins to bring a colony alive on a his^- plate was reduced.

For selection, different 3AT concentrations 10 mM, 30 mM and 50 mM were used. The colonies confirmed as positive were plated on 30 mM 3AT plates for additional 3-4 days at 30°C, and only those, which were fast in growth, were finally chosen for further analyses.

5.5.2 Plasmid isolation and sequencing of yeast plasmids

To separate plasmids from yeast two-hybrid clones an intermediate transformation in bacteria was carried out. The procedure started with plasmid isolation from a two-hybrid clone, from which a proper amount was transformed in *E. coli* (DH5α). The advantages of bacteria were the short generation time and the possibility to select only for one plasmid. For the purpose of sequencing, a special primer pair was designed. The primers were covering the multiple cloning site of the "prey" vector and labelled with two fluorescent dyes (Cy3 and Cy5). Thermo Sequenase sequencing kit (Amersham) was used together with the primers for performing the sequencing PCR. To optimize the results, all selected "prey" vectors were isolated from bacteria with Nucleobond™ AX100 plasmid kit and RNaseA treated before the sequencing reactions (protocol see methods). In total 30 clones were sequenced but only 17 could be successfully analyzed. The rest were either false positives, or failed in the sequencing reactions. The group of 17 passed sequences were investigated using Molecular Biology Online Tools for BLASTN analysis. The sequences were blasted in NCBI BLASTN and *Dictyostelium* DataBase search and the hits were chosen according to the e-value of each blasted sequence. All hits with e-value higher than 1e-05 were excluded from further investigations, because of lack of biological significance. The results are shown in Table 5-3.

Table 5-3 Overview of putative protein partners of DnmA in yeast two hybrid system

Identity	number of hits	e-value	function/annotation
cotA (SP96)	2	0	development
skipper GAG	2	0	Nucleic acid binding
EfII/a	3	0	Elongation factor 1 alpha, one of two genes for the same protein; binds and bundles F actin
RabR	2	1e-150	GTPase
Actin family	1	1e-101	Major component of the cytockeleton
Lis1	2	3e-33	DdLIS1 is a microtubule-associated protein and permanent centrosomal resident in Dictyostelium
BC5V2_0_00911	1	1e-05	contains RRM Motif; putative Ser/Arg-related nuclear matrix protein [Oryza sativa]
NADH dehydrogenase	2	3e-26	ATP synthesis coupled electron transport
BEC6V2_0_00951	1	4e-33	Contains peptidase S28 domain
rps28	1	2e-44	Ribosomal protein 28 small subunit

The highlighted proteins have an annotated nuclear localization and these could be possible partners of DnmA to form a protein forming complex *in vivo*.

Studies in different organisms have shown that H3K9 methylation acts upstream from DNA methylation. In this respect heterochromatic proteins HP 1-like proteins in *Dictyostelium* could act in a concert with DnmA in a process called heterochromatin remodelling.

Trying to display this interaction between DnmA and HcpA (heterochromatic protein A) direct yeast two-hybrid screen was performed. The coding sequence of HcpA instead of the cDNA *Dictyostelium* library was cloned in the pACT2 vector, and yeast Y190 was subsequently transformed with pGBKT7-DnmA followed by pACT2-HcpA. Yeast colonies were grown for 3-4 days at 30°C on selective medium and the faster clones which appeared on 3-AT plates were subjected to a β-galactosidase assay. Unfortunately, no single colony turned to blue. Apparently no physical interaction is required for the function of both proteins in *Dictyostelium* or at least not detectable by using yeast two-hybrid system.

5.6 Expression and purification of His-HcpA, His-HcpB and His-HP1α

At that point another topic was added to this work by investigating HcpA and HcpB (HP1 homologues in *Dictyostelium*) *in vitro*. It has been shown that both proteins are highly conserved on the level of sequence and function in *Dictyostelium* (Kaller et al. 2006). Some additional features like DNA or RNA binding of these proteins were to be proven in *Dictyostelium*. More over their implication in the DNA methylation pathway was not studied yet.

For that purpose the genes were cloned (Kaller 2006) and expressed in *E. coli*. Both expression constructs pET15b-HcpA and pET15b-HcpB were transformed independently in *E. coli* BL21 (Fig. 5.15). Single clones were then first tested for protein expression. Recombinant proteins were found to be soluble and were purified with Ni-sepharose resin under native conditions. Fractions of the purified proteins were analyzed by Western blot (Fig. 5.16), eluates were dialysed and protein amount determined by Bradford method. In parallel, a control experiment was done with the murine HP1α under the same conditions.

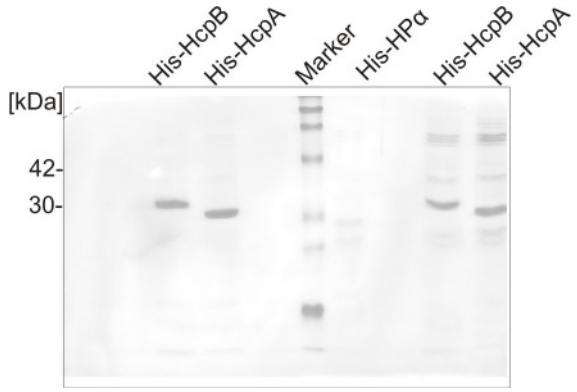

Fig. 5.15 Comassie staining showing purification of HcpA, HcpB and mouse HP1α proteins in BL21 (DE3) strain. On the right site are shown elution steps performed with 250 mM imidazole, and on the left the same but with 300 mM imidazole. Protein sizes: His-HcpA is 27 kDa, His-HcpB is 29 kDa and HP1α is 22 kDa.

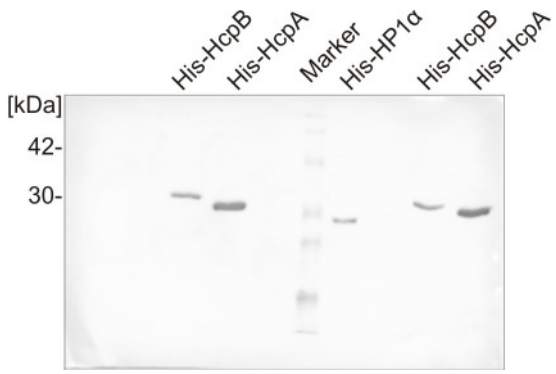

Fig. 5.16 Western blot with His-antibody of dialyzed recombinant HcpA, HcpB and murine HP1α.

5.7 EMSA with pure His-HcpA, His-HcpB and murine HP1α

It has been shown that HP1 homologues from different organisms can bind to DNA and RNA (Zhao et al. 2000; Muchardt et al. 2002; Meehan et al. 2003), suggesting that it is a conserved function of HP1 proteins. *Dictyostelium* HP1 homologues were subjected to gel retardation assay with both RNA and DNA, to investigate the relation between DnmA and HP1-like proteins in the process of heterochromatin formation, this time *via* indirect RNA/DNA binding. Since the yeast two-hybrid system did not reveal clear evidence for interaction between DnmA and any of the components involved in *Dictyostelium* heterochromatin formation, this question should be addressed by this assay.

5.7.1 RNP shift assay

The assay was performed with a radio-labelled *in vitro* transcript of 43 nt in length, using pGEM 7z vector linearized by EcoRI as a template. Incubation with purified His-HcpA, His-HcpB was done for 20 min on ice. Binding to the RNA was significant (Fig. 5.17). Here, two complexes with a different mobility were formed, which could be due to slight differences in the hinge and chromo shadow domains at the C-termini between the HcpA and HcpB proteins. These determine the differences in dimerisation behaviour with preferences in increasing order HcpA-HcpA<<HcpA-HcpB<<HcpB-HcpB (Kaller 2006). In this work, however experiments incubating both proteins together with the substrate RNA were not performed.

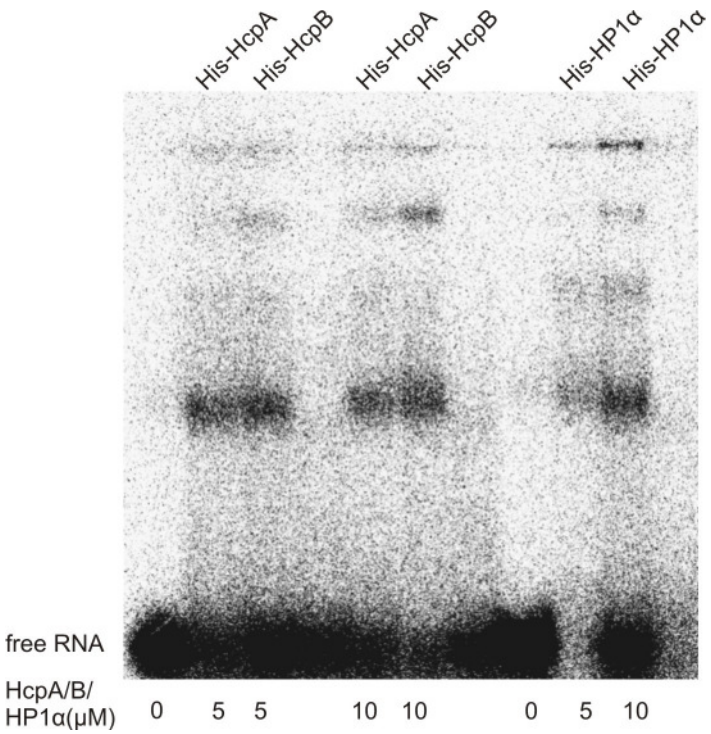

Fig. 5.17 EMSA with His-HcpA, His-HcpB and murine His-HP1α and RNA as target. RNA amounts were kept constant, and amounts of the proteins are indicated below the picture.

5.7.2 DNA-protein shift assay

It has been proposed that HP1 binds DNA under physiological conditions in a way, in which the methylated histone tails are engaged in interactions with the DNA and thus might be inaccessible (Meehan et al. 2003).

Gel shift assay showed that both *Dictyostelium* proteins can bind DNA directly, as the control HP1α does. The 93 bp PCR product was gel purified and end labelled by T4 Polynucletide Kinase. The incubation with the protein was done for 20 min at room temperature in 11 mM Tris/HCl pH 8.0 plus Mg^{2+}as buffer conditions. Protein concentration was determined by Bradford method. DNA binding was not strong, but significant. For the HcpA and HcpB proteins higher products were observed as in the case of RNA binding (Fig. 5.17), which can be explained by tendency of the proteins to dimerize and because multiple complexes bind to the probe (Fig. 5.18). In comparison to HP1, where it was shown that the amino acids composition in the hinge region is of importance, *Dictyostelium* HcpA / HcpB slightly differ in this domain from HP1. However, they still bear some of the very important, highly conserved residues, such as C-62, which is required for DNA binding.

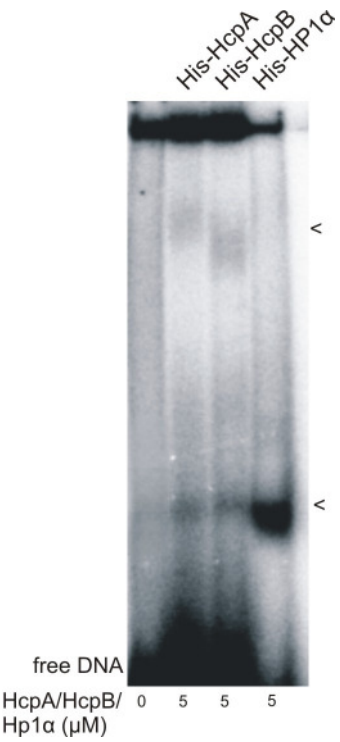

Fig. 5.18 EMSA with His-HcpA, His-HcpB and mouse His-HP1α and DNA as a template. The amounts of the used proteins are indicated below the picture.

This *in vitro* data proved the heterochromatic proteins HcpA / HcpB in *Dictyostelium* as binders of DNA and RNA. More detailed studies have been done in (Földesi 2005; Kaller 2006). However, the question whether DnmA-mediated DNA-methylation was dependent on histone modifications and the acting of the histone binding enzymes was left open. Also clarification is needed on what is first, methylation of H3K9 which is bound by HcpA / HcpB and then DNA (cytosine) methylation mediated by DnmA, or *vice versa*.

5.8 *In vivo* studies

Based on some evidence for the existence of two pathways regulating gene silencing in *Dictyostelium* - RNAi mediated gene silencing and DNA methylation, two experiments were designed with the idea to elucidate if there is an influence on the gene silencing mediated by RNAi and/or antisense pathways in a DnmA disruption strain in contrast to wild type.

5.8.1 Generation of knock-out mutant

Generation of the knock out cell line for dnmA was done by (Kaller 2002). Gene disruption and sub-cloning were done by homologous recombination (Witke et al. 1987) and the clones were

analyzed by PCR, and RT-PCR. For dnmA, primers dnmA 5' and BS^R G1 5' were used to verify site specific integration of the BS^R cassette. For screening of the disrupted wild type allele, primers MT1 and MT2 were used. In knock out clones these primers should not generate a PCR product under the same PCR conditions (Fig. 5.19).

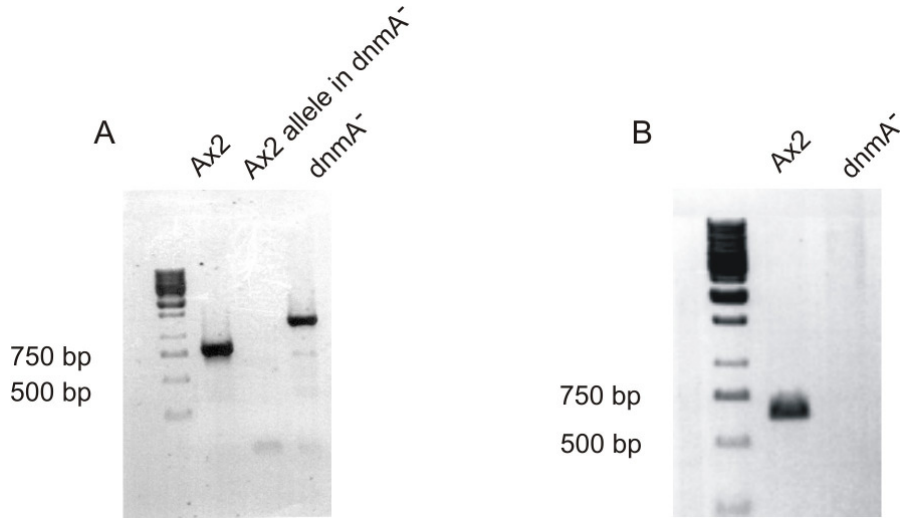

Fig. 5.19 (A) PCR using primers (left to the right) MT1, MT2 amplifying the wild type allele revealed 800 bp PCR product; MT1, MT2 in the disrupted strain no PCR product observed; and dnmA 5', BSR G1 5' primer pair revealed 1200 bp PCR product. (B) RT-PCR on dnmA gene transcripts from wild type Ax2 cells and the dnmA disruption mutant using MT1, MT2 primer pair. Resulting PCR product in the wild type has a size 600 bp, because of a 200 bp intron.

5.8.2 RNAi / antisense RNA mediated gene silencing and DNA methylation

A confirmed clone with correct integration of the BSR cassette, named DnmA knock-out, was transformed in parallel to the wild type strain with an RNAi hairpin construct directed against the endogenous discoidin gene to test putative connection between DNA methylation and RNAi. The inverted repeat sequences were transcribed from the actin 6 promoter and constructs were integrated in multi-copy tandem into the genome (Nellen and Firtel 1985). Since antisense RNA mediated gene silencing is believed to share components with the RNAi pathway, the influence of the DnmA knock-out was tested also for this mechanism. For that purpose both the mutant and the wild type were transformed with an antisense construct again directed against discoidin gene, but transcribed from the actin 15 promoter. In both experiments gene silencing was monitored by western blots and colony blots (Fig. 5.20).

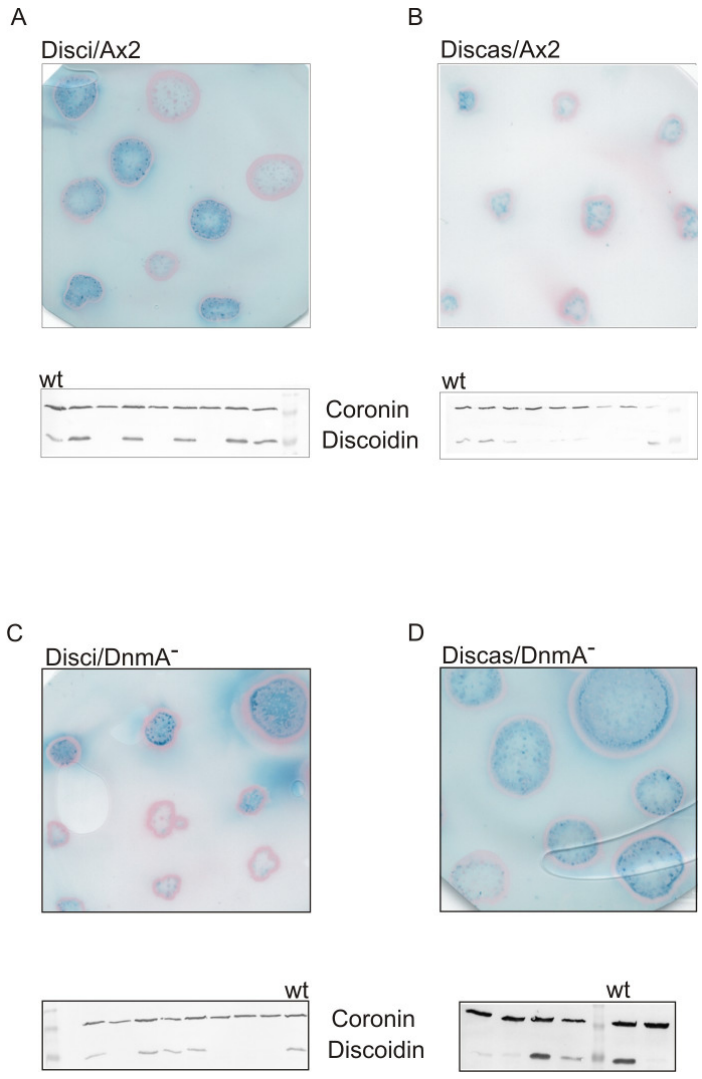

Fig. 5.20 (A) RNAi against discoidin in the wild type. (B) Antisense RNA against discoidin in the wild type. Colony blot (top) and corresponding western blot (bottom); (C) RNAi against discoidin in the DnmA ko mutant. (D) Antisense RNA mediated gene silencing in the DnmA ko strain. Colony blot (top) and corresponding western blot (bottom). For colony blots a monoclonal anti-discoidin antibody was used (blue staining) and Ponceau (pink) staining for visualizing the colony. Western blots were performed using the same anti-discoidin antibody as for the colony blots and a second monoclonal antibody against coronin as an internal control to monitor the protein loading. With wt are marked the lanes where protein samples from mock transformation were loaded, where discoidin and coronin are expressed.

In the wild type strain the discoidin gene can be silenced by both antisense RNA-mediated gene silencing and RNAi (Martens et al. 2002). However, the question arose whether the discoidin gene was also silenced on the transcriptional level by DnmA-mediated DNA methylation. This experiment showed that in the dnmA knock-out strain no significant difference in the number of silenced clones for discoidin gene was observed neither in RNAi clones (70 clones examined) nor

in antisense RNA clones (60 clones examined). About 50% of the discoidin RNAi (50 clones examined) as well as 50% of the discoidin antisense (50 clones examined) were silenced in colony blot and western blot in the wild type. This ratio stayed unchanged in the dnmA mutant, which suggested that there is no influence on silencing levels mediated by DnmA.

5.8.3 Immunodetection of 5-methylcytosine

To address the question at what level cytosines are methylated and how methylation is distributed in the *Dictyostelium* genome, an immunological detection of 5-methylcytosine was done. Similarly, this technique has been shown to be very useful for the analysis of DNA methylation during early vertebrate development (Dean et al. 2001). Genomic DNA preparations were done from wild type Ax2 cells, the dnmA ko strain and myc tagged overexpressor strain (Kaller 2006). All were RNaseA treated and then extensively denatured in 0.4 M NaOH and 10 mM EDTA, at 100°C for 10min. This procedure leaves only DNA intact and eliminates other potentially cross-reacting epitops. Subsequently, DNA preparations were spotted on the membrane and incubated with an antibody that specifically recognizes 5 – methylcytosine (Fig. 5.21).

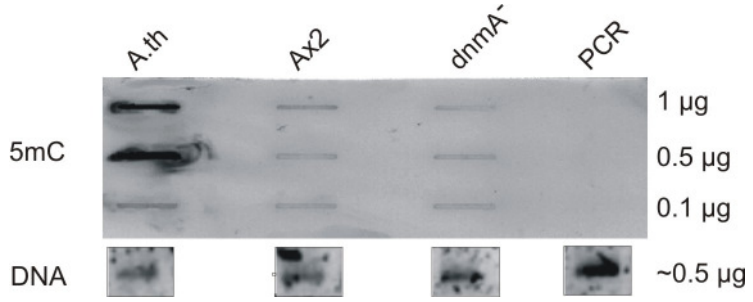

Fig. 5.21 Slot blot analysis representing the levels of methylation detected by the 5-methylcytosine antibody. *Arabidobsis thaliana* DNA was used as a positive control, as well as a PCR product as a negative control. The middle two lines are *Dictyostelium* wild type DNA and dnmA ko DNA both from vegetatively grown cells. Amounts loaded were adjusted according to the ethidium bromide stained DNA (lower panel).

The intensity of the signal was strongest for the plant DNA as expected with 6 to 30% methylation of the cytosine residues in the plant genome(Finnegan 2000). However, a slight difference was observed between the intensity of the signals for the wild type Ax2 and the dnmA knock-out strain. With levels of DNA methylation of about 0.2% for the *Dictyostelium* genome, as analyzed by capillary electrophoresis (Kuhlmann* and Borisova* et al. 2005), and the background level given by this immunological detection, the data support the assumption that C-methylation exists in *Dictyostelium*.

5.8.4 Drug treatment in *Dictyostelium*

5-azacytidine (5-azaC) is a demethylating agent, which is also known to induce mutagenesis in mammalian cells by forming adducts between DNA methyltransferase and genomic DNA, and by this blocking further DNA replication, transcription and DNA repair (Santi et al. 1984).

Dictyostelium wild type cells and DnmA myc overexpressor cell type were exposed to 5-azaC which was provided in the medium. Different concentrations of the drug were used, starting with 0.1 µg and up to 1 µg / ml cells with density of 2×10^5. The cells had a little delay in the first generation time (first 8 hours) compared to the non-treated, most probably due to the stress caused by the 5-azaC and blocked DNA replication. Cells were harvested after one generation time and protein samples for western blot from the overexpressor strain were isolated and in addition genomic DNA for slot blots analysis.

To detect if DnmA was recruited to the DNA after normal cytosine was substituted with 5-aza-cytidine, the myc-tagged DnmA was used in the experiment. The probes for western blots were collected before and after the 5-azaC treatment. Nuclear extracts were prepared together with cytoplasm samples, separated by SDS-PAGE and blotted on a nitrocellulose membrane. The detection was done with α-myc antibody, but no significant difference was observed between the cell protein extract before and after the treatment with 5-azaC Fig. 5.22. The DnmA protein was also detected in small amounts in the cytoplasm, but mainly in the nuclei as it has been seen for the DnmA-GFP fusion (Fig. 5.3). The cytoplasm traces of DnmA could be explained by insufficient separation of the nuclear from the cytoplasm fraction.

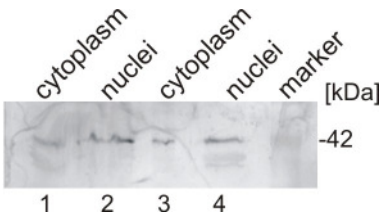

Fig. 5.22 Western blot on cytoplasm and nuclear cell extracts before the 5azaC treatment (1 and 2) and after (3 and 4). Detection was done with α-myc antibody.

To analyse whether adducts can be formed by covalent binding of the enzyme to the DNA, the *Dictyostelium* DNA before and after 5-azaC treatment was spotted onto a nitrocellulose membrane as done for the slot blot experiment. For this *in vitro* experiment, recombinant His-DnmA (crude cell extract) was used for an incubation of the membrane for 2h at room temperature after which

the protein was washed out, and the membrane was incubated with an α-his antibody for detection (Fig. 5.23). To check the specificity of the detection, the same DNA samples were not incubated with the enzyme, but subjected directly to antibody incubation or incubated with a protein preparation of the empty pET15b vector, which encodes for 6x-His.

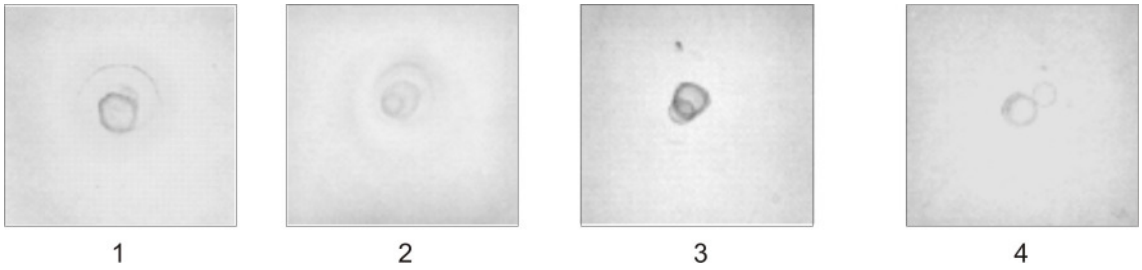

Fig. 5.23 Dot blots assays. On all dots, 1 µg of DNA was spotted and detection was carried out with an α-his antibody. Dot blot 1: non-5-azaC treated DNA, His-DnmA; Dot blot 2: same DNA as slot1, but no His-DnmA to adjust background levels; Dot blot 3: 5-azaC treted DNA, His-DnmA; Dot blot 4: same DNA as slot 3 but protein preparation of *E. coli* transformed with empty pet15b vector (negative control).

Dot blots 2 and 4 were used to assess the background detection level of the antibody. Dot blots 1 and 3 showed significantly higher signals. Comparison between dot blots 1 and 3 revealed that the signal in 3 had higher intensity than in dot blot 1. This could lead to the conclusion that 5-azaC was incorporated in the DNA *in vivo* and subsequent incubation with the recombinant protein *in vitro*, lead to its binding to the DNA, allowing for detection by the α-his antibody (dot blot 3). This result was in agreement with what has been published for mammalian cells by (Santi et al. 1984) and further supported the activity of the DNA methyltransferase *in vivo*.

5.9 Microarray analysis

To obtain insight in gene expression changes in the dnmA knock-out and DnmA-myc overexpression strains, compared to the wild type Ax2, DNA microarray experiments were performed. The method allows studying transcription levels of thousands of genes simultaneously, generating a "gene expression profile". The initial implementation of the DNA microarray technology was the comparative genomic hybridization array, which allowed high resolution analysis of the gene copy number (Solinas-Toldo et al. 1997; Pinkel D. et al. 1998). The principle of the Microarray screening is shown on Fig. 5.24.

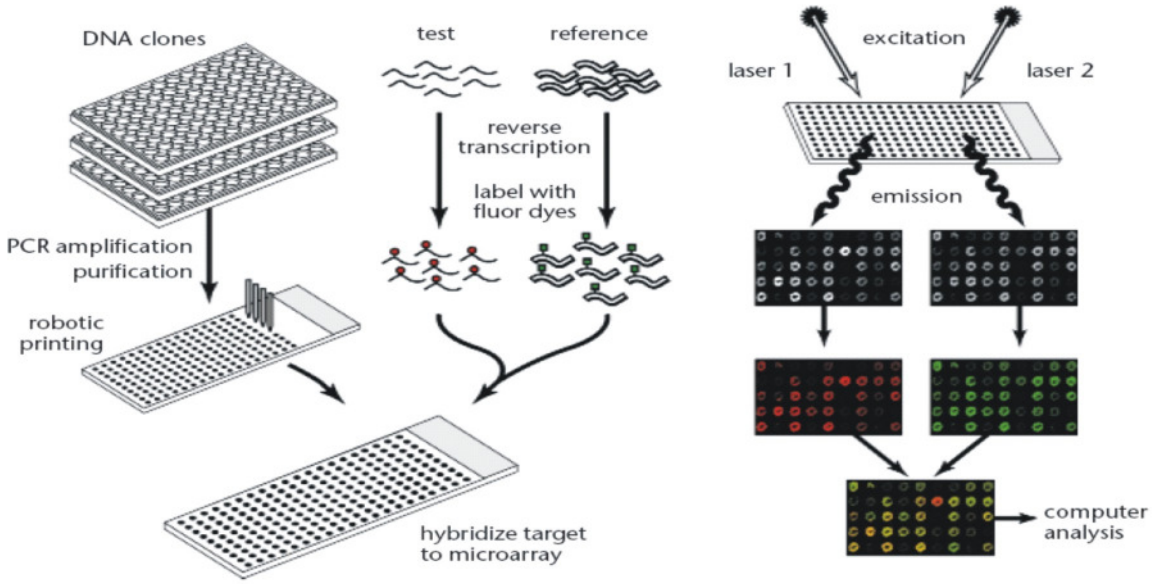

Fig. 5.24 The microarray screening principle. On the left site: the spotting procedure of the microarray. Different DNA clones are robotically spotted on the glass microscope slides together with control spots – positive and negative. The positive ones were probes for 12 genes with known expression levels in different growth stages. The negative controls were Bst EIII digested λ-phage DNA. In the middle: preparation of the probes for hybridization. Two RNA probes are used: test and reference. After reverse transcription was performed cDNA are labelled with a different fluorescent dye, and then the two samples are mixed and hybridized at the same time to the microarray. On the right: the principle of analysing the data. When the microarray is scanned, number of photons in the experimental dye's spectrum is compared to the number of photons in the reference dye's spectrum. After overlaying the two spectrums, every spot different than yellow was counted as change in gene expression.

The *Dictyostelium* microarray consisted of individual DNA sequences representing 50% of the *Dictyostelium* genome, printed in a high-density array on a glass microscope slide by a robotic arrayer (Table 5-4 and Fig. 5.24). For the microarray analysis, RNA from the wild type (Ax2) and two of the mutants, the disruption mutant for DnmA and the overexpressor DnmA-myc were used. Both of them failed to show phenotypes in axenic growth and development. They were growing and forming fruiting bodies during developmental program, within the same time frame as the wild type.

Table 5-4 Table with the probes and controls spotted on the *Dictyostelium* microarray. Abbreviations: PGS- partial gene sequences; EST-expressed sequence tags.

	Probes	Spots
PGS	450	900
EST	5423	10846
Controls	33	2874
Total	5906	14620

For every RT-PCR, 20 μg RNA was needed to be mixed with the appropriated standard controls. For generating cDNA, the RT-PCR reactions were carried out with oligo (dT) and in the presence of amino allyl modified dUTP. cDNA was coupled to red-fluorescent dye Cyanine 5 (Cy5), or green-fluorescent dye Cyanine 3 (Cy3) respectively. Every Cy3 dye-coupled cDNA derived from one of the mutants was mixed with Cy5 dye-coupled cDNA from the wild type and co-hybridized to the microarray, and *vice versa*. This so called dye-swap was needed to reduce the systematic differences in the red and the green intensities, which required correction in the normalization step. After this competitive hybridization, the slides were imaged using a scanner. The fluorescence measurements were done separately for each dye at each spot of the array. The ratio of the red and the green fluorescence intensities for each spot was indicative of the relative abundance of the corresponding cDNA probe in the two nucleic acid target samples.

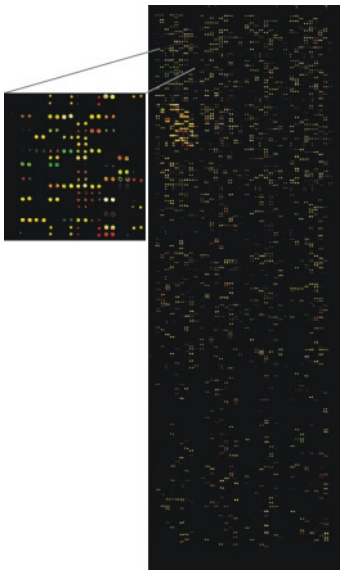

Fig. 5.25 Slide visualization with one of the 48 sub-regions zoomed. Every spot corresponds to one sample (EST, PGS or control). Slide number 15 from the DnmA-cmyc mciroarray.

To further analyze the obtained expression profiles the following set of procedures was done (Fig. 5.26)

Fig. 5.26 Bioinformatics steps used for analyzing the microarray data. The row data represented as spots with different colors had to be analyzed by quantification in a program called Scan Array Tools. Using this program every spot had to be fitted in a frame that later on can be recognized and the intensities of the spots can be correctly counted. The array tools program is one add-in for the Excel, where the data is filtered and transformed in format usable by the statistical program called R. In the R program all the data is normalized and then sent back to Array Tools. In the next step normalized data from Array Tools program representing different gene expression profiles is used by SAM program for identifying genes. The SAM (Significance analysis of Microarrays) program is also a program which is running in Excel environment.

This method identified in total 22 genes, which were up-regulated and 36 down-regulated in DnmA knock-out strain, and 125 genes up-regulated and 256 down-regulated in the DnmA-myc strain. To identify changes in gene expression that might be the key in this work was almost like to looking for a needle in a haystack. Using a factor of 1.5 fold increase or decrease in gene

expression was selected as a reasonable margin and the genes affected are summarized in Table 5-5, Table 5-6, Table 5-7, Table 5-8.

Priority was given to the up-regulated genes in the DnmA knock-out mutant. Among them, the ESTs: SLD246 and SLE355 contained sequences corresponding to the gag and RT genes respectively of the retrotransposon *skipper* (Leng et al. 1998). Initially, the analysis was concentrated on *skipper*, as this was of special interest, since transposable elements are frequently targets for methylation (Lippman et al. 2003). The possibility that methylation could control transposition was the reason that *skipper* was first chosen for further analysis.

Table 5-5 Genes with at least 1.5 fold increased expression level in the DnmA ko strain. Highlighted in grey are hits, which were chosen for further work.

Gene ID	Times fold increase	Gene name or Dicty Base accession number	Gene product or annotated function
X15387	**5,23**	*cinB*	Esterase/lipase/thioesterase domain-containing
VSA360	**4,50**	DDB0167572	Ubiquitin carboxyl-terminal hydrolase
SSC656	**4,07**	DDB0187546	Vegetative specific protein H5
SLD246	**2,23**	DDB0216526	LTR-RETROTRANSPOSON SKIPPER, GAG
SLD548	**2,14**	*abcG21*	ABC transporter G family protein
SLE355	**2,09**	DDB0218314	LTR-RETROTRANSPOSON SKIPPER, GAG.
SLD691	**1,89**	*abcG10*	ABC transporter G family protein
SSA241	**1,73**	*alrA*	Aldo-keto reductase
SLD251	**1,69**	*cfaD*	Counting Factor-Associated Protein
VSF740	**1,49**	DDB0191760	Telomerase protein-1.
SLE817	**1,48**	*mvpB*	Major vault protein B

Table 5-6 Genes with at least 1.5 fold decreased expression level in the DnmA ko strain.

Gene ID	Times fold decrease	Gene name or Dicty Base accession number	Gene product or annotated function
SSM424	**3,39**	DDB0218347	AgCP9056, Aminopeptidase
SSE346	**3,29**	DDB0167826	Nucleic acid binding
SSL845	**2,65**		Adenylyl cyclase
VSD851	**2,50**	DDB0190099	Hypotetical protein
VSA166	**2,18**	*lmcA*	Vegetative specific protein V4
SSJ758	**2,16**	DDB0167628	Hypotetical protein
X15380	**2,10**	*lmcB*	Lack of multicellularity
VSF378	**2,09**	DDB0216409	Hypotetical protein
VSI401	**1,96**	*abcG3*	ABC transporter G family protein
SSB389	**1,91**	DDB0218503	Hypotetical protein
X15381	**1,74**	*lmcA*	Vegetative specific protein V4.
SSL850	**1,70**	*DD7-1*	Discoidin I, C chain and B chain.
AF140780	**1,59**	*ctnA*	Component of the counting factor (CF) complex, countin

Table 5-7 Genes with at least 1.5 fold increased expression level in the DnmA-myc strain. The highlighted genes fall in one category (stress response) but could not be further investigated in this work.

Gene ID	Times fold increase	Gene name or Dicty Base accession number	Gene product or annotated function
SSE777	**6,98**	*hspG7*	heat shock protein Hsp70
VSE408	**4,58**	*hspG12*	heat shock protein
VSI332	**3,49**		hypotetical protein
SSJ114	**2,09**	DDB0167511	hypothetical protein
VSJ403	**1,82**	DDB0167417	hypothetical protein
VSG329	**1,77**	*hspG1*	Hsp20 domain-containing protein
SLD643	**1,75**	DDB0233607	DnaJ protein homolog
VSI350	**1,72**	*hspG4*	Hsp20 domain-containing protein
VSH742	**1,65**	DDB0203727	Similar to cell wall biosynthesis kinase; Cbk1p
VSG642	**1,62**	*hspM*	Hsp20 domain-containing protein
VSH848	**1,61**	*cafA*	calfumirin-1
SSB372	**1,60**	*culD*	culmination specific protein 45D
SSC591	**1,55**	DDB0184163	von Willebrand factor, type A
SSM347	**1,51**	*hspJ*	Heat shock protein Hsp20
SSL284	**1,50**	*rtoA*	RatioA, OsmC-like protein(stress response)
VSD302	**1,49**	*rnlA*	mitochondrial large subunit rRNA
VSB437	**1,47**	DDB0184362	Stress-induced protein sti1-like protein.
SLF309	**1,46**	DDB0187827	HEAT repeat
SLD769	**1,46**	DDB0231600	Endopeptidase Clp ATP-binding chain B, ClpB.

Table 5-8 Genes with at least 1.5 fold decreased expression level in the DnmA-myc strain. The highlighted genes were further investigated in this work.

Gene ID	Times fold decrease	Gene name or Dicty Base accession number	Gene product or annotated function
VSD851	**2,84**	DDB0190099	Hypotetical protein
VSJ869	**1,55**	DDB0204837	hydrolase activity
VSH619	**1,75**	DDB0168979	transporter activity
SSD175	**1,68**	*gtoC*	GTP cyclohydrolase I
VSF378	**1,96**	DDB0216409	negative regulation of transcription
SSL850	**1,51**	*DD7-1*	Discoidin I, C chain and B chain.
VSH428	**1,64**	DDB0229909	PUTATIVE O-METHYLTRANSFERASE
VSJ569	**1,75**	*gp130*	Lipid-anchored plasma membrane glycoprotein 130
SSH169	**1,63**	*abcG3*	ABC transporter G family protein
M64282	**1,55**	*guaA*	GMP synthetase
SSH379	**1,50**	*ancA*	ADP/ATP translocase
SSD228	**1,62**	DDB0206164	Hypothetical protein
VSB701	**1,55**	DDB0218607	Cell wall catabolism
SSM731	**1,54**	DDB0217332	Fatty acid desaturase
SSA241	**1,97**	*alrA*	Aldehyde reductase, aldo-keto reductase
AJ243946	**1,56**	*ctsD*	Cathepsin D

5.10 Northern blot analysis

The retrotransposon *skipper* has 20 copies in the *Dictyostelium* genome and consists of direct long terminal repeats and three open reading frames encoding GAG (matrix protein), PRO (protease) and RT (reverse transcriptase), POL (polymerase) and IN (invertase) (Leng et al. 1998). *Skipper* is barely expressed in cells grown in axenic suspension culture.

The retrotransposon *DIRS-1* occurs in 40 complete and approximately 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. Transcription of *DIRS-1* is developmentally regulated and expression increases substantially after the onset of starvation (Rosen et al. 1983; Zuker et al. 1984; Glockner et al. 2001).

To study the expression profiles of *skipper* and *DIRS-1*, RNA from the mutant and the wild type strain was isolated and 10 µg each were separated on a GTC gel. The same two cell lines were examined after multiple passages through the differentiation cycle, assuming that transposons could be mobilized during development. The RNA was transferred to a nylon membrane, UV-cross linked and subjected to hybridization with the probes. For that purpose the PCR fragments of *skipper GAG* and *DIRS-1* right LTR were radioactively labelled by the Klenow random priming.

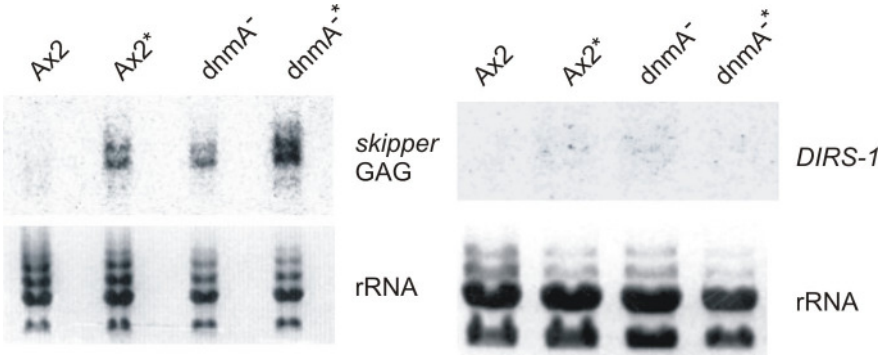

Fig. 5.27 Northern blot analysis with specific *skipper* (left) and *DIRS-1* (right) probes on RNA isolated from wild-type Ax2 and DnmA- cells after a short period of growth in axenic medium and after 16 passages of development cycle (indicated by asterisk).Ethidium bromide staining is shown for quantitative comparison.

Northern blot analysis revealed that *DIRS-1* expression during growth was unaffected in the mutant while *skipper* expression was significantly up-regulated and further increased after 16 rounds of development. *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.

Passages of development were done by growing cells on a lawn of *Klebsiella aerogenes* until they had developed mature fruiting bodies. Spores were then harvested and used to inoculate a plate for a new cycle. After 0 and 16 cycles, spores were transferred to axenic medium and cells were grown up to prepare RNA, DNA and to measure the generation time for each cell line (Fig. 5.28). After 16 cycles, mutant cells were severely compromised in growth on axenic medium in one experiment (generation time of approximately 25 hours) 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 hours over at least 20 passages in two independent experiments.

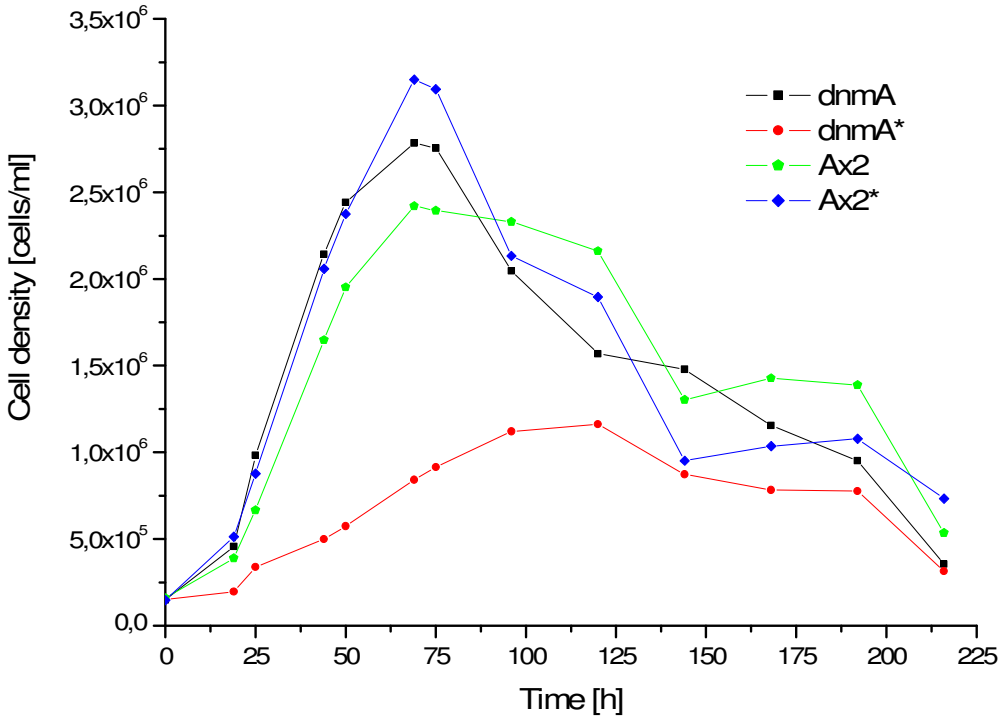

Fig. 5.28 Growth curves for the cells that had undergone development for 16 rounds are marked Ax2* for the wild type (blue) and dnmA$^-$* for the disruption mutant (red). Controls: Ax2 after 0 cycles in development (green) and dnmA$^-$ after 0 cycles (black).

In *Schizosaccharomyces pombe* siRNAs were involved in chromatin remodelling (Noma et al. 2004; Verdel et al. 2004) and in plants, de novo DNA methylation is mediated by small RNAs (Mette et al. 2000). Therefore it was examined, if *skipper* and DIRS-1 expression was impaired in mutants affecting the RNAi pathway.

As shown in Fig. 5.29, *skipper* expression was not only up-regulated in the dnmA knock-out, but also in strains where one of the three RdRP genes (Martens et al. 2002) was disrupted. A minor degree of overexpression was also observed in knock-outs of the gene dicer A (drnA) and the heterochromatin protein gene hcpA (Kaller et al. 2006). In contrast, *DIRS-1* expression was only affected in the rrpC knock-out mutant. The data showed that components of the RNAi pathway were involved in suppression of both retrotransposons but that *DIRS-1* and *skipper* are regulated by distinct mechanisms.

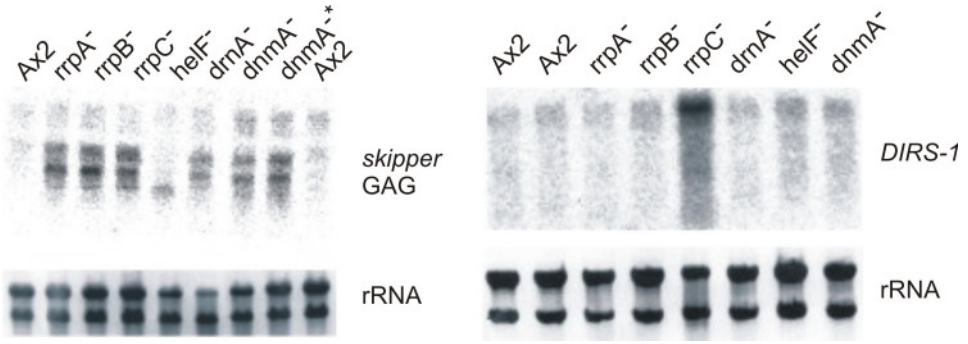

Fig. 5.29 Northern blot with a Skipper (left) and a DIRS-1 probe (right) on RNA isolated from axenically growing cells of different mutants. Ax2: wild type control, rrpA$^-$, rrpB$^-$, rrpC$^-$: knock outs of the RNA directed RNA polymerase genes rrpA, B and C respectively, helF$^-$: knock out of the 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.

5.11 siRNA coverage for *skipper* and *DIRS-1*

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 9.000 small RNAs represented in two small RNA libraries (Ambros pers. communication), approximately 600 were complementary to either the bottom or the top strand of *DIRS-1* (a more recent analysis that eliminated false sequences in the library, even show 50% of the recovered siRNAs to be complementary to *DIRS–1). DIRS-1* siRNAs were clearly over represented because the retrotransposon contributes approximately 3.2% to the total *Dictyostelium* genome (Glockner et al. 2001) but more than 7.2% of the siRNAs contain *DIRS-1* sequences. In contrast, about 1% of the genome is *skipper* sequences but no siRNAs corresponding to the complete element were found.

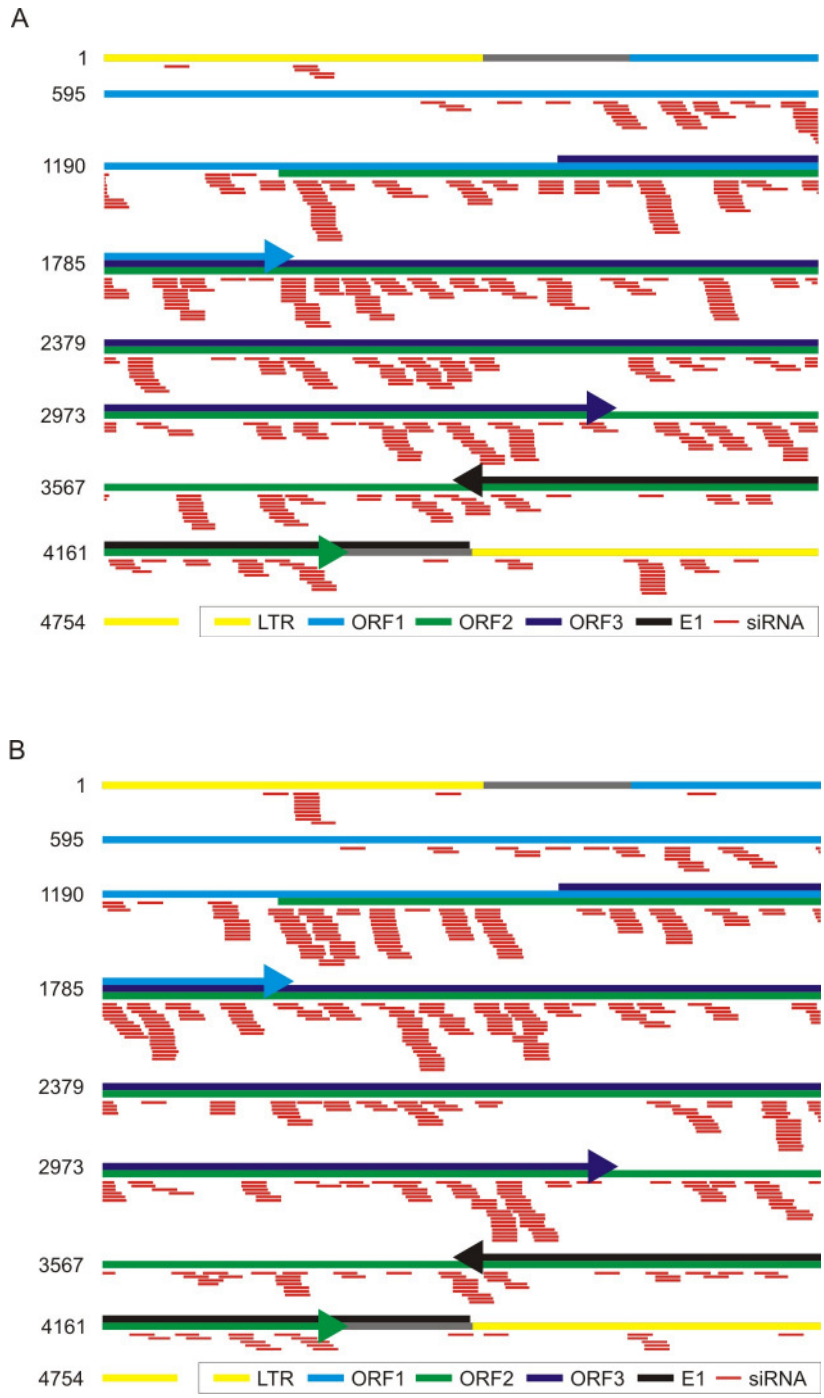

Fig. 5.30 RNAs derived from the small RNA library are aligned on the schematic depiction of DIRS-1: sense strand (A), antisense strand (B). The inverted repeats are marked in yellow. The ORFs I, II and III and the E1 antisense transcript are depicted as colored arrows. siRNAs are shown as red lines. Most of the small RNAs are in the range of 21nts, some longer molecules have been retrieved and are also shown.

5.12 Mobilization of *skipper* and *DIRS-1*

To examine if 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 done using specific *skipper* and *DIRS-1* fragments as probes (Fig. 5.31). 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. 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 transposons.

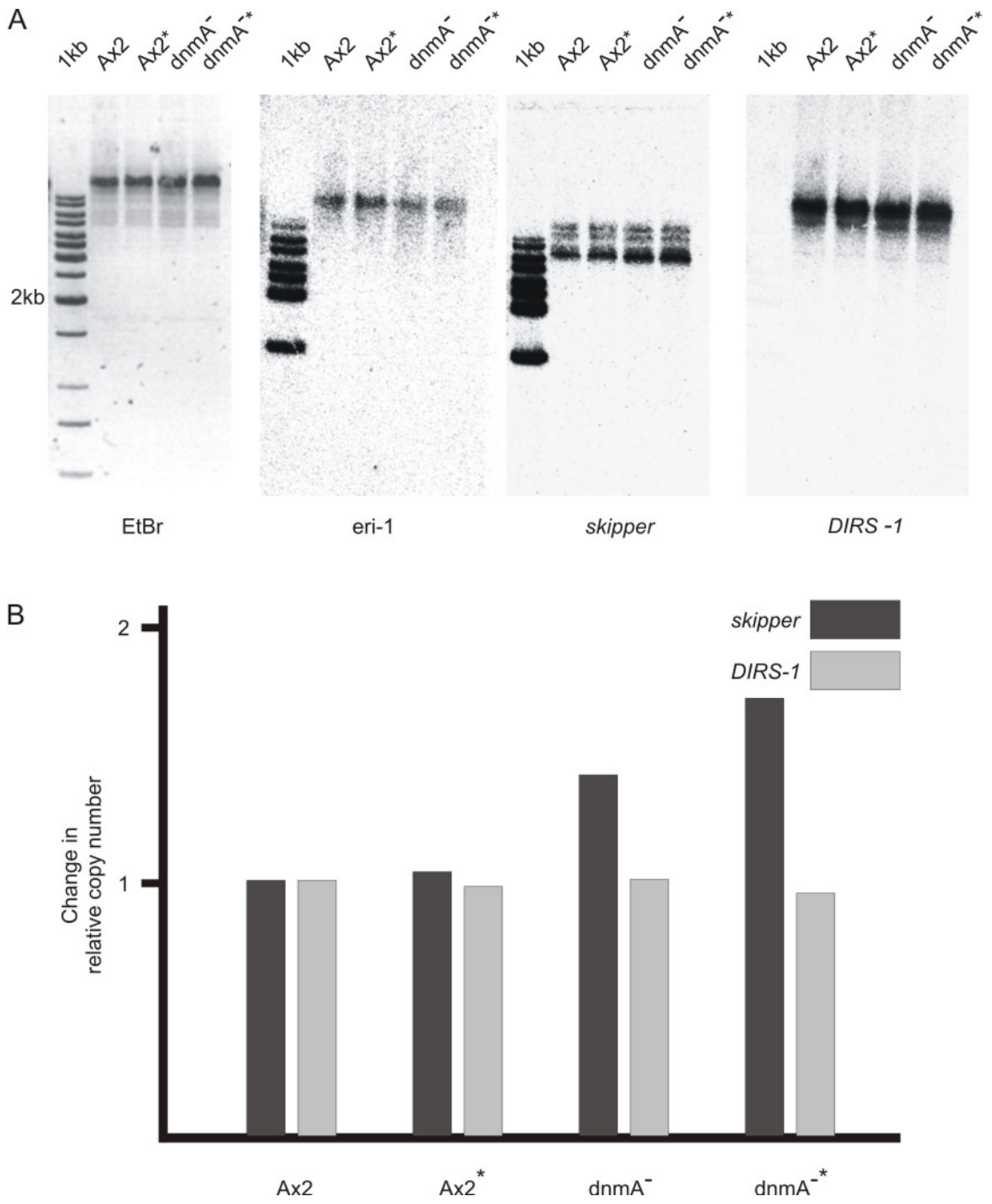

Fig. 5.31 (A) Southern blot with *skipper* and *DIRS-1* probes on wild type Ax2 and dnmA- mutant cells after 0 and 16 rounds (indicated by *) of the developmental cycle. From left: ethidium bromide staining, hybridization with an eriA probe, hybridization with a *skipper* probe, hybridization with a *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).

Since, *DIRS-1* expression was only affected in the rrpC- mutant it was thought that this could have an effect on retrotransposon mobility in this particular RNAi mutant. To test this, DNA

from individual rrpC$^-$ clones was digested with HpaII enzyme. Southern analysis was done using a specific *DIRS-1* probe. DNA from wild type cells was used as control.

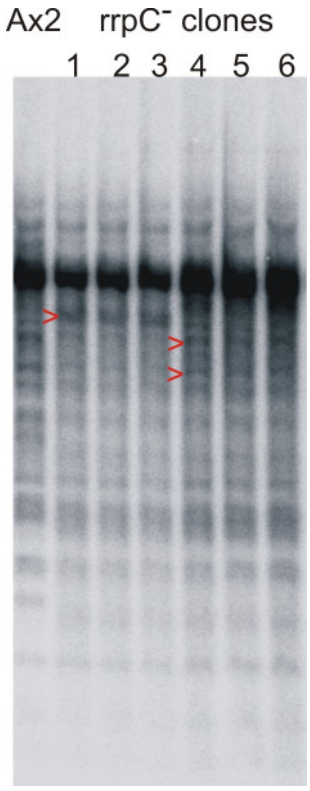

Fig. 5.32 Southern blot with *DIRS-1* probe on wild type Ax2 and rrpC$^-$ mutant-clonal isolates. The differences are pointed by red arrows, which indicate single jumping events.

Hybridization signals showed clear by additional bands that there is mobilization for *DIRS-1* in this RNAi mutant, which is consistent with the shown increased transcription on Northern blot and support our hypothesis for involvement of RNAi mechanism in retrotransposon regulation.

Mobilization of *skipper* represented by distinct jumping events in rrpC$^-$ mutant was not observed (data not shown). This suggested again for different behaviour of these two retrotransposons present in the *Dictyostelium* genome.

5.13 Bisulfite analysis

The bisulfite reaction was first described in early 1970s and was used subsequently to distinguish between cytosine and 5-methylcytosine in DNA (Clark et al. 1994).

The sodium bisulfite method was used to convert cytosine residues to uracil residues in single stranded DNA, under conditions whereby 5-methylcytosine remained non-reactive. The

converted DNA was amplified with specific primers which were complementary only to the converted DNA, while primers for not converted should not yield any product. As control PCR reactions were carried out 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 fragments were subjected to sequencing to confirm that all cytosine residues could be converted. All cytosine residues remaining in the sequence represented previously methylated cytosines in the genome and all cytosines which were not methylated were converted to uracil and further PCR and sequencing revealed them as thymidine.

5.13.1 Bisulfite analysis on *skipper* and *DIRS-1* for DnmA-mediated DNA methylation

By using global methods for DNA methylation analysis, like capillary electrophoresis, Southwestern blots and slot blot analyses, it was impossible to obtain conclusive evidence for a loss of DNA methylation in the mutant strain, presumably, because the former two methods were operating close to their detection limit (Kuhlmann* and Borisova* et al. 2005) and data not shown). Additionally, the slot blot experiment gave indication for methylation but with very high background of the immunological reaction.

For this reason, the work was focused on experiments for gene-specific methylation analysis. Based on observations in plants that RNAi could mediate transcriptional silencing by DNA methylation (Sijen et al. 2001), first one of the discoidin gene loci was investigated after silencing by an RNAi construct (Martens et al. 2002). Bisulfite 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 (Mette et al. 2000). The RNAi construct was, however, directed against the coding sequence.

In order to identify specific targets for DNA methylation, the microarray data was used. *Skipper,* which was among the ESTs with highest time fold increase and for which overexpression had been confirmed by Northern blot, was subjected for bisulfite treatment and sequencing. Using DNA derived from the wild type (Ax2) and dnmA knock-out strain bisufite analysis of a 294 bp region in the RT gene bottom strand showed that 12 out of 21 C-residues were methylated (Fig. 5.33).

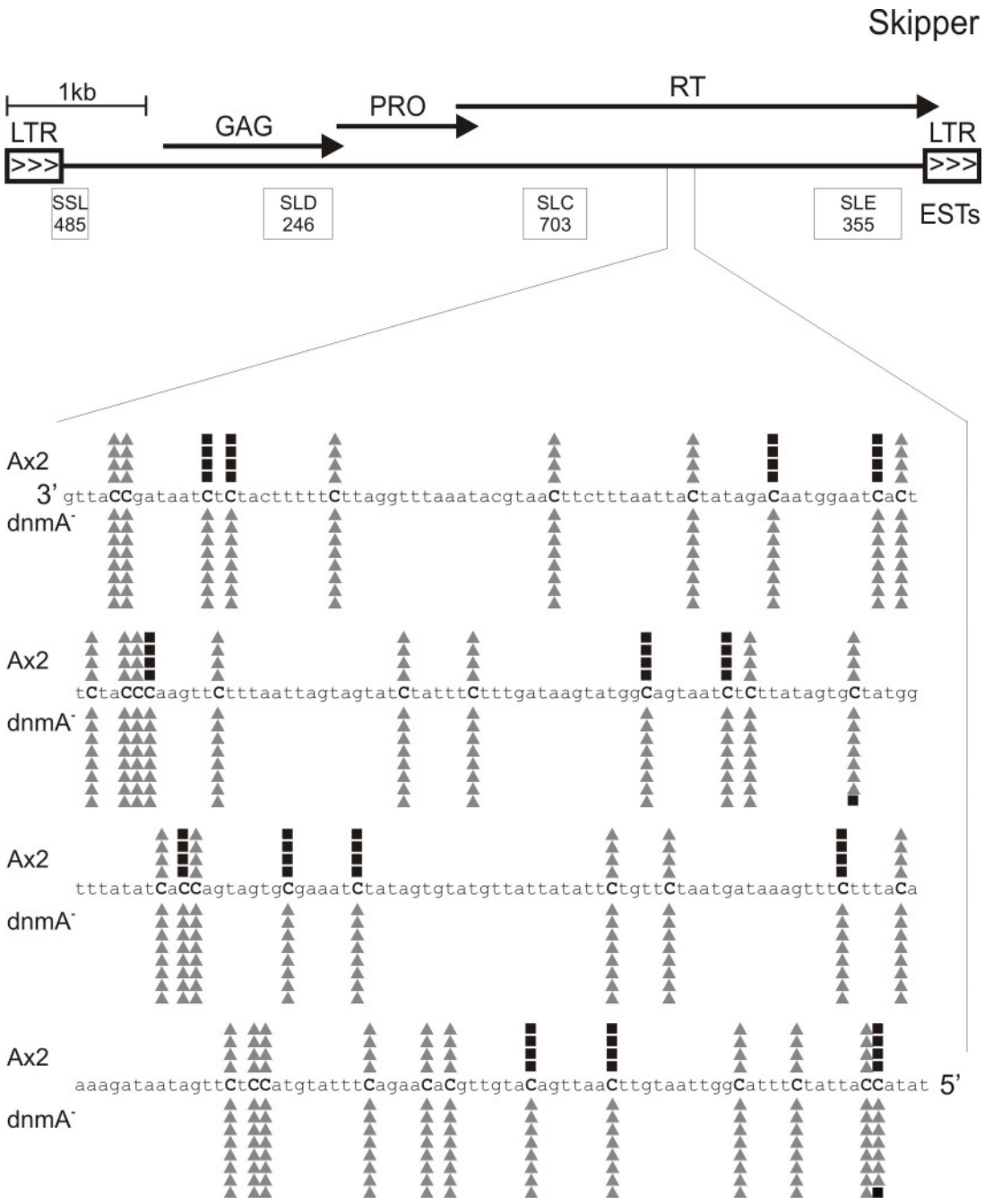

Fig. 5.33 Bisulfite sequencing of 294 bp in the Skipper RT gene (bottom strand). The top part shows the organization of the retrotransposon with GAG, PRO and RT genes and LTRs. ESTs that were included in the microarray are indicated below. Grey triangles indicate unmethylated C-residues; black squares methylated C-residues. Symbols above the sequence refer to DNA from Ax2 wild type cells, symbols below the sequence to DNA from the dnmA$^-$ strain. No symbol is shown when the sequence could not be unambiguously determined.

Methylation was mostly at asymmetric sites and could not be correlated with any obvious sequence context. 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. In contrast, bisulfite sequencing of 214 bp of the *skipper* LTR bottom strand revealed no methylation of the 12 C-residues within this sequence (Fig. 5.34). This could be due to different organization of skipper LTRs where there are direct repeats in comparison to *DIRS-1*, where there are inverted repeats and methylated (see Fig. 5.34 and Fig. 5.35).

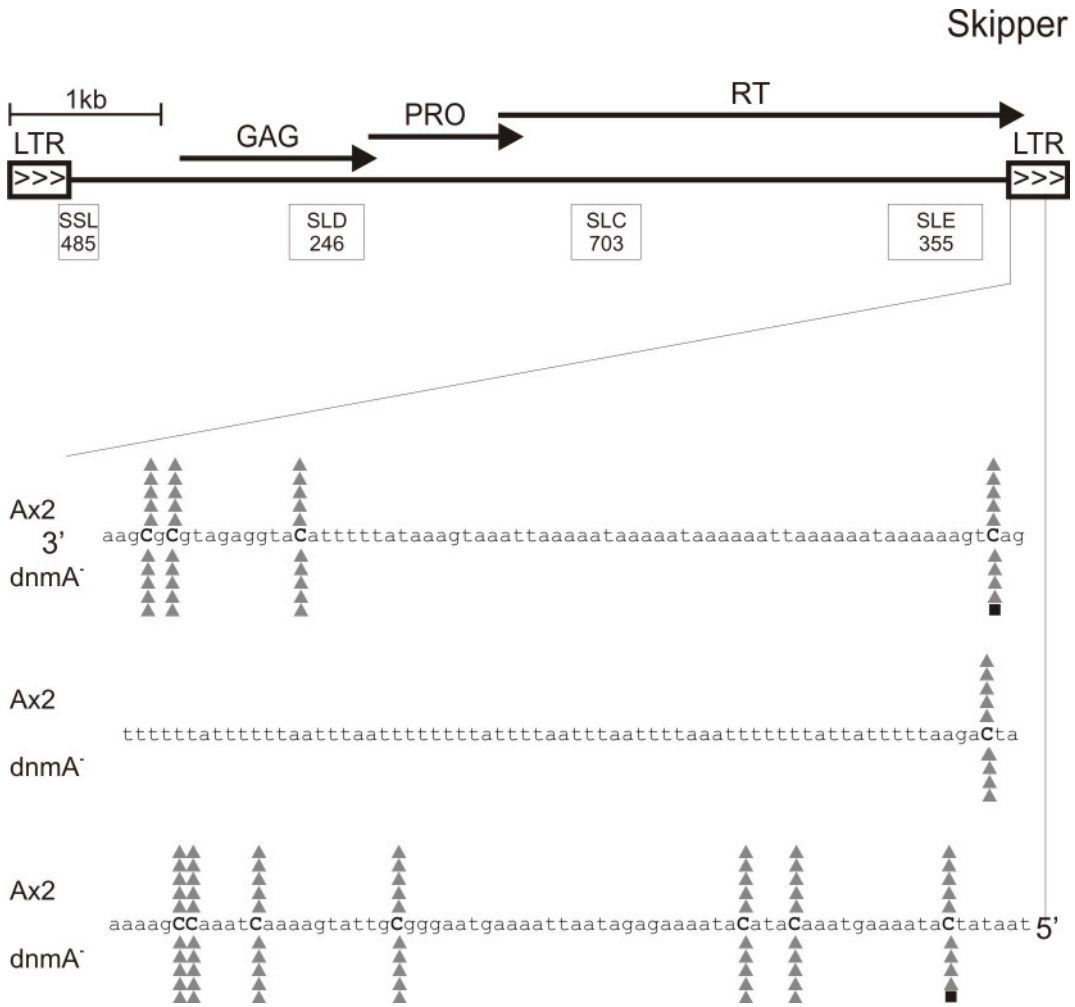

Fig. 5.34 Bisulfite sequencing of 214 bp in the Skipper LTR region (bottom strand). Grey triangles indicate unmethylated C-residues; black squares methylated C-residues. Symbols above the sequence refer to DNA from Ax2 wild type cells, symbols below the sequence to DNA from the dnmA$^-$ strain. No symbol is shown when the sequence could not be unambiguously determined.

To investigate if methylation was present in *DIRS-1* retrotransposon, 128 bp of the right LTR (bottom strand) were sequenced and it was found that 7 out of 23 C-residues in this region were

consistently methylated (Fig. 5.35). Similar to the results from *skipper*, methylation sites were mostly asymmetric and no obvious sequence context could be correlated with methylation.

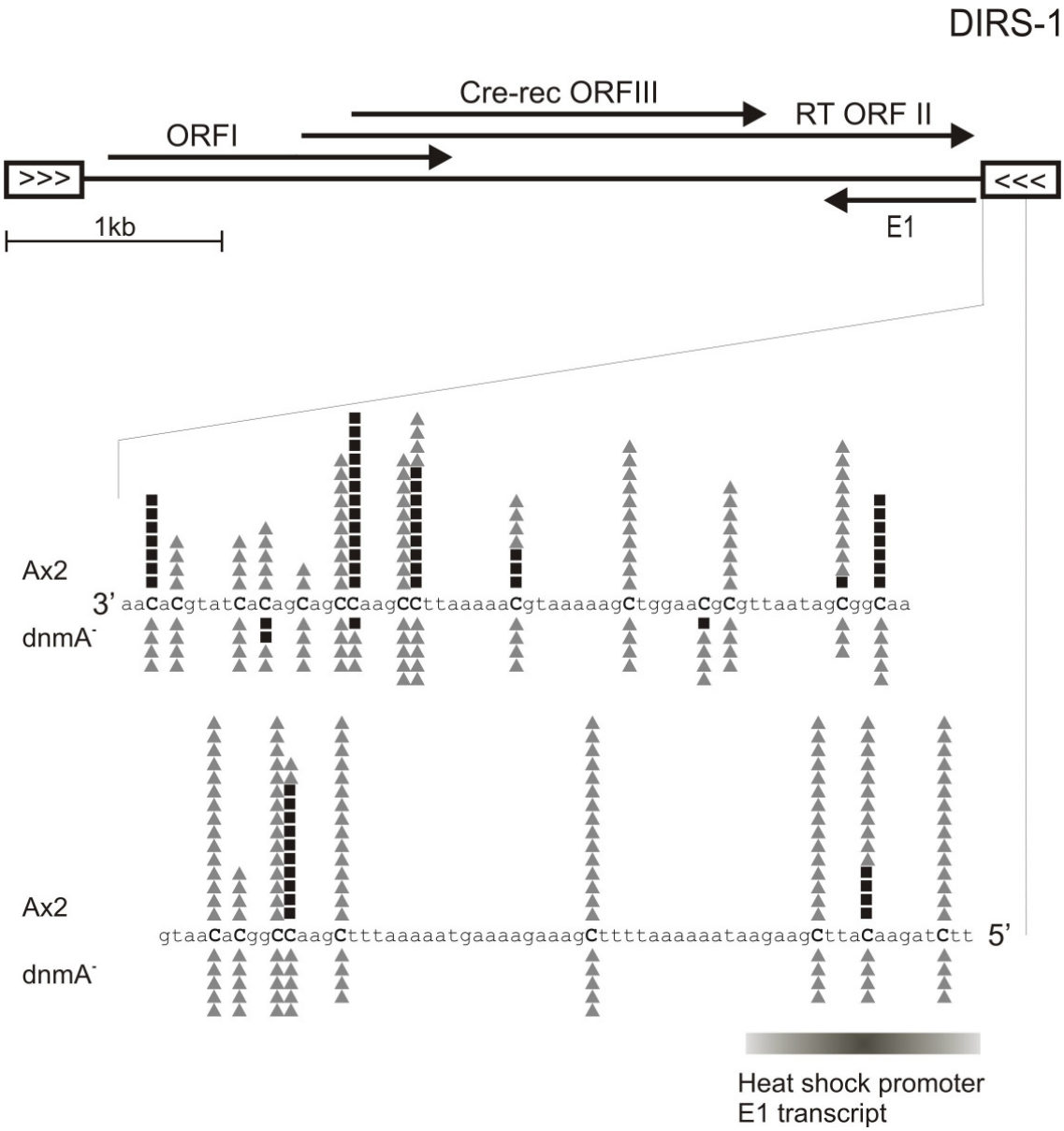

Fig. 5.35 Bisulfite sequencing of 128 bp in the right LTR of *DIRS-1* (bottom strand). The top part shows the organization of the retrotransposon with the overlapping reading frames ORF I, III and II, LTRs and the E1 antisense transcript. Grey triangles indicate unmethylated C residues, black squares methylated C residues. Symbols above the sequence refer to DNA from Ax2 wild type cells, symbols below the sequence to DNA from the dnmA$^-$ strain. No symbol is shown when the sequence could not be unambiguously determined.

Overall, eight non-converted C-residues were found by bisulfite sequencing in the dnmA$^-$ mutant strain (2 in *skipper* RT, 2 in *skipper* LTR, 4 in *DIRS*-1 LTR). This was a very small proportion of the unambiguously sequenced C-residues and it was assumed 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 represented an *in vitro* artifact. However, it was not excluded that an additional minor, non-conventional methyltransferase was active in the knock-out strain.

5.13.2 Target genes identified by microarray assay and analyzed by bisulfite sequencing for DnmA-mediated DNA methylation

To further investigate DNA methylation in *Dictyostelium,* another set of genes affected in the dnmA disruption mutant was chosen for further bisulfite analysis. ESTs - SLD 548, VSF 740 and SLE 817 representing ABC G21 transporter G family protein, Telomerase protein-1 and Major vault protein (mvpB), respectively, were among the up-regulated genes in the dnmA knock out strain (Table 5-5).

It has been shown (Emre et al. 2004; Liu et al. 2004) that MVPs are at both, the gene and protein level highly conserved among species ranging from the slime mold *Dictyostelium* to humans. In mammals, the vault complex is made of the major vault protein (MVP), vault poly (ADP-ribose) polymerase (VPARP), and telomerase-associated protein (TEP-1). The vault is involved in multi-drug resistance in cancer cells where it is highly expressed (Gopinath et al. 2005). The 13 MDa ribonucleoprotein particle (vault complex) is comprised of the three proteins and also a small untranslated vault RNA (vRNA) (Gopinath et al. 2005). In addition it has been shown that eukaryotic ABC transporters, which are mainly involved in transmembrane transport, give rise to multiple drug resistance by pumping the drugs out of the cells. (Gros et al. 1986).

In *Dictyostelium,* the most studied component of the vault complex is the major vault protein, where three isoforms were identified (MVPA, MVPB and MVPC), but only the first two were studied (Vasu and Rome 1995). At that time, the equivalent of the vRNA had not yet been identified in *Dictyostelium*. The telomerase protein was not studied, nor the ABC transporters. The *Dictyostelium* ABCs were classified according to their conservation among different species (Anjard et al. 2002), but since different drugs were unlikely to be encountered by the social amoebae naturally, the physiological function of ABC transporters is largely unknown.

Interestingly, MVP have been shown to be regulated by chromatin remodelling in mammalian cells (Emre et al. 2004). Since the ESTs for vault complex members were the next with high times fold increase expression in microarray analysis after *skipper* in dnmA knock-out strain, their expression was checked on Northern blot (Fig. 5.36).

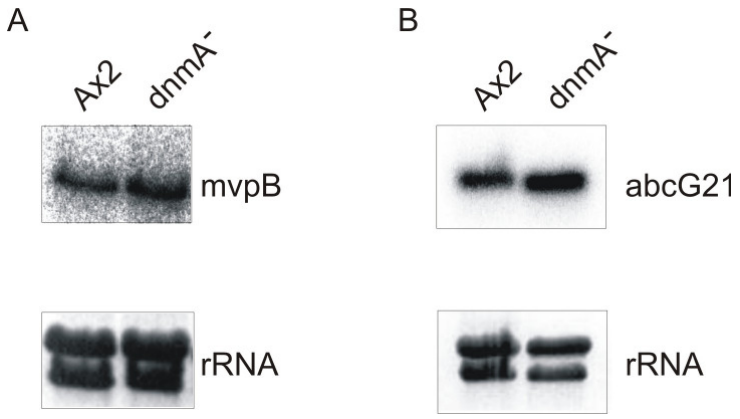

Fig. 5.36 Northern blot showing the expression profiles of (A) mvpB and (B) abcG21. The probes for both genes were complementary to parts of the genes, that were covered by corresponding ESTs.

Northern blots confirmed the microarray data, where a 1.48 times fold increase in the gene expression was observed for the major vault protein (mvpB) and a 2.14 times fold increase for the ABC transporters (abcG21). The Northern blots for telomerase protein-1 with 1.49 times fold increase on the microarray failed. Despite the fact, that telomerase protein-1 (telA) gene expression failed to show any signal on Northern blot, bisulfite treatment was carried out on this gene (Fig. 5.37) and on mvpB (Fig. 5.38). Due to problems recovering the PCR product of abcG21 after the bisulfite treatment, further sequencing could not be performed. Bisulfite data for the Major vault protein (mvpB) and the Telomerase protein-1 (telA) revealed that both these genes contained methylated C-residues. Unlike for *skipper* and *DIRS-1*, these C-residues did not show full methylation. Because DNA methylation was absent in the DnmA$^-$ strain the observed methylation of mvpB and telA in the wild type was assumed to be mediated by DnmA. As in the case of *skipper* and *DIRS-1*, most of the sites were asymmetrical and could not be correlated with any obvious sequence context.

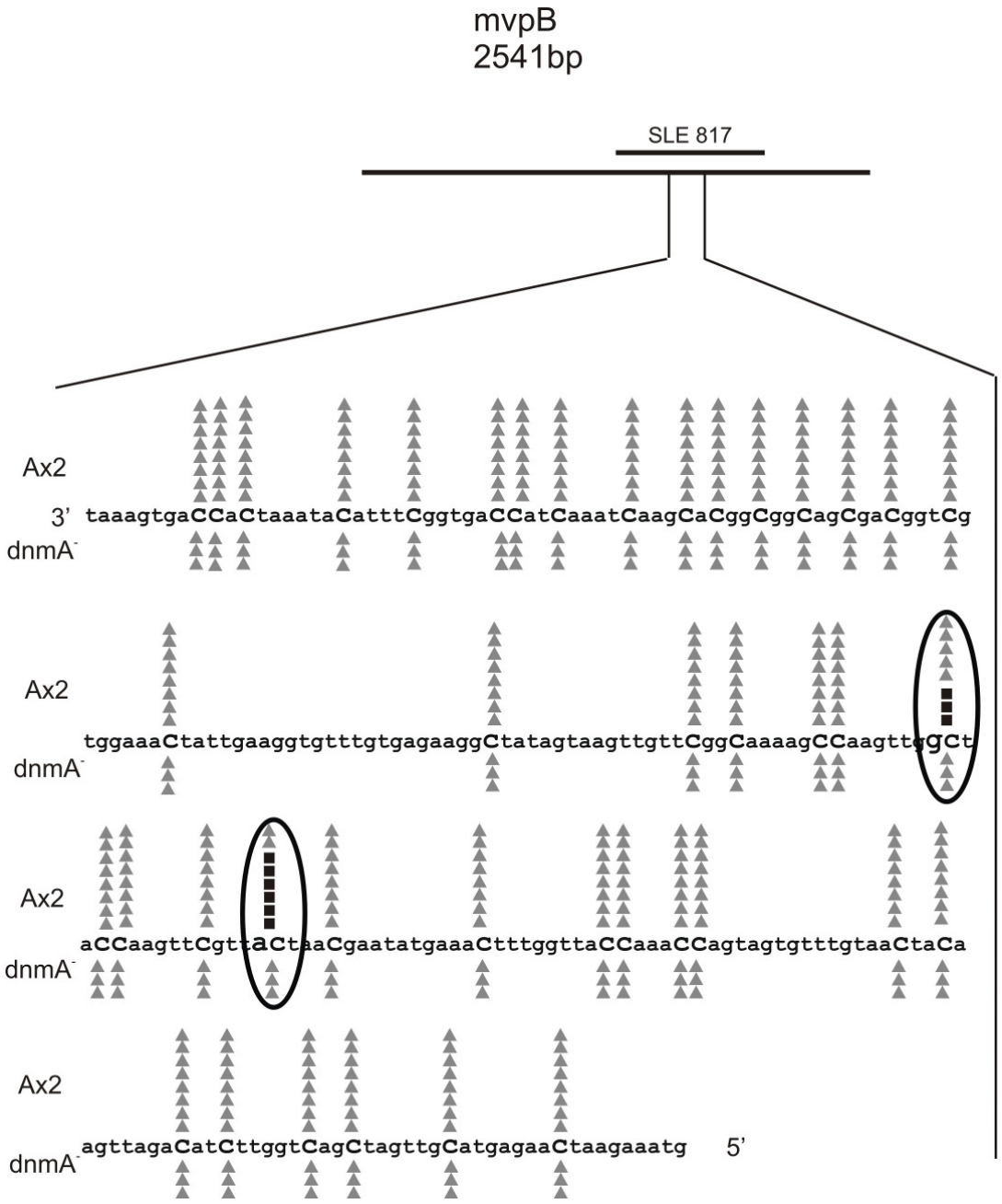

Fig. 5.37 Bisulfite sequencing of 231 bp in the SLE 817 region of the mvpB gene (bottom strand). Highlighted in red are the positions confirmed to be methylated in the wild type but not in the dnmA- strain. Grey triangles indicate unmethylated C residues, black squares methylated C residues. Symbols above the sequence refer to DNA from Ax2 wild type cells, symbols below the sequence to DNA from the dnmA- strain. No symbol is shown when the sequence could not be unambiguously determined.

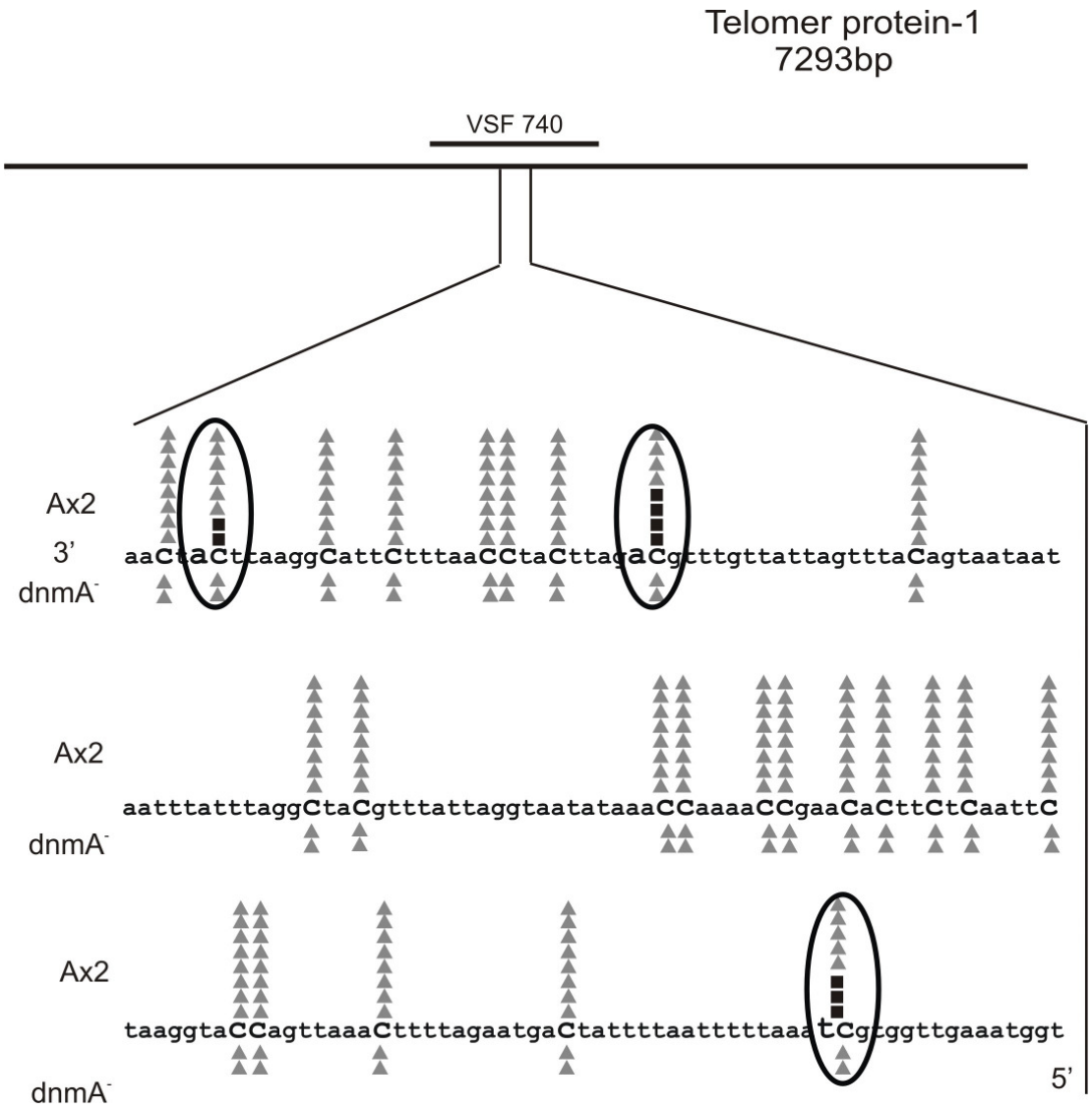

Fig. 5.38 Bisulfite sequencing of 188 bp in the VSF 740 region of the tel A gene (bottom strand). Highlighted in red are the positions confirmed to be methylated in the wild type but not in the dnmA- strain. Grey triangles indicate unmethylated C residues, black squares methylated C residues. Symbols above the sequence refer to DNA from Ax2 wild type cells, symbols below the sequence to DNA from the dnmA- strain. No symbol is shown when the sequence could not be unambiguously determined.

To come to a better understanding of the regulation of the vaults in *Dictyostelium*, further bisulfite analysis was done in the promoter region of the mvpB gene. In *Dictyostelium,* promoter regions are not well defined and, in addition, the AT richness of the *Dictyostelium* genome, especially in the promoter regions, made the design of the experiment not trivial. Nevertheless, the assay was successful, but showed no methylation in the mvpB promoter (Fig. 5.39).

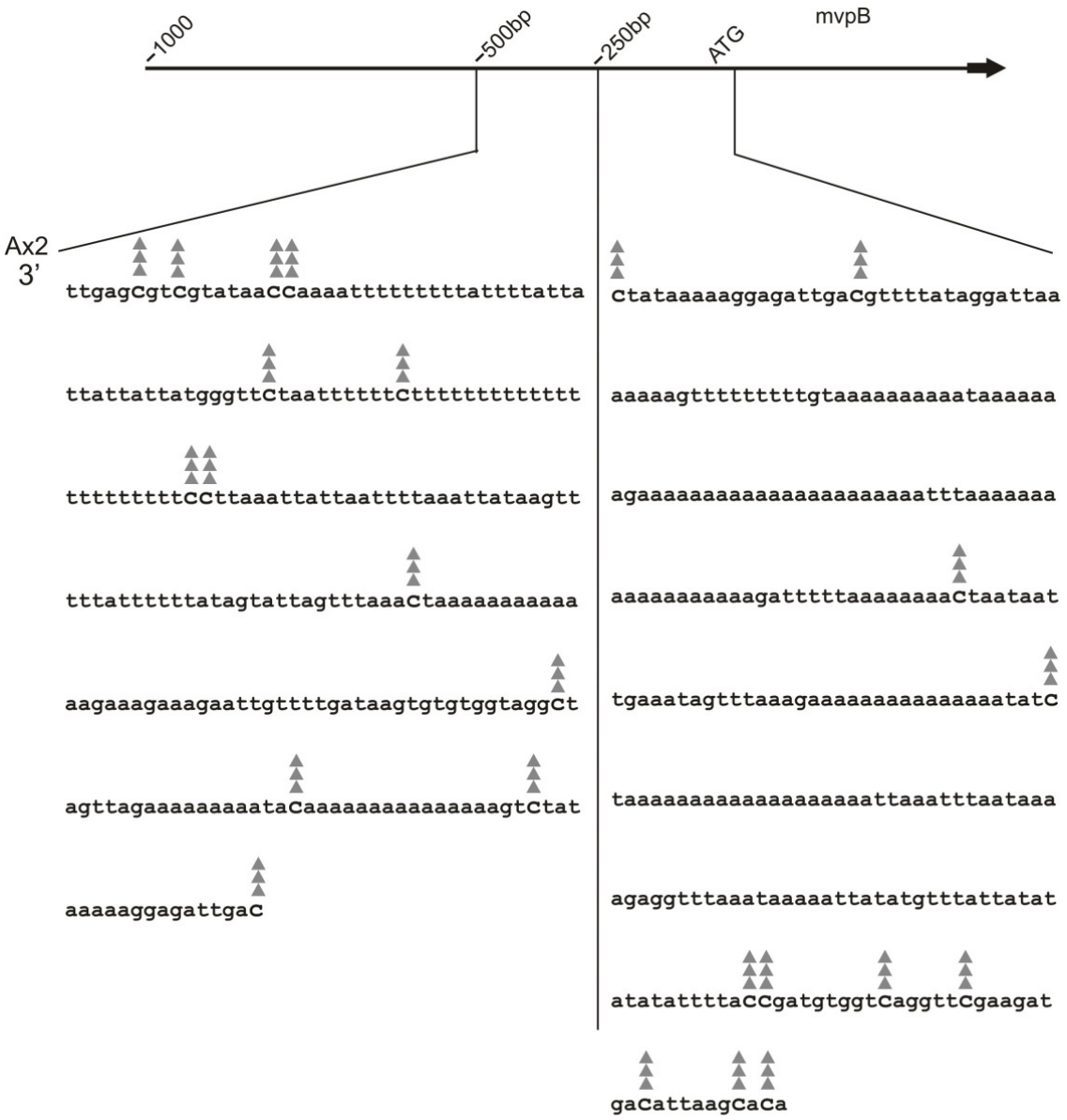

Fig. 5.39 Bisulfite sequencing of 285 bp upstream from the transcription site (ATG) of mvpB and 249 bp from the -250 bp site using wild type DNA. The entire PCR product covering 534 bp was not successfully amplified. All C-residues were unmethylated (grey triangles).

The number of clones analyzed was not as high as for the previous genes, but there was no single C-residue, which was methylated in any of the clones. Since *Dictyostelium* MVP promoter does not show in BLAST searches similarities to the mammalian MVP promoters (Emre et al. 2004), where they could be subjected for heterochromatin formation, *Dictyostelium* MVPs might have different mechanism of control. Alternatively, a methylated transcription initiation site might be further upstream and was not detected in this work.

5.13.3 Developmentally regulated DNA methylation in *Dictyostelium*

In contrast to data in this study, it was reported recently that DNA methylation was developmentally up-regulated in *Dictyostelium* (Katoh et al. 2006). Using computational analysis, the distribution of the CpG dinucleotides in *Dictyostelium* genome was found to be unusual, and some potential CpG sites were identified in the guaB locus. These authors performed quantitative analysis based on methylation sensitive restriction endonuclease digestion, which showed that 2 out of 3 sites in guaB gene were methylated and that methylation was increased in development. In addition, the deletion of DnmA resulted in a decrease in the level of DNA methylation in developing cells.

Unfortunately, the guaB gene was not spotted on the used *Dictyostelium* microarray and therefore the methylation status of the guaB gene was checked directly by bisulfite sequencing. based on what has been shown by (Katoh et al. 2006). The experiment was designed such that the bisulfite primers were covering the second potential methylation site (Katoh et al. 2006), where the methylation sensitive enzyme Ava I had shown accumulation of undigested DNA.

Genomic DNA from the wild type and dnmA$^-$ cells in vegetative growth and in development (24h) were collected. Bisulfite treatment was done according to the standard protocol (see materials and methods). Sequencing revealed that there was no detectable DNA methylation in the analyzed 250 bp guaB fragment, neither in vegetative grown cells, nor in 24 hours developed cells (Fig. 5.40). There were two sites in one clone which appeared to be methylated in vegetatively grown wild type, but these were not confirmed in the rest of the sequenced clones. In addition, there were three potential sites in one sequence, one of which was confirmed in another sequence from wild type in development, but in no other sequences. Importantly, the Ava I site was completely unmethylated in contrast to the report by Shaulsky and co-workers (Katoh et al. 2006).

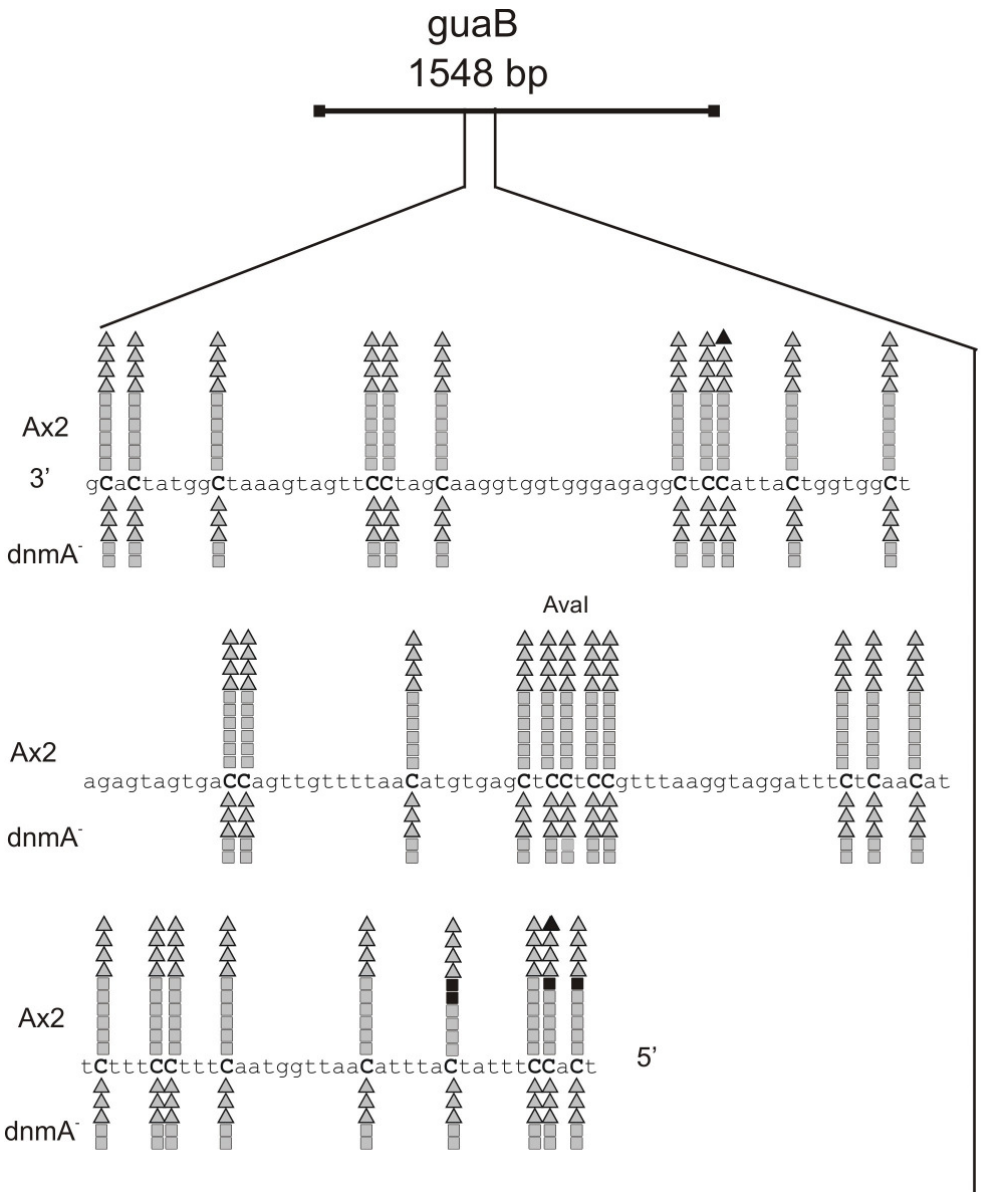

Fig. 5.40 Bisulfite sequencing on 250 bp in site 2 (bottom strand). Black squares represents methylation in vegetative cells, grey squares no methylation respectively. Black triangles indicates methylation in development, grey triangles no methylation respectively. Symbols above the sequence refer to DNA from Ax2 wild type cells, symbols below the sequence to DNA from the dnmA$^-$ strain. No symbol is shown when the sequence could not be unambiguously determined.

This result met our expectations since most of the methylated sites were asymmetrical rather than symmetrical CpG sites, accordingly to the bisulfite data in this work. Also, the clear down-regulation of dnmA in development (Fig. 5.2) raises doubts on the others studies.

6 Discussion

6.1 *In vitro* characterization of DnmA

DNA methyltransferases are involved in many cellular processes like development, X-chromosome inactivation and imprinting in higher eukaryotes (Li et al. 1992; Okano et al. 1999).

As a eukaryote and very well established model organism, *Dictyostelium discoideum* became an attractive system to study epigenetics, and in this particular work, the DNA methylation as a part of it. In addition other components, which are comprising the protein machinery required for epigenetic gene silencing in higher eukaryotes, were identified in *Dictyostelium* genome (Kaller et al. 2006; Popova et al. 2006).

Until now, *Dictyostelium* DNA was considered not to be modified by cytosine methylation (Smith and Ratner 1991). With the completion of the genome sequencing project (Eichinger et al. 2005), a gene, dnmA, highly homologous to the methyltransferases of the Dnmt2 family, was detected. In this work further analyses for determining the function were performed, such as *in vitro* electrophoretic mobility shift assays using DNA oligonucleotides and recombinant DnmA. This revealed that the bacterially expressed enzyme might not be active *in vitro*, even though different conditions and different protein preparations were tried.

In contrast, the human DNMT2 has been shown to display denaturant-resistant binding to DNA *in vitro*, but lacks observable transmethylase activity (Dong et al. 2001). To further explore the transmethylase activity of DnmA, analyses with methylation sensitive restriction enzymes were carried out. In case DnmA displays such an activity, it would be possible to prove this *in vitro*, by the sensitivity of the restriction enzymes to methylated DNA. The approach should reveal a different pattern with accumulation of undigested DNA if it was methylated and complete digest, if it was not. However restriction enzymes will only detect methylation on symmetrical sites in the sequences. No difference was observed in this experiment, which indicated that recombinant DnmA might be lacking accessory factors essential for the methyl transfer activity. This seemed to be due to the fact that DnmA (like all homologues of the DNMT2 family) lacks the large N-terminal domain of DNMT1, which is necessary for its function. Alternatively, the enzyme might not be active *in vitro*, for example due to a lack of post-translational modifications. Could also be the case that methylation is taking place but at asymmetrical sites, which are not detectable by the

restriction enzymes. Based on published data that components of the mammalian DNA methylation system (Dnmt1, Dnmt3, MeCP2) are RNA binding proteins, as an additional feature (Jeffery and Nakielny 2004), DnmA has been subjected to similar analyses. No RNA binding was observed for DnmA as had been shown for the human DNMT2 (Jeffery and Nakielny 2004).

In order to investigate the proposed need of more components for the methyl transfer activity of DnmA, a search for potential protein binding partners was done using the yeast two-hybrid system. The assay revealed several candidates, which at least had an annotated nuclear localization and thus could be partners of DnmA *in vivo*. Further investigations were not performed on these potential partners (see Table 5-3), however, since their annotated function was not related to the DNA methylation pathway.

It has been shown that there is a physical and functional link between the Suv39h-HP1 histone methylation system and the DNA methyltransferase Dnmt3b in mammals (Lehnertz et al. 2003). Furthermore, not only Dnmt3b but also Dnmt3a and Dnmt1 were shown to interact both *in vivo* and *in vitro* with Suv39-H1 and HP1β (Fucks et al. 2003). Based on this data showing the connection between histone H3-K9 methylation and DNA methylation in mammals, a directed yeast two-hybrid search was carried out for detection of interaction between DnmA and HcpA (HP1-like protein in *Dictyostelium*) (Kaller et al. 2006). No interaction between these two proteins could be observed in the β-galactosidase assay. This indicates that in *Dictyostelium* no physical contact is required for the function of these proteins.

HP1-like homologues in *Dictyostelium* might link histone methylation to DNA methylation, similarly to what has been observed in *Neurospora crasa* (Freitag et al. 2004). Also human HP1 binds to nucleosomes and DNA *in vitro* (Zhao et al. 2000). To investigate DNA binding of *Dictyostelium* HP1-like proteins gel shift experiments were done using DNA and purified recombinant HcpA, HcpB and murine HP1α protein as a positive control. Obtained *in vitro* data proved that *Dictyostelium* heterochromatic proteins HcpA / HcpB are binding DNA and RNA as well. However, the question whether DnmA-mediated DNA-methylation was connected to the histone binding enzymes could not be answered by this approach. From the directed yeast two-hybrid experiments can be inferred that DnmA does not directly interact with HcpA. This discriminates epigenetics in *Dictyostelium* from mammalian systems, where both enzymes interact in the process of chromatin remodelling.

6.2 *In vivo* studies of DnmA

For *in vivo* investigations a knock-out strain for dnmA was generated (Kaller 2002). The gene disruption revealed no obvious phenotype in axenic growth or during development, where the spore formation was normal. The only phenotype was reduced phagocytic uptake in the DnmA knock-out strain (Iris Müller, personal communication.). This effect is still to be explained. Thus the knock-out strain was subjected for several experiments together with the wild type Ax2 to investigate the function of DnmA in *Dictyostelium*. In addition to postulated transcriptional gene silencing mediated by DNA methylation, antisense (Nellen et al. 1992), as well as RNAi mediated gene silencing (Martens et al. 2002) were shown to be functional in *Dictyostelium*. Homologues of many key components of the RNAi mechanism were identified in *Dictyostelium* and some of them functionally characterized: three RdRP homologues – RrpA, RrpB and RrpC (Martens et al. 2002), two Dicer-like proteins in *Dictyostelium*, Dicer A (drnA) and Dicer B (drnB) , five Argonaute homologues – agnA - E, and recently, a homologue of eri-1, an endogenous inhibitor of RNAi in *C. elegans* (Kennedy et al. 2004) was identified in *Dictyostelium*. The function of these components has been shown in the wild type strain (Ax2) where discoidin can be silenced by both antisense RNA-mediated gene silencing and RNAi (Martens et al. 2002). However, the question arose whether the discoidin gene could also be silenced on the transcriptional level by DnmA-mediated DNA methylation. The silencing efficiency for RNAi as well as antisense stayed unchanged in the dnmA mutant strain, which showed that lack of DnmA as a potential DNA methyltransferase was not affecting the silencing level in particular for the discoidin gene. This would suggest for no direct implication of DnmA in RNAi mediated gene silencing. However, whether these mechanisms could be related in some manner or whether they share common components is to be explained.

To reveal the function of DnmA two methods were applied. One of them based on immunological detection of methylated DNA and another one based on drug treatment causing a block of the enzyme by its covalent binding to the DNA. Both methods were used to check the transmethylase activity of DnmA and additionally the second for capturing the enzyme to the DNA. It has been shown for *Drosophila* that methylated DNA could be detected by slot blot analysis with an antibody specific for methylated cytosine (5mC) (Kunert et al. 2003). In the case of *Dictyostelium*, using the same antibody on DNA from the wild type and dnmA knock-out mutant, slight differences in the signal were observed. This was a first important indication for DNA methylase activity of DnmA, which, however, was not conclusive because of the high background level of the antibody.

Using an overexpressor strain with myc - tagged DnmA, hypermethylation of the DNA was expected to be observed. Furthermore, the myc tag allowed to investigate the cellular localization of DnmA and to isolate the enzyme bound to the DNA after 5-aza-cytidine treatment. After feeding 5-aza-cytidine to the cells for one cell generation time (8 hours), the effect was monitored on western blot after isolation of nuclei and compared to non treated cells. The signal detected by α-myc antibody was predominantly in the nuclear fraction, compared to cytoplasmic isolate. Since this was observed independent of 5-azaC treatment, covalent attachment of the enzyme to DNA could not be shown by this approach, however, the experiment confirmed the predominantly nuclear localization of DnmA inferred from GFP and myc staining of the cells. In another set of experiments, DNA isolated from wild type *Dictyostelium* cells treated or non-treated with 5-azaC was immobilized and incubated with recombinant His-tagged DnmA. Incubation with α-his antibody showed that in case of using recombinant DnmA, attracting the protein to the DNA from 5-azaC treated cells was higher than all controls present on the membrane. This result supports the enzyme function by its recruitment to the DNA. A small hint for the methylation activity could be the 5mC detection in the slot blot experiment, where a signal for methylation was observed, but not lost completely in the knock-out mutant. These data and previously obtained results by capillary electrophoresis (Kuhlmann* and Borisova* et al. 2005), indicated 0.2% methylation. Detected methylation very likely is due to the function of the sole DNA methyltransferase in the *Dictyostelium* genome.

6.3 DnmA-mediated DNA methylation in the *Dictyostelium* genome

To investigate the cellular function and to identify target sequences of DnmA, a microarray analysis was carried out. In this experiment wild type cells together with the knock-out strain and DnmA-myc overexpressing cells were used. This approach aimed to investigate changes in mRNA expression, and thus to establish a molecular phenotype, since no global phenotype had been observed for either overexpressor or knock-out strain. The method resulted in a number of genes which were affected either by knocking out the DnmA, or by overexpressing it (see Table 5-5,Table 5-6,Table 5-7,Table 5-8). Clearly, the genes that were up-regulated in the knock-out strain were of particular interest, since they might represent direct targets of the enzyme. Among them was the retrotransposon *skipper,* and this work has shown that the dnmA gene product is responsible for methylation of C-residues in this sequences, which was confirmed by bisulfite analysis. As it was expected based on the microarray data, a disruption of the methyltransferase gene resulted in transcriptional activation of *skipper* and probably as a consequence, in

mobilization of the retrotransposon displayed on both Northern and Southern blots. Surprisingly, *DIRS-1* expression was not affected by a loss of DNA methylation. It was assumed 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 methylated. Furthermore, there are many incomplete copies of *DIRS-1* in the *Dictyostelium* 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 (Glockner et al. 2001). 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. Therefore it has been proposed that DNA methylation causes chromatin remodeling over the entire retroelement and thus blocks accessibility for the transcription machinery. The component(s) mediating chromatin remodeling remain elusive since a search for methyl-CpG binding proteins in the *Dictyostelium* data base 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 (Klose et al. 2005) 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 (Marhold et al. 2002) 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 (Verdel et al. 2004) and in plants DNA methylation is targeted by small RNAs (Mette et al. 2000). Therefore it was investigated if 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 (Mund et al. 2004). 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, since apparently they do not define the target of methylation. In fact, it has been shown in *Neurospora* that *de novo* DNA methylation is independent of RNAi (Freitag et al. 2004).

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 (drnA) 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 (Martens et al. 2002) it was 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 (Martens et al. unpublished). 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 up-regulated 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 (Rosen et al. 1983; Cappello et al. 1984) 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 (Sijen et al. 2001). Alternatively, an antisense strand could be generated by an RNA polymerase IV like activity that has recently been detected in *Arabidopsis* (Herr et al. 2005; Kanno et al. 2005). However, a polymerase IV like enzyme has not yet been identified in *Dictyostelium*. Though we do not know if 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 remodeling 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 (Lippman et al. 2003; Kanno et al. 2005). 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 posttranscriptional mechanisms contribute simultaneously to the silencing machinery.

In mammalian cells one of the key components involved in the RNAi pathway is the small RNA-binding protein called Argonaute (Ago). In *S. pombe* ago$^-$ mutants, dcr$^-$ (Dicer) or rdp$^-$ (RdRP, also known as RDR) mutants showed reduced H3K9 methylation (Volpe et al. 2002), which demonstrated that RNAi was responsible for heterochromatic modifications. Surprisingly, in the knock down strain for agnA (*Dictyostelium* homologue of Ago1) DNA methylation was reduced but not completely abolished for *skipper* and *DIRS*-1 (Zhang 2006). Although there are indications that AgnA is involved in RNAi, the exact function of the protein in DNA methylation is not yet well understood.

Taken together, this data established *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 *skipper* and *DIRS-1* but further experiments are required to elucidate how the two pathways interact.

In an independent approach, Shaulsky and co-workers (Katoh et al. 2006) detected low levels of DnmA -dependent DNA methylation in *Dictyostelium*. Using methylation sensitive restriction enzymes, they identified methylated CpG sites in *DIRS-1* and in the guaB gene. Here, our bisulfite sequencing data of the guaB gene showed no methylation. Because of those authors' data had shown increased level of methylation in development using methylation sensitive restriction enzymes, guaB was first chosen to be analyzed for methylation in development by bisulfite analysis. This result argue strongly against guaB as a target gene for DnmA as reported by (Katoh et al. 2006). Furthermore, it underlines the power of bisulfite sequencing as the final proof of the methylation status of a gene. The contradicting result on expression of dnmA during development can only indirectly be addressed by this experiment. The guaB gene is involved in the synthesis of guanine, and thus it was interesting, that another gene of the same pathway, guaA, appeared to be down regulated in the DnmA-myc overexpression strain as suggested by our microarray analysis. This would predict that guaA and other genes may be hypermethylate and thus suppressed when DnmA is in excess. How and why expression of guaA is affected by methylation is still to be understood. Moreover, it should be mentioned that many of the genes which appear to be up-regulated in the DnmA-myc strain were annotated to be involved in the stress response in *Dictyostelium* (see Table 5-7). Interestingly, siRNA library data revealed only few siRNAs, which were complementary to coding sequences in the *Dictyostelium* genome (Ambros

personal communication) (see Table 7-1 in supplementary material). Also most of these genes were involved in the stress response. In fission yeast, heterochromatin nucleation is induced in an RNAi-dependent manner using stress activated proteins (Jia et al. 2004). The results of the microarray analysis of DnmA-myc overexpressor strain in combination with the siRNA library data would point to a similar connection for *Dictyostelium* as observed in *S. pombe*.

6.4 Outlook

Further understanding of DnmA should benefit from characterizing its function by analyzing potential genes for methylation. Since it has been shown that Dnmt2 from mouse, *Drosophila* and *Arabidopsis* can serve as an RNA methyltransferase (Goll et al. 2006), but not DNA methyltransferase, an additional level in the discussion of its function was opened. Apparently the specificity of individual DNA methylatransferases is broader than commonly assumed. Recently it has been proposed that Dnmt2 methyltransferases display dual specificity for both DNA and RNA (Jeltsch et al. 2006). The question which can be asked is what biological meaning stays behind these findings. Could be that tRNA is used as a primer for initiation of retrotransposon reverse transcription like in the case of HIV-1 virus (Xu and Morrow 2006), and by these means they become methylated at some point. Another possibility would be that tRNA is used as primer by RdRp involved in *Dictyostelium* RNAi. This would be rather easy to be checked by gel shift experiment using recombinantely expressed RdRp and tRNA as a substrate.

For further studies another set of genes affected in the dnmA disruption mutant was chosen for bisulfite analysis. These gene products are involved in mammals in the multi-drug response (MDR) in cancer cell lines (Gopinath et al. 2005). Three of these proteins, telomerase protein, major vault protein and abc transporters are components of the MDR complex formation. Expression of the corresponding genes in *Dictyostelium* was found to be increased by microarray analysis of the dnmA knock-out mutant. Unfortunately, neither function nor regulation of these proteins in *Dictyostelium* is clear. Murine components of the MDR system are regulated by chromatin remodeling (Emre et al. 2004), and based on this observation DNA methylation of these genes in *Dictyostelium* was investigated.

Bisulfite data for the Major vault protein (mvpB) and the Telomerase protein-1 (telA) from *Dictyostelium* revealed that both genes contained a few methylated C-residues in the checked regions, but unlike for *skipper* and *DIRS-1*, these C-residues did not show full methylation. In addition, DNA methylation was absent from the same C-residues in the DnmA knock-out strain, again showing that the observed methylation in the wild type strain is due to the function of

DnmA. More sequences analysis will have to be done to support this results, but clear evidence for DNA methylation was obtained. Furthermore the promoter region of the mvpB gene was analyzed by bisulfite sequencing as a potential target for methylation. Not surprisingly no methylation in the 500 bp upstream region of the mvpB gene was observed. Promoter regions in *Dictyostelium* are very different from mammalian ones. Even though the *Dictyostelium* genome project was completed (Eichinger et al. 2005), no clear idea for the term promoter was provided. Based on very high AT composition, the *Dictyostelium* genome appears to be difficult in studies like DNA methylation, especially for intergenic regions which mostly consist of AT stretches. Either this or the possibility that methylated regulatory elements are further upstream was the reason for unsuccessful methylation analysis on the promoter region of mvpB gene. The mammalian MDR system regulation of vault components could occur by several mechanisms one of which is alterations in the chromatin structure (Emre et al. 2004). This might also be the case in *Dictyostelium*, and further analyses will be required to investigate regulation and expression of these proteins. In addition designing experiments for revealing the function of these proteins would be quite interesting. Initially, creating different fusion constructs of MVPB and Telomerase protein would show their localization, and by this getting closer to their function.

7 Supplementary material

Table 7-1 siRNA, which appeared to be complementary to coding sequences in *Dictyostelium* genom.

gene name	Sequence siRNA	gene product
JC1V2_0_00305	UACAGAAAUCGCAAACUUUAC	Pol., GAG
sigD	GAAGUGAAAGCGGUAGUGGA	spore coat protein
hspD	GAAGAUCAACUCGAUUAU	heat shock protein
JC1V2_0_00936	GUAUUCAAUAAUAAUAAUAAUGAU	response to oxidative stress
JC1V2_0_01186	UGAUUAUUCAUCCAGAUGGUA	DNA binding
JC2V2_0_00228	AUUAAAUGGUACUUCCAAUAUGUCA	polyketide synthase-fatty acids biosin.
JC2V2_0_02468	GGGAGUUUAGUCAAGAGGCU	Strand exchange protein 1
JC2V2_0_02481	CGCAAAUACCGGUGUCAAAG	CCT chaperonin theta subunit
dhkB	GAUCUCACACAUACAAGGCCA	histidine kinase
sodD	GGAGCACAUUUCAAUCCAUUUCAUGUU	superoxide dismutase
cotD	GCAAGAGGUAUGGAAU	spore coat protein SP75
BC4V2_0_00972	AUACUAUUUGGUGGCUUUGA	Probable cation-transporting ATPase
pXi	UACGUGAAUUCAAUACCCUC	CAMK1 family protein kinase
ndrD	CCUUCUUCUCCACCA	putative protein serine/threonine kinase
ndrD	CCUUCUUCUCCACCA	putative protein serine/threonine kinase
Dd5P3	GGAGGAGGUGGUGGUGGAGGUGGU	inositol 5-phosphatase
geneDDB0231281	UGAAUGGUGUUGUUCAAAUUG	putative protein serine/threonine kinase
rnlA	GAAUCACUUCGAUGAAAUU	mitochondrial large subunit rRNA
rnlA	GGCUAGUCACAUAUUAGAGUUCAUAUC	mitochondrial large subunit rRNA
rnlA	GAGGUGGGUCGAUCACUGGU	mitochondrial large subunit rRNA
rnlA	UGGAUGACGUAUUUUA	mitochondrial large subunit rRNA
rnlA	GGAUGACGUAUUUUAU	mitochondrial large subunit rRNA
rnlA	GAUGACGUAUUUUAU	mitochondrial large subunit rRNA

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