Comparative analysis of DNA methylation patterns in transgenic *Drosophila* overexpressing mouse DNA methyltransferases

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DNA methyltransferases (Dnmts) mediate the epigenetic modification of eukaryotic genomes. Mammalian DNA methylation patterns are established and maintained by co-operative interactions among the Dnmt proteins Dnmt1, Dnmt3a and Dnmt3b. Owing to their simultaneous presence in mammalian cells, the activities of individual Dnmt have not yet been determined. This includes a fourth putative Dnmt, namely Dnmt2, which has failed to reveal any activity in previous assays. We have now established transgenic *Drosophila* strains that allow for individual overexpression of all known mouse Dnmts. Quantitative analysis of genomic cytosine methylation levels demonstrated a robust Dnmt activity for the *de novo* methyltransferases Dnmt3a and Dnmt3b. In addition, we also detected a weak but significant activity for Dnmt2. Subsequent methylation tract analysis by genomic bisulphite sequencing revealed that Dnmt3 enzymes preferentially methylated CpG dinucleotides in a processive manner, whereas Dnmt2 methylated isolated cytosine residues in a non-CpG dinucleotide context. Our results allow a direct comparison of the activities of mammalian Dnmts and suggest a significant functional specialization of these enzymes.

Key words: DNA methyltransferase, Dnmt1, Dnmt2, Dnmt3a, Dnmt3b, *Drosophila*.

INTRODUCTION

DNA methyltransferases (Dnmts) play a major role in development and disease [1–3]. Targeted disruption of mouse Dnmt genes has been shown to result in embryonic lethality and developmental defects [4,5]. Similar effects have also been reported in other organisms [6–8]. In addition, mutational inactivation or aberrant expression of Dnmts has been implied in several human diseases. Genetic mutations in a human Dnmt gene have been shown to cause ICF syndrome, a rare disorder characterized by immunodeficiency, centromere instability and facial anomalies [5,9,10]. Misexpression of Dnmts has also been reported for various kinds of cancer [11–13].

Dnmts have been highly conserved during evolution and can be found in numerous organisms, ranging from bacteria to man. Eukaryotic Dnmts are known to methylate DNA only at the cytosine 5-position. All DNA (cytosine-5) methyltransferases share a characteristic set of catalytic motifs [14]. These motifs are essential for enzymic function and are generally clustered in a catalytic domain of the protein. In addition, most eukaryotic Dnmts also contain a regulatory domain that mediates diverse functions, including protein–protein interactions [15,16]. The regulatory domains are not conserved between methyltransferases and might mediate the functional specificity of individual proteins.

In mammals, there are presently three known families of Dnmts. Dnmt1 appears to function as a maintenance Dnmt [17,18]. The protein is closely associated with the DNA replication machinery [15] and might be primarily required to copy DNA methylation patterns from the parental strand to the newly synthesized strand. Dnmt2 represents an enigmatic protein whose function has not yet been fully resolved [19]. Targeting of the mouse Dnmt2 gene had no detectable effect on retroviral DNA methylation [20]. However, mouse and human Dnmt2 proteins could be trapped by 5-azacytidine, which indicated an as yet undetected Dnmt activity [21]. Lastly, the Dnmt3 family consists of two closely related proteins, Dnmt3a and Dnmt3b [22]. Both enzymes have a clear preference for unmethylated DNA and have therefore been termed *de novo* methyltransferases [22].

A major characteristic of mammalian DNA methylation patterns is the specificity for CpG dinucleotides. Whereas symmetrical CpG methylation ensures heritability of the DNA methylation signal via maintenance methylation, other organisms show a significant level of non-CpG methylation. Non-CpG methylation has also been shown to be prevalent during early mouse development and it has been suggested that it might be mediated by Dnmt3a [23]. In addition, both Dnmt3a and Dnmt3b were shown to methylate non-CpG dinucleotides *in vitro* [24,25]. Since mammalian DNA methylation patterns are established and maintained by co-operative interactions between individual Dnmts, it is difficult to determine the activity of individual methyltransferase enzymes *in vivo*.

We have previously analysed the function of the mouse Dnmt1 and Dnmt3a proteins in transgenic *Drosophila*. Transgenic flies represent a powerful tool for a direct analysis of Dnmt activities, because the level of endogenous DNA methylation is negligible in post-embryonic stages [17,26]. Thus the activity of ectopically expressed Dnmts can be analysed directly and without interference from endogenous DNA methylation. In our previous experiments, we have demonstrated a *de novo* methyltransferase activity of the Dnmt3a enzyme and also suggested a specialized maintenance activity of Dnmt1 [17]. We have now extended this analysis to also include Dnmt2 and the three major isoforms of Dnmt3b. This allowed a comparative analysis of all known mammalian Dnmts under *in vivo* conditions. We detected robust *de novo* methylation activity for Dnmt3a, Dnmt3b1 and Dnmt3b2. Dnmt2 showed a weaker, but significant, activity. Bisulphite

Abbreviations used: Dnmt, DNA methyltransferase; UAS, upstream activating sequence.

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sequencing of genomic DNA also revealed different methylation patterns for individual Dnmt proteins. Our results thus provide detailed insights into the activities of mammalian Dnmts.

EXPERIMENTAL

Transgenic fly strains

The cDNAs of mouse Dnmt2, Dnmt3b1, Dnmt3b2 and Dnmt3b3 were subcloned into the pUAST (where UAS stands for upstream activating sequence) vector and transgenic flies were established using P-element-mediated germline transformation under standard conditions. For every construct, several independent strains were established. Flies overexpressing Dnmt1 and Dnmt3a in the same experimental system have been described previously [17]. For the induction of Dnmt expression, UAS-Dnmt males were crossed with hs-GAL4 females. To avoid potential bacterial contaminations, all fly crosses were kept on standard food supplemented with tetracycline (0.25 mg/ml). For immunodetection of Dnmt proteins, flies were raised at 28 °C. For DNA methylation analysis, crosses were incubated at 28 °C and whole culture vials were heat-shocked at 37 °C for 1 h/day as soon as the first larvae had reached the third instar stage. This heat-shock treatment effectively increased the overall amount of genomic DNA methylation. At 5 days after the first heat shock, adult flies were collected and their DNA was prepared with DNAzol Reagent (Invitrogen), according to the manufacturer's instructions.

Immunodetection of overexpressed Dnmt proteins

The antibody against Dnmt1 has been described previously [27]. The anti-Dnmt2 antibody was raised by standard immunization of rabbits with a mixture of three keyhole-limpet-haemocyanin-coupled peptides (ANEVYKHNFPHTHLLC, CAESSSTQSSGKDTILFKLE and CFPPEFGFPEKTTVKQRYRL; Peptide Specialty Laboratories, Heidelberg, Germany). Antibodies against Dnmt3a and Dnmt3b were raised by immunizing rabbits with the purified proteins. Protein overexpression was confirmed by Western-blot analysis of five pairs of isolated salivary glands that had been boiled in loading buffer before separation by standard SDS gel electrophoresin. Immunostaining of third instar larval salivary gland chromosomes was performed as described previously [28].

Capillary electrophoretic analysis of genomic DNA

Genomic DNA methylation levels were determined by capillary electrophoretic analysis, as described previously [29]. All measurements were repeated at least three times and the results were found to be strictly reproducible.

Bisulphite genomic shotgun sequencing

Genomic DNA was subjected to bisulphite sequencing as described previously [23]. Briefly, 1 μg of DNA was digested with *Rsa*I and ligated to *Eco*RI linkers containing five cytosine residues. The ligated DNA was then deaminated with sodium bisulphite and amplified with 18-mer PCR primers corresponding to the deaminated linker sequence. Bulk PCR products in the 200–300 bp range were excised from an agarose gel, purified and cloned using the TopoTA cloning kit from Invitrogen. Sequences of cloned fragments were verified by alignment with a deaminated version of the *Drosophila* genome sequence and only fragments with an extensive alignment were used for further analysis. A precise evaluation of DNA methylation patterns was performed with alignments with the non-deaminated *Drosophila* genome sequence. Only 5-methylcytosine residues with a matching cytosine residue in the *Drosophila* genome sequence were considered methylated. The presence of 5-methylcytosine residues was always confirmed by sequencing from the opposite strand.

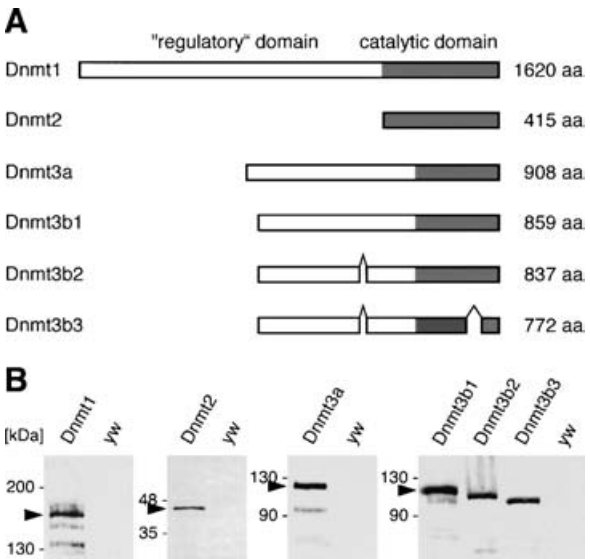

Figure 1 Overexpression of mouse Dnmts in *Drosophila*

(**A**) Schematic illustration of known mouse Dnmts. The conserved C-terminal catalytic domain is shaded in grey. (**B**) Confirmation of protein overexpression by Western-blot analysis. Specific protein bands were detected at expected molecular-mass ranges for all Dnmts analysed (arrowheads). No signals were detected in control extracts from flies not overexpressing any Dnmt (yw). Smaller bands for Dnmt1, Dnmt3a and Dnmt3b presumably represent degradation products.

RESULTS

For a comparative analysis of Dnmt activity, we used a transgenic system that allows for GAL4-dependent overexpression of Dnmts. cDNAs from all known mouse Dnmts were subcloned into a vector that can be transactivated by the yeast GAL4 protein [30]. Using P-element-mediated germline transformation, we then established various independent transgenic fly lines that allowed for the separate overexpression of Dnmt1, Dnmt2 and Dnmt3a (Figure 1A). In addition, we also established fly strains for the overexpression of each of the three major isoforms of Dnmt3b [22]: Dnmt3b1 encodes the full-length protein, Dnmt3b2 lacks amino acid residues 363–382 of the N-terminal 'regulatory' domain and Dnmt3b3 additionally lacks amino acid residues 731–813 of the C-terminal catalytic methyltransferase domain [22] (Figure 1A). For each construct, at least two independent strains were established and analysed in subsequent experiments. Protein overexpression was induced by crossing UAS-Dnmt strains to hs-GAL4 strains and keeping the flies at 28 °C. This results in a ubiquitous, but non-lethal, overexpression in the offspring (results not shown). Overexpression of Dnmts was confirmed by Western-blot analysis using Dnmt-specific antibodies (see the Experimental section for details). Distinct bands were detected at expected molecular-mass ranges in protein extracts from Dnmt-expressing flies but not in extracts from control flies (Figure 1B). This confirmed the establishment of transgenic strains for inducible overexpression of all known murine Dnmts.

We also analysed the chromosomal association of ectopically expressed Dnmt proteins. In a first experiment, we prepared

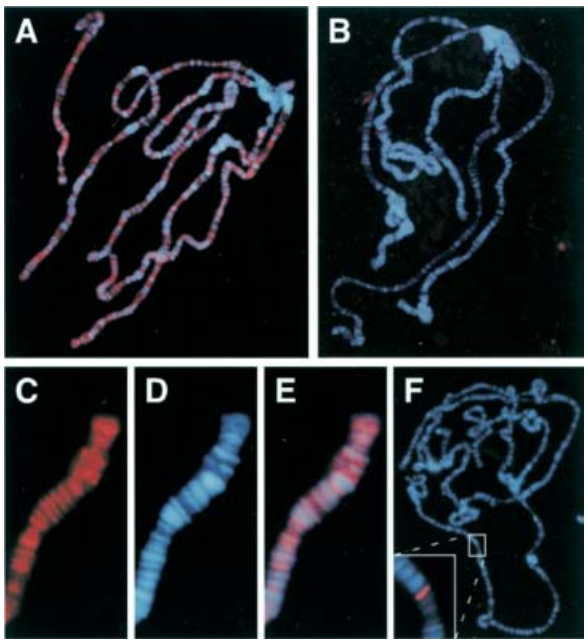

Figure 2 Association of mouse Dnmts with *Drosophila* polytene chromosomes

Exemplary results from stainings are shown with a Dnmt3b-specific antibody (red); DNA is stained blue. Staining patterns for Dnmt1, Dnmt2, Dnmt3a, Dnmt3b2 and Dnmt3b3 were found to be identical (results not shown). (**A**) Dnmt3b associates with numerous loci on polytene chromosomes. (**B**) Only background signals were detected on chromosomes from flies not overexpressing Dnmt3b. (**C**, **D**) Higher magnification images showing the association of Dnmt3b with interbands. (**E**) In contrast with ectopically expressed Dnmts, ectopically expressed GAL4 protein was found to be associated with a single locus on polytene chromosomes that presumably represents the integration site of the UAS-Dnmt3b transgene.

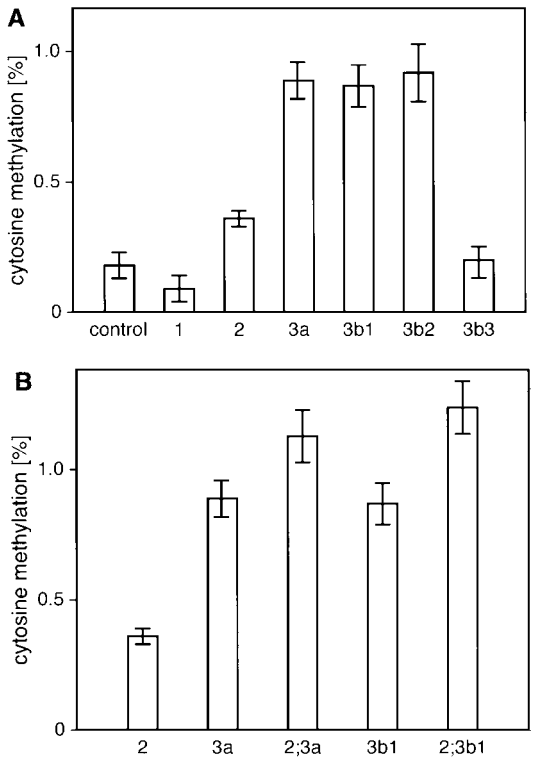

Figure 3 Genomic DNA methylation levels induced by overexpression of mouse Dnmts

Cytosine methylation levels were determined by capillary electrophoretic analysis. The non-transgenic w^{1118} strain was used as a control; levels of 0.2 % and below were considered as background. (**A**) Quantitative evaluation of electropherograms revealed comparable Dnmt activities for Dnmt3a (3a), Dnmt3b1 (3b1) and Dnmt3b2 (3b2). Overexpression of Dnmt2 (2) resulted in a less pronounced hypermethylation of genomic DNA. The difference in methylation levels between Dnmt3a-overexpressing flies and control flies was found to be highly significant ($P < 0.01$), as determined by a Student's *t* test. Dnmt1 (1) and Dnmt3b3 (3b3) caused no detectable hypermethylation. (**B**) Confirmation of Dnmt2 activity in double-transgenic flies. Cytosine methylation levels were determined in double-transgenic fly lines overexpressing Dnmt2 in combination with Dnmt3a or Dnmt3b1. The cytosine methylation level of corresponding single-transgenic lines was determined in parallel experiments. This revealed that co-expression of Dnmt2 resulted in a significant increase of DNA methylation ($P < 0.01$, as determined by a Student's *t* test).

polytene chromosomes from third instar larval salivary glands overexpressing Dnmt3b1. Polytene chromosomes are generated by several rounds of DNA replication without separation of the chromatids. Therefore the distribution of a chromatin-associated protein can be visualized at high resolution. Immunostaining with a specific antibody against Dnmt3b revealed a complex staining pattern of several hundred euchromatic bands and a weak staining of centromeric heterochromatin (Figure 2A). No chromosome staining was observed in controls that did not overexpress the Dnmt3b1 protein (Figure 2B). Subsequently, we also performed chromosome stainings for flies overexpressing Dnmt1, Dnmt2, Dnmt3a, Dnmt3b2 and Dnmt3b3. This revealed a seemingly identical staining pattern for all Dnmts (results not shown). A closer examination of immunostained chromosomes revealed that Dnmts were always associated with interbands of polytene chromosomes (Figures 2C–2E). Interbands represent polytene chromosomal areas that are less intensely stained by 4,6-diamidino-2-phenylindole and contain less-condensed chromatin fibres [31]. In addition, interbands have also been shown to contain hypoacetylated histones and the SIN3–RPD3 histone deacetylase complex [32]. Association of Dnmts with interbands appeared to be a specific characteristic of the proteins, since it is not usually observed with other overexpressed proteins. This was confirmed by immunostaining of polytene chromosomes with an antibody raised against the yeast GAL4 protein. Ectopically expressed GAL4 mediates the transactivation of UAS-Dnmt transgenes in our experimental system. Consistently, immunostained polytene chromosomes revealed a single GAL4 signal (Figure 2F) that presumably corresponded to the integration site of the UAS-Dnmt transgene. In conclusion, our results thus suggest a defined chromosomal association pattern for mouse Dnmts in *Drosophila*. This pattern was found to be identical for all Dnmts.

In the next step, we determined the level of genomic cytosine methylation in Dnmt-overexpressing flies. To this end, genomic DNA was prepared from adult flies and the level of cytosine methylation was quantified. Genomic DNA was hydrolysed to single nucleotides and derivatized with a fluorescent marker. The derivatized mixture was then separated by capillary electrophoresis and analysed through laser-induced fluorescence. This revealed readily detectable amounts of cytosine methylation in flies overexpressing Dnmt3a, Dnmt3b1 and Dnmt3b2 (Figure 3A). Surprisingly, overexpression of Dnmt2 also resulted in low but significant levels of DNA hypermethylation (Figure 3A). Only background levels of genomic 5-methylcytosine were detected in DNA preparations from control flies not overexpressing any Dnmt and from flies overexpressing Dnmt1 or Dnmt3b3 (Figure 3A). These results confirmed the *de novo* methylation activity of Dnmt3a, Dnmt3b1 and Dnmt3b2 under *in vivo* conditions. No *de novo* methylation activity could be detected for Dnmt3b3, which is in agreement with previous results from *in vitro* assays [24]. In addition, our results also suggested a low Dnmt activity for Dnmt2.

Figure 4 DNA methylation tract analysis

Genomic DNA was subjected to shotgun bisulphite sequencing. Sequences were then aligned with the corresponding regions of the *Drosophila* genome sequence and the methylation status of cytosine residues was determined. Only clones with at least one methylated cytosine residue are shown. ●, Methylated cytosine residues; ○, unmethylated cytosine residues. Different colours indicate different dinucleotide contexts of cytosine residues. Cytosine residues from flanking linker fragments were always completely deaminated and reproducible DNA methylation patterns were found in several independent experiments. Thus the possibility of bisulphite sequencing artifacts could be largely excluded.

To confirm a potential Dnmt activity of Dnmt2, we also generated double-transgenic fly strains that expressed Dnmt2 in combination with either Dnmt3a or Dnmt3b1. Protein overexpression was induced under identical conditions as for single transgenics and genomic DNA was prepared and subjected to capillary electrophoretic analysis. This revealed that Dnmt2 mediates a detectable increase in DNA methylation levels in all cases (Figure 3B). Genomic cytosine methylation levels of Dnmt2-expressing double-transgenic strains were consistently 0.4% higher than in corresponding single-transgenic strains expressing either Dnmt3a or Dnmt3b1 (Figure 3B). This increase corresponds well with the overall cytosine methylation level detected in flies overexpressing Dnmt2 only and thus provides further evidence for an enzymic activity of Dnmt2.

For a detailed analysis of methylated sequences, we subjected genomic DNA from Dnmt-overexpressing flies to bisulphite sequencing. DNA was digested with the restriction enzyme *Rsa*I and restriction fragments were ligated to linker fragments. Ligated fragments were subsequently deaminated with sodium bisulphite and then PCR-amplified using primers directed against the deaminated linkers. Lastly, PCR fragments were isolated, cloned and sequenced. After this procedure, only methylated cytosine residues were sequenced as cytosine residues, whereas unmethylated cytosine residues were converted into thymines. A BLAST alignment with the *Drosophila* genome sequence then allowed for the precise determination of the cytosine methylation state. This procedure was used to analyse genomic DNA from flies overexpressing Dnmt2, Dnmt3a, Dnmt3b1 as well as from control flies. No 5-methylcytosine was found in controls, whereas overexpression of Dnmt2, Dnmt3a and Dnmt3b1 resulted in detectable cytosine methylation (Table 1). The corresponding cytosine methylation levels were in good agreement with those obtained by capillary electrophoretic analysis (Figure 3). The higher methylation level for Dnmt3a was caused by a single, densely methylated clone from the bisulphite analysis (see Figure 4).

To analyse the sequence specificity of mouse Dnmts, we also determined the sequence context of methylated cytosine residues. Generally, methylated DNA fragments were found at various euchromatic locations with no detectable preference for particular regulatory elements, such as gene promoters or enhancers (results not shown). Heterochromatic sequences were not retrieved in any experiment, which is probably due to the inefficient cloning of repetitive elements in our bisulphite sequencing procedure. Consistent with our previous results [26], DNA from adult control flies not overexpressing any Dnmt was found to be unmethylated (Table 1). Methylation tract analysis from flies overexpressing Dnmt2 revealed several isolated 5-methylcytosine residues (Figure 4). Interestingly, all of these residues were found in the context of non-CpG dinucleotides. In contrast, the methylation pattern induced by Dnmt3a and Dnmt3b looked strikingly different. DNA methylation tract analysis showed a preferential methylation of CpG dinucleotides (Figure 4). To a lesser extent, we also observed methylation at non-CpG dinucleotides, particularly at CpA sites (Figure 4). Interestingly, methylated cytosine residues were frequently clustered in individual fragments (Figure 4). Our results thus allow a direct comparison of mammalian Dnmt activities and provide detailed insights into the methylation patterns established by individual enzymes.

Table 1 DNA methylation analysis by genomic bisulphite sequencing

The non-transgenic w^{1118} strain was used for controls; the full-length Dnmt3b1 isoform was used for the analysis of Dnmt3b-mediated methylation. The methylation level was calculated by dividing the number of methylated cytosine residues by the sum of unmethylated plus methylated cytosine residues.

	No. of clones sequenced	No. of methylated clones	No. of cytosine residues sequenced	No. of 5-methylcytosine residues sequenced	Methylation level (%)
Control	15	0	360	0	0.0
Dnmt2	23	4	458	5	1.1
Dnmt3a	20	6	462	19	4.0
Dnmt3b	17	2	500	6	1.2

DISCUSSION

Mammalian DNA methylation patterns are currently being characterized in great detail. At the same time, only little is known

about the activity and specificity of individual methyltransferases. DNA methylation patterns of a differentiated cell are established by the interdependent activities of several enzymes. Overlapping activities have made it difficult to define their function by conventional gene targeting. We have used *Drosophila* as a model system because we could analyse the enzymic activity of individual Dnmts *in vivo* and with negligible amounts of background methylation. We have compared the activities of Dnmt1, Dnmt2, Dnmt3a, Dnmt3b1, Dnmt3b2 and Dnmt3b3 and our results revealed distinct activity patterns for most of these proteins.

Interestingly, the chromosomal association of Dnmts seemed to be highly similar for all proteins. Analysis of immunostained polytene chromosomes revealed a consistent localization of Dnmts to interbands. While the function of polytene chromosome banding patterns remains to be resolved, interbands have been shown to contain relatively less condensed chromatin [31]. An interband-specific polytene chromosome staining pattern association has also been observed for hypoacetylated histones H3 and H4 and for the SIN3–RPD3 histone deacetylase complex and has been interpreted to represent a specific marker of repressed genes in transcriptionally active regions [32]. DNA methylation has also been linked to the mammalian Sin3–histone deacetylase complex via the methyl-DNA binding protein MeCP2 [33,34]. In addition, it has also been shown that Dnmt3a interacts with mammalian histone deacetylases [16]. It is possible that the interaction between some of the relevant epigenetic factors has been conserved between flies and mammals. Because the association between Dnmts and interbands was seen for all Dnmt proteins, it is probably mediated by the conserved catalytic domain, rather than by the highly divergent N-terminal domains.

Consistent with previous results we did not observe any detectable *de novo* methylation activity of Dnmt1, although the Dnmt1 protein overexpressed in flies shows a high Dnmt activity *in vitro* [17]. The maintenance methylation activity of Dnmt1 could not be analysed in our assay because it did not allow for an independent regulation of two or more transgenes. However, a predominant maintenance function of human DNMT1 has also been confirmed in recent experiments with cultured cell lines [18].

Our results also provide direct evidence for a Dnmt activity of Dnmt2. It has been previously concluded that Dnmt2 might not function as an active Dnmt but rather could play a role in centromere organization [35]. However, the strong conservation of catalytic Dnmt motifs combined with the conservation of a three-dimensional Dnmt structure [35] have always suggested an as yet undetected activity. Our experiments revealed a weaker but significant activity that we characterized further by bisulphite sequencing of genomic DNA fragments. The observed activity is different from that of Dnmt3a and Dnmt3b enzymes (see below) and apparently restricted to non-CpG dinucleotides. This is consistent with our characterization of the *Drosophila* Dnmt2 enzyme that revealed specific methylation at isolated CpT and CpA dinucleotides [36]. The function of Dnmt2-mediated methylation still remains to be determined. The absence of significant phenotypes in Dnmt2 mutant mice and embryonic stem cells [20] could argue for a more limited role in the generation of mammalian DNA methylation patterns.

The methylation tracts in *Drosophila* did not directly correspond to the methylation patterns found in vertebrates, where DNA methylation has been shown to be clustured in CpG islands. A plausible explanation is the interplay of several methyltransferases in mammals. Additionally, the absence of conventional CpG islands in the *Drosophila* genome might also contribute to reduced density of DNA methylation in transgenic flies. Regardless of these differences, our results provided novel insights into the activity of Dnmt3 enzymes. (i) Capillary electrophoretic analysis demonstrated an active Dnmt activity of Dnmt3b2 under *in vivo* conditions. This is in agreement with previous results obtained with *in vitro* assays [24,37]. (ii) The Dnmt3b3 isoform, which lacks part of the catalytic domain, did not show any detectable activity and is probably not an active Dnmt. (iii) Based on chromatographic analyses and *in vitro* assays, it has been suggested that Dnmt3 enzymes might also methylate a low amount of non-CpG dinucleotides [23–25,38]. Consistently, our results demonstrated predominant CpG methylation, but also showed readily detectable non-CpG methylation, particularly at CpA dinucleotides. (iv) Several DNA fragments from Dnmt3-overexpressing flies showed clustered methylation of associated cytosine residues. This might indicate a processive, rather than distributive, activity of these enzymes under *in vivo* conditions. Our results thus confirm previous findings from *in vitro* systems and also provide additional insights into the characteristics of mammalian Dnmts.

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