A Dnmt2-like protein mediates DNA methylation in *Drosophila*

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Summary

The methylation status of *Drosophila* DNA has been discussed controversially over a long time. Recent evidence has provided strong support for the existence of 5-methylcytosine in DNA preparations from embryonic stages of fly development. The *Drosophila* genome contains a single candidate DNA methyltransferase gene that has been termed *Dnmt2*. This gene belongs to a widely conserved family of putative DNA methyltransferases. However, no catalytic activity has been demonstrated for any Dnmt2-like protein yet. We have now established a protocol for the immunological detection of methylated cytosine in fly embryos. Confocal analysis of immunostained embryos provided direct evidence for the methylation of embryonic DNA. In order to analyse the function of Dnmt2 in DNA methylation, we depleted the protein by RNA interference. Depletion of Dnmt2 had no detectable effect on embryonic development and resulted in a complete loss of DNA methylation. Consistently, overexpression of Dnmt2 from an inducible transgene resulted in significant genomic hypermethylation at CpT and CpA dinucleotides. These results demonstrate that Dnmt2 is both necessary and sufficient for DNA methylation in *Drosophila* and suggest a novel CpT/A-specific DNA methyltransferase activity for Dnmt2 proteins.

Key words: DNA methylation, *Drosophila*, DNA methyltransferase, Dnmt2, Su(var)3-9

Introduction

Most organisms methylate their genomic DNA at cytosine residues (Bird, 2002). This modification has been linked to various aspects of epigenetic regulation (Jackson-Grusby et al., 2001; Miura et al., 2001; Stancheva and Meehan, 2000; Walsh et al., 1998). DNA methylation is essential for mammalian development (Li et al., 1992) and also plays an important role in tumourigenesis and other diseases (Jones and Baylin, 2002; Robertson and Wolffe, 2000). The majority of mammalian DNA methylation occurs at CpG dinucleotides, and involves the activities of several DNA methyltransferases and their associated factors (Li, 2002).

Based on sequence homology, animal DNA methyltransferases can be subdivided into three families: Dnmt1, Dnmt2 and Dnmt3 (Colot and Rossignol, 1999). Because of their substrate preference for hemimethylated CpG dinucleotides (Bestor and Ingram, 1983; Gruenbaum et al., 1982), Dnmt1 enzymes are generally regarded as maintenance methyltransferases. Accordingly, it has been suggested that the primary 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. The function of the second family of DNA methyltransferases, Dnmt2, has been enigmatic for a long time. Direct evidence for a catalytic activity could not be provided yet (Okano et al., 1998a) and it has been suggested that Dnmt2 proteins might not function as DNA methyltransferases (Dong et al., 2001). However, more recent data indicated a weaker DNA methyltransferase activity of Dnmt2 in mouse and human cells (Liu 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., 1998b). 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.

The question of DNA methylation in *Drosophila* has been discussed controversially for a long period of time. There are several reports that demonstrate the absence of 5-methylcytosine from pupal and adult stages of fly development (Patel and Gopinathan, 1987; Tweedie et al., 1999). In addition, it has also been shown that DNA from *Drosophila* embryos is largely unmethylated at CpG dinucleotides (Urieli-Shoval et al., 1982). This is an apparent contradiction to two more recent reports that provided evidence for low levels of DNA methylation both in embryos and in adults (Gowher et al., 2000; Lyko et al., 2000b). The contradiction could be partially resolved by analysing the sequence context of 5-methylcytosine in the fly genome (Lyko et al., 2000b). This confirmed the virtual absence of CpG methylation in *Drosophila*, but also provided strong evidence for low amounts of non-CpG methylation in embryos (Lyko et al., 2000b).

The enzyme that mediates DNA methylation in the fly has remained unknown. The *Drosophila* genome contains a single candidate DNA methyltransferase gene (Hung et al., 1999; Lyko, 2001; Tweedie et al., 1999). This gene (Mt2 – FlyBase) belongs to the widely conserved Dnmt2 family of putative DNA methyltransferases with known homologues in humans, mice, insects and fungi (Dong et al., 2001; Okano et al., 1998a; Wilkinson et al., 1995; Yoder and Bestor, 1998). *Drosophila*

Dnmt2 (like other Dnmt2 proteins) contains all the catalytic signature motifs of active DNA methyltransferases. In addition, the structure of human DNMT2 is highly similar to active bacterial DNA methyltransferases (Dong et al., 2001). However, all attempts to demonstrate a catalytic activity of Dnmt2-like proteins have failed so far, and targeting of the mouse *Dnmt2* locus showed no detectable effects on DNA methylation (Okano et al., 1998a). Similarly, *Drosophila* Dnmt2 failed to reveal any activity in standard in vitro methylation assays (Tweedie et al., 1999). Thus, the function of Dnmt2 proteins has remained enigmatic (Bestor, 2000).

In order to characterize *Drosophila* DNA methylation in higher detail we established a protocol for the immunodetection of 5-methylcytosine in embryos. Confocal analysis of stained embryos confirmed the presence of 5-methylcytosine in embryonic genomes. To analyse the function of Dnmt2, we depleted the protein by RNA interference. This demonstrated that Dnmt2 is essential for DNA methylation in *Drosophila* embryos. Additional experiments showed that overexpression of Dnmt2 resulted in significant genomic hypermethylation at CpT and CpA dinucleotides. Our results thus demonstrate that DNA methylation in *Drosophila* is mediated by Dnmt2.

Materials and methods

Immunodetection of 5-methylcytosine

A more detailed version of our embryo immunostaining protocol can be found elsewhere (Marhold et al., 2002). Briefly, wild-type Oregon R embryos were collected from a population cage. To analyse the effect of *Su(var)3-9* mutations on embryonic DNA methylation, we used the strains *Su(var)3-9*^{06} (Schotta et al., 2002) and *Su(var)3-9*^{19} (a kind gift from G. Reuter). Collected embryos were dechorionated, fixed, permeabilized and denatured for 2 hours in 2 M HCl. After washing and blocking, embryos were subsequently stained with an affinity-purified rabbit polyclonal antibody against 5-methylcytosine (Megabase Research Products, 1:200) and with a human polyclonal antibody against DNA (NatuTec, 1:200). After mounting, embryos were analyzed by confocal microscopy. For pharmacological inhibition of DNA methylation, dechorionated embryos were incubated for 2 hours in 10 mM 5-azacytidine (Sigma) in PBS and subsequently fixed and stained. Slot blot analysis was performed with a Bio-Dot SF microfiltration apparatus (BioRad), according to the manufacturer's instructions. We used purified genomic DNA from calf thymus (Sigma), *S. cerevisiae* (strain AH109) and *Drosophila* (0-6 hours Oregon R embryos).

Dnmt2 protein analysis

The Dnmt2-specific antiserum was raised by standard immunization of rabbits with a mixture of three KLH-coupled peptides (YAHNYGSNLVKTRNC, CQPHTRQGLQRDTEDK and CDTSNQDASKSEKILQ; Peptide Specialty Laboratories, Heidelberg, Germany). For developmental western blots, protein extracts were prepared from various developmental stages of wild-type flies. Equal amounts of protein were separated on a 10% SDS-polyacrylamide gel and blotted using standard procedures. A specific antibody against ribosomal protein P40 was used for loading controls (Torok et al., 1999). For the confocal analysis of subcellular Dnmt2 distribution, wild-type embryos were stained with our Dnmt2-specific antiserum (1:200).

Depletion of Dnmt2 by RNAi

Double-stranded Dnmt2 RNA was prepared by in vitro transcription of a Dnmt2 cDNA clone (Lyko et al., 2000a) using Megascript kits from Ambion. For controls, we synthesized double-stranded RNA from an EST clone (CK00414) (Kopczynski et al., 1998) of the CG11840 gene. Annealing of complementary RNA strands was verified by agarose gel electrophoresis. For microinjection, wild-type embryos were collected over 30 minutes at 18°C and directly injected under hydrocarbon oil. Development was closely monitored through the adult stage. For DNA methylation analysis, series of about 200 embryos were injected at 18°C and subsequently aged for 3 hours at 25°C and then dechorionated, fixed, permeabilized and stained as described above. All experiments were repeated several times and the results were found to be strictly reproducible.

Overexpression of Dnmt2

We generated the transgenic construct for GAL4-inducible Dnmt2 overexpression by subcloning a Dnmt2 cDNA into the pUAST vector (Brand and Perrimon, 1993). Several independent *UAS-Dnmt2* strains were generated by *P*-element mediated transformation using standard procedures and *w*^{1118} as host. For Dnmt2 overexpression, *UAS-Dnmt2* females were crossed to *hs-GAL4* (Brand et al., 1994) males, and offspring were heat shocked daily for 1 hour at 37°C in a water bath. For controls, we used *w*^{1118} instead of *UAS-Dnmt2* females. Protein overexpression was confirmed by western blotting using our Dnmt2 antiserum. For DNA methylation analysis, genomic DNA was isolated from 10-day-old adult flies. DNA samples were hydrolysed, derivatized and analyzed by capillary electrophoresis as described previously (Stach et al., 2003). Samples from independent DNA preparations were measured at least three times, and the results were found to be strictly reproducible. To minimize the possibility of bacterial contaminations, fly food was supplemented with tetracycline (0.25 mg/ml) (Holden et al., 1993) in all experiments.

Bisulfite genomic shotgun sequencing

Genomic DNA from adult flies overexpressing Dnmt2 and from control flies (see above) was subjected to bisulfite sequencing as described previously (Ramsahoye et al., 2000). Sequences were aligned to the *Drosophila* genome sequence and only sequences with an extensive alignment were used for analysis. To reduce the number of potential experimental artefacts further, only 5-methylcytosine residues with a matching cytosine residue in the *Drosophila* genome sequence were considered methylated. The presence of a methylated cytosine residue was always confirmed by sequencing from both strands.

Results

The presence of DNA methylation in *Drosophila* genomic DNA has been demonstrated by three independent methods. A sensitive chromatographic approach allowed the detection of 5-methylcytosine in embryonic DNA preparations and to a lesser extent in DNA preparations from adults (Gowher et al., 2000). Similar results were obtained by the use of nearest neighbour analysis and thin layer chromatography (Lyko et al., 2000b). Methylation of *Drosophila* embryonic DNA was also demonstrated by bisulfite sequencing, which confirmed the presence of 5-methylcytosine in its natural sequence context (Lyko et al., 2000b). However, the level of genomic cytosine methylation was rather low (<0.4%) in all experiments. This could either reflect a low overall density of 5-methylcytosine or a fractional distribution, as described for primitive vertebrates (Tweedie et al., 1997).

The characterization of *Drosophila* DNA methylation patterns by biochemical or molecular methods has been precluded by the low overall methylation level and the small amount of DNA that can be extracted from early embryonic stages. To confirm the methylation of *Drosophila* genomic

DNA by an independent method and to address the distribution of methylated DNA in the fly genome, we established a protocol for the immunological detection of 5-methylcytosine. Similar protocols have recently proved very useful for the analysis of DNA methylation during early vertebrate development (Dean et al., 2001; Mayer et al., 2000). Overnight collections of wild-type embryos were obtained from population cages; they were washed, fixed, treated with RNase and then extensively denatured in 2 M HCl. This procedure leaves only DNA intact for immunostaining and eliminates other potentially crossreacting epitopes. Subsequently, embryo preparations were double stained with antibodies that specifically recognize 5-methylcytosine and DNA, respectively. The use of the DNA-specific antibody also provided us with a valuable internal control for our staining procedure. Analysis of the developmental 5-methylcytosine pattern by confocal microscopy revealed a weak signal in early cleavage embryos (Fig. 1A). During syncytial blastoderm, the distribution of 5-methylcytosine became more inhomogeneous (data not shown). The intensity of the 5-methylcytosine signal appeared strongest in cellular blastoderm embryos (Fig. 1B). Later stages of embryonic development still showed detectable DNA methylation, but the intensity of the signal appeared to be reduced (Fig. 1C). Interestingly, pole cells also revealed clear 5-methylcytosine staining (Fig. 1D), which indicated the presence of DNA methylation in the germline. Our results thus confirm previous observations that DNA methylation is most prevalent during earlier stages of *Drosophila* embryogenesis (Lyko et al., 2000b). In addition, they suggest a fractional distribution of 5-methylcytosine during the blastoderm stages.

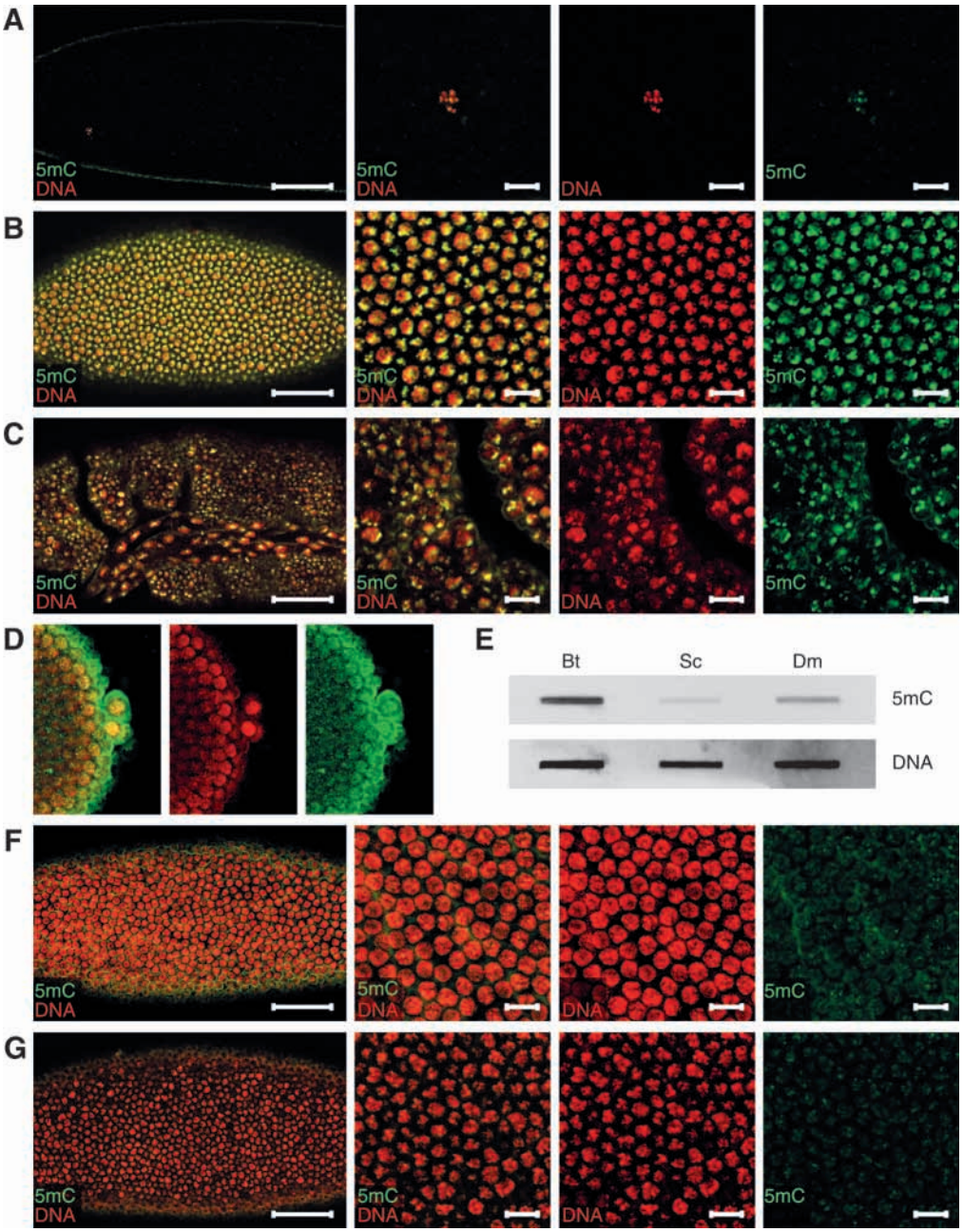

Fig. 1. Immunological detection of 5-methylcytosine in *Drosophila* embryos. Embryos were double stained with an antibody that specifically recognizes 5-methylcytosine (5mC, green) and with an antibody that stains DNA (red). The left panel shows images from confocal sections of whole embryos. Scale bars: 50 μm. The right panels show an enlargement from the same embryos. Scale bars in enlarged pictures: 10 μm. (A) Early cleavage stage embryo; (B) cellular blastoderm stage embryo; (C) post-gastrulation stage embryo. (D) Pole cells from blastoderm embryos showed a distinct staining for 5-methylcytosine. (E) The specificity of the 5-methylcytosine antibody was confirmed by slot blot analysis of methylated (*Bos taurus*, Bt) and unmethylated (*S. cerevisiae*, Sc) genomic DNA. DNA from 0-6 hour *Drosophila* embryos (Dm) showed an intermediate staining intensity. Staining with the DNA antibody indicated equal loading of all slots. (F) 5-methylcytosine staining was found to be greatly reduced in *Su(var)3-9* mutants (see text for details). (G) Incubation of dechorionated embryos with the DNA methyltransferase inhibitor 5-azacytidine reduced the 5-methylcytosine signal to background levels. The signal for DNA was not affected by the *Su(var)3-9* mutation or by 5-azacytidine.

In order to confirm the specificity of our antibodies we used slot blot analysis. Equal amounts of genomic DNA from calf thymus (methylated, positive control), *S. cerevisiae* (unmethylated, negative control) and *Drosophila* embryos were spotted onto a membrane and stained for 5-methylcytosine and DNA, respectively. This revealed a clearly differential staining pattern for 5-methylcytosine, while the DNA staining pattern was equal for all samples (Fig. 1E). As an additional control for the specificity of our immunostaining protocol, we also looked for mutations that could potentially affect embryonic methylation levels. Studies in *Neurospora* and *Arabidopsis* have shown that mutations in *Su(var)3-9* histone H3-K9 methyltransferase homologues result in reduced levels of DNA methylation (Jackson et al., 2002; Tamaru and Selker, 2001). In addition, we have shown recently that DNA hypermethylation phenotypes in *Drosophila* can be rescued by mutations in the *Su(var)3-9* gene (Weissmann et al., 2003), which suggested a preservation of this phenomenon in flies. The *Drosophila Su(var)3-9* gene has been shown to encode a major histone H3-K9 methyltransferase activity in fly embryos (Schotta et al., 2002). Null mutant fly strains are viable and fertile and can be maintained as homozygous mutant stocks (Tschiersch et al., 1994). To analyse the effect of *Su(var)3-9* mutations on DNA methylation in embryos, we obtained embryo collections from two different mutant strains and double stained them with antibodies against DNA and 5-methylcytosine, respectively. Consistent with results from other organisms (Jackson et al., 2002; Tamaru and Selker, 2001) this revealed an unambiguous reduction in DNA methylation (Fig. 1F) and confirmed that DNA methylation in *Drosophila* interacts with histone H3-K9 methylation (Weissmann et al., 2003). Finally, we also incubated wild-type embryos with the (cytosine-5) DNA methyltransferase inhibitor 5-azacytidine. This compound has been recently shown to trap both the human and the murine Dnmt2 homologues, respectively (Liu et al., 2003). Confocal analysis of immunostained embryos revealed a strong reduction of the 5-methylcytosine signal, while the signal for DNA was not affected (Fig. 1G). This result again underscored the specificity of our immunostaining protocol and demonstrated that DNA methylation in *Drosophila* can be inhibited by 5-azacytidine.

The primary candidate for a *Drosophila* DNA methyltransferase is the Dnmt2 protein. As an initial step towards the characterization of Dnmt2, we raised an antibody against the protein. We obtained a rabbit polyclonal antiserum that specifically recognizes a single band at ~45 kDa in western blots (Fig. 2A). This band was not observed with preimmune serum (data not shown) and the signal was strongly reduced upon depletion of Dnmt2 by RNA interference (see below). In order to analyse the developmental expression pattern of Dnmt2, protein extracts were prepared from all stages of *Drosophila* development and analyzed by western blotting. This demonstrated low but detectable levels of Dnmt2 in embryos (Fig. 2A). By contrast, larvae, pupae or adults contained only background levels of protein (Fig. 2A). The expression of Dnmt2 is therefore developmentally regulated. Our results are consistent with previous findings that described the presence of Dnmt2 mRNA in ovaries and in early embryos (Lyko et al., 2000a). Most of the mRNA from ovaries is probably not translated but rather deposited in embryos as a maternal component. The specificity of the antibody was further confirmed by analysis of protein extracts derived from adult flies overexpressing Dnmt2. Western blots revealed a strong ectopic band that co-migrated with the weaker, embryo-specific bands at ~45 kDa (Fig. 2A, right lane).

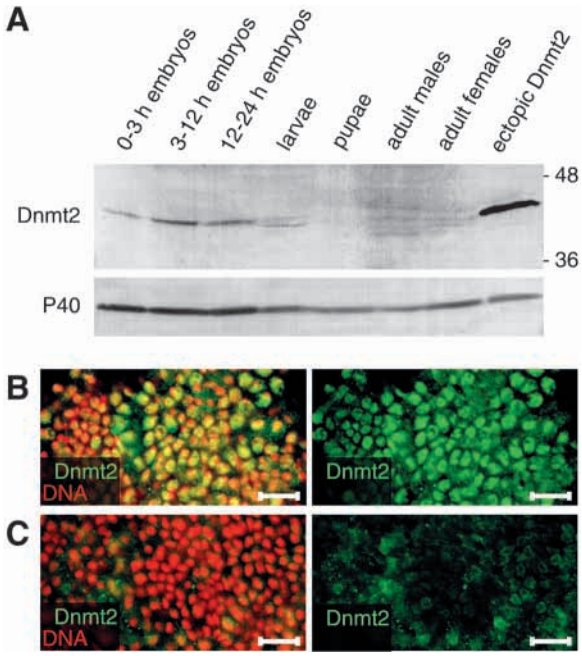

Fig. 2. Expression pattern of Dnmt2. (A) Protein extracts were prepared from the developmental stages indicated and analyzed by western blotting. Significant amounts of Dnmt2 protein were found only in embryos. The position of marker proteins (in kDa) is indicated. A protein extract from adult females overexpressing Dnmt2 was included as an additional control for the specificity of our antibody (right lane). P40 signals are shown to indicate equal loading of all lanes. (B) Subcellular Dnmt2 localization in embryos. Stained embryos were analyzed by confocal microscopy. The protein is green, DNA is coloured red. Scale bars: 10 μm. (C) Depletion of Dnmt2 by RNA interference. Double-stranded RNA was injected into syncytial blastoderm embryos and embryos were stained after a 3 hour staging period. This procedure strongly reduced the amount of Dnmt2 protein.

Next, we sought to analyse the subcellular localization of the Dnmt2 protein. Therefore we stained wild-type embryos with our Dnmt2 antibody and with propidium iodide to visualize DNA. Confocal analysis then revealed a nuclear localization of the Dnmt2 protein (Fig. 2B). To again confirm the specificity of our antibody, we depleted Dnmt2 by RNA interference (RNAi). Injection of double-stranded RNA (dsRNA) into *Drosophila* embryos has previously been shown to be an efficient tool for the generation of knock-down mutants (Kennerdell and Carthew, 1998). Dnmt2 dsRNA was synthesized by in vitro transcription and injected into syncytial blastoderm embryos. Three hours after injection, only background levels of Dnmt2 protein were detectable in embryos (Fig. 2C). This result underscores the specificity of our antibody and also provided an opportunity for a functional analysis of Dnmt2.

Because fly strains with a mutation in the *Dnmt2* gene are

not available, we used RNAi to analyse the function of Dnmt2. After injection of Dnmt2 dsRNA at the syncytial blastoderm stage, embryos were aged for 3 hours. This procedure efficiently removed most of the Dnmt2 protein from embryos (Fig. 2C). Double staining of dsRNA-injected embryos with antibodies against 5-methylcytosine and DNA revealed a strong reduction of 5-methylcytosine signals (Fig. 3A). The effect was specific for the 5-methylcytosine signal and the staining pattern for DNA was not affected (Fig. 3A). For controls, we injected embryos with dsRNA from an independent gene. CG11840 encodes a *Drosophila* signal peptide peptidase homologue (Weihofen et al., 2002) and has no conceivable function in epigenetic regulation. Embryos injected with CG11840 dsRNA showed the characteristic nuclear 5-methylcytosine staining pattern also observed in wild-type embryos (Fig. 3B). From these results, we concluded that Dnmt2 is required for embryonic DNA methylation. Interestingly, RNA interference of Dnmt2 appeared not to have a detectable effect on embryonic viability (Fig. 3C). Similarly, injection of a 5 μM solution of 5-azacytidine caused pronounced demethylation but showed only minimal effects on the survival rates of embryos (data not shown). By contrast, control injection with CG11840 dsRNA caused detectable embryonic lethality (Fig. 3C).

To confirm the DNA methyltransferase activity, we also established transgenic fly strains for ectopic overexpression of Dnmt2. Because post-embryonic stages of *Drosophila* development show very little endogenous DNA methylation (Lyko et al., 2000b) overexpression during later stages of development would provide a sensitive system with which to analyse the methyltransferase activity of the protein. To this end, transgenic *UAS-Dnmt2* flies were generated by *P*-element mediated germline transformation. Subsequently, several independent fly strains were established. Protein overexpression was then induced with a heat shock inducible transgene (*hs-GAL4*) and confirmed by western analysis (Fig. 4A). In order to analyse Dnmt2-mediated DNA methylation, we also used *UAS-Dnmt2* flies that had been induced with *hs-GAL4*. For controls, we set up parallel crosses with no *UAS-Dnmt2* transgene. To minimize the possibility of bacterial contamination, flies were raised on tetracycline-containing medium (Holden et al., 1993). Adult offspring flies were heat-shocked to induce transgene expression and their genomic DNA was prepared. DNA was then hydrolysed, derivatized and subjected to biochemical analysis by capillary electrophoresis (Stach et al., 2003). This method is highly specific for

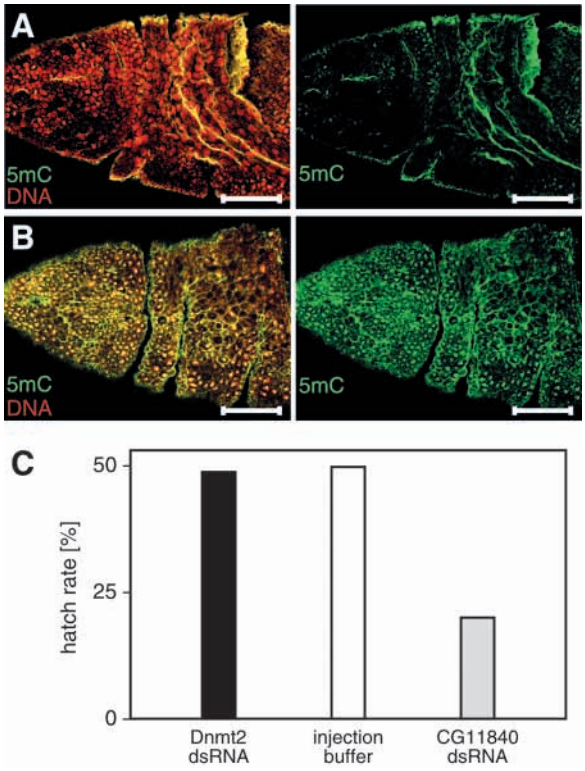

Fig. 3. Dnmt2 RNA interference results in loss of embryonic DNA methylation. Embryos were injected with Dnmt2 dsRNA and then staged, fixed and double stained with antibodies against 5-methylcytosine (5mC, green) and DNA (red). The left panel shows an overlay of both the 5-methylcytosine and the DNA signal, the right panel shows the 5-methylcytosine signal only. Scale bars: 50 μm. (A) Injection of Dnmt2 dsRNA resulted in a complete loss of nuclear 5-methylcytosine signals. The signal for DNA was not affected. (B) Injection of CG11840 dsRNA revealed a nuclear staining similar to wild-type embryos. (C) The effect of RNA interference on embryonic development. The percentage of hatching first instar larvae (hatch rate) did not differ significantly between embryos injected with Dnmt2 dsRNA (black bar) and embryos injected with injection buffer only (white bar). Embryos injected with CG11840 dsRNA showed detectable embryonic lethality (grey bar). For each experiment, about 300 embryos were analyzed.

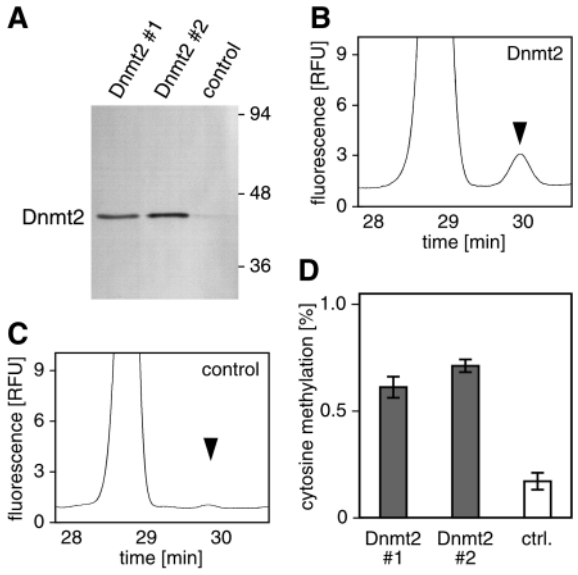

Fig. 4. Transgenic overexpression of Dnmt2 causes hypermethylation of the *Drosophila* genome. (A) Western analysis demonstrates significant levels of Dnmt2 protein in transgenic flies (left lanes). No protein was detectable in controls (right lane). (B) Genomic DNA from transgenic flies was analyzed by capillary electrophoresis. This revealed readily detectable amounts of 5-methylcytosine. (C) Very little 5-methylcytosine was present in matched control samples that did not overexpress Dnmt2. (D) The cytosine methylation level was quantified in several independent measurements from several independent experiments and the average was determined. Standard deviations are indicated by error bars.

deoxyribonucleotides and sufficiently sensitive to detect even low levels of DNA methylation (Stach et al., 2003). Our results demonstrated the unambiguous presence of 5-methylcytosine in the genomic DNA from Dnmt2-overexpressing flies (Fig. 4B). Significantly less 5-methylcytosine was detected in DNA from control flies (Fig. 4C). This result was confirmed in several independent crosses and in several independent measurements. The relative amount of cytosine methylation was then calculated from each individual measurement and mean values were determined. This revealed that overexpression of Dnmt2 results in a strong increase of genomic DNA methylation (Fig. 4D). The maximum cytosine methylation level induced by Dnmt2 overexpression was 0.7% (Fig. 4D). The cytosine methylation level of control flies was distinctly lower, but it still appeared to be significant (Fig. 4D). These results are in agreement with our earlier conclusions and confirm that DNA methylation in *Drosophila* is mediated by Dnmt2.

Finally, we used bisulfite genomic shotgun sequencing to analyse the target sequences of Dnmt2. Under appropriate conditions, sodium bisulfite converts cytosine, but not 5-methylcytosine, to uracil. Thus, all bases sequenced as cytosine actually represent 5-methylcytosine. We subjected genomic DNA from adult flies overexpressing Dnmt2 as well as from control flies to sodium bisulfite treatment and random fragments were cloned and sequenced. Sequences were analyzed by alignment to the *Drosophila* genome sequence and only sequences with a consistent alignment were used for further analysis. This left a total of 40 clones containing 5.6 kbp of *Drosophila* genomic DNA and eight 5-methylcytosine residues. Consistent with our results from capillary electrophoretic analysis, most of the 5-methylcytosine residues were found in DNA from Dnmt2-overexpressing flies (data not shown). The methylated sequences did not reveal an extended consensus sequence for Dnmt2-mediated DNA methylation (Fig. 5). However, all 5-methylcytosine residues were found in the context of CpT and CpA dinucleotides (Fig. 5). This is in agreement with earlier results obtained by chromatography (Lyko et al., 2000b) and suggests that Dnmt2 is a CpT/A-specific DNA methyltransferase.

Discussion

The presence of 5-methylcytosine in the *Drosophila* genome has been discussed controversially for a long time. We have recently provided definite evidence for DNA methylation in the fly (Lyko et al., 2000b), but the low overall methylation level raised additional questions. Using a sensitive protocol for the immunological detection of methylated DNA we have now confirmed the presence of 5-methylcytosine in *Drosophila* embryonic DNA using a completely independent method. In addition, we could also show that DNA methylation in *Drosophila* shares two characteristic features with DNA methylation in other organisms.

(1) Our experiments revealed a strong dependency on the Su(var)3-9 histone H3-K9 methyltransferase. A similar dependency has been recently described to involve the Su(var)3-9 homologues dim-5 in *Neurospora* (Tamaru and Selker, 2001) and KRYPTONITE in *Arabidopsis* (Jackson et al., 2002) and has been interpreted to reflect a role of DNA methylation in heterochromatin stability. Our observations are also consistent with our own previous results that demonstrated a strong effect of *Su(var)3-9* mutations on ectopically induced hypermethylation in the fly (Weissmann et al., 2003).

(2) DNA methyltransferase activity in *Drosophila* embryos could be effectively inhibited by 5-azacytidine. This compound functions as a suicide substrate for cytosine-5 DNA methyltransferases by covalently trapping the enzymes (Santi et al., 1984). It has been shown recently that 5-azacytidine also forms covalent bonds with the human and mouse Dnmt2 homologues (Liu et al., 2003). This result had provided the first indication for an active DNA methyltransferase function of Dnmt2 proteins.

The primary candidate DNA methyltransferase gene for *Drosophila* has been *Dnmt2*. Based on antibody stainings, the presence of a second Dnmt1-like methyltransferase protein has also been suggested (Hung et al., 1999). However, there is no corresponding open reading frame in the *Drosophila* genome sequence (Adams et al., 2000). The complete loss of DNA methylation in embryos injected with double-stranded Dnmt2 RNA also argues against the presence of a second DNA methyltransferase. While these results provided unambiguous evidence for a DNA methyltransferase activity of Dnmt2, they did not reveal a function of DNA methylation in the fly. Depletion of Dnmt2 by RNA interference appeared not to have any detectable consequences for embryonic development. We also monitored development during later stages and observed only minor phenotypic aberrations that could not be unambiguously linked to the demethylation of the genome (data not shown). The apparent lack of phenotypic effects is consistent with the absence of major phenotypes in null mutant *Su(var)3-9* fly strains (Tschiersch et al., 1994) that we have shown to lack most, if not all, DNA methylation. In this context it is noteworthy that experimental demethylation of the *Neurospora* genome also has no major phenotypic consequences (Kouzminova and Selker, 2001).

Dnmt2-like genes are widely conserved during evolution, but their function has been elusive for a considerable period of time (Dong et al., 2001). No catalytic activity could be demonstrated by standard in vitro methylation assays and by gene targeting of the mouse *Dnmt2* gene (Okano et al., 1998a; Tweedie et al., 1999; Wilkinson et al., 1995; Yoder and Bestor, 1998). Our results revealed a comparatively low activity of Dnmt2 that was specific for CpT and CpA dinucleotides. Both characteristics could have contributed to the apparent lack of DNA methyltransferase activity in previous assays. The

gene	location	sequence
CG12149	8D	AGGAGAAACC**CA**ATAGGCTTA
subtelomeric	41F	TTTTGCGACC**CT**TTCTGGTAT
ImpL3	65A	ATTTTGAATT**CT**CCATCGCCT
CG7257	68E	TGTGACCACT**CT**CACCTACAT
intergenic	86C	ATCGGTGCTG**CT**ATCCGCTGG
intergenic	86C	TAGCGGCTCA**CA**AGACCGAAG
CG13661	96C	AGAAAGTATT**CA**AGATTAATA
Gprk2	100C	AAAAGCGCAT**CA**AGAAGCGCG

Fig. 5. Target sequences of Dnmt2-mediated DNA methylation. Methylated sequences were identified in *Drosophila* DNA by bisulfite sequencing of random genomic fragments. This revealed that overexpression of Dnmt2 results in specific methylation at CpT and CpA dinucleotides. Methylated cytosine residues are shown in red and their 3′-neighbours are indicated in green.

specificity for CpT and CpA distinguishes Dnmt2 from all other known animal DNA methyltransferases and confirms our previous suggestion of predominant non-CpG methylation in *Drosophila* (Lyko et al., 2000b). CpT/A methylation has also been reported in various mammalian test systems (Clark et al., 1995; Lorincz et al., 2000; Toth et al., 1990; Woodcock et al., 1997). Most notably, the *B29* gene promoter in human B cells has been found to be methylated in the context of CCTGG or CCAGG (Malone et al., 2001), and it has been hypothesized that Dnmt2 might be the responsible enzyme (Lorincz and Groudine, 2001). Our data did not reveal a consensus sequence that extended beyond the CpT/A dinucleotide. However, mammalian CpT/A methylation is not restricted to CCTGG or CCAGG pentanucleotides (Ramsahoye et al., 2000). Thus, it will be interesting to analyse the methylation of CpT/A sites in other animal systems for their dependency on Dnmt2-like proteins.

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