Human DNMT2 methylates $tRNA^{Asp}$ molecules using a DNA methyltransferase-like catalytic mechanism

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ABSTRACT

Although their amino acid sequences and structure closely resemble DNA methyltransferases, Dnmt2 proteins were recently shown by Goll and colleagues to function as RNA methyltransferases transferring a methyl group to the C5 position of C38 in $tRNA^{Asp}$. We observe that human DNMT2 methylates tRNA isolated from Dnmt2 knock-out *Drosophila melanogaster* and *Dictyostelium discoideum*. RNA extracted from wild type *D. melanogaster* was methylated to a lower degree, but in the case of *Dictyostelium*, there was no difference in the methylation of RNA isolated from wild-type and Dnmt2 knock-out strains. Methylation of in vitro transcribed $tRNA^{Asp}$ confirms it to be a target of DNMT2. Using site directed mutagenesis, we show here that the enzyme has a DNA methyltransferase-like mechanism, because similar residues from motifs IV, VI, and VIII are involved in catalysis as identified in DNA methyltransferases. In addition, exchange of C292, which is located in a CFT motif conserved among Dnmt2 proteins, strongly reduced the catalytic activity of DNMT2. Dnmt2 represents the first example of an RNA methyltransferase using a DNA methyltransferase type of mechanism.

Keywords: Dnmt2; catalytic mechanism; RNA methylation; $tRNA^{Asp}$

INTRODUCTION

Dnmt2 was initially assigned a member of the DNA methyltransferase family on the basis of its extensive homology with eukaryotic and prokaryotic DNA-(cytosine C5)-methyltransferases (Yoder and Bestor 1998). However, in the apparent absence of a phenotype in *dnmt2* knock-out cells, Dnmt2's possible biological function remained unknown (Okano et al. 1998), even though Dnmt2 is strongly conserved and it is found in species ranging from *Schizosaccharomyces pombe* to human. Later very weak, residual DNA methylation activity was found with enzymes from different species (Hermann et al. 2003; Kunert et al. 2003; Tang et al. 2003; Fisher et al. 2004; Kuhlmann et al. 2005). The finding that Dnmt2 is an active RNA methyltransferase capable of methylating the C38 position of the $tRNA^{Asp}$ came as a surprise (Goll et al. 2006). However, still no cellular function of the $tRNA^{Asp}$ methylation has been found, although in Zebrafish Dnmt2 knock-down caused a developmental phenotype (Rai et al. 2007). It is very intriguing that an enzyme that looks like a DNA methyltransferase can methylate RNA, in particular since the RNA and DNA specific m^5C methyltransferases use different catalytic residues and a different mechanism for the methyl transfer reaction (Liu and Santi 2000).

Although, the cofactor *S*-adenosyl-*L*-methionine (AdoMet) is a very effective donor of methyl groups, methylation of cytosines at position 5 is not a trivial reaction, because cytosine is an electron-poor heterocyclic aromatic ring system and the carbon 5 of cytosine is not capable of making a nucleophilic attack on the methyl group of AdoMet. Therefore, the reactions catalyzed by RNA and DNA m^5C methyltransferases follow the reaction pathway of a Michael addition. The catalytic mechanism of DNA m^5C methyltransferases was first suggested by Santi (Santi et al. 1984) and later refined by Wu and Santi (1987).

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According to this mechanism (for review, see Jeltsch 2002), DNA methylation is initiated by a nucleophilic attack of an SH group from a catalytic cysteine residue located in the conserved amino acids sequence motif IV (GPPC) (Kumar et al. 1994) on the C6 position of the target cytosine, yielding a covalent intermediate between the base and the enzyme. Thereby, the C5 position of the cytosine gets activated and becomes capable of performing a nucleophilic attack on the methyl group bound to the cofactor substrate AdoMet. The enzyme facilitates the nucleolytic attack on the C6 atom by a transient protonation of the cytosine ring at the endocyclic nitrogen atom N3 (Chen et al. 1991), which is stabilized by the glutamate residue from a highly conserved motif VI (ENV). The covalent complex between the methylated base and the DNA is resolved by deprotonation at the C5 position, which leads to the elimination of the cysteinyl group and the reestablishment of aromaticity. Then, the methylated base together with the cofactor product, *S*-adenosyl-*L*-homocysteine, is released. In addition to the residues already mentioned, the second arginine residue in motif VIII (RXR) plays an important role in the catalytic mechanism of DNA m^5C methyltransferases (O'Gara et al. 1996; Gowher et al. 2006).

The fact that RNA and DNA m^5C methyltransferases employ different mechanisms for the methyl transfer was discovered by Liu and Santi (2000) by showing that RNA m^5C methyltransferases do not use the cysteine from motif IV for the initial attack on the base, but rather one located in motif VI (TCS in RNA MTases). Later, King and Redman (2002) provided evidence that also the cysteine in motif IV has a role in catalysis. Moreover, instead of using the glutamate residue located in motif VI of DNA

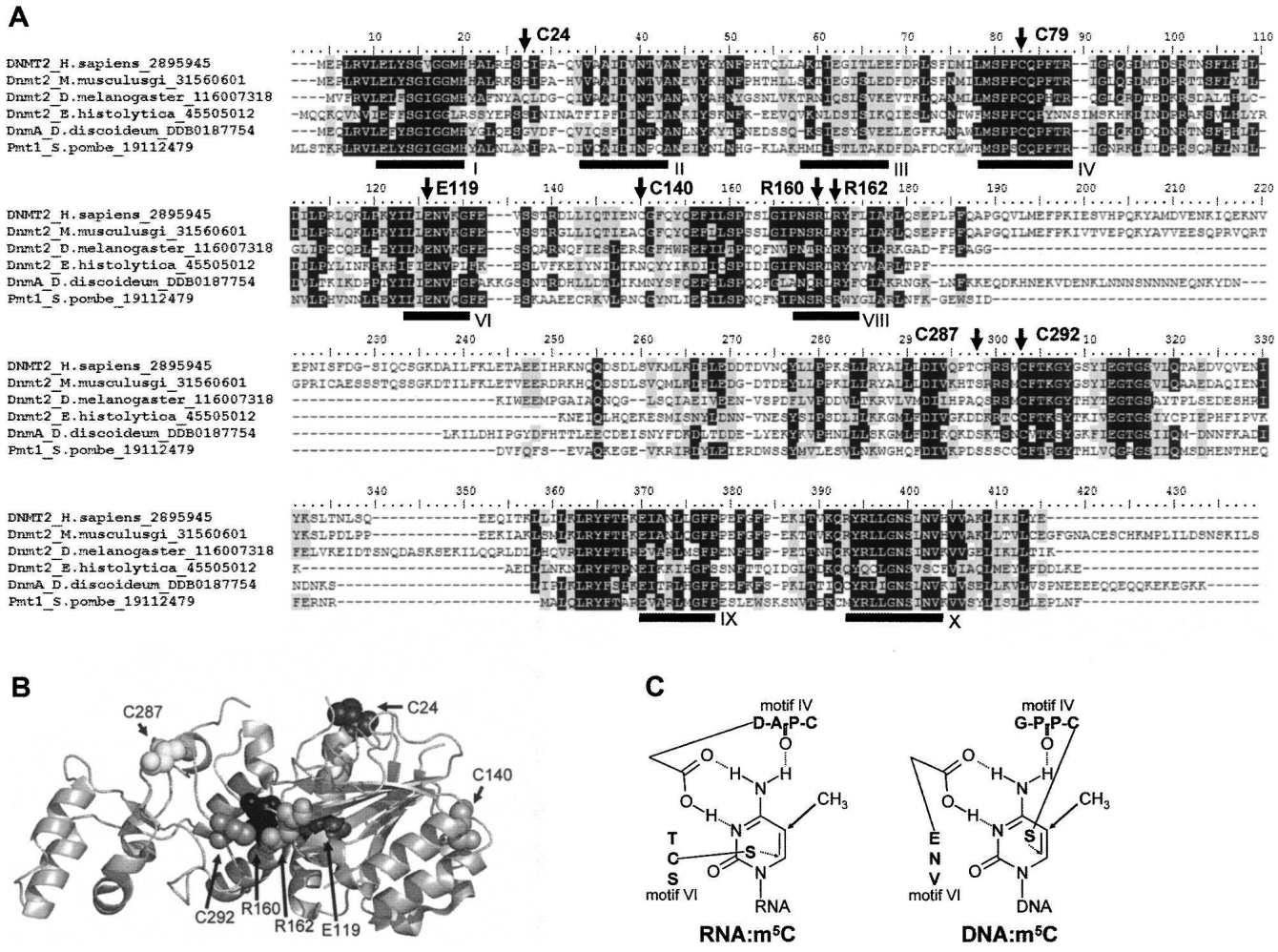

FIGURE 1. Alignment of Dnmt2 family. (*A*) Multiple sequence alignment of Dnmt2 proteins from human, mouse, *D. melanogaster*, *E. histolytica*, *D. discoideum*, and *S. pombe*. The regions corresponding to catalytic motifs of DNA MTases are labeled. The residues selected for analysis are marked with arrows and labeled. (*B*) Structure of human DNMT2 protein (Dong et al. 2001). The residues studied in this work are shown in space fill representation. Note that the loop containing C79A is not ordered in the structure, such that this residue is not visible. (*C*) Schematic picture of the catalytic mechanisms proposed for DNA MTases and RNA MTases. For details see the text (data adapted from Bujnicki et al. 2004).

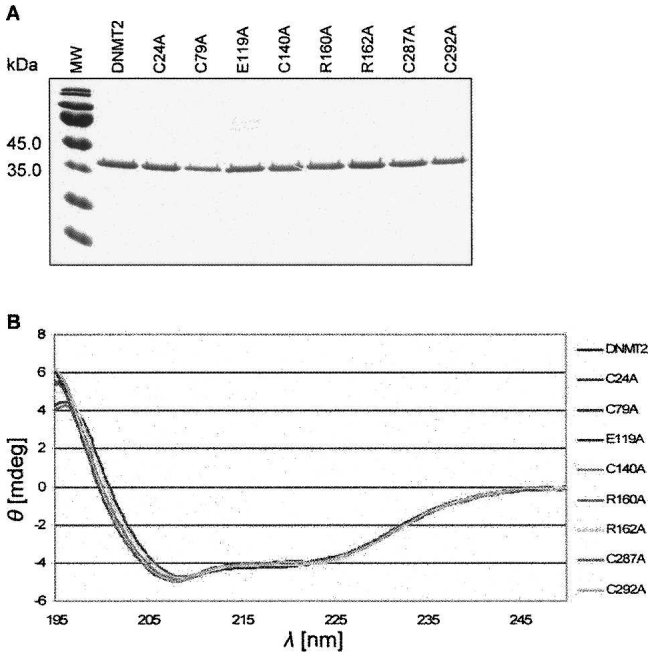

FIGURE 2. Protein purification and circular dichroism spectra of the DNMT2 wild type and all variants. (*A*) Coomassie stained SDS polyacrylamide gel showing the purified wild-type and mutant DNMT2 proteins. (*B*) Far UV circular dichroism spectra of DNMT2 and its variants recorded using 10 μM enzyme in 10 mM Tris/HCl (pH 7.5), 200 mM KCl solution. The figure shows a superposition of the spectra measured with the wild-type and mutant proteins.

methyltransferases (ENV), it uses an aspartate residue from motif IV (DAPC) to stabilize the transition state of the reaction (Bujnicki et al. 2004).

Dnmt2 proteins contain all the conserved residues that are used for catalysis by DNA m^5C methyltransferases. However, they also contain a cysteine residue (C292 in human DNMT2-CFT), which is strongly conserved in the Dnmt2 family but absent in the DNA m^5C methyltransferase family. Based on the structure of the human DNMT2 enzyme (Dong et al. 2001), this residue is located near the putative catalytic pocket, such that it could be involved in catalysis. It was the aim of this study to determine if DNMT2 methylates RNA following a DNA or RNA MTase like catalytic mechanism. We conclude from our data that DNMT2 is the first example of an RNA MTase that uses the catalytic mechanism of DNA MTases.

RESULTS AND DISCUSSION

In order to identify the principal catalytic mechanism of the human DNMT2 enzyme for the transfer of methyl groups to RNA, we have created and purified the alanine exchange mutants of residues possibly involved in catalysis. To differentiate the catalytic mechanism of DNMT2 between the m^5C RNA and m^5C DNA types, we have selected nine residues for this mutagenesis study. The C79 (from motif IV–PCQ) and C292 residues (conserved in the Dnmt2 family and close to the putative active site) were chosen to be studied by mutagenesis. Additionally, we have decided to exchange all other the cysteine residues in the protein for alanines to investigate their hypothetical involvement in catalysis. Moreover, we have selected E119 (from motif VI–ENV), R160 and R162 (from motif VIII–RXR) by their homology with the catalytic residues found in DNA m^5C methyltransferases that were experimentally confirmed in different systems (see Fig. 1; Klimasauskas et al. 1994; O'Gara et al. 1999; Sankpal and Rao 2002; Reither et al. 2003; Gowher et al. 2006; Shieh et al. 2006; Shieh and Reich 2007). We have purified these variants, confirmed their correct folding, and studied their RNA methylation activity and RNA binding.

Cloning, site-directed mutagenesis, protein expression, and purification

Using site-directed mutagenesis, all nine amino acid exchanges were performed and the mutant genes sequenced to confirm introduction of the desired mutation and the lack of additional ones. DNMT2 mutant proteins carrying a C-terminal His_6-Tag along with the wild-type enzyme were expressed in *Escherichia coli* and purified in soluble form to 100–500 μM concentrations. The purity of DNMT2 wild-type and mutant proteins was >95% as determined by SDS-PAGE electrophoresis and Coomassie staining (Fig. 2).

Since single amino acid exchanges can disrupt the proper folding of a protein, we have determined the far UV circular dichroism (CD) spectra (which reflect the secondary structure composition of the protein) of all the DNMT2 protein mutants and compared them with the spectra obtained with the wild-type protein. As shown in Figure

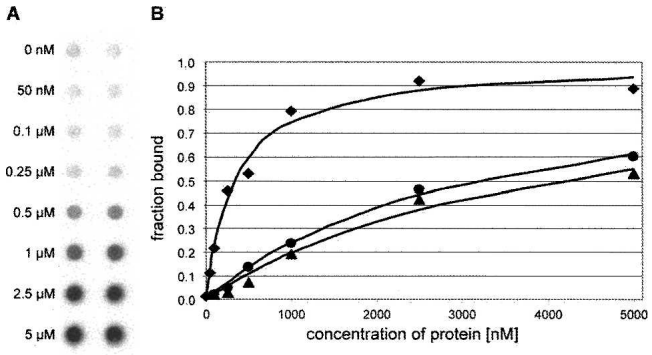

FIGURE 3. RNA binding by DNMT2 and its variants analyzed by the nitrocellulose filter binding assay. (*A*) Dot blot analysis of the wild-type DNMT2 binding the ^{32}P labeled in vitro transcribed $tRNA^{Asp}$. (*B*) Binding curves of DNMT2 wild type and C79A and E119A mutants to in vitro transcribed and ^{32}P-labeled $tRNA^{Asp}$. Diamonds denote E119A mutant, triangles wild-type DNMT2, circles C79A. The experimental data points were fitted to a bimolecular binding equilibrium to determine the K_{Ass} values.

TABLE 1. Equilibrium binding constants and catalytic activities of the human DNMT2 and its variants

Enzyme variant	K_{Ass} (M^{-1})	Relative activity
DNMT2	6.1×10^5	+ + +
C24A	5.2×10^5	+ +
C79A	2.4×10^5	−
E119A	2.9×10^5	−
C140A	1.1×10^5	+
R160A	1.0×10^5	−
R162A	6.2×10^5	−
C287A	4.3×10^5	+ +
C292A	2.7×10^5	(+)

RNA binding was analyzed using in vitro transcribed $tRNA^{Asp}$. RNA methylation was investigated using total RNA extract prepared from Dnmt2 KO *D. melanogaster* flies. Activity ranges were classified as following: + + +, full activity or less than twofold reduction in activity; + +, 2- to 10-fold reduced activity; +, 10- to 50-fold reduced activity; (+), 50- to 250-fold reduced activity; −, no activity detectable (>5000-fold reduced activity).

2, all the single exchange variants' CD spectra were superimposable with the spectrum of the wild-type protein. This result indicates that all the mutant proteins were properly folded. All spectra were fitted by 32% α-helix and 17% β-strand, which is in good agreement with the secondary structure composition calculated from the crystallographic structure of human DNMT2 (35% α-helix, 15% β-strands).

RNA binding of wild-type Dnmt2 and its variants

We have investigated the RNA binding affinity of the wild-type DNMT2 protein as well as of all the mutants using a nitrocellulose filter binding assay. ^{32}P-labeled in vitro transcribed $tRNA^{Asp}$ was used as a substrate, since $tRNA^{Asp}$ was identified as a Dnmt2 target (Goll et al. 2006). In these experiments, constant amounts of labeled in vitro transcribed tRNA (0.4 nM) were incubated with increasing amounts of DNMT2 and sucked through a nitrocellulose filter (Fig. 3). The apparent equilibrium binding constant of the wild-type DNMT2 was 6×10^5 M^{-1} (Table 1). This value is low, when compared with nucleic acid interaction of other DNA or RNA modifying enzymes (for example, the K_{Ass} for M.EcoRV binding to 40 bp DNA substrate is 1.2×10^6 M^{-1} [Beck et al. 2001], and the K_{Ass} for Trm4p binding to $tRNA^{Phe}$ is 2.2×10^7 M^{-1} [Walbott et al. 2007]). The weak tRNA binding of DNMT2 may be explained by the fact that the substrate used in the experiment was an in vitro transcribed $tRNA^{Asp}$, which lacks additional modifications like the mannosylqueosine base (manQ) present at the wobble position (34) of endogenous $tRNA^{Asp}$ (Kuchino et al. 1981; Johnson et al. 1985). Most of the variants had equilibrium RNA binding constants similar to the wild type (Table 1). The weakest binding was observed with C79A and C292A, which both showed about threefold reductions in binding affinity. The E119A mutant bound stronger to the tRNA, as shown in Figure 3B, which could be explained, because the removal of negative charge from the protein could reduce the electrostatic repulsion of the RNA. We conclude that all variants bind to the RNA to a similar degree as the wild-type DNMT2 does.

RNA methylation activity of the wild-type DNMT2

To detect the $tRNA^{Asp}$ methylation activity of human DNMT2 protein reported by Goll et al. (2006), we have constructed a *Drosophila melanogaster* Dnmt2 null allele strain (Fig. 4). Total RNA was extracted from *D. melanogaster* wild-type flies and the Dnmt2 knock-out strain and used as substrate for in vitro methylation experiments using the purified enzymes. In these experiments the incorporation of tritiated methyl groups from *S*-[*methyl*-^{3}*H*]-AdoMet into the RNA was detected. After the methylation reactions, the RNA was ethanol precipitated, dissolved in formamide, heat denaturated, and subjected to gel electrophoresis on denaturating polyacrylamide gels. As shown in Figure 5A, the wild-type DNMT2 enzyme methylated RNA molecules extracted from Dnmt2 knock-out cells, which migrated in the gel in the size range of the tRNA fraction. In contrast, the RNA extracted from *D. melanogaster* cells that contained the active *dnmt2* gene was methylated to a much lower extent (about 0.1% in Fig. 5B), which confirmed the specificity of the assay for DNMT2. The residual methylation observed with RNA isolated from wild-type flies suggests that either the tRNA substrates of Dnmt2 are not fully methylated in the wild-type *D. melanogaster* or

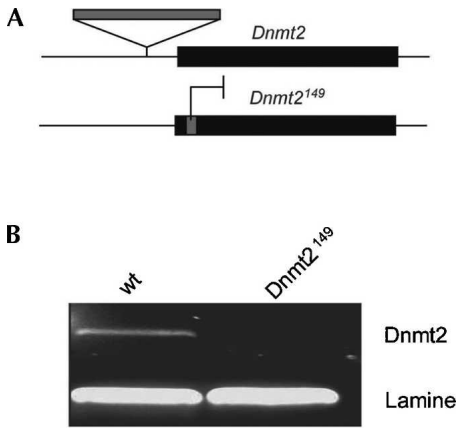

FIGURE 4. Generation of a dDnmt2 knock-out *Drosophila melanogaster* strain. (*A*) Schematic drawing of the structure of the dDnmt2^{149} null allele. A null allele of dDnmt2 was generated after remobilization of the P element GE15695 inserted 128 bp upstream of the dDnmt2 gene. dDnmt2^{149} shows an insertion of 59 bp of 5′ P element sequences 9 bp downstream of first ATG position within the Dnmt2 ORF introducing an early stop codon. (*B*) RT-PCR detection of dDnmt2 mRNA. No specific transcript is detected in dDnmt2^{149} null embryos. Lamine mRNA was used as internal control.

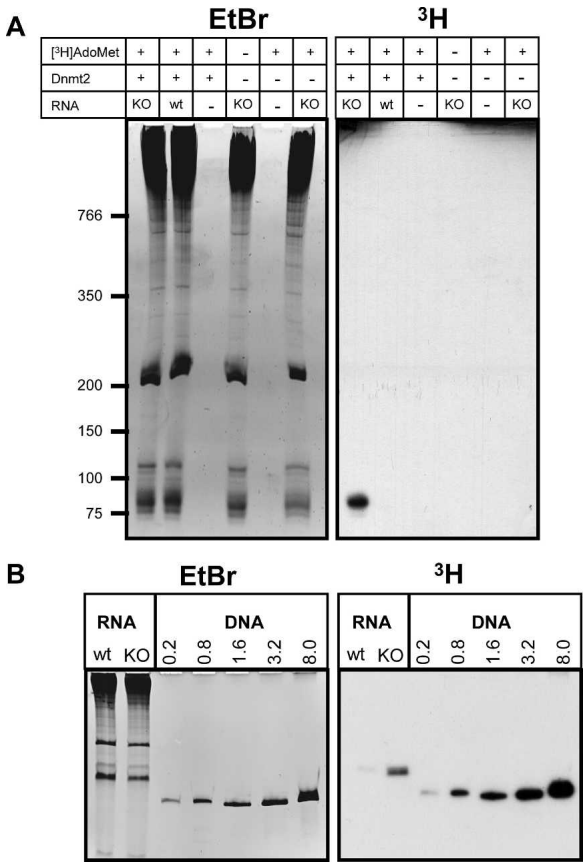

FIGURE 5. Methylation of total RNA extracted from the *dnmt2* knock-out *D. melanogaster* cells by wild-type DNMT2 and its variants. (*A*) Human DNMT2 methylates RNA molecules in the size range of tRNA. A DNA molecular weight marker was used and the approximate positions of the DNA fragments of different lengths (in nucleotides) are indicated. (*B*) Quantification of DNMT2 catalyzed methyl group transfer to RNA. In this experiment, DNA methylated by M.SssI was used to calibrate the RNA methylation by DNMT2. Different amounts of methylated DNA were loaded into the different lanes of the gel. The amount of methyl groups in each lane (in picomoles) is indicated *above* the corresponding well. In this experiment, 10 μg of total RNA were loaded into each well.

that the DNMT2 shows relaxed specificity in vitro and methylates targets that are not methylated in vivo.

In order to estimate the amount of methyl groups incorporated into the tRNA, we used a 60mer DNA fragment methylated with M.SssI using the same radiolabeled AdoMet as methyl group donor and loaded in different amounts on the denaturing polyacrylamide gel together with the tritiated RNA samples (Fig. 5B). Quantification of the radioactivity observed in the DNA and in 10 μg of DNMT2 modified RNA revealed that about 1 pmol of methyl groups was transferred to the RNA. Although this is only a rough estimate, the result fits to the estimated amount of $tRNA^{Asp}$ used in the experiment, if one assumes that tRNA represents about 10% of the total RNA extract and $tRNA^{Asp}$ is about 1/40 of all tRNAs. To measure the rate of RNA methylation by DNMT2, time courses of the incorporation of radioactivity into the RNA were determined. The data were fitted to a single exponential reaction progress curve, revealing an apparent rate of RNA methylation of 4.4 ($\pm$0.2) h^{-1} under our experimental conditions (Fig. 6A).

Since $tRNA^{Asp}$ was identified as Dnmt2 target, we used in vitro transcribed $tRNA^{Asp}$ for the methylation assays as well. As shown in Figure 7A, in vitro transcribed $tRNA^{Asp}$ was methylated by DNMT2, albeit at reduced efficiency, as indicated by the fact that the methylation signal was similar to that observed with the whole RNA extract, although 0.6 μg of the in vitro transcribed tRNA were used, which is about 10 times more than the amount of $tRNA^{Asp}$ present in 20 μg of total RNA extract. Therefore, either native $tRNA^{Asp}$ is the preferred substrate of DNMT2 or DNMT2 methylated other targets in the whole RNA preparation as well. Preferential methylation of native tRNAs could be due to the presence of additional modifications. Additionally, methylation of in vitro transcribed $tRNA^{Asp}$ was only observed after Mg^{2+} ions were added to the buffer, presumably because this supports native folding of the tRNA. This detail in the experimental procedures, or the lower activity of DNMT2 on in vitro transcribed $tRNA^{Asp}$, might explain why this activity was not observed before (Goll et al. 2006).

We have also tested whether the human DNMT2 can methylate total RNA isolated from other organisms. To this end, RNA was extracted from *Dictyostelium discoideum* Ax2 wild-type strain and from *dnmA* knock-out cells (Kuhlmann et al. 2005) (DnmA is the homolog of Dnmt2 in *D. discoideum*). As shown in Figure 7B, the RNA isolated from both wild-type and *dnmA* knock-out cells was methylated by the human DNMT2 enzyme in vitro. We conclude that human, mouse, and *Dictyostelium* tRNA all are substrates for human DNMT2. Our results suggest, however, that tRNA methylation in vivo appears to be less complete or even absent in *Dictyostelium*; whether this depends on growth conditions and/or cell cycle remains an open question.

Methylation activity of the DNMT2 variants

In order to investigate the catalytic mechanism of DNMT2, we have performed in vitro methylation reactions of the total RNA extracts from Dnmt2 deficient *D. melanogaster* flies with all the purified DNMT2 mutant enzymes. Four of the variants (namely, C24A, C140A, C287A, and C292A) methylated the RNA to a detectable amount (Fig. 6B). For more detailed analysis, time courses of incorporation of radioactivity were determined, and the level of RNA methylation by mutants compared with the wild-type enzyme (Table 1). Our data show that C292 is important for catalytic activity. However, this residue cannot be directly involved in the catalytic mechanism, because the C292A

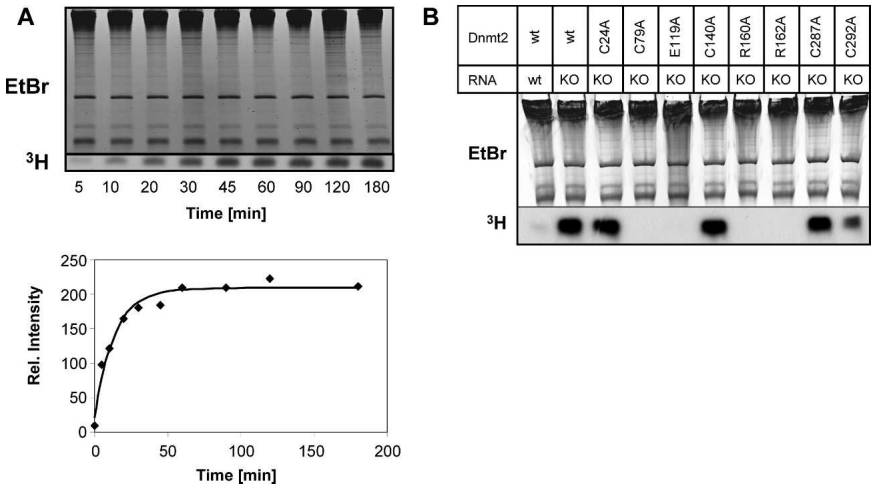

FIGURE 6. In vitro methylation of RNA by DNMT2 and its variants. (*A*) Time course of RNA methylation by wild-type DNMT2. The *upper* panel shows the time course of incorporation of radioactivity into RNA. The radioactive bands were analyzed quantitatively and the data fit to a single exponential reaction progress curve as shown in the *lower* panel. (*B*) Example of RNA methylation by the DNMT2 variants after 3 h reaction time.

variant showed significant residual activity. The C79A, E119A, R160A, and R162A mutants showed no detectable in vitro methylation activity. These inactive mutants did not show any detectable methylation activity even when the concentration of the protein in the methylation reaction was fivefold increased (data not shown). These experiments were done using one preparation of RNA and reproduced with two independent batches. As described above, the lack of activity cannot be explained by loss of RNA binding or misfolding of the proteins. We conclude that C79, E119, R160, and R162 are essential for the catalytic mechanism of DNMT2. These residues correspond to the residues that have most important roles in the catalytic mechanism of DNA MTases, suggesting that DNMT2 methylates RNA with a DNA MTase-like mechanism.

In conclusion, we have shown here that DNMT2 methylates RNA with a mechanism that follows DNA MTases and differs from mechanisms previously established for RNA MTases. This result is in agreement with the high similarity of DNMT2 to DNA MTases, with respect to both primary sequence and structure (Dong et al. 2001). Therefore, DNMT2 is the RNA MTase most similar to DNA MTases, which raises interesting questions of the evolutionary origin of both enzyme families and their substrate interaction.

MATERIALS AND METHODS

Site-directed mutagenesis, protein expression, and protein purification

Human DNMT2 wild type and its C79A variant (both in fusion with a C-terminal His_6-tag) were cloned into the pET28a(+) vector at HindIII-XhoI sites (Hermann et al. 2003). The variants of human DNMT2 were created by the megaprimer site-directed mutagenesis method as described previously (Jeltsch and Lanio 2002). All created mutants were sequenced to check for absence of undesired additional mutations. The oligonucleotides used for site-directed mutagenesis were purchased from Thermo Hybaid. The human DNMT2-His_6 fusion proteins were expressed in *E. coli (DE3) Rosetta2 pLysS* cells. The cells were induced at OD(600 nm) = 0.6 with 1 mM IPTG and harvested 3 h after induction. The protein purification was performed as described previously (Hermann et al. 2003).

Generation of a *D. melanogaster* Dnmt2 null allele

The $Dnmt2^{149}$ null allele was generated by remobilization of the P element GE15695 inserted 128 bp upstream of the Dnmt2 ORF using the P[ry^+ $\Delta 2$-3] transposase producing element. Homozygote *GE15695* females were mated with *SM6, al*^{2} *Cy sp*^{2}*/+; Dr P[ry*$^+$ *Δ2-3]/+* males. The resulting *GE15695/SM6, al*^{2} *Cy sp*^{2} *Dr P[ry*$^+$ *Δ2-3]/+* single male was mated with *SM6, al*^{2} *Cy sp*^{2}*/Sco* females and w^- Cy exceptional progenies selected and analyzed for lesions in the Dnmt2 gene. $Dnmt2^{149}$ shows an insertion of 59 bp of 5′ P element sequences 9 bp downstream of first ATG position within the Dnmt2 ORF introducing an early stop codon.

For RT-PCR analysis cDNA was prepared from the total RNA isolated from wild-type and $Dnmt2^{149}$ embryos. cMT2_fwd (TTG GTCGACTCATGCCTTTAATTGTGAG) and cMT2_rev (ATG TCGACGTTTTATCGTCAGCAATTTAATA) primers amplifying the complete Dnmt2 ORF were used. Lamine cDNA amplified with the primers Lamine_fwd (GAGACCACCAACATCAAGAAC)

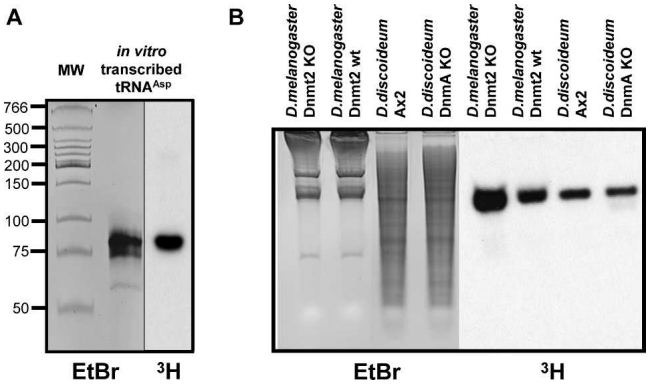

FIGURE 7. In vitro methylation of different RNA substrates by DNMT2. (*A*) In vitro transcribed $tRNA^{Asp}$ can be methylated by DNMT2 wild-type protein. A DNA molecular weight marker was used and the approximate positions of the DNA fragments of different lengths (in nucleotides) are indicated. (*B*) Methylation of the *D. discoideum* total RNA extract from the Ax2 wild-type cells and the *dnmA* knock-out cells.

and Lamine_rev (AGGGACTGGATTTCATCGC) was used as internal control.

RNA substrates

Overnight collections of *D. melanogaster* wild-type and mutant embryos were dechorionated in 50% bleach and flash frozen in liquid nitrogen. Total RNA was prepared using Trizol (Invitrogen) following the manufacturer's instructions. Pellets were dissolved in DEPC treated water.

The DNA template encoding for $tRNA^{Asp}$, the hammerhead ribozyme and the T7 promoter (TGGCTCCCCGTCGGGGAATC GAACCCCGGTCTCCCGCGTGACAGGCGGGGATACTCACCA CTATACTAACGAGGAGACGGTACCGGGTACCGTTTCGTCC TCACGGACTCATCAGTCCTCGTTATCTCCCTATAGTGAGTC GTATT) was amplified in a standard PCR reaction using T7 primer and $tRNA^{Asp}$ primer (TGGCTCCCCGTCGGGGAATCG). For in vitro transcription, 100 μL of the PCR reaction were incubated with 200 μL 2× transcription buffer (80 mM Tris-HCl at pH 8.1, 2 mM Spermidine, 10 mM DTT, 0.02% Triton-X-100, 60 mM $MgCl_2$, 4 μg/mL BSA), 5 mM of each NTP (final concentration), and 10 μL of T7-Polymerase (Fermentas) in a total volume of 400 μL for 3 h at 37°C. Transcripts were purified over 12% denaturing PAGE and bands of correct size were excised, eluted in 0.5 M ammonium acetate, and precipitated with two volumes of 100% ethanol. After centrifugation, pellets were washed once with 80% ethanol and then dissolved in water. Radioactively labeled $tRNA^{Asp}$ was prepared by phosphorylating the in vitro transcribed $tRNA^{Asp}$ with polynucleotide kinase (NEB) and γ-^{32}P-ATP (Amersham) according to the NEB protocol.

In vitro RNA methylation assay

The methylation of the total RNA extracts with the DNMT2 wild-type and its variants was performed similarly as previously described (Goll et al. 2006). Briefly, 20 μg of *D. melanogaster* total RNA extracts were incubated with 1 μM of the DNMT2 protein for 3 h in 40 μL of methylation buffer (100 mM Tris/HCl at pH 7.5, 5% glycerol, 5 mM $MgCl_2$, 1 mM DTT, and 100 mM NaCl) containing 4.2 μM labeled [methyl-^{3}H] AdoMet (NEN). After the reaction, samples were phenol extracted, ethanol precipitated, resuspended in formamide, and run on a 7 M urea 12% denaturating polyacrylamide gel. Afterward, the gels were stained with ethidium bromide, fixed with 10% acetic acid, 10% methanol solution, immersed for 1 h in Amplify solution (Amersham), dried, and exposed to Hyperfilm film (Amersham) for 1 d to 1 wk at −70°C. All RNA methylation assays were conducted at least in triplicate. Quantification was done by densitometric analysis of the films. Data were fitted to a single exponential reaction progress curve using the following equation: $\text{intensity}(t) = BL + A \times [1 - \exp(-t \times k)]$, where BL is the background signal, A is the scaling factor relating densitometric staining and methylation, and k is the apparent turnover rate constant of RNA methylation.

Circular dichroism

Far UV circular dichroism spectra were obtained with a Jasco J-815 circular dichroism spectrophotometer, using protein concentrations of 10 μM DNMT2 in 10 mM Tris/HCl (pH 7.5), 200 mM KCl solution. The spectra were recorded in a cell with a 0.1-mm path length in the wavelength window between 190 nm and 250 nm with a step size of 0.1 nm and bandwidth of 1 nm. For all spectra, 25 scans were performed and averaged. The buffer baseline was recorded and consequently subtracted from the protein spectra. All experiments were carried out at least twice.

Nitrocellulose filter binding assay with in vitro transcribed $tRNA^{Asp}$

The $tRNA^{Asp}$ binding of the DNMT2 wild type and the mutants was analyzed by nitrocellulose filter binding assay experiments using the in vitro transcribed and ^{32}P labeled $tRNA^{Asp}$. The experiment was carried out using a Bio-Dot apparatus (BioRad). The reaction mixtures contained 0–5 μM DNMT2 protein in 100 mM Tris/HCl (pH 7.5), 5 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT, 5 mg/mL BSA, 2 units RNasin ribonuclease inhibitor (Promega), 10 μM sinefungin, and 0.4 nM in vitro transcribed ^{32}P labeled $tRNA^{Asp}$. The reaction mixtures were first preincubated at ambient temperature for 20 min to allow the system to reach equilibrium, then loaded on a prewashed nitrocellulose membrane and immediately sucked through the membrane. The membrane was washed three times with the wash buffer (100 mM Tris/HCl at pH 7.5, 5 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT) and dried, and the bound radioactivity was quantified using a phosphorimager (FLA-300, Fuji). The data were fitted to a bimolecular binding equilibrium model using the Excel Solver module. All binding experiments were reproduced at least twice.

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