The Dnmt2 RNA methyltransferase homolog of *Geobacter sulfurreducens* specifically methylates tRNA-Glu

Raghuvaran Shanmugam1, Muktak Aklujkar2, Matthias Schäfer3, Richard Reinhardt4, Olaf Nickel5, Gunter Reuter5, Derek R. Lovley2, Ann Ehrenhofer-Murray6, Wolfgang Nellen7, Serge Ankri8, Mark Helm9, Tomasz P. Jurkowski1 and Albert Jeltsch1,*

1Institute of Biochemistry, Stuttgart University, 70569 Stuttgart, Germany, 2Department of Microbiology, University of Massachusetts, Amherst, MA 01003-9298, USA, 3Division of Epigenetics, DKFZ-ZMBH Alliance, German Cancer Research Center, 69120 Heidelberg, Germany, 4Max-Planck-Genomzentrum, 50829 Köln, Germany, 5Institute of Biology, Developmental Genetics, Martin Luther University Halle, 06120 Halle, Germany, 6Institute of Biology, Humboldt University, 10115 Berlin, Germany, 7Department of Genetics, University of Kassel, 34132 Kassel, Germany, 8Department of Molecular Microbiology, The Bruce Rappaport Faculty of Medicine, Technion, Haifa 31096, Israel and 9Institute of Pharmacy and Biochemistry, Johannes Gutenberg-Universität Mainz, 55128 Mainz, Germany

Received November 26, 2013; Revised March 14, 2014; Accepted March 17, 2014

ABSTRACT

Dnmt2 enzymes are conserved in eukaryotes, where they methylate C38 of tRNA-Asp with high activity. Here, the activity of one of the very few prokaryotic Dnmt2 homologs from *Geobacter* species (Gs-Dnmt2) was investigated. GsDnmt2 was observed to methylate tRNA-Asp from flies and mice. Unexpectedly, it had only a weak activity toward its matching *Geobacter* tRNA-Asp, but methylated *Geobacter* tRNA-Glu with good activity. In agreement with this result, we show that tRNA-Glu is methylated in *Geobacter* while the methylation is absent in tRNA-Asp. The activities of Dnmt2 enzymes from *Homo sapiens*, *Drosophila melanogaster*, *Schizosaccharomyces pombe* and *Dictyostelium discoideum* for methylation of the *Geobacter* tRNA-Asp and tRNA-Glu were determined showing that all these Dnmt2s preferentially methylate tRNA-Asp. Hence, the Gs-Dnmt2 enzyme has a swapped transfer ribonucleic acid (tRNA) specificity. By comparing the different tRNAs, a characteristic sequence pattern was identified in the variable loop of all preferred tRNA substrates. An exchange of two nucleotides in the variable loop of murine tRNA-Asp converted it to the corresponding variable loop of tRNA-Glu and led to a strong reduction of GsDnmt2 activity. Interestingly, the same loss of activity was observed with human DNMT2, indicating that the variable loop functions as a specificity determinant in tRNA recognition of Dnmt2 enzymes.

INTRODUCTION

Several chemical modifications of the bases in deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) have been discovered in the last decades of research, which include methylation, pseudouridylation, hydroxymethylation, formylation and carboxylation (1–4). In eukaryotes, three families of enzymes with strong sequence similarity to DNA-(cytosine C5)-methyltransferases have been found, namely Dnmt1, Dnmt2 and Dnmt3 (5–7). Dnmt2 enzymes are highly conserved with homologs present from lower eukaryotes like *Schizosaccharomyces pombe* to higher eukaryotes like *Homo sapiens* (8,9). These enzymes contain all motifs characteristic for DNA methyltransferases, but lack the large N-terminal domain found in the Dnmt1 and Dnmt3 enzymes. Later, in a seminal paper by Goll et al., Dnmt2 enzymes were reported to have a robust methylation activity on tRNAAsp at position C38 (6). After the initial discovery of tRNAAsp methylation by Dnmt2, many studies have confirmed this activity in different organisms (10–16). However, even though Dnmt2 methylates transfer RNA (tRNA), it utilizes a DNA methyltransferase-like catalytic mechanism in the methyl transfer reactions (10), which also explains the conservation of the corresponding amino acid motifs in the catalytic center.

Dnmt2 knockout studies have indicated a connection to stress response and methylation of small RNAs (13,17,18), non-random sister chromatid segregation in stem cells...
(19), mobile element and RNA virus control in *Drosophila melanogaster* (20,21), paramutation in mice (22) and adaptation to different growth conditions in lower eukaryotes (12,15,16). However, it is still unclear whether these effects stem from the loss of methylation of C38 in tRNA\textsuperscript{Asp} or whether other Dnmt2 targets are responsible for the phenotypes. Many of the studies have also investigated methylation of alternate tRNA substrates by Dnmt2. In *Dicyostelium discoideum*, Dnmt2 was reported to methylate tRNA\textsuperscript{Glu}(CUC/UUC) and tRNA\textsuperscript{Glu}(GCC) very weakly in *vitro* (15). The *S. pombe* Dnmt2 homolog, Pmt1, was shown to have a weaker methylation activity on tRNA\textsuperscript{Glu} as well (16). *D. melanogaster* Dnmt2 was found to methylate tRNA\textsuperscript{Asp}(AAC) and tRNA\textsuperscript{Gly}(GCC) in *vivo* in addition to tRNA\textsuperscript{Asp} (13). However, in all enzymatic studies, tRNA\textsuperscript{Asp} was the most preferred substrate for Dnmt2. The molecular basis for the specific interaction of Dnmt2 with its tRNA substrates is not yet known. Previously, we have mapped the binding of tRNA\textsuperscript{Asp} to human DNMT2 by mutating several conserved lysine and arginine residues in human DNMT2 and concluded that the anticodon stem/loop of tRNA\textsuperscript{Asp} is a main region of contact for human DNMT2 for the methyl transfer reaction by human DNMT2 (23). Supporting this notion, Muller et al. proposed a role of the C32, A37 and C40 nucleotides in tRNA recognition (15).

A recent phylogenetic analysis revealed that the Dnmt2 gene family was most likely derived from a prokaryotic DNA-(cytosine C5)-methyltransferase (9). As mentioned above, the enzyme is highly conserved in all eukaryotic phyla. Strikingly, there are only a few putative Dnmt2 homologs in bacteria, one of which is found in *Geobacter* species (6,9,24), another putative representative is present in *Holophaga foetida* (Supplementary Figure S1). These enzymes are clearly defined by the presence of a CFT amino acid motif (CFI in *Holophaga*), which is characteristic for Dnmt2 enzymes and involved in the tRNA interaction (9,10). A bioinformatics analysis indicated that the Geobacter Dnmt2 enzyme is likely the outcome of a horizontal gene transfer from eukaryotes to Geobacter (9), which is known for its ability of lateral gene transfer (25,26). This conclusion is supported by the additional identification of a putative Dnmt2 homolog in *Holophaga*, because these bacteria are not closely related to Geobacter (*Holophaga* is a member of the Fibrobacteres/Acidobacteria group while Geobacter belongs to the δ-Proteobacteria).

As part of a larger initiative aiming to investigate the function of Dnmt2 in many species including humans, mice, flies and uncellular eukaryotes and to define its biological role, we set out to investigate the biochemical properties and substrate recognition of *Geobacter sulfurreducens* Dnmt2 (GsDnmt2). Since it has been shown that eukaryotic Dnmt2 enzymes are specific for tRNA\textsuperscript{Asp} C38 methylation, our work was started in this direction. However, we surprisingly found that GsDnmt2 specifically methylates Geobacter tRNA\textsuperscript{Glu} *in vitro* and *in vivo* and not Geobacter tRNA\textsuperscript{Asp}. This represents the first example of a Dnmt2 enzyme with a completely switched substrate specificity. Based on this finding, we further identify the variable loop as a sequence determinant in tRNA recognition of Geobacter Dnmt2 and the human enzyme.

**MATERIALS AND METHODS**

**Cloning and site-directed mutagenesis**

The gene for *G. sulfurreducens*, GsDnmt2 (GSU0227), was amplified from genomic DNA of *G. sulfurreducens* obtained from DSMZ (Braunschweig, Germany) using a primer set flanking the gene (forward 5′-GCC GCA TAT GAG GCg GGT CGA GCT CTT CTG-3′ and reverse 5′-GAT CGG AAT TCT CAC CCC TCC TCA GCC GGT AAC-3′). The amplified gene was cloned into pET28a(+) using NdeI and EcoRI sites and successful cloning validated by DNA sequencing. The catalytic site variant C74A of GsDnmt2 was created by the megaprimer site-directed mutagenesis method as described previously (27). This residue corresponds to C79 in human DNMT2, which is a key catalytic residue, mutation of which has been shown to inactivate the enzyme (10). The primer used for the site-directed mutagenesis was 5′-GTA AGG CTG CGC AGG AGG CGA C-3′.

The presence of the desired mutation was confirmed by sequencing. Constructs for other Dnmt2s are as described: human DNMT2 (28), *S. pombe* Pmt1 (16) and *D. discoideum* DnmtA (15). The gene for *D. melanogaster* Dnmt2 was amplified from complementary DNA (cDNA) using the following primer pair: 5′-GCC GTG GTG CTC GAG TTA TTT TAT CAG C-3′ and 5′-GCC GCA GCC ATA TGG TAT TTC GGG TGC TTA-3′, and cloned into pET28a+ using XhoI and NdeI sites. The cDNA was prepared using RNA isolated from 0–6-h-old embryos of *D. melanogaster* BerlinWild strain. The cloned sequence was found to contain an insertion present in the Isoform A of the *D. melanogaster* Dnmt2 gene, which was subsequently excised by polymerase chain reaction (PCR) mutagenesis performed with the following primers: 5′-ATG CCC AAT TGG ATG GAC AAC TAG TTG CCG CCT TGG-3′ and 5′-ACT AAA TAG TTC TAA GAC CCC AAA TAC CAT-3′.

**Protein expression and purification**

The wild-type and mutant C74A GsDnmt2 were expressed as His\textsubscript{6}-tagged proteins in *Escherichia coli* Rosetta 2 (DE3) cells. Protein expression and purification was performed as described previously (28). Briefly, transformed Rosetta 2 (DE3) cells were grown to OD\textsubscript{600} = 0.6. Protein expression was induced with 1-mM IPTG (Isopropyl-β-D-1-thiogalactopyranosid) and cells were further incubated at 37°C for 3 h. The cells were sonicated in lysis buffer (30-mM potassium phosphate pH 7.5, 500-mM KCl, 0.1-mM DTT (dithiothreitol), 10-mM imidazole and 10% glycerol). Recombinant proteins were purified on Ni-NTA beads (Genoxon) and eluted with 200-mM imidazole in lysis buffer. The protein was then dialyzed against dialysis buffer I (30-mM potassium phosphate pH 7.5, 300-mM KCl, 10% glycerol, 1-mM ethylenediaminetetraacetic acid (EDTA) and 0.1-mM DTT) and dialysis buffer II (30-mM potassium phosphate pH 7.5, 100-mM KCl, 60% glycerol, 1-mM EDTA and 0.1-mM DTT). A similar procedure was followed for human DNMT2 purification. For *D. melanogaster* Dnmt2, the protein expression was conducted at 28°C. For the DnmA recombinant protein, expression was carried out at 22°C. The amounts of all recombinant
proteins were determined by their specific absorbance and the concentrations and purities were verified by Coomassiestained sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels.

**In vitro transcription of tRNA substrates**

The tRNA sequences were derived from the Genomic tRNA data base (http://gtrnadb.ucsc.edu/) (29). The tRNA substrates for methylation reactions were prepared by in vitro transcription basically as described (10). The DNA templates for the in vitro transcription were purchased from MWG. The template DNA and primer sequences are given in Supplemental Table S2. The templates were amplified by PCR with T7 and tRNA-specific primers and the yield and purity of the PCR was inspected on an 8% acrylamide-agarose gel. PCR with T7 and tRNA-specific primers and the yield and purity of the PCR was inspected on a native 8% acrylamide-agarose gel.

The template DNA and primer sequences are given in Supplemental Table S2. The templates were amplified by PCR with T7 and tRNA-specific primers and the yield and purity of the PCR was inspected on a native 8% acrylamide-agarose gel.

**In vitro methylation kinetics**

The in vitro methylation reactions of tRNAs were carried out as described before (30) with few modifications. Before the methylation reactions, the substrate tRNAs were refolded by heating to 65°C and slowly cooled to ambient temperature in the presence of 2 mM MgCl₂ and 2 U/μl of RNAsin plus (Promega). The subsequent methylation reaction was performed with 0.5 μM of the tRNA substrates and 1 μM of the Dnmt2 enzyme in methylation buffer (20 mM Tris-HCl (pH 8), 20 mM NH₄OAc, 2 mM MgCl₂, 2 mM DTT and 0.02 mM bovine serum albumin), 20 mM NTPs (nucleoside triphosphates) mix, 1 U/μl of RNAsin plus (Promega), 1 U/μl of T7 RNA polymerase (Fermentas) and 35 μl of PCR-amplified template DNA. The reaction was incubated at 37°C for at least 3 h, followed by addition of 15 U DNase I and incubation for another 30 min at 37°C. Then tRNAs were separated on a 12% acrylamide/7-M urea gel and the tRNA bands were excised from the gel (Figure 1B). The tRNA was eluted with elution buffer (50 mM Tris-HCl pH 7.5, 300 mM sodium acetate and 0.5% SDS) overnight at room temperature followed by ethanol precipitation.

**Total RNA isolation from G. sulfurreducens**

*G. sulfurreducens* was grown anaerobically (N₂:CO₂ 80:20) at 30°C in NBFA medium with acetate (15 mM) as the electron donor and fumarate (40 mM) as the electron acceptor as previously described (31). Total RNA isolation from *G. sulfurreducens* was carried out with the MasterPure RNA purification kit (EpiTect Bisulfite Kit, Qiagen) and T4 RNA ligase (NEB) in 50 mM ligation buffer (50 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 1 mM DTT and 1 mM adenosine triphosphate) and 10% DMSO (dimethyl sulfoxide) overnight at 4°C. The labeled tRNAs were purified from unincorporated nucleotides by using Micro-Bio-Spin 6 columns (BioRad). The purified tRNAs were refolded and incubated with increasing Dnmt2 concentrations in methylation buffer. After 30 min the reactions were spotted onto a nitrocellulose membrane and washed three times with the reaction buffer. The membranes were exposed to X-ray films and the intensity of the spots was measured using ImageJ software. Data were fitted using the Excel solver module to an equation describing a bimolecular binding equilibrium.

**tRNA binding analysis**

The tRNA-binding analysis was performed using 3'-end-labeled tRNAs. For labeling, 1 μg of the in vitro transcribed Gs-tRNA<sup>Asp</sup> and Gs-tRNA<sup>Glu</sup> was incubated with 5 μl [5'-<sup>32</sup>P] Cytidine 3', 5'-bisphosphate (pCp) (Hartmann Analytic) and T4 RNA ligase (NEB) in 50 mM ligation buffer (50 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 1 mM DTT and 1 mM adenosine triphosphate) and 10% DMSO (dimethyl sulfoxide) overnight at 4°C. The labeled tRNAs were purified from unincorporated nucleotides by using Micro-Bio-Spin 6 columns (BioRad). The purified tRNAs were refolded and incubated with increasing Dnmt2 concentrations in methylation buffer. After 30 min the reactions were spotted onto a nitrocellulose membrane and washed three times with the reaction buffer. The membranes were exposed to X-ray films and the intensity of the spots was measured using ImageJ software. Data were fitted using the Excel solver module to an equation describing a bimolecular binding equilibrium.

**tRNA bisulfite sequencing**

The tRNA bisulfite sequencing was performed as described previously (32). For this study, tRNA<sup>Asp</sup>, tRNA<sup>Glu</sup>, tRNA<sup>Glu</sup><sup>2</sup> and tRNA<sup>Val</sup> were selected because they contain cytosine at position 38 in their sequence. Briefly, 3 μg of total RNA was treated with bisulfite following the instructions of the manufacturer (EpiTect Bisulfite Kit, Qiagen). The bisulfite-treated RNA was later used to generate a cDNA by reverse transcription using a stem loop primer (5'-CTC AAC TGG TGT CTG GGA GTA GCC AAC TCA GTA GTG TGG TAA CAA AATC-3', 5'-CTC AAC TGG TGT CTG GGA GTA GCC AAC TCA GTA GTG TGG TAA TAA TCCAG-3' and 5'-CTC AAC TGG TGT CTG GGA GTA GCC AAC TCA GTA GTG TGG TAA TAA TCCAG-3').
RESULTS

GsDnmt2 is an active enzyme capable of methylating C38 in tRNA\textsuperscript{Asp}

After successfully cloning the \textit{GsDnmt2} gene into the pET28a+ vector, expressing and purifying the protein (Supplementary Figure S3), its activity was tested using \textit{in vitro} transcribed tRNAs (Supplementary Figure S4). It was known from many previous reports that Dmnt2 from all the eukaryotic organisms methylate tRNA\textsuperscript{Asp} (6,10,12–16). To test whether the bacterial Dmnt2 also methylates tRNA\textsuperscript{Asp}, \textit{in vitro} transcribed tRNA\textsuperscript{Asp} of \textit{D. melanogaster} was used as the initial substrate. Since the tRNA methylation rate of human DNMT2 is in the range of few turnovers per hour (10) and the Dmnt2 enzymes from other species studied here showed similar or lower activity, it was not possible to conduct steady-state methylation experiments. Therefore, we followed the initial time course of tRNA methylation re-
GsDnmt2 shows unusual preference for tRNA\textsuperscript{Glu} over tRNA\textsuperscript{Asp}

After the initial observation that \textit{G. sulfurreducens} Dnmt2 showed lower methylation of its \textit{Geobacter}-encoded tRNA\textsuperscript{Asp} than of tRNA\textsuperscript{Glu} from \textit{Drosophila} or \textit{M. musculus}, the question arose whether any other \textit{Geobacter} tRNA might be more preferred as a substrate. To this end, \textit{Geobacter} tRNAs were inspected for the presence of a cytosine at position 38 of the tRNA sequence. Using the UCSC Genomic tRNA Data Base (http://gtrnadb.ucsc.edu/) \cite{29} we identified tRNA\textsuperscript{His} as well as two isodecoders of each tRNA\textsuperscript{Ala}, tRNA\textsuperscript{Glu} and tRNA\textsuperscript{Val} as Dnmt2 candidate substrates in \textit{Geobacter}, in addition to tRNA\textsuperscript{Asp}. Among them, both isodecoders of tRNA\textsuperscript{Glu} were selected for methylation analysis, because they show high similarity to tRNA\textsuperscript{Asp}, as well as tRNA\textsuperscript{His} and one of the tRNA\textsuperscript{Val} and tRNA\textsuperscript{Ala} sequences (Supplementary Table S1). All of these tRNAs were synthesized by \textit{in vitro} transcription and assayed for methylation by GsDnmt2. Our results showed that tRNA\textsuperscript{Asp} was weakly methylated as stated above (Figure 2), but the initial reaction rate for methylation of tRNA\textsuperscript{Glu} (tRNA32) was 10-fold higher than methylation of tRNA\textsuperscript{Asp}. The methylation of the second isodecoder of tRNA\textsuperscript{Glu-2} (tRNA20) was intermediate between tRNA\textsuperscript{Asp} and the other tRNA\textsuperscript{Glu} (Figure 2) and methylation of other tRNAs was not detectable (data not shown). These results motivated us to continue our study using the tRNA\textsuperscript{Glu} (tRNA32) isodecoder. A mutant tRNA\textsuperscript{Glu} carrying a C38U mutation was prepared and we observed that the mutation caused a complete loss of methylation, indicating that the methylation in tRNA\textsuperscript{Glu} happens only at position C38 (Figure 2). In addition, the C74A mutation in GsDnmt2 led to loss of methylation on tRNA\textsuperscript{Glu} (Figure 2). The preferential methylation of Gs-tRNA\textsuperscript{Glu} was also confirmed using a gel-based methylation assay \cite{10} in which the methylated tRNA is directly observed (Supplementary Figure S5).

To test if the substrate preferences of human and \textit{Geobacter} Dnmt2 were based on preferential binding of one type of tRNA, we determined their equilibrium constants of binding to Gs-tRNA\textsuperscript{Glu} and Gs-tRNA\textsuperscript{Asp} (Supplementary Figure S6). Our data show that the human enzyme binds tRNA about four to five times stronger than the \textit{Geobacter-
Figure 4. Dnmt2 enzymes from various species prefer methylation of *G. sulfurreducens* tRNA<sup>Asp</sup>. (A) Examples of methylation kinetics are shown for wild-type Dnmt2 enzymes from *S. pombe* (Pmt1), *D. discoideum* (DnmA), *D. melanogaster* and *H. sapiens* using *in vitro* transcribed Gs-tRNA<sup>Asp</sup> and Gs-tRNA<sup>Glu</sup> and their respective C38U variants. (B) Average methylation rates and SEM based on two repeats of the experiments.

**Cellular methylation of tRNAs in *G. sulfurreducens***

As described above, we have found that GsDnmt2 has a different specificity than other Dnmt2 enzymes in *in vitro*. Therefore, the cellular methylation patterns of tRNAs in *G. sulfurreducens* were investigated. For this analysis, total RNA was isolated from wild-type *G. sulfurreducens* and the cytosine methylation investigated by bisulfite conversion, reverse transcription and cloning of the converted tRNA<sup>Asp</sup>, tRNA<sup>Glu</sup> and tRNA<sup>Val</sup> followed by sequencing of individual clones. As shown in Figure 3, the methylation at the C38 position of tRNA<sup>Glu</sup> in wild-type *G. sulfurreducens* was 22%. The tRNA<sup>Glu</sup>-2 isodecoder was methylated by 36% at the same site. In contrast, signals for tRNA<sup>Asp</sup> and tRNA<sup>Val</sup> at C38 methylation were at background levels of about 3%. Hence, both tRNA<sup>Glu</sup> isodecoders were efficiently methylated in *in vitro* and they are also methylated in cells, while tRNA<sup>Asp</sup>, which showed the weakest *in vitro* methylation among all substrates tested, was not methylated in cells.

We conclude that cellular tRNA methylation at C38 mirrors the *in vitro* specificity GsDnmt2. The same observation has been made with human DNMT2. In human cells tRNA<sup>Asp</sup> but not tRNA<sup>Glu</sup> is methylated at C38 (34,35), which is in agreement with the *in vitro* specificity of the human DNMT2 enzyme. The incomplete methylation of the tRNA<sup>Glu</sup> in *Geobacter* is similar to what has been observed for tRNA<sup>Asp</sup> in other species by RNA bisulfite (16,32,34). It is possible that the reverse transcription step of the RNA bisulfite method is more efficient with unmodified, immature tRNAs, which may influence the results.

**Comparison of methylation specificities of Dnmt2 homologs from various species**

We concluded in the last paragraphs that GsDnmt2 strongly prefers to methylate Gs-tRNA<sup>Glu</sup> not Gs-tRNA<sup>Asp</sup> both *in vitro* and *in vivo*. However, as shown above, tRNA<sup>Asp</sup> from other species like *D. melanogaster* and *M. musculus* were good GsDnmt2 substrates. This observation suggested that there may be changes in the *Geobacter* tRNAs that increase methylation of Gs-tRNA<sup>Glu</sup> but reduce the reaction with Gs-tRNA<sup>Asp</sup>. Hence, the specificities of Dnmt2 enzymes from other species were investigated for the methylation of *Geobacter* tRNA<sup>Asp</sup> and tRNA<sup>Glu</sup> also using the C38U mutant tRNA versions as controls (Figure 4). Dnmt2 enzymes from different species that represent key eukaryotic phyla were expressed and purified (Supplementary Figure S3) and their methylation activities were analyzed. The assays were done in parallel for a specific enzyme with all tRNA substrates. The results showed that human DNMT2,
to CGGC) which introduced the GG dinucleotide sequence into tRNA^{Asp}. The mutant tRNA^{Asp} was synthesized by in vitro transcription. Equal amounts of the wild-type and mutant tRNAs were used in methylation reactions with GsDnmt2 (Supplementary Figure S4). Strikingly, the alteration of these two bases in the variable loop of murine tRNA^{Asp} led to a strong decrease in the methylation rate (Figure 7A). This result supports the hypothesis of an involvement of the variable loop in the enzyme-substrate recognition of GsDnmt2. To further extend this finding, methylation reactions were performed with the same substrates using the human DNMT2 enzyme. Here, also, a similar reduction of activity was observed with the mutant tRNA^{Asp} compared to the wild type tRNA (Figure 7A), indicating that the variable loop is also important for tRNA^{Asp} recognition by human DNMT2. Next, we investigated if the variable loop of Gs-tRNA^{Glu} also has a role in recognition. As shown in Figure 7B, replacement of the original Gs-tRNA^{Glu} variable loop by either Gs-tRNA^{Asp} or murine tRNA^{Glu} resulted in a strong reduction of its methylation by GsDnmt2. These data indicate that the GG dinucleotide in the variable loop functions as a universal anti-determinant for methylation by Dnmt2. However, replacing the variable loop of murine tRNA^{Glu} with the corresponding loop from murine tRNA^{Asp} did not result in a significant increase of its methylation by human DNMT2 (data not shown), indicating that the variable loop is not the only recognition determinant of Dnmt2.

DISCUSSION

Dnmt2 enzymes are conserved in most eukaryotic species (6,9) and methylate tRNA^{Asp} with high activity. Here, the activity of one of the few known bacterial putative Dnmt2 enzymes was studied, which is found in Geobacter strains including *G. sulfurreducens* (GsDnmt2). We show that GsDnmt2 actively methylates tRNA^{Asp} from *M. musculus* and *Drosophila* that were used as model substrates in previous work. This result confirms that GsDnmt2 is a member of the Dnmt2 family as expected on the basis of the amino acid alignment. However, the endogenous Geobacter tRNA^{Asp} was methylated only very inefficiently. Instead, GsDnmt2 methylates *Geobacter* tRNA^{Glu} at the corresponding site, indicating a complete swap in its specificity (Figure 5)—a surprising and unexpected result that was confirmed in a methylation analysis of tRNA isolated from *G. sulfurreducens*. There are two isodecoders of tRNA^{Glu} in Geobacter (tRNA20, here called tRNA^{Glu-2}, and tRNA32, here called tRNA^{Glu}), which both were methylated *in vitro* and *in vivo*, although the relative methylation levels of the two isodecoders varied in the two data sets. *In vitro*, tRNA^{Glu} was methylated three times faster while in cells the methylation level of tRNA^{Glu-2} was 1.6 times higher. There could be several explanations for this difference: (i) tRNA^{Glu-2} methylation could be less efficient under *in vitro* conditions, (ii) additional modifications could modulate the tRNA^{Glu} or tRNA^{Glu-2} methylation *in vivo* or (iii) additional modifications could affect the recovery of methylated tRNA^{Glu} and tRNA^{Glu-2} differently after bisulfite conversion. However, in all data sets both isodecoders of Gs-tRNA^{Glu} were more methylated than Gs-tRNA^{Asp}.

**Figure 5.** Comparison of the specificities of Dnmt2 homologs from different species for methylation of *Geobacter* tRNA^{Asp} and tRNA^{Glu}. Data are replotted from Figures 2B and 4B and the relative activities of the Asp/Glu pairs were always normalized to the more active substrate for a better comparison. Error bars were based on normalized individual experiments and correspond to the SEM of two to three repeats.

The variable loop functions as a sequence determinant in the tRNA recognition of Dnmt2

To investigate the molecular reason for the inversion of the substrate preference of the GsDnmt2 enzyme with its matching *Geobacter* tRNAs, we compared the secondary structures of tRNA^{Asp} and tRNA^{Glu} from several species and noticed that Gs-tRNA^{Asp} has a longer variable loop with an extra guanine (Figure 6). Furthermore, the variable loops of all preferred Dnmt2 substrates (namely tRNA^{Asp} from all species but *Geobacter* and Gs-tRNA^{Glu}) have at least one A at position 45 or 46 of the variable loop, while all the inactive counterparts contain a GG dinucleotide. Based on this, we speculated that the variable loop may be a specificity determinant of GsDnmt2. To test this hypothesis, the variable loop of murine tRNA^{Asp} was swapped with that of murine tRNA^{Glu} by an exchange of two nucleotides (AGAC
We have shown here that Geobacter Dnmt2 has an \( \sim 100 \)-fold swapped specificity for Geobacter tRNAs, because it prefers tRNA\(^{Glu} \) roughly 10-fold over tRNA\(^{Asp} \), while all other Dnmt2 enzymes showed an at least 10-fold preference for tRNA\(^{Asp} \) over tRNA\(^{Glu} \) (Figure 5). What could be the molecular basis of the unexpected loss of Geobacter tRNA\(^{Asp} \) methylation by GsDnmt2 and the corresponding gain of methylation of tRNA\(^{Glu} \)? So far, various amino acids involved in tRNA recognition of the human enzyme could be mapped (23), but the sequence elements needed on the tRNA side were largely unknown. Initially, the G34 base at the wobble position of the anticodon, which is modified to mannosylqueuosine in eukaryotic cells, was associated with Dnmt2 activity (6). However, the results with G. sulfurreducens are not in favor of that model, because Gs-tRNA\(^{Asp} \) contains the G34, but it is a very weak substrate, while Gs-tRNA\(^{Glu} \), which is the preferred substrate, does not have a G34. By comparing substrate and nonsubstrate tRNAs of GsDnmt2, it became apparent that all non-substrates contain a GG dinucleotide in the variable loop as a characteristic feature. Indeed, introduction of this sequence into murine tRNA\(^{Asp} \) and Geobacter tRNA\(^{Glu} \) drastically reduced their methylation by GsDnmt2. We cannot rule out the possibility that the two point exchanges in the variable loop might disrupt tRNA folding. However, the initial discovery that the GG dinucleotide is inhibiting GsDnmt2 was made with natural tRNAs, which are all fully functional in protein biosynthesis, which rules out loss of structure in these cases. Therefore, when taken together, our results strongly suggest that the variable loop functions as an important specificity determinant in GsDnmt2. Interestingly, the exchange in the murine tRNA\(^{Asp} \) also reduced its methylation by the human DNMT2, indicating that the variable loop is a critical specificity determin-
nant for the tRNA recognition of human DNMT2 as well. However, introduction of the favorable variable loop into murine tRNA\textsubscript{Glu} did not increase its methylation. Hence, our data indicate that Dnmt2 enzymes use further tRNA-specific sequence determinants in addition to the variable loop. These (so far unknown) contacts mediate the preference of the human DNMT2 enzyme for Gs-tRNA\textsuperscript{Asp}, despite the presence of the unfavorable variable loop. Furthermore, they prevent methylation of murine tRNA\textsubscript{Glu}, even after introducing a favorable variable loop. The contribution of additional specificity determinants (besides the GG dinucleotide in the variable loop) to tRNA recognition by Dnmt2 enzymes is also supported by the observation that Dnmt2 enzymes from other species still strongly prefer Gs-tRNA\textsubscript{Asp} over Gs-tRNA\textsubscript{Glu}.

The role of the variable loop as a specificity determinant in tRNA recognition by Dnmt2 is not without precedent. Examples for tRNA-interacting enzymes, which use the variable loop for tRNA recognition, include a bacterial tRNA (m7G46) methyltransferase (36) and a prokaryotic tRNA\textsubscript{dependent} amidotransferase (37), and the same strategy is also used by several aminocarboxyl-tRNA-synthetases (38). Recognition of nucleic acids by proteins, in general, is based on direct and indirect readout, i.e. direct contacts of amino acids to nucleobases, which detect the nature of the base, and contacts to the nucleic acid backbone, which detect the structure of the nucleic acid. The GG dinucleotide in the variable loop of the tRNA could interfere with either of these processes. However, the observation that the variable loop of preferred tRNA substrates is only characterized by the absence of the GG dinucleotide but it does not contain a specific nucleotide sequence might argue against direct base contacts. This suggests that an indirect readout mechanism is operational in which the presence of the GG dinucleotide induces a structural change in the tRNA which then prevents its methylation.

The finding that GsDnmt2 methylates tRNA\textsubscript{Asp} from other species (as all other members of the Dnmt2 family do) but not from \textit{Geobacter} provides strong support for the hypothesis that the Gs-Dnmt2 was acquired by horizontal gene transfer, because there is no reason for this enzyme to methylate tRNA\textsubscript{Asp}, which is not a substrate in \textit{Geobacter} itself. In the horizontal gene transfer view, the activity toward tRNA\textsubscript{Asp} can be interpreted as an evolutionary relic. Based on our data, one may speculate that initially a Dnmt2 gene with tRNA\textsubscript{Asp} preference, but also the ability to methylate tRNA\textsubscript{Glu}, was acquired by \textit{Geobacter}. Precedence for such side activity can be seen in many species including \textit{D. melanogaster}, \textit{S. pombe} and \textit{D. discoideum} (13,15,16). Methylation of the Gs-tRNA\textsubscript{Glu} was favorable, which led to the stable integration of the Dnmt2, whereas methylation of the endogenous tRNA\textsuperscript{Asp} was lost in a co-evolution of GsDnmt2 and the Gs-tRNA\textsubscript{Asp}. At present, one can only speculate about the role of Dnmt2 in \textit{Geobacter}. In previous studies, tRNA methylation by Dnmt2 was found to improve the stability of the tRNA (13) or affect tRNA charging (R. Shanmugam et al., in preparation), and prevent tRNA fragmentation (13). Which of these roles, if any, is important for \textit{Geobacter} tRNA\textsuperscript{Glu} cannot be said at present. The generation of a Dnmt2 knockout strain was unsuccessful (M. Aklujkar, unpublished information), which may indicate that Dnmt2 is an important gene in \textit{G. sulfurreducens}, which is in line with the evolutionary arguments. This would be an interesting finding, since Dnmt2 enzymes are non-essential in other model organisms under normal growth conditions (6,12,13,15,21).

CONCLUSIONS

The first enzymatic characterization of the specificity of a bacterial Dnmt2 homolog, the enzyme from \textit{G. sulfurreducens}, revealed that it is the only known Dnmt2 enzyme that does not prefer methylation of tRNA\textsubscript{Asp}. Instead, an unexpected swap of specificity of this enzyme was observed toward methylation of \textit{Geobacter} tRNA\textsubscript{Glu} which was confirmed \textit{in vitro} and \textit{in vivo}. However, GsDnmt2 still methylates tRNA\textsubscript{Asp} from other species, and Dnmt2 enzymes from other species still methylate \textit{Geobacter} tRNA\textsubscript{Asp}. We provide an evolutionary scenario that can explain this interesting finding. We noticed that the \textit{Geobacter} tRNA\textsubscript{Asp} has a modified variable loop, which serves as a specificity determinant and causes the loss of methylation activity. Furthermore, we show that this mechanism could be extended to the human enzyme as well, indicating that work with a bacterial Dnmt2 homolog, finally, has led to important insight into the mechanism of the corresponding human enzyme.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

FUNDING

Deutsche Forschungsgemeinschaft (DFG) Research Group FOR1082 and Deutscher Akademischer Austauschdienst (DAAD). Funding for open access charge: DFG and University Stuttgart.

Conflict of interest statement. None declared.

REFERENCES
