The human Dnmt2 protein is one member of a protein family conserved from Schizosaccharomyces pombe and Drosophila melanogaster to Mus musculus and Homo sapiens. It contains all of the amino acid motifs characteristic for DNA-(Cytosine-C5) methyltransferases, and its structure is very similar to prokaryotic DNA methyltransferases. Nevertheless, so far all attempts to detect catalytic activity of this protein have failed. We show here by two independent assay systems that the purified Dnmt2 protein has weak DNA methyltransferase activity. Methylation was observed at CG sites in a loose ttnCggag(a) consensus sequence, suggesting that Dnmt2 has a more specialized role than other mammalian DNA methyltransferases.

In mammals, DNA methylation is the only known physiological modification of DNA. It primarily occurs at CG sites, which are methylated to 70–80% and encode epigenetic information on the DNA. DNA methylation is implicated in the regulation of gene expression, control of development, X chromosome inactivation, parental imprinting, and the protection of the genome against parasitic genetic elements such as transposons, retrotransposons, and viruses (for review see Refs. 1, 2–4). Ablerrant methylation is among the most important causes of the inactivation of tumor suppressor genes in cancer (5). DNA-(Cytosine-C5) methyltransferases (MTases) are characterized by a set of highly conserved amino acid motifs (6) that easily allows the identification of putative enzymes based on genomic sequences. In mammals, four candidate enzymes have been identified (1, 7). Dnmt1 is known to have a high preference for the motifs characteristic for prokaryotic Cytosine-C5 MTases. Dnmt2, Dnmt3a, and Dnmt3b do not recognize preexisting patterns of methylation and are de novo MTases (10–12). However, it is also clear that in vivo the functions of maintenance and de novo methylation overlap, because accurate maintenance of the methylation at certain sequences also requires the presence of Dnmt3a and 3b and de novo methylation also relies on support by Dnmt1 (13–16). Dnmt1, Dnmt3a, and Dnmt3b are large proteins comprising an N-terminal part implicated in protein targeting and regulation and a C-terminal part that contains all 10 motifs characteristic for DNA-(Cytosine-C5) MTases. All three MTases are essential in mammals. Knock-out mice die during embryogenesis or shortly after birth (8, 11). The fourth putative DNA MTase in the genome of mammals, Dnmt2, has been discovered in 1998 (17, 18). Expression of Dnmt2 has been found in many human and mouse tissues albeit at low levels (10, 17, 18). Lacking a large N-terminal part, the human protein comprises 391 amino acid residues, which show clear homology to prokaryotic DNA-(Cytosine-C5) MTases. Members of the Dnmt2 family are found in many species including man and mice, which also contain other DNA MTases, but also in D. melanogaster and S. pombe where these enzymes are the only obvious DNA MTase candidate genes. The strict conservation of all of the motifs characteristic for prokaryotic Cytosine-C5 MTases in Dnmt2 including the catalytically important amino acid residues suggests a function as DNA MTase. This hypothesis was supported by the finding that D. melanogaster DNA contains methylated cytosines that most probably is attributed to the activity of Dnmt2 (19, 20). The structure of Dnmt2 was solved in complex with AdoMet, the cofactor for DNA methylation (21). It shows strong similarities to other DNA MTases, supporting the assumption that the protein is a DNA MTase. Interaction with DNA could be detected in gel shift experiments. In addition, it has been reported that Dnmt2 covalently binds to DNA (21). This observation also connects the protein to DNA MTases, which form a covalent enzyme-DNA intermediate during catalysis (for review see Refs. 1, 22, 23). Nevertheless, despite considerable efforts no catalytic activity of Dnmt2 could be detected so far (10, 17, 18, 21). In addition, Dnmt2-deficient embryonic stem cells are viable and do not show any obvious difference in the DNA methylation pattern (10) and Dnmt2 knock-out animals are viable and fertile with minor defects.2

EXPERIMENTAL PROCEDURES

Cloning, Site-directed Mutagenesis, and Protein Purification—
Dnmt2 cDNA was obtained from the Deutsches Ressourcenzentrum für Genomforschung GmbH (Berlin, Germany) (clone number IMAGp998C184250Q2) amplified by PCR and cloned into pET28a+ (Novagen). A Dnmt2 variant in which the highly conserved active site residues Cys-79 and Glu-119 were exchanged by Ala was prepared by site-directed mutagenesis following standard procedures (24). The sequence of all of the expression constructs was verified by DNA sequencing. Dnmt2 and the Dnmt2 mutant were expressed in BL21 Rosetta(DE3) pLysS cells (Novagen) and purified to homogeneity by nickel-nitrilotriacetic acid affinity chromatography following a procedure described for Dnmt3a with the exception that no protease inhibi-

2 E. Li, personal communication.
Fig. 1. A. Purification of Dnmt2. The figure shows a Coomassie Blue-stained SDS gel. The molecular size markers are indicated. B, MALDI-TOF mass spectrometry analysis of the purified Dnmt2. The theoretical mass of the H+ ion of Dnmt2 after cleavage of the initial Met residue is 46292.7.

RESULTS

We have cloned human Dnmt2 into a bacterial expression vector and expressed and purified the protein to homogeneity (Fig. 1A). A MALDI-TOF mass spectrometry analysis confirmed the mass of the protein within the experimental error (experimental mass of M+H+: 46631.0 Da; theoretical mass: 46629.7 Da) (Fig. 1B). No significant peaks were detected at higher masses. This result shows that the purified protein does not carry covalently bound nucleotides or DNA. The protein was incubated with different radioactively labeled DNA fragments generated by PCR in the absence of cofactor as well as in the presence of AdoMet or S-adenosyl-l-homocysteine and analyzed on denaturing SDS-polyacrylamide gels. However, no coherent complex with DNA could be detected (data not shown). This result suggests that the covalent complex formation reported previously (21) might occur only with special DNA sequences.

HPLC/TLC Activity Assay — The catalytic activity of Dnmt2 was investigated using A-DNA or a PCR fragment derived from pAT153 as substrate. No catalytic activity could be detected (data not shown). Because we have used purified Dnmt2 enzyme at high concentrations in several restriction endonucleases sensitive to methylation of cytosine residues. However, in agreement with previous studies (10, 18, 21), no catalytic activity could be detected. Because we have used purified Dnmt2 enzyme at high concentrations in these assays, this result indicates that the specific activity of Dnmt2 is severalfold lower than that of Dnmt1 (9) or Dnmt3a or Dnmt3b (10, 12).

However, there are several possible explanations for this negative result, because the sensitivity of tritium transfer assays is limited and many DNA MTases carry tightly bound cofactor after purification. Bound cofactor interferes with the tritium transfer assay, in particular, if high amounts of the purified enzyme are used, which is required if low activities are to be detected. In addition, the conclusions that can be drawn from restriction protection experiments are restricted because methylation can only be detected if the recognition sequence of the radioactive sample were applied to cellulose TLC plates (Cellulose CEL 400-10, 20 × 20 cm, Macherey-Nagel, Düren, Germany) at ambient temperature. The first dimension was run in isobutyric acid/water/ammonia (66:33:1, v/v/v). The plate was dried in air and developed in the second dimension with isopropyl alcohol/concentrated HCl/water (70:15:15, v/v/v). The plate was dried, and the radioactivity was analyzed using an instant imager (Canberra Packard).

bisulfite Sequencing Analysis — Bisulfite sequencing was carried out as described previously (27, 28). Purified methylated or unmethylated DNA (20 ng) was incubated NaOH (0.3 M) for 15 min at 37 °C, sodium metabsulfite and hydroquinone were added to final concentrations of 2.4 μM and 0.5 μM, and the mixture was incubated for 16 h at 55 °C. The DNA was purified over PCR spin columns and incubated with NaOH (0.3 M) for 15 min at 37 °C. The solution was neutralized by the addition of ammonium acetate, pH 7, and precipitated with ethanol. The lower strand of the converted DNA was amplified by PCR using Taq DNA polymerase and cloned using TOPO TA cloning kit for sequencing (Invitrogen). Finally, 50 clones of the DNA incubated with Dnmt2 and 50 clones of the control DNA were sequenced.
the restriction enzyme overlaps with that of the MTase. Therefore, we have also investigated the activity of Dnmt2 using an HPLC/TLC assay that is very sensitive and general (19, 26). DNA was methylated in vitro and hydrolyzed to nucleosides, and the nucleosides were separated by HPLC. The nucleosides then were labeled with deoxynucleoside kinase (dNK) using \(^{32}\)P-ATP, and the nature of each nucleotide was confirmed by two-dimensional TLC.

As shown with methylated reference DNA, 5-methylcytidine elutes from the HPLC column after 12.9 min (Fig. 2). The elution profile of the nucleosides from the HPLC column was monitored by UV spectroscopy at 260 nm. No 5-methylcytidine was detected from the elution profile of the compound from the HPLC column. As additional control, a Dnmt2 variant was prepared in which the highly conserved active site residues Cys-79 and Gly-119 were exchanged by Ala. The variant was purified, and its identity was confirmed by MALDI-TOF mass spectrometry (experimental mass of M + H\(^{+}\) : 46537.1 Da; theoretical mass: 46538.6 Da). After incubation of the DNA with the active site variant, a much lower amount of 5-methylcytosine was detected (Fig. 5). A quantitative analysis revealed that the amount of 5-methylcytosine obtained after incubation with the Dnmt2 variant was 10-fold lower than the amount obtained with Dnmt2. This result demonstrates that the active site residues of Dnmt2 are important for catalysis, confirming the conclusion that Dnmt2 is an active DNA MTase. Because in the Dnmt2 variant the two most important catalytic residues are removed, one might have expected a larger reduction in activity. However, we recently obtained similar results with the Dnmt3a MTase where also residual activity was detectable even after the removal of these residues (30). This result indicates that in addition to the established catalytic mechanism DNA MTases also have other means to accelerate the methylation reaction such as correct positioning of the flipped base and the cofactor and binding to the transition state of methyl group transfer.

**Bisulfite Sequencing Activity Assay**—To investigate the catalytic activity of Dnmt2 with an independent assay, DNA was incubated with Dnmt2 in vitro and then subjected to bisulfite treatment (31, 32). This procedure converts all of the cytosine residues to uracil, giving rise to thymine after amplification by PCR. Only methylated cytosines are refractory to the deamination. Bisulfite-treated DNA was cloned, and 44 clones were sequenced (~350 nucleotides/clone) (Supplemental Fig. 1). As a control, 41 clones of DNA incubated with the Dnmt2 mock preparation were sequenced. In the Dnmt2-treated DNA, 24 cytosine residues were detected in comparison to 11 in the control DNA (Table 1). However, because of polymerase errors in the PCR of the bisulfite-treated DNA, not all of these cytosines correspond to methylated cytosines in the original DNA. Eight of the cytosines detected in the control DNA and...
nine in the Dnmt2-treated DNA were observed at positions corresponding to a thymine in the original DNA. Thus, these cytosines must have been introduced by polymerase errors, which cannot be avoided, because TagDNA polymerase must be used to amplify uracil-containing DNA (28). In addition, three cytosines in the control DNA occur at positions corresponding to cytosine in the original DNA. Because the control was not methylated, they can be the result of incomplete deamination during bisulfite treatment or polymerase errors after successful conversion of the original cytosine to uracil. However, the distribution of cytosines observed at the position of T or C in the original DNA is highly biased, whereas in the control DNA only three cytosines were observed at positions corresponding to a cytosine in the original DNA. In the Dnmt2-treated DNA, 15 cytosines were found at such sites. This distribution of cytosines found at the place of cytosine versus cytosine found at the place of thymine (15:9 for Dnmt2-treated DNA but 3:8 for the control DNA) cannot be due to statistical fluctuations \( (p = 1.1 \times 10^{-4}) \) and is a strong indication that Dnmt2 is an active DNA MTase. Since we observed 15 5-methylcytosine residues in the 44 clones sequenced, the ratio of 5-methylcytosine/cytosine can be estimated to be 1:250 on the basis of the bisulfite sequencing assay, close to what has been modified and sharpened as a result of further studies.

**FIG. 5. Analysis of the catalytic activity of a Dnmt2 active site variant.** 0.2 \( \mu \)g of DNA was incubated with Dnmt2 degraded to nucleosides and subjected to HPLC analysis. Fractions were collected between 12' and 13', labeled with dNK, and analyzed by two-dimensional TLC. The activity of the active site variant was >10-fold reduced as compared with Dnmt2.

**TABLE I**

Results of the bisulfite sequencing methylation analysis

\( n_{seq} \) number of clones sequenced; \( n^C \) (at C), number of cytosine residues found at positions where a C was present in the original DNA; \( n^T \) (at T), number of cytosine residues found at positions where a T was present in the original DNA.

|    | Dnmt2 | Control |
|----|-------|---------|
| \( n_{seq} \) | 44     | 41      |
| \( n^C \) (at C) | 15     | 3       |
| \( n^T \) (at T) | 9      | 8       |

**TABLE II**

Consensus sequence for DNA methylation by Dnmt2

The table compiles the numbers of occurrences of each base at each position relative to the methylated cytosine. The last line indicates the consensus sequence.

| Position | -3 | -2 | -1 | 0 | +1 | +2 | +3 | +4 |
|----------|----|----|----|---|----|----|----|----|
| A        | 2  | 3  | 1  | 0 | 1  | 6  | 1  | 6  |
| T        | 6  | 6  | 5  | - | 2  | 5  | 2  | 2  |
| G        | 4  | 5  | 5  | - | 8  | 6  | 5  | 6  |
| C        | 3  | 1  | 4  | 15| 2  | 3  | 2  | 1  |
| consensus| t  | t  | N  | C | G  | g  | g/a|    |
Catalytic Activity of Dnmt2 Methyltransferase

Liang, G., Chan, M. F., Tomigahara, Y., Tsai, Y. C., Gonzales, F. A., Li, E., Gowher, H., and Jeltsch, A. (2001) Cell 107, 491–494.

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