CXXC Domain of Human DNMT1 Is Essential for Enzymatic Activity

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ABSTRACT: DNA cytosine methylation is one of the major epigenetic gene silencing marks in the human genome facilitated by DNA methyltransferases. DNA cytosine-5 methyltransferase 1 (DNMT1) performs maintenance methylation in somatic cells. In cancer cells, DNMT1 is responsible for the aberrant hypermethylation of CpG islands and the silencing of tumor suppressor genes. Here we show that the catalytically active recombinant DNMT1, lacking 580 amino acids from the amino terminus, binds to unmethylated DNA with higher affinity than hemimethylated or methylated DNA. To further understand the binding domain of enzyme, we have used gel shift assay. We have demonstrated that the CXXC region (C is cysteine; X is any amino acid) of DNMT1 bound specifically to unmethylated CpG dinucleotides. Furthermore, mutation of the conserved cysteines abolished CXXC mediated DNA binding. In transfected COS-7 cells, CXXC deleted DNMT1 (DNMT1$^{\Delta CXXC}$) localized on replication foci. Both point mutant and DNMT1$^{\Delta CXXC}$ enzyme displayed significant reduction in catalytic activity, confirming that this domain is crucial for enzymatic activity. A permanent cell line with DNMT1$^{\Delta CXXC}$ displayed partial loss of genomic methylation on rDNA loci, despite the presence of endogenous wild-type enzyme. Thus, the CXXC domain encompassing the amino terminus region of DNMT1 cooperates with the catalytic domain for DNA methyltransferase activity.

In the human epigenome, DNA methylation is involved in regulation of tissue specific gene expression, chromatin structure stability, embryonic development, X chromosome inactivation in females and imprinted gene silencing (*1*). Silencing of retrotransposon elements by DNA methylation in the normal human genome dissuades unwanted transposon activity. DNA methylation in mammals is initiated and maintained by a class of DNA (cytosine-5) methyltransferases: DNMT1,[1] DNMT3a, DNMT3b, and a methyltransferase-like protein known as DNMT3L. DNMT3a and DNMT3b are fundamental during normal development, as they establish de novo methylation patterns in ES cells and early embryos. However, neither DNMT3a nor DNMT3b has any effect on maintenance of imprinted methylation patterns (*2*). Although DNMT3L does not possess the catalytic conserved domain of methyltransferases, it can still interact and influence the activity of DNMT3a and DNMT3b. Structural studies on DNMT3a suggest that it interacts with DNMT3L for catalytic activation, and that DNMT3L binds to histone H3 lysine 4 using its PHD domain (*3, 4*). DNA (cytosine-5) methyltransferase 1 (DNMT1) is the major maintenance methyltransferase in mammals, and it localizes on the DNA replication foci via PCNA loading factor (*5*). DNMT1 is crucial for mammalian development, proliferation and survival of cancer cells (*6–9*). It attaches a methyl group to the carbon-5 (C5) position of cytosine residues using AdoMet as a cofactor. With an unmethylated DNA substrate, the full-length DNMT1 is catalytically poor compared to its activity on the same hemimethylated DNA sequence (*10*). However, in cancer cells, DNMT1 facilitates aberrant DNA methylation of vital tumor suppressor genes, repressing them and, thereby, progressing carcinogenesis (*11, 12*).

DNMT1 is a versatile protein consisting of ~1616 amino acids in mouse and human. The first 1100 amino acids compose its large amino terminus or regulatory region, and the other 500 amino acids represent its small catalytic region (*13, 14*). Both regulatory and catalytic portions are joined by a series of Gly-Lys repeats. The catalytic region contains all the conserved motifs found in prokaryotic methyltransferases (*15*). Both regulatory and catalytic regions have been separated by V8 protease digestion of the mouse Dnmt1 (*16*); however, separate expression of either regulatory or catalytic regions does not yield a catalytically active enzyme (*17*). Therefore, interaction and interplay between both regions is necessary for methyltransferase activity. The amino terminus region of the enzyme is also involved in methylation independent gene repression through recruitment of transcriptional repressors, such as retinoblastoma, histone deacetylase 1 (*18*), G9a (*19*), MeCP2 (*20*) and HP1 (*21*). These signature repressor elements could facilitate chromatin silencing in a cooperative manner. Furthermore, the amino terminus also possesses DNA binding motifs that can bind to methylated DNA and participate in allosteric activation of the enzyme (*22*). In the amino terminus of DNMT1, embedded between the PCNA-binding and BAH regions, lies

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[1] Abbreviations: DNMT1, DNA (cytosine-5) methyltransferase 1; Znf, zinc finger; HM, hemimethylated; UM, unmethylated; CG, CpG; RSL1, Rheoswitch ligand 1; TRD, target recognition domain.

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a cysteine rich CXXC repeat region that resembles a zinc-finger-binding domain. Generally, zinc fingers are located in the catalytic region of enzymes, aiding with substrate binding. The 40 amino acid long CXXC motif of DNMT1 is shown to bind radioactive zinc ions in vitro (*16*). Similar CXXC domains have also been discovered in a number of other chromatin-associated proteins. These include methyl CpG binding protein MeCP1 (*23*), component of the mammalian Set1 H3K4 methyltransferases CpG binding protein CGBP (*24, 25*), FBXL11, a recently characterized histone demethylase specific for H3K36 and MLL histone methyltransferase, a SET domain dependent H3K4 methyltransferases that exists as a multiprotein super complex of at least 29 different proteins that is required for *HOX* gene expression in mammalian development (*26, 27*). Several of the aforementioned proteins contain CXXC domains that are capable of binding unmethylated CpG (*23, 25*−*27*).

The CXXC domain containing human DNMT1 prefers hemimethylated DNA for catalysis, whereas de novo methyltransferases DNMT3a and DNMT3b, that lack the CXXC domain naturally, do not display methylation preference on substrate DNA in vitro. Therefore to determine the effect of the CXXC domain upon methyltransferase activity of DNMT1, we have undertaken biochemical, mutational and cytochemical approaches to analyze the enzyme, and its CXXC domain. We created mutations in conserved cysteine residues to study their function in protein−DNA interaction. The cellular localization of the fluorescent-fusion CXXC domain mutant DNMT1 (DNMT1$^{\Delta CXXC}$) was investigated to perceive the impact of the CXXC domain during replication foci loading. Our results confirmed that both DNMT1 and the isolated CXXC domain of DNMT1 prefer binding to unmethylated DNA. The CXXC domain and the conserved cysteine residues are essential for enzymatic activity of DNMT1. Overexpression of a CXXC deleted DNMT1 mutant acted as a dominant negative enzyme in mammalian cells and resulted in impairment of maintenance methylation. This demonstrates the importance of an intact CXXC domain for the conservation of enzymatic function and housekeeping cellular processes.

EXPERIMENTAL PROCEDURES

PCR Amplification and Cloning of CXXC Motif from Human DNMT1. The cDNA sequence encoding human DNMT1 was from the NCBI database (accession no. x63692.gb_pr) and the CXXC motif was identified based on the translated sequence. A pair of forward (GAATTC-CCATGGAGAACGCCTTTAAGCGCCGGCG) and reverse primers (GAATTCCTCGAGACCGGTGGCTTCTC-CGACCCAAGAGATGCG) encoding the CXXC motif, with restriction enzyme (RE) site *Nco*I on the forward primer and *Age*I plus *Xho*I on the reverse primer, were obtained. The RE sites are underlined. The PCR reaction was performed using Vent DNA polymerase from New England Biolabs (NEB) in a total reaction volume of 50 μL, using a ABI2720 thermocycler (Applied Biosystem). The PCR reaction conditions were 94 °C 5 min, 55 °C 30 s, 72 °C for 25 s for 25 amplification cycles. The PCR products were analyzed on a 1% TBE agarose gel, and purified by spin columns (Qiagen). The purified DNA was digested with *Nco*I−*Xho*I and ligated into pET28b. The ligation mix was transformed into T7 Express *Escherichia coli* competent cells (NEB). The clone containing the CXXC motif of DNMT1 was named pET28hCXXC.

Bacterial Culture, Expression, and Purification of the Recombinant Zinc-Finger-like Motif. The plasmid pET28hCXXC was freshly transformed into T7 Express *E. coli* competent cells (NEB) and incubated overnight on a LB-Kan plate. Four colonies from the LB-Kan plate were inoculated into 50 mL of LB-Kan media and incubated in a shaker at 225 rpm for 12 h at 37 °C. The culture was diluted with 500 mL of LB-Kan and incubated in a shaker at 225 rpm at 16 °C for 3 additional hours, followed by an addition of 0.3 mM IPTG. The flask was kept at 37 °C for 4−5 h. The cells were harvested by centrifugation at 2700 rpm for 30 min at 4 °C. The pellets were frozen at −20 °C overnight. For protein purification, the *E. coli* cells were kept on ice and thawed in the presence of 10 mL of resuspension buffer M (50 mM Tris pH 7.4, 150 mM NaCl). The cells were sonicated at 50% duty cycle, using a microtip in pulse mode, for 5 min on ice. The cell extracts were incubated on a rotor for 15 min at 4 °C and centrifuged for 30 min at 4 °C at 14,000 rpm. The supernatant was transferred to a 50 mL tube, recentrifuged, and was loaded to a pre-equilibrated (buffer M) nickel-bound chelating Sepharose IMAC (immobilized metal ion affinity) (G.E. Healthcare) column at 1 mL/min. The unbound protein was removed with a wash of 50 column volumes of buffer. The bound proteins were eluted with buffer M supplemented with 500 mM imidazole. The eluted proteins were dialyzed with buffer M, containing 250 mM NaCl and a protease inhibitor cocktail (Sigma), and were stored at −20 °C.

Recombinant DNMT1 Purification from Baculovirus Infected Cells, DNA Methyltransferase Assay and Data Analysis. All the baculovirus constructs were made using PCR. Cell culture, recombinant viral particle isolation, recombinant protein expression and purification were carried out as described previously (*10, 29*).

DNMT1 assays were carried out at 37 °C, for 30 min in duplicate, with a total volume of 25 μL of reaction mix. A typical reaction contained *S*-adenosyl-L-[methyl-^{3}H]methionine (AdoMet) (sp. act. 15 Ci/mmol, G.E. Healthcare), substrate DNA and enzyme, suspended in assay buffer (50 mM Tris-HCl, pH 7.8, 1 mM Na_2EDTA, pH 8.0, 1 mM DTT, 7 μg/mL PMSF, 5% glycerol and 100 μg/mL BSA). Typically 200 ng of the Poly IC (Poly dI-dC) (G.E. Healthcare) substrate DNA was used per assay. The methyltransferase reactions were stopped by transferring the reaction tubes to an ethanol−dry ice bath and were processed by spotting the reaction mix on DE81 paper circles (Whatman) as described before (*10*). Data was plotted by using the GraphPad PRISM program (GraphPad Software Inc.).

Gel Shift Assay. Purified DNMT1$^{\Delta 580}$ was used with ^{32}P labeled FMR-1 DNA (*10*). The reaction was assembled on ice and incubated for 30 min at 37 °C before loading. The gel was run at 4 °C.

Various amounts of purified CXXC protein (0−117 μM) were incubated with 300 ng of fluorescent-labeled duplex DNA (FGTAGGCGGTGCTACACGGTTCCTGAAGTG, where F is fluorescein) in 1X gel shift assay buffer (5 mM Tris pH7.4, 5 mM $MgCl_2$, 1 mM DTT, 3% v/v glycerol, 100 mM NaCl) at 25 °C for 30 min. The mixture was loaded on a 6% polyacrylamide DNA retardation gel (Invitrogen).

Electrophoresis of the samples was performed in 0.5X TBE at 4 °C for 40 min at 140 V. The complexes were visualized under UV light.

Point Mutation of Conserved Cysteine of Zinc-Finger Domain. All point mutations were performed with inverse PCR approach using the pET28hCXXC plasmid, in conjunction with, PCR primers containing the mutation. There were three steps. The plasmid (pET28hCXXC) was first methylated by M.SssI (NEB) and was used as template DNA for an inverse PCR reaction using the mutagenic primers. The PCR reaction was transformed into McrBC-containing NEB5α *E. coli* strain. This step ensured the destruction of the mother plasmid post-transformation into the McrBC strain and that the PCR amplified DNA, containing the mutation, was ligated in vivo to make a mutant clone. For the C667G mutation, the forward primer was AGCAGCCTGAGTGTGGGAAA**G**GTAAAGCCTGCAAG. The reverse primer was: TTTCCCACACTCAGGCTGCTGACACACCTC. For the C667G and C670G mutations, the mutant plasmid C667G was subjected to another round of mutagenic inverse PCR as described before with forward primer: AGTGTGGGAAA**G**GTAAAGCC**G**GCAAGGACATGGTT. The reverse primer was GGCTTTACCTTTCCCACACTCAGGCTGCTGAC. The nucleotide substituted for mutation is in bold.

Cloning of Point Mutant and Deletion Mutant DNMT1 into the baculovirus and DsRed Vector. Zinc-finger-domain point mutant and deletion mutant DNMT1 clones were cloned separately into a baculovirus transfer vector pVIC1 (NEB). The clone pVICHMT3mut designates point mutations within C653G, C656G, C659G, C664F, C667G and C670G amino acids residues. The zinc-finger deletion clone is pVICDNMT1$^{\Delta CXXC}$ lacking 647−690 amino acids. Both DNMT1 point mutant and DNMT1$^{\Delta CXXC}$ were amplified using a set of gene specific primers.

Forward primer DNMT1: CAGGGAGTCGACATGCCGGCGCGTACCGCCCCAG; *Sal*I cloning site. Reverse primer DNMT1: CAGCTGGGTACCCTAGTCCTTAGCAGCTTCCTCCTC; *Kpn*I cloning site. Both pDsRed and PCR products were digested with *Kpn*I and *Sal*I and cloned.

Mammalian Cell Culture, Transfection, Fluorescence Localization and Permanent DNMT1 Overexpression Cell Line Establishment. COS-7 cells were purchased from ATCC and were grown in DMEM media (Invitrogen), with 10% fetal bovine serum (Sigma) in 6 well plates at a density of 1×10^6. The DsRed-DNMT1 wild-type or DsRed-DNMT1$^{\Delta CXXC}$ constructs were transfected using Transpass D2 (NEB) transfection reagent at a ratio of DNA to Transpass D2 at 1:3 μg/μL. The cells were grown on glass coverslips for 24 h before transfection. The transfected cells were grown for 48 h more and fixed with 4% paraformaldehyde (EM Science) for 20 min at room temperature (RT). The coverslips in the well were washed with 1 mL of 1X PBS (phosphate buffer saline, Invitrogen). The cells were permeabilized with 1X PBS supplemented with 0.2% Triton X-100 for 5 min and then washed with 1 mL of 1X PBS. The nuclear staining was performed by adding 1 mL of Hoechst 33342 solution (1 μg/mL) for 15 min at RT. The stained cells on the coverslip were washed with 1X PBS three times and air-dried. A drop of ProLong Antifade (Molecular Probe) solution was applied to the center of the coverslip, and then the slide was mounted. The mounted slide was allowed to dry overnight. The cells were observed using a fluorescence microscope (Zeiss AxioVision). The permanent cell lines containing the Rheoswitch (NEB) inducible promoter driven DNMT1$^{\Delta CXXC}$ were made as per the protocol described by the supplier. DNMT1$^{\Delta CXXC}$ was amino terminus HA tagged. Two clones (clones 4 and 5) were selected after Western blot validation and grown to confluency. The recombinant gene was induced with RSL1 ligand (NEB) for 10 days. The genomic DNA isolation was performed using DNeasy kit (Qiagen). Bisulfite modification of the genomic DNA was performed using Epitect kit (Qiagen). PCR amplification of the rDNA loci was performed using sense TTTGTTAGTTTGTTAGATATATT and antisense primer AAAACTAACATTACAAAACCTAC. The PCR products were cloned and sequenced to determine the methylation changes.

RESULTS

Amino Terminus Deletion Recombinant DNMT1 Strongly Binds to Unmethylated DNA. DNMT1 functions as the maintenance DNA (cytosine-5) methyltransferase in mammalian cells. Previous studies with DNMT1 revealed the presence of a methylated DNA binding region at the amino terminus of the enzyme. DNMT1 deletion mutants lacking the first 501 or 580 amino acids respectively were catalytically active, and retained their preference for hemimethylated DNA substrate (*22*). To determine if DNA binding affinity and DNA methylation could be correlated, we performed gel shift analysis of substrate DNA and recombinant human DNMT1 lacking the first 580 amino acids (DNMT1$^{\Delta 580}$). The DNA sequence selected was a short stretch of the *FMR*-1 locus (fragile X mental retardation syndrome) that is methylated during disease progression. Three sets of duplex DNAs representing the *FMR*-1 locus were made; either both strands unmethylated, one strand methylated (hemimethylated) or both strands methylated. The unmethylated *FMR*-1 locus contained 12 symmetrical CpG pairs versus 12 hemimethylated CpGs in the hemimethylated duplex. In the gel shift assay, a constant but low concentration of DNA was titrated with a range of DNMT1 enzyme concentrations. As the enzyme concentration gradually increased, the majority of DNA binding occurred. An initial complex between enzyme and DNA was formed with <6 nM DNMT1$^{\Delta 580}$ (Figure 1A,B,C). The formation of DNMT1$^{\Delta 580}$ complex with unmethylated duplex DNA was robust as compared to either hemimethylated or methylated DNA. A hyperbolic dependency of enzyme concentration was observed. The dissociation constant of DNMT1$^{\Delta 580}$ and DNA concentration remained between 2.3 and 6.1 nM (CGUM, 2.3 ± 0.7; CGHM, 6 ± 2.5; and CGDM, 6.1 ± 1.2). Addition of cold AdoMet or AdoHcy did not affect the formation of DNA−protein complex, suggesting that these complexes are independent of cofactors (data not shown). Therefore, it appeared that DNMT1$^{\Delta 580}$ prefers binding to unmethylated DNA instead of hemimethylated or fully methylated DNA. To find out if the binding discrimination has any bearing on DNA methylation of DNMT$^{\Delta 580}$, we performed a methylation assay under steady-state kinetic conditions with the same sets of oligonucleotide duplexes. As expected DNMT1$^{\Delta 580}$ showed ~6-fold more methylation on hemimethylated DNA, as compared to unmethylated DNA (Figure 1C). Therefore, a stronger enzyme substrate binding does not reflect higher

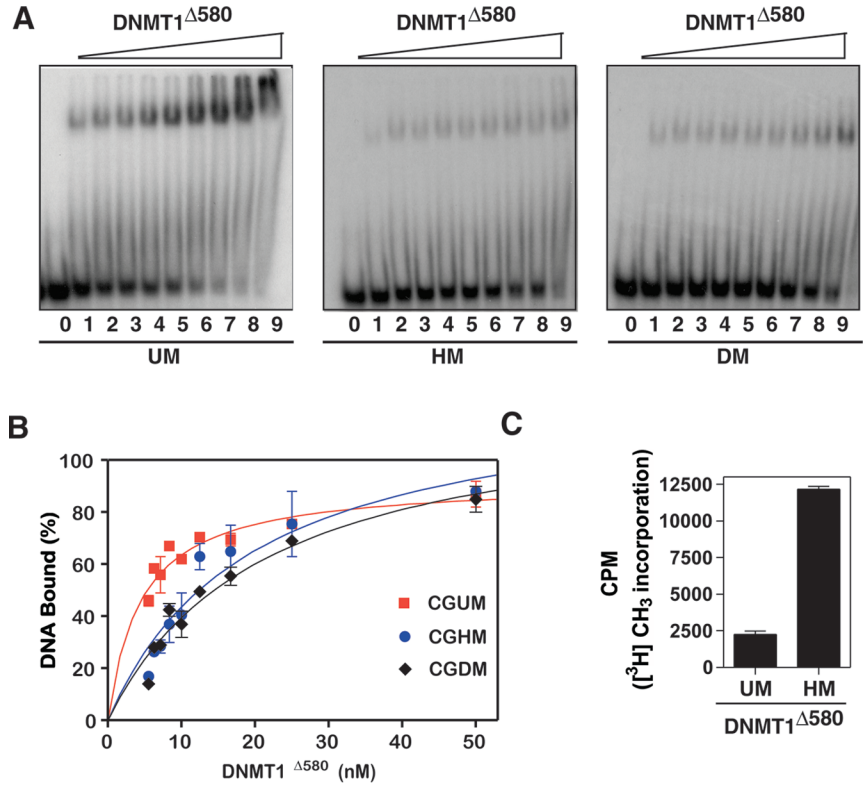

FIGURE 1: Gel mobility shift assay of DNMT1$^{\Delta 580}$ and DNA substrate. (A) Representative figures showing a fixed concentration of radioactive double stranded DNA (4.4 nM) and an increasing concentration of DNMT1$^{\Delta 580}$ (0, 5.5, 6.25, 7.15, 8.35, 10, 12.5, 16.65, 25 and 50 nM) in the assay. The lane numbers are indicated at the bottom with methylation status of DNA; UM (unmethylated), HM (hemimethylated) and DM (symmetrical doubly methylated) are indicated. (B) Nonlinear regression analysis of % bound DNA in the *y*-axis and DNMT1$^{\Delta 580}$ concentrations in the *x*-axis are shown. Each data point was obtained in triplicate from three independent gel mobility shift assays. (C) Methylation analysis of human recombinant DNMT1$^{\Delta 580}$ with hemimethylated (HM) and unmethylated (UM) DNA is shown. The methyltransfer values were calculated in duplicate, and each experiment was conducted in triplicate.

turnover number as in the case of the unmethylated substrate DNA. Furthermore, previous deletion analysis of the amino terminus of the human DNMT1 revealed that a deletion beyond amino acids 580 (672) yielded a catalytically inactive enzyme (DNMT1$^{\Delta 672}$) (*22*), suggesting amino acids between 580 and 672 may be crucial for enzymatic activity.

The CXXC Domain of Human DNMT1 Is Conserved among Other DNMT1s and Proteins. The CXXC domain of the human DNMT1 contains a cluster of eight cysteine residues to form 651$CX_2CX_2CX_4CX_2CX_2CX_{15}CX_4C$697. It resides between amino acids 580 and 697, and previous studies have shown that this region binds radioactive zinc (*16*). Therefore, it is a zinc-finger-like or CXXC motif-containing domain that lies between the proliferating cell nuclear antigen (PCNA) binding site and the catalytic domain (Figure 2). To study the conservation of the CXXC domain across different species, a blast search using the CXXC-like motif of human DNMT1 as a query in NCBI (National Center for Biotechnology Information) protein database was performed. It identified several other CXXC containing proteins, including DNMT1, from monkey, pig, cow, opossum, mouse, chicken and sea urchin (Figure 2A). Furthermore, the CXXC region is also conserved in methyl-DNA binding protein 1 (MBD1), human CG binding protein 1 (CGBP1) and the MLL histone methyltransferase. Indeed, MBD1 contains three different CXXC domains that exhibit a strong homology with CXXC/zinc-finger-like motif of DNMT1 (Figure 2B), and one of those CXXC motifs (CXXC-3) can bind unmethylated DNA (*28*). Similarly, the CXXC domain of MLL1 histone methyltransferase also binds to unmethylated CG sequences. Thus, the functional significance of the CXXC domain of DNMT1 may be stabilization of DNA binding, which would influence methyltransferase activity.

CXXC Domain of Human DNMT1 Binds to Unmethylated CpG. In order to investigate whether or not the CXXC domain binds to DNA and what specificity it encodes, we overexpressed a portion of DNMT1. This portion contained the CXXC motif (amino acids 645−737) as 6xHis fusion. The purified protein, CXXC-His6, was ~17 kDa (Figure 3A). This protein was used in gel shift assay to determine its DNA binding affinity. Five sets of fluorescein labeled duplex oligonucleotides containing unmethylated CG (UMCG), hemimethylated upper/lower strand CG (HMUS/LSCG), no CG (-CG) and methylated CG (DMCG) sequences were used for the gel shift assay (Figure 3B). The recombinant protein CXXC-His6 formed a complex with UM probe (Figure 3C), and did not bind to either DMCG or −HMUS/LS CG probe (data not shown). The UM and -CG probes were identical DNA sequences, except for a C to T substitution within the CG sequence. Therefore, it is possible that the binding activity of the CXXC domain is unmethylated CG dependent. To confirm the binding results, individual probes at equimolar concentrations were mixed into reaction cocktails, with or without CXXC-His6. Then these complexes were resolved on the gel simultaneously. Here again, only the UM probe was able to form a specific DNA−protein complex (Figure 3D). This data demonstrates that unmethylated CG is required

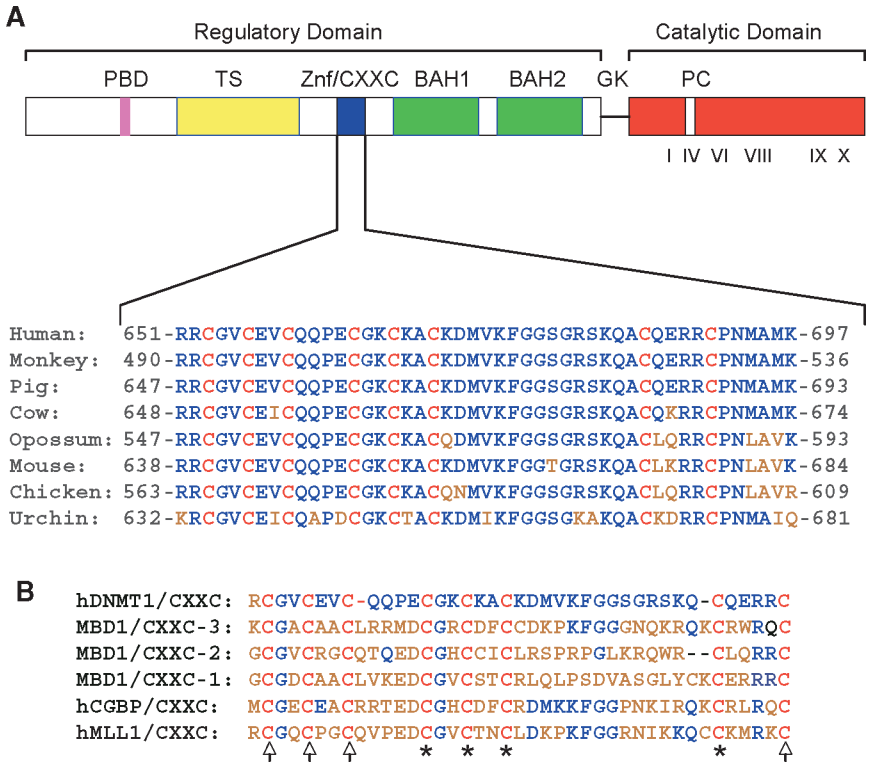

FIGURE 2: CXXC "zinc-finger-like" domain in vertebrate DNMT1. (A) A schematic diagram representing the DNMT1, its various domains, and the embedded zinc-finger-like region. The PCNA binding domain (PBD), targeting sequence (TS), CXXC motif (Znf), bromo adjacent homology domain (BAH) are shown along with regulatory and catalytic regions of the enzyme. The numbers of the amino acids are indicated. Similarity of amino acids at the CXXC motifs (Znf) of different DNMT1 from human (P26358), monkey (XP 001104704), pig (AAY45793), cow (AAO44952), opossum (AAM89252), mouse (EDL25143), chicken (BAA07867) and sea urchin (CAA90563) are compared. (B) Other CXXC domain containing proteins such as mouse MBD1 (Q9Z2E2), hCGBP (Q9P0U4) and MLL1 (Q03164). The arrows (first) and asterisks (second) show the Zn^{2+} binding amino acids residues as reported by Allen et al. (*30*). The accession numbers are in parentheses. Both in (A) and (B) the conserved cysteines are in red and all other conserved residues are in blue.

for DNA binding, and that the CXXC domain of DNMT1 can discriminate between CG, hemimethylated and symmetrically methylated CG containing DNA.

Mutation of Conserved Cysteine in the CXXC Domain Abolishes Unmethylated DNA Binding Activity. Since the CXXC domain is a zinc-coordinating domain (*16*) that selectively binds unmethylated DNA (Figure 3D), we investigated the role of conserved cysteines in protein−DNA complex formation. To determine the target cysteine to be mutated, we referred to the solution structure of the CXXC domain of MLL that is homologous to the CXXC domain of DNMT1. The MLL histone methyltransferase contains 8 cysteine residues at positions 1155, 1158, 1161, 1167, 1170, 1173, 1189 and 1194 that correspond to all 8 cysteine residues of DNMT1 (Figure 2B). The cysteines at positions 1155, 1158, 1161, 1194 and 1167, 1170, 1173, 1189 of the CXXC motif bind separately to two zinc ions. As shown, both C1170A and C1173A mutation abolishes Zn^{2+} ion binding (*30*). The amino acids corresponding to C1170 and C1173 of MLL are C667 and C670 of human DNMT1. Therefore, we mutated C667G and C667/670G (Figure 4A) and expressed the mutated proteins in *E. coli* for further characterization. Both the wild-type and mutant proteins were purified to homogeneity (Figure 4B), and then were used in gel shift assays with UM DNA. As the protein concentration increased, the wild-type CXXC-His6-DNA complex increased. However, a single mutant CXXC (C667G) or a double mutant CXXC (C667/670G) did not display protein−DNA complex formation (Figure 4C). Copious addition of mutant protein resulted in nonspecific smearing of UM DNA (data not shown). Neither of the mutant proteins displayed specific complex formation with -CG, HMUS/LSCG or DMCG duplex DNAs (data not shown). These experiments suggested that the cysteine residues within CXXC domains are essential for DNA binding activity as well as unmethylated CG specificity.

CXXC Domain of DNMT1 Is Crucial for Enzymatic Activity. Since the CXXC domain of human DNMT1 binds to unmethylated CG, we decided to examine how this specificity would affect a methyltransferase reaction. Vertebrate DNMT1 is a maintenance DNA methyltransferase that produces its highest turnover numbers catalyzing hemimethylated CG substrates. Thus, mutation or deletion of the CXXC domain in DNMT1 may affect its function. This hypothesis was supported further in a previous study, because $DNMT1^{\Delta 672}$ was unable to form covalent adduct with hemimethylated DNA when the target cytosine was substituted with a suicidal 5-fluoro-2′-deoxycytidine (*22*). To test our hypothesis, we created several baculovirus constructs with various point mutations or a whole CXXC domain deletion mutant DNMT1. These constructs were then integrated into the baculovirus genome, and recombinant proteins were produced using the Sf9 insect cell culture system. The $hDNMT1^{\Delta 580}$ lacked the first 580 amino acids, but included the CXXC motif. The $hDNMT1^{\Delta 672}$ lacked the first 672 amino acids that did not contain the CXXC domain. We also made a CXXC domain point mutant DNMT1 that had six cysteine mutations ($DNMT1^{C\text{-}Gmut}$: C653G, C656G, C659G,

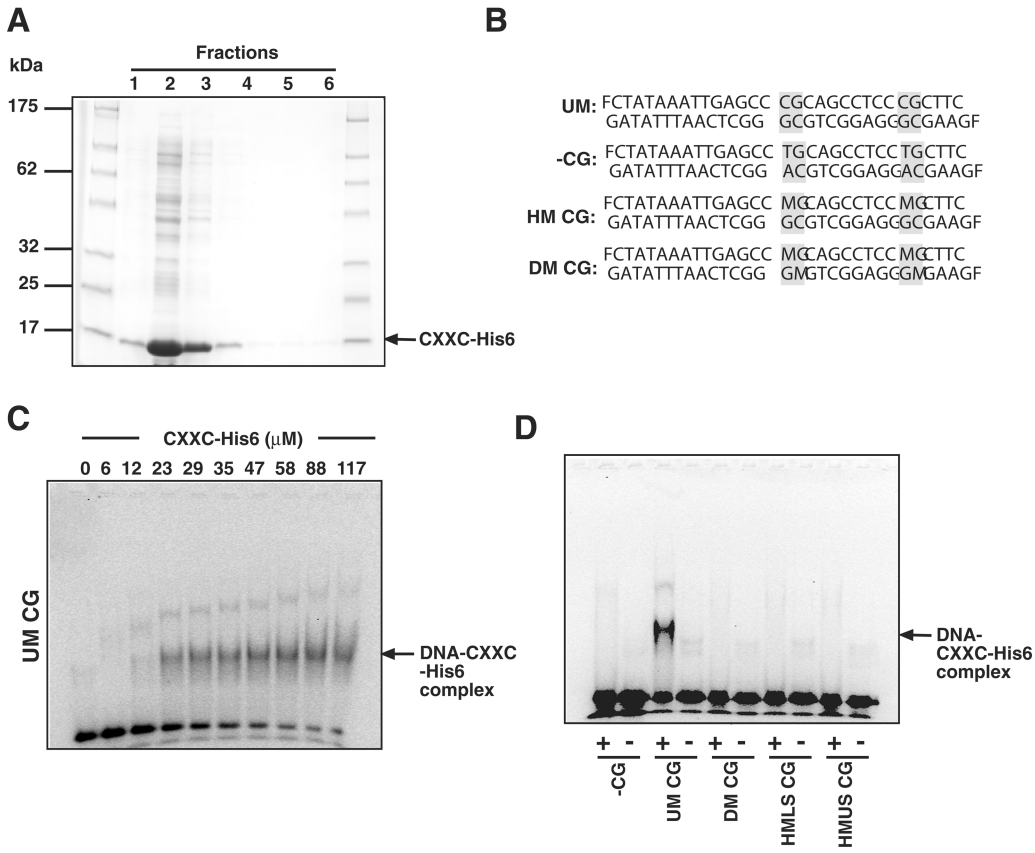

FIGURE 3: Characterization of DNA binding activity of the CXXC domain of DNMT1. (A) Hexa-His tag purification profile of CXXC domain across an IMAC column. The 17 kDa purified CXXC-His6 protein is shown. (B) The fluorescein labeled oligonucleotide duplexes used for the gel mobility shift assay are shown. M is 5-methylcytosine on the DNA. UM (unmethylated), -CG (TG instead of CG), HM CG (hemimethylated CG) and DM CG (doubly methylated CG) duplex DNA. (C) Gel mobility shift assay with an increasing concentration of the purified CXXC-His6 (shown on top) with unmethylated DNA. The DNA−protein complex is indicated. (D) Gel shift assay of various methylated and unmethylated DNA in the presence (+) or absence (−) of the CXXC protein. The CXXC-His6 concentration was ~38 μM. An arrow shows the DNA−protein complex.

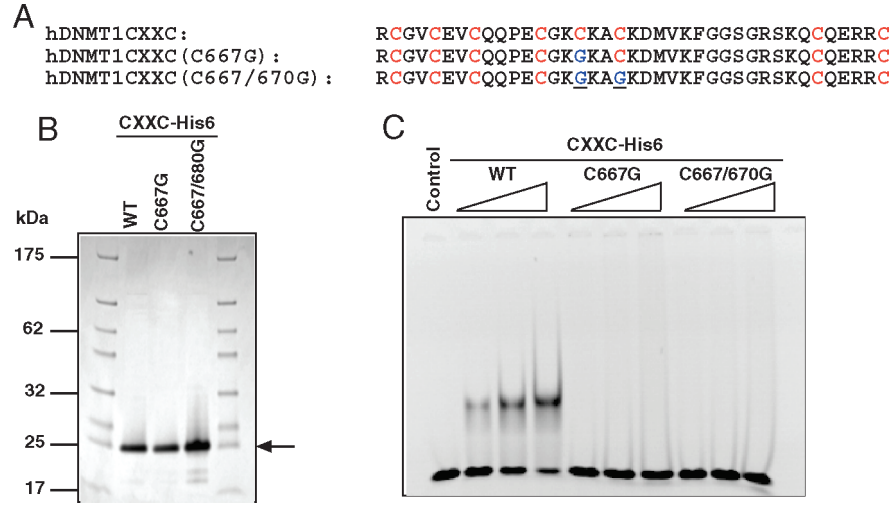

FIGURE 4: Role of the conserved Cys in DNA−protein interaction. (A) CXXC/zinc-finger-like domain of human DNMT1 is shown on the top, and its mutants are shown at the bottom. The amino acids mutations are shown and underlined. (B) The purified CXXC-His6 (WT) and its mutants are shown on a SDS gel. (C) Gel mobility shift assay of the CXXC-His6 and its mutant with unmethylated CG DNA. The control lane does not contain any protein. The protein concentrations were 24, 60 and 120 μM respectively.

C664F, C667G and C670G) in the CXXC region. The CXXC deletion mutant (DNMT1$^{\Delta CXXC}$) lacked the entire CXXC domain, encompassing residues between amino acids 647 to 690 (Figure 5A). All these proteins were purified and assayed with unmethylated and hemimethylated DNA.

Hemimethylated DNA is a proven substrate for wild-type full length DNMT1, but neither DNMT1$^{C\text{-}Gmut}$ nor DNMT1$^{\Delta CXXC}$ displayed robust catalytic activity (Figure 5B). Unmethylated DNA was a poor substrate for all the enzymes as expected (data not shown). To compare and characterize these enzymes further, we performed steady-state kinetic analysis with poly dI-dC substrate that provides a large number of CI sites for DNA methylation to occur. It has also been shown that this substrate behaves similarly to

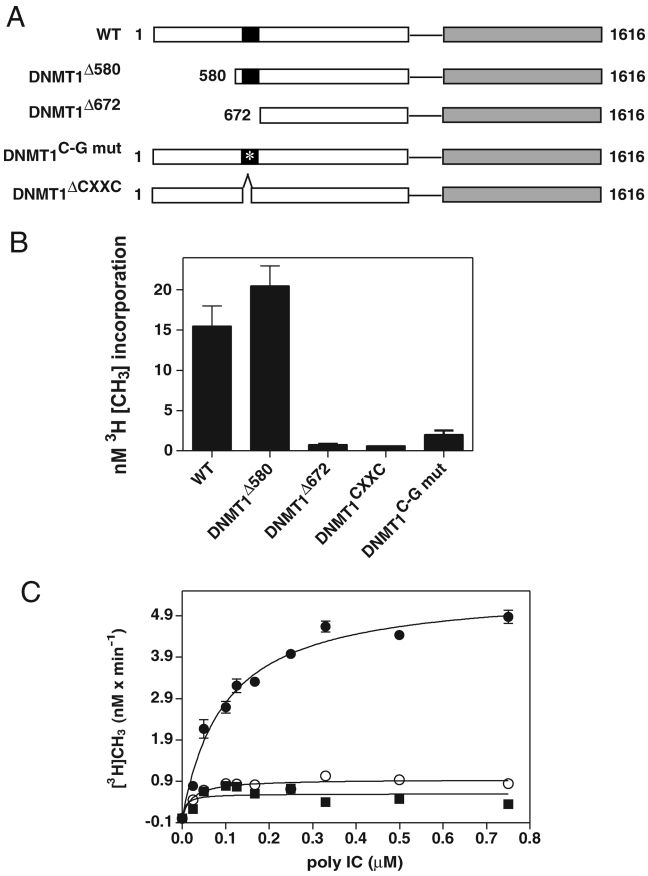

FIGURE 5: Functional role of the CXXC-like region of the DNMT1 in methyltransferase reaction. (A) Schematic diagram of deletion and point mutant DNMT1 used in this study. Deletion mutants are indicated with the first and last amino acid numbers. The point mutant on the zinc-finger-like region (C-G mut) and the CXXC deletion mutants are shown. (B) Methyltransferase assay with 40 nM of the purified protein with 5 μM tritiated AdoMet and 100 ng of poly IC. Methyl group incorporation in nM is shown at the *y*-axis, and various DNMT1s are in the *x*-axis. (C) Steady-state kinetic analysis of DNMT1 full length with mutants. The reaction progression curves for the full length DNMT1 (filled circle), C-G mut (empty circle) and CXXC del (filled square) are shown. Substrate poly IC concentrations in the reaction are shown at the bottom. Each reaction was performed in duplicate.

hemimethylated DNA (*29*). Under linear methylation reaction conditions, a series of Michaelis−Menten plots of velocity, as a function of increasing substrate concentration and saturated AdoMet, were performed. The representative plots are shown in Figure 5C. From these plots, the V_{max}, K_m and k_{cat} were calculated for wild-type, DNMT1$^{C\text{-}Gmut}$ and DNMT1$^{\Delta CXXC}$ enzymes. The value for k_{cat} of the wild-type recombinant DNMT1 was 163 (160−166) h^{-1}, as compared to 14 h^{-1} for DNMT1$^{C\text{-}Gmut}$ and 9 h^{-1} for DNMT1$^{\Delta CXXC}$ enzyme. The catalytic efficiency also varied significantly among the wild-type and mutant enzymes (data not shown). These experiments confirmed that the zinc-finger domain is essential for the overall catalytic function of the enzyme.

Deletion of CXXC Domain in DNMT1 Does Not Affect Replication Foci Occupancy during DNA Replication. DNMT1 methylates newly synthesized DNA during DNA replication. Therefore, it remains in the close vicinity of the replication foci along with PCNA, a crucial DNMT1 loading factor. Since mutation or deletion of CXXC domain of DNMT1 renders it catalytically inactive (Figure 5C), we investigated if the CXXC domain would be associated with DNMT1's replication foci loading. COS-7 cells were transfected with expression constructs of DsRed-DNMT1or DsRed-DNMT1$^{\Delta CXXC}$, synchronized with aphidicolin, fixed and visualized for fusion DNMT1s in the nucleus by fluorescence microscopy. The nuclear DNA was stained blue, and as DNA replication progressed, red punctate nuclear spots appeared throughout the nucleus except in the nucleolus. This observation was similar to a previous Dnmt1 localization study (*31*). We did not observe any significant differences in replication fork occupancy between the wild-type and mutant enzymes during S-phase. During prometa and metaphase, both mutant and wild-type enzymes remain diffused throughout the cytoplasm and the chromatin remains condensed (Supp. Figure 1 in the Supporting Information). The similarity in nuclear distribution between CXXC mutant and wild-type DNMT1 suggests that the CXXC region is not involved in enzymatic loading during DNA replication.

CXXC Domain Deleted DNMT1 Is Dominant-Negative. Since CXXC domain mutant DNMT1 is catalytically inactive in vitro, we investigated its role in maintenance DNA methylation. For selective regulation of the mutant DNMT1, an inducible expression construct of DNMT1$^{\Delta CXXC}$ was made in the RheoSwitch vector, pNEBRX1, and transfected into the Rheoswitch cell line HEK293-A7 (NEB). The cell lines were selected with hygromycin, and clones (4 and 5) were chosen. To monitor the expression of mutant DNMT1, an amino terminus HA tag was incorporated. In the presence of ligand RSL1, HA-DNMT1$^{\Delta CXXC}$ expression was observed (Figure 6A). The cells did not show any visible stress or apoptosis due to ligand addition or overexpression of the mutant enzyme (Figure 6B), and the mutant enzyme was observed at the replication foci during DNA replication (Supp. Figure 1 in the Supporting Information). To examine the effect of overexpression of DNMT1$^{\Delta CXXC}$ on genomic methylation, we extracted DNA before and after DNMT1$^{\Delta CXXC}$ induction and performed a McrBC digestion assay. McrBC is an enzyme that selectively digests DNA with methylated CG. If the DNA becomes unmethylated due to the dominant negative effect of mutant DNMT1, a fraction of the genomic DNA will appear as a higher molecular weight. As mentioned, in the presence of GTP cofactor, DNA isolated from DNMT1$^{\Delta CXXC}$ overexpressing cells displayed higher molecular weight DNA fragments and less smearing in the presence of GTP cofactor, as compared to the control genomic DNA (Supp. Figure 2 in the Supporting Information).

Since the cells contain endogenous wild-type DNMT1, overexpression of DNMT1$^{\Delta CXXC}$ may impact overall genomic DNA methylation by competing with the endogenous enzyme. Therefore, we also performed bisulfite sequencing of the rDNA repeat elements from both control and DNMT1$^{\Delta CXXC}$ overexpressing genome. The rDNA repeats are naturally methylated in the human genome. Any disruption of maintenance methylation would result in hypomethylation of this repeat region. To test this phenomenon, the control cells were not induced with the RSL1 ligand, barring expression of the constructs. Indeed, the induced cell lines genomic DNA displayed lower percentage (~25%) of methylated CpG, as compared with control cells (Figure 6C,D) for rDNA. This data conclusively proves that CXXC domain deleted DNMT1 can disrupt DNA methylation in

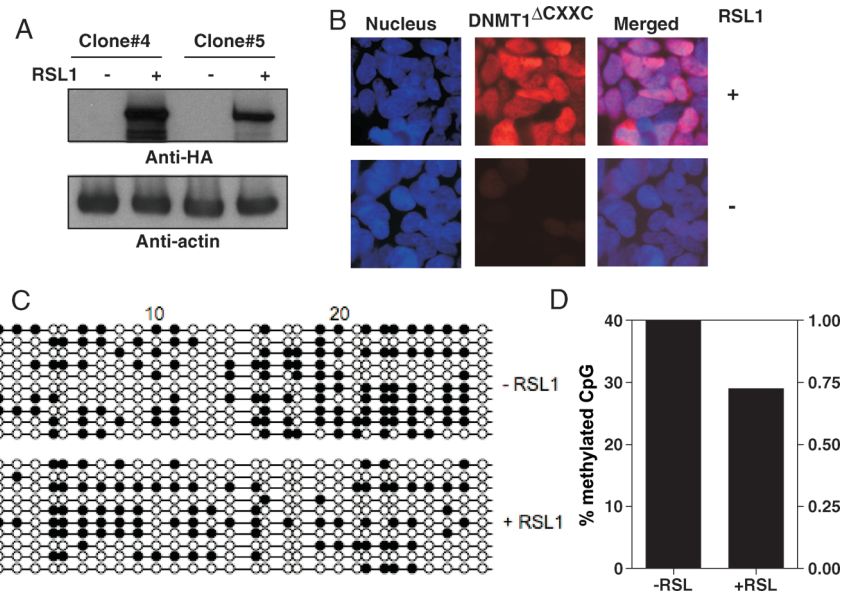

FIGURE 6: Overexpression of HA tagged CXXC deletion DNMT1 mutant and its role in maintenance methylation. (A) Stably expressing DNMT1$^{\Delta CXXC}$ mutant clones in mammalian cells in the presence (+) or absence (−) of Rheoswitch ligand (RSL1). The antibodies used for Western blot are shown below the blots (B) Characterization and uniformity of inducible DNMT1$^{\Delta CXXC}$ mutant proteins in the cells with (+) and without (−) RSL1. The cells were stained with nuclear (purple) and anti-HA (red) or merged for validation. (C). Bisulfite sequencing analysis of the rDNA repeats with (+) or without (−) RSL1 in cell genome. Each filled circle is methylated CpG and empty circle indicates unmethylated CpG. (D) % methylated CpG with (+) or without (−) RSL1 on rDNA repeat. A total of 18 clones each were sequenced for the percentage determination.

naturally methylated rDNA loci, and the CXXC domain plays an integral role in maintenance methylation in human cells.

DISCUSSION

DNMT1 is the major maintenance DNA methyltransferase in mammals. The carboxyl terminal domain of the enzyme is homologous with most of the prokaryotic cytosine-5 DNA methyltransferases, as it contains conserved motifs for catalysis in this region. However, the amino terminus is still an integral part of the enzyme, because it contains many protein−protein interaction sites. A small portion of the amino-terminal domain is indispensable for enzyme functionality because it encodes the major nuclear import signal and comprises tissue-specific exons (*32*). Surprisingly the CXXC domain, located within the amino terminus, is conserved in most of the eukaryotic DNMT1s discovered to date (Figure 2). In the mammalian genome, proteins containing CXXC domains are common. The crystal structure of mammalian DNMT1 has not been resolved, so it is currently not possible to determine whether or not the CXXC domain of DNMT1 is a true zinc finger, conforming to the standard structure of two antiparallel beta sheets and an alpha helix. This CXXC domain bears many characteristics of a zinc-finger motif: the ability to bind radioactive zinc and, specifically, unmethylated CG rich DNA. Zinc-finger motifs facilitate a variety of enzymatic and protein functions such as DNA binding, RNA recognition, and mediating protein−protein interactions (*33*). Furthermore, the zinc-finger regulated structural interaction of transcription factors on target DNA has been well studied. Yet, studies on catalytically active enzymes containing zinc-finger motifs are scarce. Therefore, the precise role of zinc-finger motifs in enzymatic catalysis remains unknown. One of the reasonable assumptions regarding the function of these motifs is that they guide the host protein or enzyme to predetermined sites, either within DNA or on chromatin. During replication, the CXXC domain may act as a sensor for unmethylated CG, and therefore avoid aberrant DNA methylation. Previous reports have discovered the presence of multiple DNA binding regions within the amino terminus region of DNMT1, including a methylated DNA binding allosteric activation domain (*22*). Therefore, it is plausible that these DNA binding domains may be responsible for sensing and distinguishing between methylation-status of CGs. As reported here, the CXXC domain of DNMT1 is crucial for enzymatic activity. Previous reports of independent expression of both amino terminus and catalytic region of mammalian DNMT1 did not yield a catalytically competent enzyme (*17, 34*). Therefore, the CXXC domain of DNMT1 may play a vital role in regulating DNA binding, prior to catalysis.

Traditionally it is believed that DNMT1 binds to PCNA during DNA synthesis and that PCNA aids DNMT1 in maintenance methylation of the newly synthesized daughter strand. Indeed, DNMT1 is localized on the replication foci during DNA replication as evident by the BrdU pulse chase experiments (*31*). To be functional on a replication fork, the enzyme must be able to maintain the rapid kinetics of replication. The kinetics of DNMT1 has been studied in detail in vitro (*35*−*38*), and the calculated turnover numbers indicate that the enzyme cannot keep pace with replication kinetics. Previous studies in vitro, in the absence of PCNA, have demonstrated that longer DNA was a better substrate for the enzyme (*39*) and a hemimethylated or poly dI-dC substrate can be methylated more efficiently than an unmethylated DNA. The level of processivity reaches up to 99% on hemimethylated DNA. Recent cell based DNA methylation studies of a PCNA binding mutant DNMT1s raises an interesting question about the absolute need of PCNA interaction with DNMT1 for DNA methylation propagation (*40*). Cells expressing

a PCNA binding mutant of DNMT1 that does not load into the replication foci displayed a 2-fold lower DNA methylation efficiency (*41*). Therefore, DNMT1 is catalytically active without PCNA in vitro, and may be able to perform bulk of maintenance methylation in cells without PCNA interaction. This suggests that other DNA binding motifs may be responsible for DNA methylation during replication and/or in a postreplicative manner. Indeed, there are two more DNA binding motifs on DNMT1. The CXXC region (this work) and the TRD (region between motifs VIII and IX) of the catalytic region have been shown to bind DNA (*22*). Furthermore, deletion of the CXXC region of DNMT1 resulted in a catalytically impaired enzyme. These observations demonstrate a central role of the CXXC domain in DNA binding and methylation. For example, in cancer cells, where aberrant DNA methylation of the unmethylated CG islands is common, the CXXC domain of DNMT1 may facilitate de novo methylation via selective localization of DNMT1. However, this hypothesis does not exclude the contribution of DNMT3A and DNMT3B in de novo methylation mediated gene silencing. A similar mechanism of targeted histone methylation by MLL histone methyltransferase using its unmethyl CG binding CXXC domain on transcriptionally active promoter containing CG islands and H3K4me3 has also been hypothesized (*30*).

DNMT1 is a general transcriptional repressor. It recruits a series of other transcriptional repressors, such as histone deacetylase (HDAC), histone methyltransferases and heterochromatic proteins (HPs), for transcriptional gene silencing (*42*), through binding sites that are located within its amino terminus. DNMT1 can also bind to unmethylated CpG DNA through its CXXC domain and thus induce transcriptional gene silencing in a DNA methylation independent manner. Once bound to unmethylated CpG DNA, DNMT1 can recruit histone H3 K9 methyltransferase G9a, to further lock chromatin into a repressed state (*19*). These modifications of chromatin that include histone deacetylation, methylation and DNA methylation make chromatin less accessible for transcription factors, which is observed during tumor suppressor gene repression in cancer cells. During replication of chromatin, DNMT1 plays a dual role of maintenance methylation and histone modification enzyme recruitment. Therefore, an effective association between DNA and DNMT1 is important for the propagation of the epigenetic information during cell division. A potential application of these findings is to target the CXXC/zinc-finger-like domain of DNMT1 using small molecule inhibitors or physiologically stable unmethylated CpG DNA/hairpins that will interfere with DNA binding and, therefore, impair DNMT1's catalytic abilities. Inactivation of this enzyme will aid in reactivation of crucial tumor suppressor genes in cancer cells.

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SUPPORTING INFORMATION AVAILABLE

Figure depicting localization of DNMT1 during cell cycle and figure depicting McrBC analysis of DNMT1$^{\Delta CXXC}$ clone genomic DNA. This material is available free of charge via the Internet at http://pubs.acs.org.

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