DNA 5-Methylcytosine Demethylation Activities of the Mammalian DNA Methyltransferases*

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Methylation at the 5-position of DNA cytosine on the vertebrate genome is accomplished by the combined catalytic actions of three DNA methyltransferases (DNMTs), the de novo enzymes DNMT3A and DNMT3B and the maintenance enzyme DNMT1. Although several metabolic routes have been suggested for demethylation of the vertebrate DNA, whether active DNA demethylase(s) exist has remained elusive. Surprisingly, we have found that the mammalian DNMTs, and likely the vertebrate DNMTs in general, can also act as Ca\(^{2+}\) and redox state-dependent active DNA demethylases. This finding suggests new directions for reinvestigation of the structures and functions of these DNMTs, in particular their roles in Ca\(^{2+}\) and redox state-dependent active DNA demethylases. This finding suggests new directions for reinvestigation of the structures and functions of these DNMTs, in particular their roles in Ca\(^{2+}\) and redox state-dependent active DNA demethylases. This finding suggests new directions for reinvestigation of the structures and functions of these DNMTs, in particular their roles in Ca\(^{2+}\) and redox state-dependent active DNA demethylases.

**Background:** Whether active DNA demethylase(s) exist in vertebrates is under debate.

**Results:** Mammalian DNA methyltransferases (DNMTs) can directly convert 5-methylcytosine in DNA to cytosine in vitro.

**Conclusion:** Vertebrate DNMTs can function as Ca\(^{2+}\) - and redox state-dependent DNA demethylases.

**Significance:** The DNA demethylase activities of vertebrate DNMTs could regulate gene expression, development, neuroplasticity, carcinogenesis, etc., through global and/or local genomic demethylation.
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EXPERIMENTAL PROCEDURES

Recombinant Plasmids and Recombinant Proteins—Construction of the expression plasmids used in this study was described previously (26). The DNA methylation-inactive mutants of the DNMTs, i.e. DNMT1-PSC, DNMT3A-PS, and DNMT3B-PS, were generated by insertion of a serine residue before Cys-1229 in the catalytic site of DNMT1 or by replacing Cys-706 and Cys-657 in the catalytic domains of DNMT3A and DNMT3B, respectively, with a serine residue. All of the recombinant DNMTs, including human DNMT1 (hDNMT1; purity of ~78%), hDNMT3A (purity of ~90%), and mouse DNMT3B (purity of ~50%) (supplemental Fig. S1), were purchased from BPS Bioscience.

Cell Culture and DNA Transfection—293T cells were cultured under 5% CO₂ at 37 °C in DMEM (Invitrogen) supplemented with 10% FBS (Biological Industries) and 1% penicillin/streptomycin (Invitrogen). For DNA transfections, the different expression plasmids were transfected into cells using either Lipofectamine 2000 (Invitrogen) or MAXiFect (Omic Bioscience). The cells were collected 2 days later for further experimentation.

Preparation of Nuclear Extracts—Nuclear extracts were prepared from porcine sperm and 293T cells by a modified method (27). Briefly, the porcine semen was washed three times with PBS, and the sperm pellet was isolated using Ficoll (GE Healthcare). The pellet was resuspended in hypotonic buffer (10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 1 mM EDTA, and EDTA-free protease inhibitors (Roche Applied Science)) on ice for 15 min. The resuspended sperm was passed 10 times through a 21-gauge needle and then centrifuged at 13,200 rpm for 10 min at 4 °C. The supernatant was removed, and the pellet of the nuclei was resuspended in resuspension buffer (10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1.5 mM MgCl₂, and EDTA-free protease inhibitors), and an equal volume of 1 M NaCl was added, followed by a 30-min incubation on ice. The solution was centrifuged at 13,200 rpm for 30 min at 4 °C, and the supernatant (nuclear extract) was dialyzed at 4 °C in buffer B (10 mM Tris-HCl (pH 7.4), 50 mM NaCl, 1.5 mM MgCl₂, and EDTA-free protease inhibitors) (26) overnight with 2 changes of the dialysis buffer.

Preparation of the nuclear extract from 293T cells followed the procedures described above. The transfected cells were washed three times with PBS and resuspended in hypotonic buffer on ice for 10 min. The solution was centrifuged at 4000 rpm for 10 min, and the supernatant was removed. The nuclear pellet was resuspended in resuspension buffer, and an equal volume of 1 M NaCl was added, followed by a 30-min incubation on ice. The lysate was centrifuged at 13,200 rpm for 30 min at 4 °C, and the supernatant was collected as the nuclear extract, which was then dialyzed overnight at 4 °C in buffer B.

DNA Substrates for in Vitro DNA Demethylation Assay—The 5-mC-containing substrate for DNA demethylation assay of the porcine sperm nuclear extract was prepared from the 2819-bp pMR1-8 plasmid containing 185 CpG dyads and 11 MspI restriction sites. The unmodified pMR1-8 plasmid was amplified in SCS110 bacteria (Stratagene) and then methylated by the bacterial methyltransferase M.SsII (New England Biolabs) in NEB Buffer 2 supplemented with 160 μM SAM. The extent of methylation of the plasmid was checked by HpaII digestion. C-5-methylated double-stranded DNA substrate (26) was used in the demethylation reactions with 293T nuclear extract or recombinant DNMTs (see below) and then analyzed by the hydrolysis-TLC assay.

In Vitro Reactions of Conversion of 5-mC to C on DNA—For DNA demethylation reactions of the porcine sperm nuclear extract, 40 ng of the methylated pMR1-8 plasmid was incubated with 100 μg of the nuclear extract in 50 μl of buffer B with 100 μg/ml BSA at 37 °C for 1–4 h. When needed, one of three divalent cations (Ca²⁺, Mg²⁺, or Fe²⁺; 10 μM to 10 mM), CRT0044876 (10 μM to 1 mM), 3-aminobenzamide (0.5 μM to 50 μM), or tetrahydrodouridine (30 μM to 1 mM) was added to the reaction mixtures.

For DNA demethylation reactions of recombinant DNMT proteins or nuclear extracts from 293T cells overexpressing different DNMTs, 40 ng of the 5-mC-containing double-stranded DNA substrate was incubated with 40 nM recombinant DNMTs or 100 μg of the 293T nuclear extracts in 50 μl of buffer B with 100 μg/ml BSA at 37 °C for 0.5–8 h. When required, 10 μM to 10 mM CaCl₂, 5 mM DTT, 2 mM tris(2-carboxyethyl)phosphine hydrochloride, or 160 μM SAM was included in the reaction mixtures.

To understand the effect of the oxidation state of the enzymes, 40 nM recombinant mouse DNMT3B was pretreated with 10 μM to 10 mM H₂O₂ in 49 μl of buffer B at 15 °C for 30 min or with 1–5 mM oxidized glutathione (GSSG) in 49 μl of buffer B at 37 °C for 1 h. After preincubation, 40 ng of the 5-mC-containing double-stranded DNA substrate was added, and the incubation was continued for another 4 h at 37 °C.

All reactions were stopped with 1.3% SDS and then treated with proteinase K for 20 min at 50 °C. The DNA substrates were isolated using the QIAquick nucleotide removal kit (Qiagen) and subjected to the restriction digestion-PCR assay or hydrolysis-TLC assay as described previously (26).

In Vitro Reactions of Conversion of C to 5-mC on DNA—Methylation in vitro of unmodified pMR1-8 plasmid DNA by the DNMTs was carried out and analyzed by the hydrolysis-TLC assay. When needed, 1 mM CaCl₂ or 5 mM DTT was also included in the reaction mixture.

Restriction Digestion-PCR Assay of C-5 Methylation on Double-stranded DNA Substrate(s)—The procedures used were those described previously (26). See the legend of supplemental Fig. S2A for more details.

Hydrolysis-Thin Layer Chromatography (TLC) Assay of 5-mC, 5-hmC, and C on DNA—The experimental procedures were similar to those described previously (26). For more details, see the legend of supplemental Fig. S2B.

RESULTS

In Vitro DNA Demethylation by the Porcine Sperm Extract—Because the level of 5-mC on the paternal genome in the mammalian pronuclei decreases rapidly after fertilization without the need for DNA synthesis, and sperm-derived factor(s) appear to be involved in this demethylation process (28–30), we carried out in vitro DNA C-5 demethylation reactions using nuclear extract prepared from porcine sperm. We also tested...
the effect of Ca\textsuperscript{2+} ion in view of the calcium wave in the oocyte upon fertilization (31). Remarkably, inclusion of 1–10 mM Ca\textsuperscript{2+}, but not Mg\textsuperscript{2+} (Fig. 1, compare lanes 7–10 with lane 6) or Fe\textsuperscript{2+} (compare lanes 12–15 with lane 11), significantly reduced the extent of DNA methylation by 20–50% (compare lanes 4 and 5 with lane 1).

Both DNA cytosine deamination and the BER pathway (in particular, through its two components APE1 and poly(ADP-ribose) polymerases) are involved in active DNA demethylation (22, 32–34). However, inclusion of inhibitors of either the BER pathway (CRT0044876 for APE1 and 3-aminobenzamide for poly(ADP-ribose) polymerases) or the cytidine deaminase (tetrahydrouridine) in the reactions had little effect on the in vitro DNA demethylation activity of the sperm nuclear extract (Fig. 1, B and C). The data of Fig. 1 suggested that Ca\textsuperscript{2+} ion stimulated a BER- and cytidine deaminase-independent DNA demethylation activity in the nuclear extract of porcine sperm.

DNA 5-mC Demethylase Activities of Wild-type (but Not Mutant) Murine DNMT1, DNMT3A, and DNMT3B with Intact Catalytic Domains—It was not trivial to purify the factor(s)/enzyme(s) in the nuclear extract of the porcine sperm that was responsible for the in vitro conversion of 5-mC to C on DNA. Because the porcine sperm nuclear extract contained DNMT1/DNMT3A/DNMT3B (data not shown), and the murine/human orthologs of the latter two DNMTs act in vitro as DNA 5-hmC dehydroxymethylases under oxidative conditions in the absence of SAM (26), we suspected that under appropriate conditions, these DNMTs might also be capable of converting other modified forms of cytosine, e.g. 5-mC, to C.

In view of the data of Fig. 1, we carried out in vitro DNA demethylation reactions in the presence of 10 mM Ca\textsuperscript{2+}. The 5-mC-containing double-stranded DNA substrate was incubated with nuclear extracts prepared from 293T cells transfected with plasmids overexpressing enhanced green fluores-
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![Diagram showing DNA 5-mC demethylation activities of mammalian DNMTs](image)

FIGURE 2. DNA 5-mC demethylation activities of mammalian DNMTs. The 5-mC-containing DNA substrates were subjected to incubation in 293T nuclear extracts in buffer B with 10 mM CaCl₂ and exogenously expressed EGFP (lanes 1 and 2) and mouse DNMT1 (lanes 3 and 4), DNMT3A (lanes 5 and 6), and DNMT3B (lanes 7 and 8), as well as their catalytic mutants (site-directed mutants of the DNA C-5 methylation catalytic sites of the three enzymes): DNMT1-PSC, DNMT3A-PS, and DNMT3B-PS (lanes 9–14). The extent of conversion of 5-mC to C was analyzed by the hydrolysis-TLC assay and is shown quantitatively in the histogram. The amounts of the exogenous wild-type enzyme in lanes 4, 6, and 8 were similar to those of the mutant enzymes in lanes 10, 12, and 14, respectively (Western blotting data not shown). M, mock control without incubation; R, with incubation. Error bars indicate S.D.; *p < 0.05 by t test comparing bars 4, 6, and 8 with bar 2.

cent protein (EGFP) and mouse DNMT1, DNMT3A, and DNMT3B, as well as their mutants. After the reactions, the DNA products were hydrolyzed as depicted in supplemental Fig. S2B, and the nucleotides were analyzed by TLC (Fig. 2). As shown in Fig. 2, under the reaction conditions tested, the DNA methylation activities of the three DNMTs might share the same catalytic domain(s).

Activities of Partially Purified Recombinant DNMTs—To further confirm the results of Fig. 2, recombinant mouse DNMT3B, hDNMT1, and hDNMT3A partially purified from recombinant baculovirus-infected S9 insect cells were examined for their DNA demethylation activities. First, recombinant mouse DNMT3B (~50% purity) (supplemental Fig. S1) was subjected to incubation with the 5-mC-containing DNA substrate in buffer B containing increasing concentrations (0, 10, and 100 μM and 1, 5, and 10 mM) of CaCl₂ (Fig. 3A). As shown in Fig. 3A, recombinant DNMT3B exhibited significant DNA demethylation activity only in the presence of Ca²⁺ (compare lanes 3–7 with lane 2), with the activity highest in the presence of 1 mM Ca²⁺ (lane 5). We next tested and compared the DNA demethylation activities of DNMT3B, hDNMT1 (~70% purity) (supplemental Fig. S1), and hDNMT3A (~90% purity) (supplemental Fig. S1) in buffer B containing 1 mM CaCl₂ (Fig. 3B). Both hDNMT1 and DNMT3B exhibited significant DNA demethylation activity, converting at least 50% of 5-mC to C at the MspI-cleaved ends of the DNA substrates.

Remarkably, the DNA demethylation activities of the three mouse DNMTs were greatly diminished (by ~73–88%) when amino acid substitutions or insertions were introduced into the known catalytic sites of C-5 methylation of these enzymes (Fig. 2, compare lanes 10, 12, and 14 with lanes 4, 6, and 8, respectively). The data of Fig. 2 suggested that the two de novo DNMTs, as well as the maintenance enzyme DNMT1, could act as active DNA 5-mC demethylases under appropriate conditions. In addition, the methylation and demethylation activities of the three DNMTs might share the same catalytic domain(s).

Ca²⁺- and Redox State-dependent DNA 5-mC Demethylation Activities of Partially Purified Recombinant DNMTs—To confirm the results of Fig. 2, recombinant mouse DNMT3B, hDNMT1, and hDNMT3A partially purified from recombinant baculovirus-infected S9 insect cells were examined for their DNA demethylation activities. First, recombinant mouse DNMT3B (~50% purity) (supplemental Fig. S1) was subjected to incubation with the 5-mC-containing DNA substrate in buffer B containing increasing concentrations (0, 10, and 100 μM and 1, 5, and 10 mM) of CaCl₂ (Fig. 3A). As shown in Fig. 3A, recombinant DNMT3B exhibited significant DNA demethylation activity only in the presence of Ca²⁺ (compare lanes 3–7 with lane 2), with the activity highest in the presence of 1 mM Ca²⁺ (lane 5). We next tested and compared the DNA demethylation activities of DNMT3B, hDNMT1 (~70% purity) (supplemental Fig. S1), and hDNMT3A (~90% purity) (supplemental Fig. S1) in buffer B containing 1 mM CaCl₂ (Fig. 3B). Both hDNMT1 and DNMT3B exhibited significant DNA demethylation activity, converting at least 50% of 5-mC to C at the MspI-cleaved ends of DNA to C (Fig. 3B, lanes 2 and 4), whereas recombinant hDNMT3A showed significantly lower activity (lane 3). The demethylation reaction with recombinant mouse DNMT3B also decreased the resistance to HpaII digestion of the methylated DNA substrate (data not shown). Finally, DNA demethylation by the DNMTs was most likely the result of direct conversion of 5-mC to C because similarly low levels of 5-hmC were present in the mock control samples (Figs. 2 and 3), as well as throughout the time course of the DNA demethylation reactions in vitro (supplemental Fig. S3). These data together demonstrate that the mammalian DNMTs can function as active DNA demethylases in vitro.

We showed previously that the redox state of de novo DNMT3A and DNMT3B can influence their DNA dehydroxymethylase activities (26). Interestingly, the DNA demethylase activities of the DNMTs also appeared to be affected by the redox state of the enzymes. As exemplified by DNMT3B, preincubation of the enzyme with the reducing DTT (Fig. 4A, compare lane 5 with lane 3) or tris(2-carboxyethyl)phosphine hydrochloride (supplemental Fig. S4A, compare lanes 2 and 3) greatly decreased the extent of conversion of 5-mC to C. On the other hand, preincubation with 10 mM H₂O₂ or 5 mM GSSG did
not affect the DNA demethylation activity of the recombinant enzyme (data not shown). In interesting contrast to 5-mC demethylation, the C-5 methylation reaction of DNMTs did not require Ca$^{2+}$ (data not shown) (3, 35, 36), nor was it affected by DTT (Fig. 4B, compare lane 6 with lane 4) (26).

Reversibility of the DNA 5-mC Demethylation and 5-C Methylation Reactions Catalyzed by DNMTs—As exemplified for DNMT3B in Fig. 4A, the inclusion of SAM, the methyl donor need for DNA 5-C methylation by the DNMTs, in the reaction mixture greatly reduced the extent of conversion of 5-mC to C.

FIGURE 3. Calcium dependence of DNA 5-mC demethylation activities of recombinant DNMTs. A, results from the hydrolysis-TLC assay of conversion of 5-mC to C by recombinant DNMT3B. The DNA demethylation activity of recombinant mouse DNMT3B was assayed by incubation of 40 ng of 5-mC-containing DNA substrate with 40 nM enzyme in buffer B containing 100 μg/ml BSA and increasing concentrations (0, 10, and 100 μM and 1, 5, and 10 mM) of CaCl$_2$. The incubations were all performed at 37 °C for 4 h. The quantitative results are presented in the histogram. M, mock control without incubation. Error bars indicate S.D. *, p < 0.05; **, p < 0.01 by t test comparing bars 3–7 with bar 2. B, comparison of the DNA demethylation activities of recombinant hDNMT1, hDNMT3A, and DNMT3B by the hydrolysis-TLC assay. The 5-mC-containing substrate was incubated at 37 °C for 4 h with 40 nM each recombinant hDNMT1 (lane 2), hDNMT3A (lane 3), and DNMT3B (lane 4) in buffer B containing 100 μg/ml BSA and 1 mM CaCl$_2$ and then analyzed by the hydrolysis-TLC assay. The quantitative analysis is presented in the histogram. M, mock control without incubation; R, with incubation. Error bars indicate S.D. *, p < 0.05; ***, p < 0.005 by t test comparing bars 2–4 with bar 1.

FIGURE 4. Inhibition effects of reducing reagents and SAM on DNA demethylation activity of DNMT3B. A, the 5-mC-containing DNA substrate was subjected to the demethylation reactions with 40 nM recombinant DNMT3B in buffer B containing 100 μg/ml BSA with or without the inclusion of 1 mM CaCl$_2$, 5 mM DTT, or 160 μM SAM. After incubation at 37 °C for 4 h, the DNA products were analyzed by the hydrolysis-TLC assay. The results are presented qualitatively in the histogram. Error bars indicate S.D. *, p < 0.05 by t test comparing bars 4–6 with bar 3. B, unmethylated pMR1-8 plasmid DNA was incubated with 40 nM recombinant DNMT3B in buffer B containing 100 μg/ml BSA with or without 1 mM CaCl$_2$, 5 mM DTT, or 160 μM SAM. After incubation at 37 °C for 4 h, the extent of C methylation of the DNAs from the different reactions was determined by the hydrolysis-TLC assay and is compared quantitatively in the histogram. Error bars indicate S.D. C, strategy of a series of reactions testing the reversibility of DNA demethylation and methylation (see “Results” for more details). Briefly, the 5-mC-containing DNA substrate was incubated at 37 °C for 2 h with 40 nM recombinant DNMT3B in buffer B containing 100 μg/ml BSA and 1 mM CaCl$_2$ (reaction 1). Then, 160 μM SAM was added, and the incubation was continued for another 1 h (reaction 2). Finally, 1 mM H$_2$O$_2$ was added to the reaction mixture, and the incubation was continued for another 2 h (reaction 3). Rx, reactions. The DNA products from the three reactions outlined in the upper panel were purified and analyzed by the hydrolysis-TLC assay. The data are quantitatively compared in the histogram. M, mock control without incubation. Error bars indicate S.D. *, p < 0.05; ***, p < 0.005.
Finally, H$_2$O$_2$, which is known to inhibit the methylation reaction catalyzed by the mammalian DNMTs was further studied by an analysis of the dynamic changes of the DNA methylation in vitro. As outlined in Fig. 4C, double-stranded DNA substrate containing 5-mC was first incubated with recombinant DNMT3B in the demethylation buffer for 2 h. DNA, as catalyzed by DNMTs, is reversible (Fig. 4A, bar M). The continued 1-h incubation in the presence of SAM converted >60% of C back to 5-mC (Fig. 4C, compare lane 2/bar 2 with lane 1/bar 1). Finally, the addition of H$_2$O$_2$ led to an apparent switch in the enzyme activity of DNMT3B from methylation to demethylation again (Fig. 4C, compare lane 3/bar 3 with lane 2/bar 2), presumably due to loss of the DNA methylation function of the oxidized enzyme. The data of Figs. 3 and 4 and supplemental Fig. S4 suggest that the switch in the catalytic functions of the DNMTs between DNA methylation and demethylation is flexible, subjected to regulation by a range of factors, including the local concentration of Ca$^{2+}$, the presence of SAM, and the redox state of the DNMTs.

DISCUSSION

This study has revealed an unexpected and novel characteristic of the mammalian DNMTs and likely those of vertebrates in general, i.e. the vertebrate DNMTs, in addition to converting C to 5-mC on DNA, can also demethylate 5-mC on DNA under specific conditions (Figs. 2 and 3), in particular in the presence of Ca$^{2+}$ ion and in the absence of reducing reagents (Figs. 3 and 4A and supplemental Fig. S4A). In other words, the covalent addition of the methyl group to the 5-position of cytosine on DNA, as catalyzed by DNMTs, is reversible (Fig. 4C). The loss of the DNA demethylation activities of the mutant forms in comparison with the wild-type enzymes (Fig. 2) also suggests that each of the three DNMTs utilizes the same domain or overlapping domains to catalytically methylate and demethylate DNA.

The Ca$^{2+}$ ion and redox state dependence of the DNA demethylation activities of the DNMTs is especially intriguing. With respect to this point, it is interesting to note that a change in the intracellular concentration of Ca$^{2+}$ ion presents a key cellular signal, and the Ca$^{2+}$ flux/influx wave in the cytosol could eventually communicate with the nucleus through a number of ways (37). Significantly, dynamic changes in the DNA methylation patterns, globally or locally, occur in close association with calcium signaling in fertilization/early embryonic development (31), synaptic transmission (38, 39), and tumorigenesis (40). Among these processes, the Ca$^{2+}$ wave during fertilization triggers activation of the oocyte and its cell cycle resumption (41). In addition, this increased concentration of Ca$^{2+}$ in the zygote is maintained for at least 6 h (42), during which time, genome-wide demethylation of both the paternal and maternal genomes occurs (43, 44). Notably, DNMT1, DNMT3A, and DNMT3B are all expressed in the zygote (45).

In addition, the elevation of calcium at synapses can serve as a spark for signal transmission among the neuronal network (46). Concurrently, transcription of specific neuronal genes could be activated, and this is associated with promoter DNA demethylation (39). In view of the above, we suggest that, besides the other previously known pathways of DNA demethylation, the DNA demethylation events in activated neurons could also be accomplished by the Ca$^{2+}$-ion-stimulated DNA demethylase activities of DNMT3A, DNMT3B, and/or DNMT1.

Finally, calcium signaling in cancer cells, as the result of increased influx of the ion by the Ca$^{2+}$-channel/pumps and release from the endoplasmic reticulum (40), is involved in aberrant transcription, the disregulated cell cycle, genotoxicity, and tumor invasion/metastasis (47, 48). In parallel, imbalance of CpG methylation in cancer cells leads to a genome-wide hypomethylation (8) accompanied by regional hypermethylation on the promoters of specific tumor suppressor genes (49). Curiously, the elevation, instead of reduction, of the levels of DNMT1, DNMT3A, and DNMT3B in cancer cells (50–52) could not be easily correlated with the global hypomethylation of the cancer genomes (53). The current finding of the Ca$^{2+}$-dependent oxidation state-facilitated DNA demethylase activities of the three enzymes, in contrast to their DNA methyltransferase activities, provides a plausible basis for the above seemingly paradoxical correlation. Future studies should also be carried out to look into the likelihood of demethylation, in addition to methylation (8), of the promoters of specific tumor suppressor genes/oncogenes by the DNMTs during carcinogenesis.

In relation to the dependence of the DNA demethylation activities of the three DNMTs on Ca$^{2+}$ and on the redox condition, it is interesting to note that elevation of the Ca$^{2+}$ ion concentration and the redox state of the cells are interdependent in vivo. For instance, the calcium wave induced during fertilization stimulates the ATP supply and the generation of the reactive oxygen species from the mitochondria during oocyte activation and maintenance (54). The high concentration of intracellular Ca$^{2+}$ in cancer cells is also associated with the oxidation stress (55). Furthermore, the reciprocal relation...
between the excitatory event and reactive oxygen species generation exists in neuronal cells (56, 57).

In summary, we have discovered that mammalian DNMT1, DNMT3B, and, to a much less extent, DNMT3A, contrary to the conventional thought of their being mainly DNA methyltransferases, can also act in vitro as DNA demethylases in a Ca\(^{2+}\) ion- and redox state-dependent manner, albeit at relatively low efficiencies under the current conditions. How the DNA methylation patterns, globally and locally, of the vertebrate genomes in different cell types under various physiological conditions are generated and maintained may need to be re-evaluated in relation to the interplay between the two totally opposite DNA modification activities of the enzymes. It should be noted that the Ca\(^{2+}\) concentration (\(\geq 10 \mu M\)) needed for the in vitro DNA demethylation reaction (Fig. 3A) is relatively high in comparison with the intracellular levels of Ca\(^{2+}\), which range from nM to \(\mu M\) even during activation of different Ca\(^{2+}\)-mediated biological processes, e.g. fertilization (58). Thus, an efficient in vivo demethylation reaction may require other essential cofactors and/or specific signal transduction pathways. How the Ca\(^{2+}\) ion, the redox condition of the environment, and other factors modulate the structures/functions, in vitro and in vivo, of the vertebrate DNMTs as DNA methyltransferases and DNA demethylases will need to be investigated.

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