Preferential Methylation of Unmethylated DNA by Mammalian de Novo DNA Methyltransferase Dnmt3a*

DNA methylation is an epigenetic modification of DNA. There are currently three catalytically active mammalian DNA methyltransferases, DNMT1, -3a, and -3b. DNMT1 has been shown to have a preference for hemimethylated DNA and has therefore been termed the maintenance methyltransferase. Although previous studies on Dnmt3a and -3b revealed that they act as functional enzymes during development, there is little biochemical evidence about how new methylation patterns are established and maintained. To study this mechanism we have cloned and expressed Dnmt3a using a baculovirus expression system. The substrate specificity of Dnmt3a and molecular mechanism of its methylation reaction were then analyzed using a novel and highly reproducible assay. We report here that Dnmt3a is a true de novo methyltransferase that prefers unmethylated DNA substrates more than 3-fold to hemimethylated DNA. Furthermore, Dnmt3a binds DNA nonspecifically, regardless of the presence of CpG dinucleotides in the DNA substrate. Kinetic analysis supports an Ordered Bi Bi mechanism for Dnmt3a, where DNA binds first, followed by S-adenosyl-l-methionine.

DNA methylation in mammals occurs predominantly at cytosine within the CpG dinucleotide. Numerous studies have revealed that DNA methylation is critical for proper embryonic development (1, 2), genome stability (3), X chromosome inactivation (4), genomic imprinting (5), and the suppression of the detrimental effects of parasitic elements (6). Certain genomic regions, like CpG islands, are usually hypomethylated regardless of whether the associated gene is transcriptionally silent or active (7). Other genomic regions, such as centromeres and parasitic DNA sequences, are hypermethylated in normal cells (6). During the process of tumor formation, these methylation patterns are frequently disrupted, resulting in improper gene silencing by hypermethylation of promoter regions and genomic instability by global hypomethylation (8, 9).

It remains unclear how cellular DNA methylation patterns are established during development and properly maintained in somatic cells. One mechanism may be through the use of several differentially regulated, independently encoded, DNA methyltransferases (DNMTs) (10). To date, four mammalian DNMTs have been cloned and characterized. Of these four, three have been found to be essential for proper embryonic development in murine knockout models (Dnmt1, -3a, and -3b) (1, 2). DNMT1 is the most abundant DNA methyltransferase in somatic cells (11) and is targeted to replication foci during S phase (12, 13). DNMT1 has been shown to have a 7–21-fold preference for hemimethylated DNA and is therefore believed to be the primary enzyme responsible for copying methylation patterns from the parental to the daughter strand following DNA replication (14). The DNMT3 family of enzymes appears to be responsible for the de novo establishment of DNA methylation patterns during embryonic development. These enzymes are more highly expressed during early developmental stages where de novo methylation is believed to occur. Limited substrate preference studies revealed that these enzymes have an equal affinity for unmethylated and hemimethylated DNA. The sequence specificity of the mammalian DNMTs is less restricted than most bacterial methylases, requiring only a CpG dinucleotide (2, 15).

Structurally, mammalian DNMTs can be divided into an N-terminal regulatory domain and a C-terminal catalytic domain. The N-terminal domains of the DNMT3 family enzymes and DNMT1 are unrelated to each other and are completely lacking in bacterial cytosine methylases (16). The N-terminal region of the Dnmt3 family contains a domain with similarity to the plant homeodomain, common to many chromatin-associated proteins, which may mediate protein-protein interactions (15, 17). The N-terminal domain of Dnmt3a participates in the interaction with histone deacetylases (18–20), pRb (19), co-repressors (20), and proliferating cell nuclear antigen (21). An allosteric DNA-binding site in this domain was shown to influence enzymatic activity of the C-terminal catalytic domain (22) that is highly conserved in all cytosine methyltransferases from bacteria to mammals. Structural studies with bacterial cytosine methylases have shown that the cytosine base is extruded from the DNA double helix into the active site of the enzyme where it can be reacted upon without steric hindrance (23). The active site cysteine attacks the 6-position of the pyrimidine ring and then activates the 5-carbon for nucleophilic attack of the methyl group donor S-adenosyl-l-methionine (AdoMet) (24).

Unlike the maintenance methyltransferase DNMT1, mamm-
malian Dnmt3a has been poorly characterized biochemically, because the enzymatic activity of Dnmt3a is considerably lower than that of DNMT1 (15, 25, 26). To understand how cellular DNA methylation patterns are regulated enzymatically, we have cloned and purified both enzymes using a baculovirus expression system. Also, we have established a novel and highly reproducible methyltransferase assay system utilizing magnetic beads to determine the precise enzymatic activity of Dnmt3a. Here we report the substrate specificity and molecular mechanism for the methylation reaction of Dnmt3a compared with DNMT1. Most importantly, our results show that Dnmt3a prefers unmethylated DNA more than 3-fold over hemimethylated DNA. Initial velocity analysis, product inhibition, and substrate inhibition studies indicated that the methylation reaction mediated by Dnmt3a followed an Ordered Bi Bi reaction mechanism, with DNA as the first substrate to bind and methylated DNA as the last product to be released.

EXPERIMENTAL PROCEDURES

Materials—Radioactive materials ([3H]AdoMet, [32P]dATP, and [32P]dCTP) were purchased from Amersham Bioscience. Streptavidin-coated magnetic beads were purchased from Dynal. General chemicals were purchased from Sigma, Invitrogen, and Roche Molecular Biochemicals. DNA oligonucleotides were synthesized by MWG Biotech, Sangon, and New England Biolabs. Sequences of Oligonucleotides are listed in Table I. Equal amounts of complementary single-stranded oligonucleotides were mixed, heated at 95 °C for 5 min, and annealed by slowly cooling to room temperature to make double-stranded oligonucleotides. Annealing was confirmed by electrophoresis of samples on a 20% nondenaturing PAGE gel. The DNMT1 anti-peptide antibody has been previously described (19). The Dnmt3a antibody was raised against the synthetic peptide DILLPGDFLEKRSEFPQ(C) injected into rabbits and affinity purified against the same peptide (Affinity BioReagents).

Construction and Infection of Recombinant Baculoviruses—Recombinant His-tagged DNMT1 and Dnmt3a were produced in Sf9 cells using the Bac-to-Bac Baculovirus expression system (Invitrogen). The full-length human DNMT1 cDNA was cloned into pFastBacHisAT as an EcoRI-Sall fragment from plasmid pKB30-1. The Dnmt3a cDNA was created by PCR using primers (sense) 5’-GAATCTACTAGTATGCGCCTCTCAGCCGCCCCGGG and (antisense) 5’-TCTAGACTAGTTACACATAAAGAAATATTTCC, full-length murine Dnmt3a cDNA as template, and Pfu polymerase (Stratagene). The PCR product was cloned into the SpeI site of pFastBacHisC and clones were screened for the proper insert orientation. The cloned genes were sequenced entirely to ensure the absence of mutations (Promega). Restriction endonuclease and Sall sites were engineered into the manufacturer’s instructions and clones were amplified twice to achieve a high titer stock (≈10^9 pfu/ml). Sf9 cells grown in SF900II medium containing 5% fetal calf serum (Invitrogen) were infected with baculovirus stocks. The multiplicity of infection was varied between 1 and 10 and times of infection were varied between 24 and 96 h. For both DNMT1 and Dnmt3a, a multiplicity of infection of 5 and an infection time of 72 h were determined to be optimal.

Purification of Recombinant DNA Methyltransferases—Infected Sf9 cells were pelleted and washed once with 1 × phosphate-buffered saline. The cell pellet was sonicated in RIPA buffer (1 × phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10% glycerol, 0.5 μM phenylmethylsulfonyl fluoride, 5 μg/ml peptatin A, 5 μg/ml antipain, 5 μg/ml chymostatin, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml E-64) and centrifuged at 3000 rpm for 20 min. The supernatants were saved as whole cell extracts. Nickel-agarose resin (Novagen) was charged with 50 μl NISO4 for 3 h and washed with distilled water and then binding buffer (20 mM phosphate, pH 7.6, 10% glycerol, 500 mM NaCl, 5 mM imidazole). Whole cell extract (250 μl) was mixed with Ni-agarose resin (200 μl in bed volume) and rotated gently for 30 min at 4 °C. Nickel-agarose resin was washed three times with 400 μl binding buffer (100 mM phosphate, 50 mM NaCl, 40 mM imidazole) and then twice with 400 μl of washing buffer (200 mM phosphate, pH 7.6, 10% glycerol, 10 mM NaCl). His-tagged protein was eluted with 280 μl of elution buffer (20 mM phosphate, pH 7.6, 10% glycerol, 10 mM NaCl, 1 μM imidazole) and snap-frozen in liquid nitrogen. Protein solutions were stored at −70 °C. Enzyme concentration was determined with the Protein Assay Kit (Bio-Rad).

FIG. 1. Expression and purification of His-tagged recombinant human DNMT1 and murine Dnmt3a from insect cells. Sf9 cells were infected with recombinant baculoviruses and whole cell extracts were prepared as described under “Experimental Procedures.” Recombinant proteins were purified in a single step using nickel-agarose resin. Samples of whole cell extract (WCE) or purified proteins (Ni) were analyzed on an 8% SDS-PAGE gel followed by staining with Coomassie Brilliant Blue (lanes 1–6). Purified proteins were transferred to polyvinylidene difluoride membrane and probed with antibodies specific for DNMT1 (lane 7) or Dnmt3a (lane 8).

DNMT Magnetic Beads Assay—A 96-well microtiter plate was pre-treated with a 10 mg/ml bovine serum albumin solution for 30 min just prior to use. Magnetic beads (10 mg/ml, Dynal) were washed with TENT2M (20 mM Tris, pH 8.0, 2 mM EDTA, 0.01% Triton X-100, 2 mM NaCl) three times. To prepare magnetic beads suspension, the final concentration of magnetic beads was adjusted to 2.5 mg/ml with TENT2M and 1/10 volume of 100 mg/ml nonradioactive AdoMet (Sigma) was added. A typical methylation reaction (40 μl) contained enzyme (30 μM for DNMT1 or 300 μM for Dnmt3a), 125 μM DNA oligonucleotides, and 900 nM tritium-labeled AdoMet (Amersham Bioscience, 1 μCi/ml) in reaction buffer (50 mM Tris, pH 8.0, 5 mM EDTA, 10% glycerol, 10 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride). After incubation at 37 °C for 30 min, reactions were quenched with an equal volume of the magnetic beads suspension and gently shaken on a rocking shaker for 15 min at room temperature. Magnetic beads were separated on a magnet and washed with TENTIM (10 mM Tris, pH 8.0, 1 mM EDTA, 0.005% Triton X-100, 1 mM NaCl) as follows: 300, 200, 150, and 100 μl. Magnetic beads were suspended in 25 μl of TENTIM and tritium incorporation was measured by scintillation counting. A detailed protocol will be published elsewhere. All experiments were performed in duplicate or triplicate independently. For a subtracted DNA inhibition experiment (Fig. 5C, left panel), the concentration of magnetic beads was increased 4-fold (10 mg/ml) to ensure a steady recovery of large amounts of DNA substrate.

Gel Shift Assay—The reaction mixture (20 μl) contains 50 mM Heps (pH 7.9), 5 mM EDTA, 10% glycerol, 100 mM DNMTs, and deDNA (2 × 10⁵ counts/min). 10 mM AdoMet, 10 mM AdoHey, or 1.2 μg of poly d(C)-d(G) (Roche Molecular Biochemicals) was added to selected reactions. After incubation at room temperature for 30 min, an equal volume of a loading buffer (0.5 × TBE, 10% glycerol, 0.02% bromphenol blue) was added and protein-DNA complexes were resolved on an 6% nondenaturing PAGE gel run in 0.5 × TBE. The gel was dried and visualized by autoradiography.

RESULTS

Construction, Expression, and Purification of Mammalian DNA Methyltransferases—The murine de novo methyltransferase Dnmt3a and the human maintenance methyltransferase DNMT1 were cloned, expressed, and purified to directly compare their substrate specificities and kinetic behaviors in vitro. Since we observed that the expression of DNMT1 in bacteria is highly inefficient, both enzymes were expressed in Sf9 insect cells using a baculovirus expression system to ensure a similar condition for protein production. Recombinant enzymes were purified via nickel-agarose resin by virtue of a His tag at their N termini. This method yielded recombinant proteins that were nearly homogenous as determined by SDS-PAGE followed by Coomassie Blue staining (Fig. 1, left panel). Further
ther confirmation of the identity of the purified recombinant proteins was carried out by Western blotting with antibodies specific for each DNMT (right panel).

Establishment of a Novel Quantitative DNMT Assay System—The low level of methyltransferase activity for Dnmt3a reported by others (15, 25, 26), coupled with our data, necessitated the development of a reproducible assay with a higher signal-to-noise ratio than currently available methods. A flow chart of the DNMT magnetic beads assay is shown in Fig. 2A, top panel. Briefly, 5′-terminal biotinylated DNA oligonucleotides are incubated with DNA methyltransferase in the presence of AdoMet that has a tritium-labeled methyl group. After the reaction, biotinylated DNA is immobilized onto streptavidin-coated magnetic beads. DNA containing tritium can be easily separated from the unreacted radioactive AdoMet using a magnet and washed with the buffer containing 1 M NaCl and a detergent. Tritium incorporation is then counted using a liquid scintillation system. Synthetic ssDNA and dsDNA oligonucleotides utilized in this report are listed in Table I.

Mammalian maintenance DNA methyltransferase DNMT1 has been extensively studied biochemically and enzymatically (14, 27–31). DNMT1 prefers hemimethylated dsDNA and its kinetic constants have been reported (14, 28, 29). Therefore, we employed recombinant human DNMT1 as a reference enzyme to evaluate our new DNMT assay system. To demonstrate the efficiency and reproducibility, the substrate specificity of DNMT1 (Fig. 2A, bottom panel) and kinetics analysis (Fig. 2B–E) were examined. DNMT1 showed a 20-fold preference for hemimethylated dsDNA substrates as compared with unmethylated dsDNA and ssDNA, whereas no methylation was detected in fully methylated DNA controls (Fig. 2A, bottom panel, columns 1–6). These results are consistent with previous work (14, 28, 29). Since poly d(I-C) has been used as the standard DNA substrate for traditional DNMT assays (14), a synthetic d(I-C) oligonucleotide was also tested. It was, however, shown that a synthetic d(I-C) substrate was less efficiently methylated (Fig. 2A, bottom panel, column 7). Kinetic analysis of DNMT1 was pursued to examine the accuracy of this assay system. The linearity of time course and the dose dependence of the methylation reaction by DNMT1 were investigated first. The reaction was found to be linear up to 140 nM DNMT1 and for the first 60 min (Fig. 2B and C). Initial enzymatic velocities were then measured by varying one substrate concentration while holding the other substrate concentration constant. The data are displayed in double-reciprocal plots (Fig. 2D and E). The kinetic constants obtained from secondary plots (not shown) are summarized in Table II. These results showed that the DNMT magnetic beads assay is very reproducible and confers a higher signal-to-noise ratio. Therefore, it is applicable to investigate the enzymatic properties of any DNA methyltransferase that may have relatively weak activity.

Preferential Methylation of Unmethylated dsDNA by Dnmt3a—Using the DNMT magnetic beads assay, the substrate specificity of Dnmt3a was examined in vitro. As expected, Dnmt3a efficiently incorporated tritium into unmethylated dsDNA (Fig. 3A, column 1). Interestingly, the enzymatic activity of Dnmt3a with hemimethylated DNA was over 3-fold lower than unmethylated dsDNA under the same conditions (columns 2 and 3). The methylation efficiency with a hemimethylated dsDNA substrate was even lower than ssDNA substrates (column 5). These results strongly suggest that Dnmt3a can transfer a methyl group preferentially to unmethylated dsDNA in the presence of AdoMet. In the absence of DNA substrates, less than 100 cpm were detected as the background, which is comparable with the signal detected using fully methylated DNA substrate controls (columns 4, 6, and 8). Synthetic d(I-C) was also tested as a substitute for poly d(I-C), however, no significant stimulation was observed (column 7). In this experiment, saturating conditions for DNA substrates were essential to minimize the difference in CpG concentrations between different substrates. Since the number of CpG dinucleotides in unmethylated dsDNA is twice that of hemimethylated dsDNA or unmethylated ssDNA substrates, it was possible that the difference in the activity between unmethylated and hemimethylated dsDNA by Dnmt3a was a reflection of these different CpG concentrations. To exclude this possibility, we carried out reactions containing twice the concentration of hemimethylated dsDNA and unmethylated ssDNA substrates (columns 9–11, compare with columns 2, 3, and 5). We found little difference in the methylation efficiency when 125 or 250 nM DNA (corresponding to 1.25 or 2.5 μM CpG dinucleotides) was used, indicating that the DNA concentrations were close to saturating. Since the methylation activity was shown to be independent of DNA dosage in these reaction conditions, preferential methylation of unmethylated dsDNA was indeed caused by the substrate specificity of Dnmt3a, rather than resulting from a dose dependence artifact.

To further confirm this property, we examined internal substrate competition assays under nonsaturating conditions (Fig. 3B). The methylation reactions were carried out in the presence of both unmethylated and hemimethylated dsDNA substrates, in which one DNA substrate concentration is varied while the other is held constant. Since only the variable substrate is conjugated to biotin, this assay measures the competitive methylation efficiency of the biotinylated dsDNA in the presence of a fixed amount of another substrate. Increasing amounts of unmethylated dsDNA (with biotin) versus a fixed amount of hemimethylated dsDNA (without biotin) gave higher methylation activities than increasing amounts of hemimethylated dsDNA (with biotin) versus a fixed amount of unmethylated dsDNA (without biotin), suggesting that Dnmt3a has a small but significant preference for unmethylated dsDNA even in the presence of hemimethylated dsDNA.

Steady-state Kinetic Analysis of Dnmt3a—The methylation reaction by Dnmt3a involves two substrates, AdoMet and DNA. To evaluate the enzymatic properties of Dnmt3a quantitatively, bi-substrate steady-state kinetic analysis was performed using the DNMT magnetic beads assay. The rate of methylation catalyzed by Dnmt3a was linear at protein concentrations between 30 nM and 1.5 μM and for the first 90 min (data not shown). Initial velocity experiments were carried out at varying or fixed concentrations of AdoMet and either of two DNA substrates, unmethylated (Fig. 4A) or hemimethylated dsDNA (Fig. 4B). The kinetic parameters such as $k_{\text{cat}}$, $K_m$ for AdoMet, and $K_m$ for CpG were estimated from these double-reciprocal plots of initial velocity versus substrate concentration, followed by secondary plots with their slopes and intercepts (not shown). Values for all kinetic constants for the two different DNA substrates and AdoMet are listed in Table II.

The purpose of kinetic analysis is not only to represent the catalytic activity of an enzyme as numbers such as $k_{\text{cat}}$ and $K_m$, but also to elucidate the molecular mechanism of the enzymatic reaction (32). Kinetic patterns of bi-substrate analysis provide a good clue to distinguish between a ternary complex mechanism and a ping-pong mechanism. The double-reciprocal plots of the initial velocities of Dnmt3a obtained at various AdoMet and fixed CpG concentrations yielded an intersecting pattern (Fig. 4, A and B, top panels). The same pattern was obtained when various concentrations of CpG dinucleotide and fixed concentrations of AdoMet were used (Fig. 4, A and B, bottom panels).
Together, these results suggest that Dnmt3a proceeds by a random or compulsory ordered Bi Bi type rather than a ping-pong type reaction mechanism. Unlike bacterial cytosine DNA methylases such as M.HhaI (33) and M.MspI (34), the binding of substrates to mammalian Dnmt3a cannot be described by a rapid equilibrium-ordered mechanism during turnover because the intersection points do not lie on the 1/v axis. Although the data clearly indicate the requirement for a linear concentration of enzyme, the data also indicate that the reaction is order-dependent. Substrate specificity of Dnmt3a was determined as a demonstration of the novel DNMT assay. The enzymatic properties of DNMT1 were determined as a demonstration of the novel DNMT assay. The mean values of independent triplicate experiments are plotted for the steady-state kinetics unless noted. A, top panel: a flow chart of the DNMT magnetic beads assay. Bottom panel: substrate specificity of DNMT1. DNMT1 (30 nM), AdoMet (900 nM), and synthetic DNA oligonucleotides (125 nM) were incubated at 37 °C and tritium incorporation was measured. Values are expressed as the mean of triplicate experiments and error bars represent the standard deviation. Unmethylated, hemimethylated-1 (bottom strand methylated), hemimethylated-2 (top strand methylated), and fully methylated DNA were used as double-stranded substrates (columns 1–4). Unmethylated or methylated DNA was used as single-stranded substrates (columns 5 and 6). Synthetic d(I-C) dsDNA was also used (column 7). B, linearity of the methylation reaction as a function of enzyme concentration. Triplicate reactions contain 900 nM AdoMet, and 125 nM unmethylated (triangles) or hemimethylated (circles) dsDNAs as DNA substrates. C, time course of the methylation catalyzed by DNMT1. Duplicate reactions (400 μl) containing 30 nM DNMT1, 125 nM unmethylated dsDNA, and 900 nM AdoMet were incubated at 37 °C. Aliquots (40 μl) were removed for analysis at 0, 2, 15, 30, 45, 60, 90, 120, and 150 min. D, double-reciprocal plots for unmethylated dsDNA. Each reaction contains 30 nM DNMT1. Top panel, the CpG ( unmethylated dsDNA) concentration was fixed at 250 nM (squares), 310 nM (circles), 420 nM (triangles), and 560 nM (diamonds). Bottom panel, the AdoMet ( unmethylated dsDNA) concentration was fixed at 600 nM (squares), 900 nM (circles), 1.2 μM (triangles), and 1.8 μM (diamonds). E, double-reciprocal plots for hemimethylated dsDNA. Each reaction contains 30 nM DNMT1. Top panel, the CpG (hemimethylated dsDNA) concentration was fixed at 62.5 nM (squares), 100 nM (circles), 190 nM (triangles), and 310 nM (diamonds). Bottom panel, The AdoMet concentration was fixed at 300 nM (squares), 450 nM (circles), 600 nM (triangles), and 900 nM (diamonds).
ternary complex, the order of substrate addition and product release cannot be elucidated from these kinetic plots alone.

Product Inhibition and Substrate Inhibition—Product inhibition analysis is useful in distinguishing between random and compulsory ordered Bi Bi mechanisms (32, 35, 36). The methylation reaction by DNMTs generates two products, methylated DNA and AdoHcy. Thus, these products were utilized as reversible inhibitors against both DNA and AdoMet to determine the binding mechanism for Dnmt3a. When methylated dsDNA was examined as an inhibitor against the substrates, unmethylated dsDNA and AdoMet, double-reciprocal plots of the initial velocity versus varying concentrations of unmethylated dsDNA, with respect to different fixed concentrations of methylated dsDNA at a fixed concentration of AdoMet, gave a series of lines intersecting to the left (Fig. 5A, left panel). Therefore, methylated DNA inhibition was competitive with respect to variations in unmethylated DNA. On the other hand, the inhibition by AdoMet was noncompetitive with respect to variations in both substrates. This inhibition pattern, summarized in Table III, suggested that Dnmt3a followed an Ordered Bi Bi reaction mechanism with DNA as the leading substrate (Fig. 5B). The $K_{I}$ value for AdoHcy was determined to be 183 ± 13 nm and the $K_{I}$ value for methylated DNA was 28 ± 3 nm.

To further verify the binding order of substrates in a compulsory ordered mechanism, studies at high substrate concentration were examined (32, 36). This analysis, also known as substrate inhibition, has been successfully utilized to identify the binding order of substrates for many enzymes (for example, Refs. 37–39) including T4 Dam adenine methyltransferase (40). In a compulsory ordered mechanism, if the initial concentration of the second substrate to bind were sufficiently high, an inhibitory effect on the enzyme would be observed due to the formation of nonproductive binary and/or dead-end ternary complexes. On the contrary, the first substrate to bind does not have such an inhibitory effect even at a high concentration, because the binary complex is productive and the dead-end complex is not formed. As predicted, no substrate inhibition of Dnmt3a was observed at CpG dinucleotide concentrations up to 9 μM (Fig. 5C, left panel). On the other hand, a severe inhibition of Dnmt3a was exclusively observed by AdoMet at high concentrations above 1 μM (Fig. 5C, right panel). These results clearly prove that Dnmt3a does not obey a random Bi Bi mechanism and that AdoMet is the second substrate to bind. The data are consistent with the result of the ordered mechanism as concluded from the product inhibition analysis described above. A detailed interpretation of these results will be presented under “Discussion.”

Relationship between DNA Binding and Catalysis—Human DNMT1 can form a covalent complex with dsDNA in which a cytosine in non-CpG sequences is substituted with 5-fluoro-2-deoxycytidine (14), suggesting that DNMT1 can methylate non-CpG sequences with low efficiency. Is the reduced methyl accepting capability of non-CpG cytosines equivalent to the reduced binding affinity of DNMTs to such sequences? In other words, will DNMTs bind to DNA substrates lacking CpG? To investigate the functional relationship between the DNA binding affinity and catalytic ability of Dnmt3a, we first assayed its DNA binding properties by gel-shift analysis. Radioisotope
labeled dsDNA containing CpNPG triplet repeats ((CGG/CCG)₇ or (CAG/CTG)₇, Table I) was mixed with Dnmt3a, and the resulting DNA-protein complexes were fractionated on a 6% nondenaturing PAGE gel. Dnmt3a formed multiple stable DNA-protein complexes with both DNA substrates in the absence or presence of AdoMet and AdoHcy (Fig. 6B, lanes 1–4 and 6–9). The complexes were reduced in the presence of excess amounts of poly d(I-C) (Fig. 6B, lanes 5 and 10). These results suggest that Dnmt3a bound nonspecifically to dsDNA. However, (CGG/CCG)₇ was efficiently methylated as expected, while Dnmt3a could methylate (CAG/CTG)₇ with much lower efficiency (Table IV). The methylation activity on AT/TA dsDNA substrate lacking cytosine residues was at background level (Table IV), while Dnmt3a could bind this DNA substrate (data not shown). Similar experiments were performed with DNMT1 as a comparison (Fig. 6A; Table IV). Therefore, we concluded that Dnmt3a, as well as DNMT1, has nonspecific binding affinity to dsDNA regardless of the presence of CpG cytosines. It should also be noted that both Dnmt3a and DNMT1 were able to methylate non-CpG cytosine (Table IV), although they were minor activities in vitro.

**FIG. 3.** Dnmt3a is a true de novo methyltransferase in that it prefers unmethylated dsDNA over hemimethylated dsDNA. A, substrate specificity of Dnmt3a under saturating conditions. Dnmt3a (300 nM), AdoMet (900 nM), and synthetic DNA oligonucleotides (125 or 250 nM) were incubated at 37 °C. The mean values of triplicate experiments are presented with error bars representing the standard deviation. Columns 1–7 contain the indicated DNA substrates at 125 nM. Column 8 is a no DNA control. Columns 9–11 contain DNA substrates at 250 nM. B, internal substrate competition under non-saturating conditions. Each reaction contains 300 nM Dnmt3a, 900 nM AdoMet, and two DNA substrates (unmethylated and hemimethylated dsDNAs). One DNA substrate concentration was fixed at 250 nM (concentrations in CpG) while the other was varied (125, 250, 375, and 500 nM CpG). Hemimethylated dsDNA without biotin was fixed and unmethylated dsDNA was varied (squares). Unmethylated dsDNA without biotin was fixed and hemimethylated dsDNA was varied (circles). The mean values of duplicate experiments are presented.

**FIG. 4.** Bi-substrate steady-state kinetic analysis of Dnmt3a. The mean values of independent triplicate experiments were plotted for double-reciprocal plots. Each reaction contains 300 nM Dnmt3a, AdoMet, and either unmethylated dsDNA or hemimethylated dsDNA. A, double-reciprocal plots for unmethylated dsDNA. Top panel, the CpG (unmethylated dsDNA) concentration was fixed at 325 nM (squares), 500 nM (circles), 675 nM (triangles), and 1.25 μM (diamonds). Bottom panel, the AdoMet concentration was fixed at 600 nM (squares), 750 nM (circles), 900 nM (triangles), and 1.8 μM (diamonds). B, double-reciprocal plots for hemimethylated dsDNA. Top panel, the CpG (hemimethylated dsDNA) concentration was fixed at 160 nM (squares), 250 nM (circles), 340 nM (triangles), and 625 nM (diamonds). Bottom panel, the AdoMet concentration was fixed at 600 nM (squares), 750 nM (circles), 900 nM (triangles), and 1.8 μM (diamonds).
DISCUSSION

We report here the first quantitative biochemical analysis of Dnmt3a, a mammalian de novo DNA methyltransferase, which was expressed in eukaryotic cells. Dnmt3a demonstrated preferential methylation of unmethylated double-stranded DNA relative to a hemimethylated DNA substrate in vitro. These results clearly indicate that mammalian Dnmt3a is an enzyme worthy of being termed a de novo methyltransferase.

**Substrate Specificity of Dnmt3a**

No eukaryotic DNA methyltransferase has thus far been shown to prefer unmethylated dsDNA over hemimethylated dsDNA, while the existence of a de novo methyltransferase in eukaryotes has been predicted. Mammalian DNMT1 can distinguish between these methylation states but prefers hemimethylated dsDNA over unmethylated dsDNA, thus giving rise to the term “maintenance methyltransferase” (41). Therefore, this is the first report to conclude that a eukaryotic DNMT exhibits a substrate specificity (above 3-fold) for unmethylated dsDNA. Several reports demonstrated that bacterial methylases show 1.5–2-fold preferences for dsDNA substrates by $k_{cat}/K_m$ estimation (for example, Refs. 42–46), although none of these reports concluded that these enzymes could discriminate between unmethylated and hemimethylated target sequences.

**TABLE III**

| Product inhibition analysis of the reaction catalyzed by Dnmt3a |
|---------------------------------------------------------------|
| Product inhibitor | Variable substrate | Fixed substrate | Type of inhibition |
| Methylated dsDNA | Unmethylated dsDNA | AdoMet | Competitive |
| Methylated dsDNA | AdoMet | Unmethylated dsDNA | Noncompetitive |
| AdoHcy | Unmethylated dsDNA | AdoMet | Noncompetitive |
| AdoHcy | AdoMet | Unmethylated dsDNA | Noncompetitive |

**FIG. 5.** Kinetic analyses to elucidate the molecular mechanism of the methylation reaction catalyzed by Dnmt3a. The mean values of independent duplicate experiments are plotted. A, product inhibition by methylated dsDNA. Reactions contain 300 nM recombinant Dnmt3a, AdoMet, unmethylated dsDNA, and methylated dsDNA as a product inhibitor. Concentrations of methylated dsDNA are indicated at the left of each panel. Left panel, AdoMet concentration was fixed at 900 nM and CpG (unmethylated dsDNA) concentrations were varied. Right panel, CpG concentration was fixed at 500 nM (concentration in CpG dinucleotides) and AdoMet concentrations were varied. B, product inhibition by AdoHcy. Reactions contain 300 nM recombinant Dnmt3a, AdoMet, unmethylated dsDNA, and AdoHcy as a product inhibitor. Concentrations of AdoHcy are shown at the left of each panel. Left panel, double-reciprocal plots of AdoHcy product inhibition with varying CpG concentrations and a fixed amount of AdoMet (900 nM). Right panel, double-reciprocal plots of AdoHcy product inhibition with varying AdoMet concentrations and a fixed amount of DNA substrate (500 nM, concentration in CpG dinucleotides). C, substrate inhibition analysis of the Dnmt3a methylation reaction. Each reaction contains recombinant Dnmt3a (100 nM), AdoMet, and unmethylated dsDNA. Left panel, the substrate (DNA) saturation curve of Dnmt3a. AdoMet concentration was fixed at 900 nM and DNA concentrations were varied (indicated as the concentration in CpG dinucleotides). Right panel, the substrate (AdoMet) inhibition of Dnmt3a by high concentrations of AdoMet. DNA concentration was fixed (2.5 μM, concentration in CpG dinucleotides) and AdoMet concentrations were varied. D, a proposed scheme of the enzymatic mechanism for the methylation reaction of mammalian Dnmt3a. E, enzyme. CpG, CpG dinucleotides in DNA strand. MpG, CpG dinucleotides in which a cytosine residue is methylated.
other words, the term "substrate" for a DNMT as the whole DNA strand molecule. In unmethylated dsDNA by Dnmt3a be revealed? Here we define wrong orientation to the DNA. How can a real preference for expected to be non-productive, because the enzyme binds in the vitro presence of DNMT1 is somewhat more relaxed than Dnmt3a. In contrast, DNMT1 expressed in this Drosophila system yielded enzymatic properties inconsistent with this and previous work (14, 28, 29). Consistent with this observation, we could detect Dnmt3a catalyzed non-CpG methylation in vitro, although it is not the major substrate specificity of Dnmt3a. In contrast, DNMT1 expressed in this Drosophila system yielded enzymatic properties inconsistent with this and previous work (14, 28, 29). DNMT1 expressed in Drosophila had no detectable activity toward CpG or non-CpG methylation in vitro (25, 48). Our in vitro study does not support this notion. Our results showed that DNMT1 methylates CGG/CCG (CpG cytosine) and CAG/CTG (non-CpG cytosine) 4- and 16-fold better, respectively, than Dnmt3a (Table IV). The relative activity of DNMT1 on non-CpG versus CpG substrates is 4-fold better than that of Dnmt3a. Thus, the substrate preference of DNMT1 is somewhat more relaxed than Dnmt3a in vitro.

One-half of all binding events on hemimethylated DNA are expected to be non-productive, because the enzyme binds in the wrong orientation to the DNA. How can a real preference for unmethylated dsDNA by Dnmt3a be revealed? Here we define a substrate for a DNMT as the whole DNA strand molecule. In other words, the term "preference" not only refers to the catalytic efficiency on an active-center in the transition state, but also takes into account the entire methylation reaction including steps such as recognition of substrates, binding, catalysis, and release of products. Efficiency at all steps affects the enzymatic activity of a DNMT and defines the substrate preference of an enzyme. Viewed in this light, a 50% probability of nonproductive binding should be considered as one of the reasons for reduced activity on hemimethylated dsDNA. It is important, however, that the productive binding effect is distinguished from a dose effect of a methylation-acceptable nucleotide. Such a dose effect can be minimized in several ways. First, for a large excess of the DNA substrate, the reaction velocity is nearly independent of the substrate concentration. The formation of an enzyme-DNA intermediate is maximized at equilibrium under such a saturating DNA condition. Hence, one can ignore the difference in the concentration of the CpG dinucleotide in distinct DNA molecules under saturating conditions, because the amount of substrate no longer affects the apparent reaction velocity. Second, the catalytic efficiency of an enzyme is best defined by the ratio of the kinetic constants, $k_{cat}/K_m$. This ratio is generally used to compare the efficiencies of different enzymes, but can also be used to compare the utilization of different substrates for a particular enzyme, since the ratio $k_{cat}/K_m$ captures the effects of differing substrates on both $k_{cat}$ and $K_m$ (49). In the third approach, the enzymatic activity of Dnmt3a with unmethylated dsDNA is measured in the presence of hemimethylated dsDNA. The competitive activity of the enzyme for both substrates can be observed in one reaction solution at the same time. The difference in methylation efficiency on each substrate will directly reflect the preference of an enzyme for a particular substrate. These three approaches, use of saturating substrate conditions (Fig. 3A), comparison of $k_{cat}/K_m$ values (Table II), and substrate competition studies (Fig. 3B), have been utilized in this report and support our conclusion that Dnmt3a has a preference for unmethylated dsDNA.

DNA substrates of non-fixed length such as poly d(I-C) and genomic DNA fragments have often been utilized for biochemical analysis of DNA methyltransferases. In contrast, we employed synthetic DNA oligonucleotides of fixed length as the DNA substrate. Since it has been reported that DNMT1 is a processive enzyme (50), the length of a substrate molecule may affect the apparent enzymatic activity. Thus, a DNA substrate of fixed length should be utilized to estimate the substrate specificity and kinetic parameters of DNMTs.

We expressed both DNMT1 and Dnmt3a in S99 insect cells using a baculovirus expression system. Although it is known that insect cells do not always reproduce the natural pattern of post-translational modifications seen in mammalian cells (51–53), a baculovirus system is still regarded as the best alternative (54). It is noteworthy that Dnmt1 was reported to be post-translationally modified in vivo (29, 55). The enzymatic activity of recombinant Dnmt3a expressed in Escherichia coli has recently been reported by other groups (26, 56). Their Dnmt3a demonstrated properties different from our baculovirus-expressed Dnmt3a. In particular, one group concluded that their Dnmt3a showed no preference for unmethylated dsDNA (26). Furthermore, the Dnmt3a expressed by these groups possessed relatively strong methylation activity toward non-CpG cytosines, particularly CpA, which was methylated only 2- (26) or 10-fold (56) slower than CpG sites. These seemingly inconsistent results strongly suggest that post-translational modifications may dictate the true substrate specificity of mammalian Dnmt3a. This idea is currently under study.

Gel shift analysis revealed that multiple protein-DNA complexes were formed with both DNMTs (Fig. 6). This is a common characteristic for a relatively nonspecific DNA-binding protein. An obvious difference in the banding patterns was observed between (CGG/CCG)$_7$ and (CAG/CTG)$_7$ probes for both DNMT1 and Dnmt3a. Dnmt3a clearly acted as a de novo methyltransferase. It has also been postulated that Dnmt3a may be capable of non-CpG cytosines, particularly CpA, which was methylated only 2- (26) and 10-fold (56) slower than CpG sites. These seemingly inconsistent results strongly suggest that post-translational modifications may dictate the true substrate specificity of mammalian Dnmt3a. This idea is currently under study.
and Dnmt3a. We assume that these DNA binding profiles could represent cooperative binding of the DNMTs to dsDNA. Binding to a preferable substrate containing CG dinucleotides may induce a conformational change in the protein and may promote a cooperative protein-protein interaction. A titration analysis can be utilized to conclude the cooperativity of DNMTs and to determine the exact structures of these complexes. Such studies will be the subject of future work.

**Molecular Mechanism of Dnmt3a**

**Implications of Kinetic Analysis**—The methylation reaction catalyzed by Dnmt3a utilizes two substrates to produce two products and is thus referred to as a Bi Bi reaction. This type of enzymatic reaction raises several important questions regarding its molecular mechanism. Do these two substrates bind to the enzyme randomly or in an ordered manner? If it proceeds in an ordered fashion, then which substrate binds to Dnmt3a first? There are at least three distinct mechanisms for an enzyme reaction with multiple substrates; sequential ordered (ternary complex), ping-pong (covalent intermediate; double-displacement; substituted-enzyme), and rapid equilibrium ordered mechanisms (57). Furthermore, the ordered mechanism can be classified as random or compulsory ordered mechanisms. A major goal of steady-state kinetic measurements is to differentiate between these various mechanisms.

It is first necessary to determine the basic kinetic mechanism utilized by Dnmt3a before a detailed analysis of the order of substrate binding and product release can be done. Double-reciprocal plots for Dnmt3a resulted in intersecting line patterns (Fig. 4), which are characteristic of a ternary complex mechanism. Consistent with this notion, there is previous structural and mechanistic data demonstrating that DNMTs form a ternary complex (40, 47, 58). To determine the order of substrate binding and product release, product inhibition studies were pursued. Both products, AdoHcy and methylated dsDNA, were examined as inhibitors versus two substrates, AdoMet and DNA, respectively (Table III). AdoHcy demonstrated noncompetitive inhibition with respect to AdoMet. Also, AdoHcy exhibited noncompetitive inhibition with respect to DNA at a fixed and nonsaturating concentration of AdoMet. Increasing amounts of DNA under these conditions could not overcome the effect of AdoHcy inhibition, indicating that AdoHcy binds to different enzyme forms. In a ternary complex mechanism, this inhibition pattern requires that AdoMet does not bind to free enzyme. Another product inhibitor for DNA methyltransferase is methylated CpG dinucleotides. Methylated dsDNA showed competitive inhibition with DNA as a variable substrate (40) may account for the slight discrepancy in the estimated substrate preference of Dnmt3a between the ratio under saturating conditions (3.6-fold) and k_{cat}/K_m under nonsaturating conditions (2.4-fold). Similar to the analyses described in previous reports (14, 22, 31), secondary plots were generated to analyze the kinetic constants presented in this report. It was confirmed that these values are not significantly different from the results analyzed by the program Sigma Plot/Enzyme Kinetics Module (not shown). Kinetic constants in this report are considered as apparent values, since the concentration of active enzyme in a sample was not determined.

Dnmt3a possessed a very low turnover number. Can such a slow enzyme mediate the wave of de novo methylation during

**Table IV**

**Methylation sequence specificity of DNMT1 and Dnmt3a**

All reactions contain AdoMet (900 nm) and DNA substrate (125 nm) except no DNA controls.

| DNA methyltransferase (protein concentration) | Substrate | Specific activity | Relative value | cpm | % | cpm/mg protein |
|---------------------------------------------|-----------|------------------|----------------|-----|----|----------------|
| DNMT1 (30 nm)                               | (CGG/CGG)_7 | 253 | (100) | 3,335 ± 25 | 111 ± 0.8 |
|                                             | (CAG/CTG)_7 | 245 ± 4 | (7.4) | 11.2 ± 0.0 |
|                                             | AT/TA      | 50 ± 5 | (1.5) | 1.7 ± 0.2 |
|                                             | No DNA     | 49 ± 7 | (1.4) | 1.6 ± 0.2 |
| Dnmt3a (400 nm)                             | (CGG/CGG)_7 | 11,001 ± 689 | (100) | 28 ± 1.7 |
|                                             | (CAG/CTG)_7 | 185 ± 11 | (1.6) | 0.46 ± 0.03 |
|                                             | AT/TA      | 66 ± 8 | (0.6) | 0.17 ± 0.02 |
|                                             | No DNA     | 42 ± 6 | (0.4) | 0.11 ± 0.02 |
Substrate Specificity of Dnmt3a

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REFERENCES
1. Li, E., Bestor, T. H., and Jaenisch, R. (1992) Cell 69, 915–926
2. Okano, M., Bell, D. W., Haber, D. A., and Li, W. (1999) Cell 99, 247–257
3. Chell, E. Z., Pettersson, U., Beard, C., Jackson-Grusby, L., and Jaenisch, R. (1998) Nature 395, 89–93
4. Beard, C. L., Li, E., and Jaenisch, R. (1995) Genes Dev. 9, 2325–2334
5. Reik, W., and Walter, J. (2001) Nat. Rev. Genet. 2, 21–32
6. Yoder, J. A., Walsh, C. P., and Bestor, T. H. (1997) Trends Genet. 13, 335–340
7. Bird, A. (1986) Nature 321, 209–213
8. Baylin, S. B., and Herman, J. G. (2000) Trends Genet. 16, 168–174
9. Jones, P. A., and Laird, P. W. (1999) Nature Genet. 21, 163–166
10. Robertson, K. D., and Wolff, A. P. (2000) Nat. Rev. Genet. 1, 11–19
11. Robertson, K. D., Uzvolygi, E., Liang, G., Talmadge, C., Sumegi, J., Gonzales, V. A., and Jones, P. A. (1999) Nat. Genet. 22, 2291–2296
12. Leonhardt, H., Page, A. W., Weier, H., and Bestor, T. H. (1992) Cell 71, 865–873
13. Liu, Y., Oakeley, E. J., Sun, L., and Jost, J.-P. (1998) Nucleic Acids Res. 26, 508–515
14. Pradhan, B., Bacalla, A., Wells, R. D., and Roberts, R. J. (1999) J. Biol. Chem. 274, 33002–33010
15. Okano, M., Xie, S., and Li, E. (1998) Nature Genet. 19, 219–220
16. Kumar, S., Cheng, X., Klimasauskas, S., Mi, S., Pospisil, J., Roberts, R. J., and Wilson, G. G. (1994) Nucleic Acids Res. 22, 1–10
17. Asauland, R., Gibson, T. J., and Stewart, A. F. (1995) Trends Biochem. Sci. 20, 56–59
18. Fuks, F., Bergers, A., Brehm, A., Hughes-Davies, L., and Kouzarides, T. (2000) Nature Genet. 24, 88–91
19. Robertson, K. D., Ait-Si-Ali, S., Yokochi, T., Wade, P. A., Jones, P. L., and Wolff, A. P. (2000) Nature Genet. 25, 338–342
20. Rountree, M. R., Bachman, K. E., and Baylin, S. B. (2000) Nature Genet. 23, 359–377
21. Chuang, L. S. H., Ihan-I., Koh, T.-W., Ng, H.-H., Xu, G., and Li, B. F. L. (1997) Science 277, 1996–2000
22. Bacalla, A., Pradhan, S., Larson, J. E., Roberts, R. J., and Wells, R. D. (2001) J. Biol. Chem. 276, 18508–18513
23. Klimasauskas, S., Kumar, S., Roberts, R. J., and Cheng, X. (1994) Cell 76, 357–369
24. Smith, S. S., Kaplan, B. E., Sowers, L. C., and Newman, E. M. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 4744–4748
25. Lyko, F., Ramasahoye, B. H., Kashevsky, H., Tudor, M., Mastrangelo, M. A., Orr-Weaver, T. L., and Jaenisch, R. (1999) Nature Genet. 23, 363–366
26. Giguere, H., and Jeltsch, A. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 9776–9781
27. Flynn, J., Glickman, J. F., and Reich, N. O. (1996) Biochemistry 35, 7308–7315
28. Glickman, J. F., Flynn, J., and Reich, N. O. (1997) Biochem. Biophys. Res. Commun. 239, 280–284
29. Pradhan, S., Talbot, D., Sha, M., Benner, J., Hornstra, L., Li, K., Jaenisch, R., and Roberts, R. J. (1997) Nucleic Acids Res. 25, 4666–4673
30. Flynn, J., and Reich, N. (1998) Biochemistry 37, 15162–15169
31. Bacalla, A., Pradhan, S., Roberts, R. J., and Wells, R. D. (1999) J. Biol. Chem. 274, 33011–33019
32. Segel, I. H. (1983) Enzyme Kinetics, John Wiley & Sons, Inc., New York
33. J. C. and Santi, D. V. (1987) J. Biol. Chem. 262, 4776–4786
34. Bhattacharya, S. K., and Dubey, A. K. (1999) J. Biol. Chem. 274, 14743–14749
35. Cleland, W. W. (1977) Adv. Enzymol. Relat. Areas. Mol. Biol. 45, 273–387
36. Caskey-Cowan, A. P. (2001) Fundamentals of Enzyme Kinetics, Portland Press, London
37. Andersen, R. B., and Neuhard, J. (2001) J. Biol. Chem. 276, 3031–3034
38. Flugel, R. S., Hwangbo, Y., Lambalot, B. H., Cronan, J. E., Jr., and Walsh, Vectors: A Laboratory Manual, 2nd ed., Freeman, New York
39. Hsieh, P. C., Kowalczyk, T. H., and Phillips, N. F. (1996) Biochemistry 35, 9772–9781
40. Evdokimov, A. A., Zinoviev, V. V., Malysin, E. G., Schlagman, S. L., and Hattman, S. (2002) J. Biol. Chem. 277, 279–286
41. Bestor, T. H. (2000) Hum. Mol. Genet. 9, 2395–2402
42. Reich, N. O., and Danzitz, M. J. (1991) Nucleic Acids Res. 19, 6587–6594
43. Jeltsch, A., Friedrich, T., and Roth, M. (1998) J. Mol. Biol. 275, 747–758
44. Zinoviev, V. V., Evdokimov, A. A., Gorburon, Y. A., Malysin, E. G., Kossykh, V. G., and Hattman, S. (1998) Biochem. Biophys. Res. Commun. 257, 481–486
45. Lindstrom, W. M., Jr., Flynn, J., and Reich, N. O. (2000) J. Biol. Chem. 275, 4912–4919
46. Gowher, H., and Jeltsch, A. (2000) J. Mol. Biol. 303, 93–110
47. Malysin, E. G., Lindstrom, W. M. Jr., Schlagman, S. L., Hattman, S. and Reich, N. O. (2000) Nucleic Acids Res. 28, 4207–4211
48. Ramasahoye, B. H., Biniszkiewicz, D., Lyko, F., Clark, V., Bird, A. P., and Jaenisch, R. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 5237–5242
49. Cipelka, R. A. (2000) European Patent EP 0624 744
50. Bestor, T. H., and Ingram, V. M. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 5559–5563
51. Cho, K., Bruel, C., Inglese, J., and Khorana, H. G. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10577–10582
52. Piwnica-Worms, H., Williams, N. G., Chen, S. H., and Roberts, T. M. (1990) J. Virol. 64, 61–68
53. Fang, N. X., Frazer, I. H., and Fernandez, G. J. (2000) Biotechnol. Appl. Biochem. 32, 27–33
54. O’Reilly, D. R., Miller, L. K., and Luckow, V. A. (1992) Baculovirus Expression System, 2nd ed., London
55. Glickman, J. F., Pavlovich, J. G., and Reich, N. O. (1997) J. Biol. Chem. 272, 17861–17867
56. Aoki, A., Suetake, I., Miyagawa, J., Fujito, T., Chijiiwa, T., Sasaki, H., and
Tajima, S. (2001) *Nucleic Acids Res.* **29**, 3506–3512
57. Cleland, W. W. (1989) *Biochim. Biophys. Acta* **213**, 213–220
58. Vilkaitis, G., Merkiene, E., Serva, S., Weinhold, E., and Klimasauskas, S. (2001) *J. Biol. Chem.* **276**, 20924–20934
59. Szilak, L., Der, A., Deak, F., and Venetianer, P. (2001) *Eur. J. Biochem.* **218**, 727–733
60. Berdis, A. J., Lee, I., Coward, J. K., Stephens, C., Wright, R., Shapiro, L., and Benkovic, S. J. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 2874–2879
61. Lei, H., Oh, S. P., Okano, M., Juttermann, R., Goss, K. A., Jaenisch, R., and Li, E. (1996) *Development* **122**, 3195–3205
62. Graff, J. R., Herman, J. G., Myohanen, S., Baylin, S. B., and Vertino, P. M. (1997) *J. Biol. Chem.* **272**, 22322–22329
63. Fuks, F., Burgers, W. A., Godin, N., Kasai, M., and Kouzarides, T. (2001) *EMBO J.* **20**, 2536–2544
64. Mizuno, S., Chijiwa, T., Okamura, T., Akashi, K., Fukumaki, Y., Niho, Y., and Sasaki, H. (2001) *Blood* **97**, 1172–1179