Peptide Mapping of the Murine DNA Methyltransferase Reveals a Major Phosphorylation Site and the Start of Translation*

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The murine DNA methyltransferase catalyzes the transfer of methyl groups from S-adenosylmethionine to cytosines within d(CpG) dinucleotides. The enzyme is necessary for normal embryonic development and is implicated in a number of important processes, including the control of gene expression and cancer. Metabolic labeling and high pressure liquid chromatography-electrospray ionization-mass spectrometry (HPLC-ESI-MS) were performed on DNA methyltransferase purified from murine erythroleukemia cells. Serine 514 was identified as a major phosphorylation site that lies in a domain required for targeting of the enzyme to the replication foci. These results present a potential mechanism for the regulation of DNA methylation.

HPLC-ESI-MS peptide mapping data demonstrated that the purified murine DNA methyltransferase protein contains the N-terminal regions predicted by the recently revised 5′ gene sequences (Yoder, J. A., Yen, R.-W. C., Vertino, P. M., Bestor, T. H., and Baylin, S. B. (1996) J. Biol. Chem. 271, 31092–31097). The evidence suggests a start of translation at the first predicted methionine, with no alternate translational start sites. Our peptidemapping results provide a more detailed structural characterization of the DNA methyltransferase that will facilitate future structure/function studies.

An essential mechanism for tissue-specific differentiation during embryonic development in mammals is the post-synthetic methylation of d(CpG) dinucleotides in DNA (1). During embryogenesis, 70–80% of d(CpG) dinucleotides become methylated in a developmentally regulated, tissue-specific fashion (2). The methylation of regulatory DNA elements frequently results in the transcriptional silencing of proximal genes (3). Although no general mechanism for this silencing has been identified, the methylation status of several DNA sequences is known to modulate binding by regulatory proteins (4, 5).

The tissue-specific methylation patterns in mammalian cells presumably result from the action of the DNA methyltransferase in concert with other unidentified cellular factors (6), such as chromatin packaging proteins (7), active demethylation systems (8), or particular DNA structures (9) that dictate this enzyme’s specificity for certain d(CpG)/s within the genome.

The DNA methyltransferase enzyme interacts with p23, a known component of the progesterone receptor complex (10), and it is likely that control of genomic DNA methylation is coupled to receptor-mediated signaling systems in some, as yet unknown, way. A detailed knowledge of the factors involved in the regulation of the methyltransferase enzyme will be necessary to know how genomic methylation patterns are established.

The murine DNA methyltransferase is an approximately 180–190-kDa enzyme that transfers a methyl group from S-adenosylmethionine (AdoMet) onto the C-5 position of cytosine within the d(CpG) dinucleotides of double-stranded DNA. Targeted disruption of the gene for this enzyme is lethal to embryos at the middle stages of gestation (1). A number of functional domains of the enzyme have been identified and are presented in Fig. 1. The catalytic domain lies in the C-terminal third of the protein and contains regions of homology with the prokaryotic DNA methyltransferases, including a conserved AdoMet binding site and catalytic center. The N-terminal two-thirds of the enzyme is separated from the catalytic domain by a flexible hinge region consisting of glycine-lysine repeats (11) and contains sequences that bind zinc and DNA independently (11, 12). A minor groove DNA-binding motif (SPKK) shown to undergo phosphorylation-dependent attenuation in other proteins (13, 14) is located in the non-catalytic region of the protein. The DNA methyltransferase localizes to the replication foci during S phase of the cell cycle (15), and a 200-amino acid segment of the protein is necessary for this cell cycle-dependent localization.

Various sizes of the murine DNA methyltransferase enzyme (20, 21, 35), its cDNA (16, 17), and its mRNA (18, 40) have been reported. Based on SDS-PAGE analysis the protein is estimated to be from 170 to 190 kDa. Post-translational processing (20, 21) as well as alternate transcriptional start sites have been suggested to account for this range in size (18, 40). However, when purified in the presence of protease inhibitors, the DNA methyltransferase from murine erythroleukemia (MEL) cells is detected as a single protein band on SDS-PAGE (22), casting doubt upon the presence of multiple enzyme forms. The original cDNA predicted a protein of 1573 amino acids, corresponding to a mass of 175 kDa (16), but was later revised to 1502 amino acids (169 kDa) (Genbank accession X14805). Further revisions to the 5′ regions of the human and murine DNA methyltransferase genes now predict a protein of 182 kDa (17). These data implicate a new translational start site resulting in an enzyme 118 amino acids longer than previously determined. Furthermore, these data suggest that a previously identified DNA methyltransferase promoter (19) would lie in an intron (17), upstream of the originally predicted start of translation in MEL cells.

We have used sensitive mass spectroscopic techniques to map the peptides in the purified MEL DNA methyltransferase,
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Materials and Methods

Electrophoresis, blotting, and DNA methyltransferase preparation—SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 7.5% polyacrylamide, using broad range molecular weight protein standards (New England Biolabs). Transfer of proteins from polyacrylamide gels to nitrocellulose (MSI Inc.) or PVDF membranes (Millipore, Inc.) was performed using an Integrated Separation Systems semi-dry electroblotting apparatus set at 200 mA for 50 min.

Purification of DNA methyltransferase from MEL cells was performed as described previously (22). Protein concentrations were determined with a Bio-Rad protein assay kit using bovine serum albumin as a standard.

Glycosylation analysis—Purified homogeneous MEL DNA methyltransferase was subjected to SDS-PAGE and transferred to nitrocellulose membranes. N-Glycosylated and O-glycosylated polypeptides were detected by using a Glycotrack (Oxford Glycosystems) carbohydrate standards on Coomassie-stained SDS-PAGE gels.

Proteolytic digestion of 32P-labeled murine DNA methyltransferase—In situ digestion and extraction of the DNA methyltransferase in SDS-PAGE gels was performed according to the method of Williams (26). Briefly, DNA methyltransferase-containing bands (5–10 μg) were excised from Coomassie-stained SDS-PAGE gels of immunoprecipitates from 32P-labeled MEL cells. The bands were washed by shaking for 30 min in ice-cold acetone, dried in a speed-vac, and then re-hydrated in 100 μl of 100 mM NH4HCO3, pH 7.8, for 4 h with gentle agitation. The buffer was changed once, and the bands were cut into three equal pieces followed by the addition of either trypsin (Sigma), trypsin (Sigma), or V8 protease (Boehringer Mannheim), or V8 protease (Boehringer Mannheim) (1 μg) and incubated for 16 h at 37 °C. The supernatant was collected, and the remaining gel slices were extracted by shaking with an additional 100 μl of 100 mM NH4HCO3 for 4 h. The supernatant was dried, and a further extraction was performed for 2 times with 150 μl of 100 mM NH4HCO3.

Proteolytic digestion of murine DNA methyltransferase purified from MEL Cells—Murine DNA methyltransferase was purified to homogeneity at a concentration of 1.7 μM (300 μg/ml) from MEL cells as described previously (21). This preparation (500 μl) was precipitated with 5 volumes of ice-cold acetone and centrifuged at 20,000 × g, and then the washed pellet was passed through a small Dounce homogenizer. The suspension was centrifuged at 50,000 × g for 60 min and the supernatant processed as described below.

Immunoprecipitation—Cell-free extracts were added to 0.8–4.0 ml of immunoprecipitation buffer (50 mM Tris, pH 8.0, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS with protease inhibitors, and phosphatase inhibitors as described in the previous section), and the final NaCl concentration was brought to 150 mM.

Immunoprecipitations were carried out by the addition of 5–30 μl of pATH 52 antisera directed against a Trp E-methyltransferase N-terminal fusion encoding amino acids 256–754 (11) or non-immune rabbit antiserum to the cell-free extracts, incubated for 1 h on ice, then addition of 50–60 μl protein A-Sepharose beads (Pharmacia Biotech Inc.), and incubated with frequent agitation for 1 h on ice. The suspension was pelleted by centrifugation at 2000 × g for 5 min and washed three times at room temperature in 150 mM NaCl, 20 mM Tris, pH 8.0, 0.1% Tween 20. The final pellet was dissolved in 20 μl of SDS-PAGE sample buffer supplemented with 2 mM dihtiothreitol, heated at 70 °C for 10 min, and subjected to SDS-PAGE for analysis.

Phosphoamino Acid Analysis—SDS-PAGE gels of the immunoprecipitates were transferred onto Immobilon PVDF membranes as described previously (23). The membrane was air-dried and exposed to Fuji RX film at −70 °C for 24 h. The [32P]orthophosphate-labeled DNA methyltransferase was excised and extracted from the filter, acid-hydrolyzed as described previously (24), and then subjected to thin layer cellulose chromatography as described (25) using 0.5 M NH4OH/iso- butyric acid (5:3, v/v) as a solvent. Negative controls were performed by the excision of nonspecifically labeled bands from a lane on the PVDF membrane that was immunoprecipitated with non-immune rabbit serum. Phosphoserine, phosphothreonine, and phosphotyrosine standards (500 ng, Sigma) were detected by ninhydrin spray (Sigma), and the experimental lanes were air-dried and detected by autoradiographic exposure to Fuji RX film at −70 °C for 36 h.

C-18 Reverse Phase Chromatography/Electrospray Mass Spectrometry—A typical sample contained 20 pmol of 32P-labeled DNA methyltransferase digested with protease in the gel, containing 300–1700 dpm, mixed with 200 pmol of purified DNA methyltransferase digested in the solution as described in the previous section. All chromatography was performed using a Michrom Ultrafast HPLC system (Michrom Bioresearches, Sunnyvale, CA), equipped with a micro-flow cell UV absorbance detector set at 215-nm wavelength. A 1.0-mm inner diameter × 15.0 cm long C18, 300-μm reverse phase HPLC column (Michrom Bioresearches, Sunnyvale, CA) was used to separate peptides derived from proteolytic digests of the DNA methyltransferase. Chromatography solvents were 0.1% trifluoroacetic acid, 1% acetonitrile (solvent A)
**RESULTS**

Post-translational Modification: Serine Phosphorylation and Absence of Glycosylation—The glycosylation state of the purified MEL DNA methyltransferase was analyzed using a GlycoSure carbohydrate detection kit. Our results showed that glycosylation of the homogeneous enzyme was not detectable. We were able to detect glycosyl moieties in 35 ng of ovalbumin which is 5% glycosylated by weight, whereas glycosylation was not detected in 4 μg of either MEL-DNA methyltransferase or recombinant DNA methyltransferase (data not shown).

Post-translational phosphorylation was detected by $^{32}$P labeling of the DNA methyltransferase in MEL cells. Immunoprecipitations with anti-methyltransferase antibodies from recombinant DNA methyltransferase (data not shown).

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Absence of Glycosylation—

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**Fig. 2.** Immunoprecipitation, purification, and post-translational phosphorylation of the MEL-derived DNA methyltransferase. A, autoradiogram showing immunoprecipitated DNA methyltransferase is metabolically labeled with $[^{32}]P$orthophosphate. MEL cells were cultured for 12 h with 0.5 mCi of $[^{32}]P$orthophosphate and immunoprecipitated with either a non-immune rabbit serum (lane 1) or anti-methyltransferase antiserum (lane 2). The immunoprecipitated proteins were separated by 7.5% SDS-PAGE. The gel was dried and autoradiographed for 24 h. B, Coomassie-stained SDS-PAGE gel of the immunoprecipitated DNA methyltransferase (MT). Large and small IgG subunits are indicated. MEL cells were grown to mid-log phase and immunoprecipitated from nuclear extracts with anti-pATH52 antibodies as described under "Materials and Methods." C, purified DNA methyltransferase from MEL cells. Lane 1, Coomassie-stained 7.5% acrylamide gels loaded with molecular weight size standards; lane 2, 2 μg of purified DNA methyltransferase. Purification of the enzyme was performed as described previously (21). Size standards in descending order: 212, 158, 116, 97.2, 66.4, 55.6, and 42.7 kDa.

and 0.1% trifluoroacetic acid, 95% acetonitrile (solvent B). After loading the column with the proteolyzed DNA methyltransferase, a 5–10-min isocratic wash of 0% B was followed by a gradient of 0 to 95% solvent B over 30 min to 100 min at a flow rate of 50 μl/min.

For analyses that required Cerenkov counting, the outlet of the UV absorbance detector flow cell was connected to a Pharmacia fraction collector with 30 cm of PEEK tubing (0.005-inch diameter, Upchurch Scientific Inc., Oak Harbor, WA). Fractions were collected at 1-min intervals and were counted for 2 min in a Beckman scintillation counter.

For analyses that required electrospray mass spectrometry, the outlet of the UV absorbance detector flow cell was connected to the electrospray probe with 20 cm of PEEK tubing (0.005-inch diameter). Mass spectrometry was performed using a Fisons VG Platform II quadrupole mass spectrometer, equipped with a pneumatically assisted ESI source. The mass spectrometer was scanned repetitively over a mass to charge ratio (m/z) range of either 300–1500 or 400–1500, at a scan time of 2 s/scan, a 25–35 V orifice potential, and a cone temperature of 70 °C. Data were collected in centroid mode.

MassLynx (Micromass Inc.) software provided with the mass spectrometer permitted mass spectra to be displayed for any observed peak of ion detection events, allowed background subtraction, as well as extraction of a defined input m/z from the data set. A Biolynx peptide analysis algorithm (Micromass Inc.) was used to predict the peptide products of proteolytic digests of the murine DNA methyltransferase (Genbank accession number X14805) and to search ESI-MS data for these products. Manual searching of data sets was also used.

**Fig. 3.** Phosphoamino acid analysis of DNA methyltransferase SDS-PAGE gels from metabolic labeling experiments shown in Fig. 2 were transferred to PVDF membranes. The $[^{32}]P$phosphate-labeled DNA methyltransferase band was excised and analyzed by thin layer cellulose chromatography, followed by autoradiography. Lane 1, phosphoamino acid standards, detected by ninhydrin; lane 2, DNA methyltransferase; lane 3 non-specific band excision from the non-immune immunoprecipitate lane. PT, phosphotyrosine; PT, phosphothreonine; PS, phosphoserine.

$[^{32}]P$orthophosphate-treated MEL cells resulted in the recovery of $^{32}$P-labeled DNA methyltransferase as detected by x-ray film exposed to SDS-PAGE gels of the immunoprecipitates (Fig. 2A).

The DNA methyltransferase band was excised from the gel along with the nonspecific gel slice from a non-immune rabbit antiserum. In 10-mCi labeling experiments using 2 × $10^5$ to 5 × $10^5$ MEL cells, the DNA methyltransferase band repeatedly contained 9000–11,400 dpm by Cerenkov counting, whereas negative control gel slices, produced by a non-immune antiserum, resulted in 680 dpm, indicating that the $^{32}$P label was specifically associated with the Coomassie-detectable DNA methyltransferase and not with any other portion of the gel. The phosphorylation state of the DNA methyltransferase was not affected by serum starvation for 30 h prior to labeling, serum starvation, and replenishment or by treatment of MEL cells with the phosphatase inhibitor, okadaic acid (0.1 μM), for 24 h prior to labeling.

The major sites of protein phosphorylation in vertebrate cells are on serine, threonine, and tyrosine residues (27). To determine which type of amino acid was phosphorylated, the $^{32}$P-labeled DNA methyltransferase was acid-hydrolyzed into individual amino acids, and the amino acids were separated by thin layer chromatography. The presence of $[^{32}]P$phosphate was detected by x-ray film. Fig. 3 shows that the primary sites of phosphorylation reside on serine or threonine, because the majority of label co-migrated with the phosphoserine and phosphothreonine standards.

**Peptide Mapping and Identification of Serine 514 as a Major Phosphorylation Site—HPLC was used to separate peptides derived from proteolytic digests of the DNA methyltransferase. Cerenkov counting of the HPLC column fractions was used to detect $^{32}$P-labeled peptides, and electrospray ionization mass spectrometry was used to measure the masses of these peptides. These studies required the use of highly pure DNA methyltransferase (Fig. 2C), since other proteins in the preparation might result in the detection of spurious masses. $^{32}$P-Labeled DNA methyltransferase was obtained from immunoprecipitated nuclear extracts of metabolically labeled MEL cells, which was excised from SDS-PAGE gels (Fig. 2B). The $^{32}$P-labeled DNA methyltransferase was separated from the IgG
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subunits of the antibody by excision of the methyltransferase from the SDS-PAGE gel (see “Materials and Methods”). Because only a small amount (3–10 pmol) of the DNA methyltransferase-derived peptides were extracted for any single digest, the samples were supplemented with digested, non-labeled (Fig. 2C) DNA methyltransferase purified from MEL cells (50–200 pmol). In these experiments, 60–95% of the total 32P label (200–1200 dpm) was eluted in a small retention window (3 min), and the remaining 5–40% of loaded counts were distributed throughout the HPLC gradient. Over 60% of the predicted masses were detected in each digest, resulting in the assignment of over 80% of the DNA methyltransferase sequence, spanning the entire protein. Less than 8% of the detected m/z’s were not predicted by the cDNA.

Two criteria were used to identify phosphorylated peptides as follows: (i) the peak of [32P] in the HPLC chromatogram, and (ii) an 80-atomic mass unit increase from the predicted mass of peptides generated by sequence-specific proteases. Our data identified serine 514 as a major phosphorylation site. Digests with trypsin, chymotrypsin, and Staphylococcus aureus V8 proteases demonstrated masses that differed by 80 atomic mass units from the predicted peptides containing serine 514. A summary of the mass spectrometry data showing the structure of the phosphorylated peptide, and its predicted cleavage sites by S. aureus V8, trypsin, and chymotrypsin is shown in Fig. 7. In tryptic and chymotryptic digests the non-phosphorylated peptide was detected at a retarded retention time consistent with its increased hydrophobicity.

The V8, chymotryptic, and tryptic phosphopeptides containing serine 514 were co-retained with the 32P label on C18 columns. Figs. 4–6 present the data from peptide mapping experiments using C18 chromatography to separate digests of the 32P-labeled DNA methyltransferase. The tryptic digest (Fig. 4A) produced a peak of 32P label in the pre-gradient volume, indicating that the peptide had very low affinity for the stationary phase. The phosphopeptide and peak of 32P label eluted with many unassigned large molecule contaminants in the pre-gradient volume. This fraction was collected and purified on the same C18 column coupled to the mass spectrometer. This additional step served to separate the phosphopeptide from these contaminants. Fig. 4 shows the co-retention of m/z 702 with the 32P label, corresponding to the singly charged phosphopeptide, IYISpK(T84 + P), containing serine 514.

Similar experiments with S. aureus V8 and chymotrypsin are presented in Figs. 5 and 6. In these experiments, 32P-labeled digests were divided into two equal aliquots and separated by HPLC. The first aliquot was subjected to HPLC, and the fractions were collected and Cerenkov counted. The second aliquot was subjected to HPLC and mass spectrometry. In the
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Identification of the N-terminal Region of the DNA Methyltransferase—Reports on the size of the DNA methyltransferase have been variable with respect to the migration on SDS-PAGE gels, the length of its messenger RNA (18, 40), and the 5' ends of the gene (17, 19). Recently, a revision of the 5' regions of the methyltransferase gene was reported, suggesting new 5' exons and a new start of translation (17). The formerly predicted start of translation would lie 357 base pairs downstream and code for an internal methionine. Our HPLC-ESI-MS results are consistent with the start of translation residing at the newly identified start codon (Table I, Fig. 8). Peptide A1-2 includes the start methionine and peptide T19 spans the first internal methionine, suggesting that translation of the DNA methyltransferase begins prior to this previously suggested start site. The detection of peptides corresponding to the newly determined 5' region suggests that the DNA methyltransferase from MEL cells is not significantly proteolyzed.

Our HPLC-ESI-MS analysis was unable to assign any masses consistent with N-terminal acetylation, formylation, or pyroglutamation. Furthermore, we were unable to find any evidence for clipping of the N terminus. Peptide T2 is isomassive with an acetylated T1 peptide (Table I). Since the non-acetylated N-terminal peptide was detected in the V8 digests, we assigned the m/z 516 to T2.

DISCUSSION

Post-translational Modification of the Murine DNA Methyltransferase—The post-translational modification of proteins plays a central role in the function of many critical cellular processes, including modulation of catalytic activity (30), alterations of the affinity of proteins for DNA (31, 32), and alteration of subcellular localization (33). Since DNA methylation is an essential component of embryonic development and the mechanisms of regulation of DNA methylation in metazoans are poorly understood, a detailed characterization of the structure of the mammalian DNA methyltransferase is critical. Our
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Peptides are lettered according to the protease used (A, V8; T, trypsin; Y, chymotrypsin), and numbered according to their position in the primary sequence of the DNA methyltransferase cdNA (Genbank accession X14805). Chromatographic peaks were background subtracted and analyzed for multiple charge states.

Table I

| Peptide | Retention time (min) | Residues (position) | Mass of MH* (Da) |
|---------|----------------------|---------------------|------------------|
| T2      | 2.8                  | 5–9                | 516.0            |
| T7      | 25.39                | 30–33              | 531.5            |
| T8      | 33.26                | 34–39              | 662.95           |
| T11     | 40.75                | 46–58              | 1598.2           |
| T12     | 29.5                 | 59–67              | 1034.9           |
| T14     | 11.5                 | 71–81              | 1266.8           |
| T18     | 21.01                | 103–116            | 1486.1           |
| T19     | 33.92                | 117–124            | 893.2            |
| T21     | 14.0                 | 128–133            | 740.9            |
| T7–8    | 30.17                | 30–39              | 1175.4           |
| T20–21  | 31.80                | 125–133            | 1079.5           |
| T12–14  | 21.34                | 59–81              | 2663.2           |

* This peptide is isomassive with acetylated T1, the predicted N-terminal peptide.

** This peptide spans the second predicted start methionine.

*** This peptide corresponds to the predicted N terminus from the first methionine.

Post-translational phosphorylation is a plausible means for regulating some aspect of the DNA methyltransferase’s function. Because the phosphorylation site lies in a domain required for protein targeting to the replication foci during S phase of the cell cycle, phosphorylation may affect the subcellular localization of the enzyme. The large N-terminal two-thirds of the protein is unnecessary for catalysis and contains distinct DNA and zinc binding sites (Fig. 1) (11, 12). We recently showed that an allosteric site on the enzyme is involved in both substrate inhibition and the binding of inhibitory single-stranded DNA sequences; phosphorylation might serve to attenuate this allosteric effect. Preliminary studies of the enzyme treated with alkaline phosphatase, λ- phosphatase, and casein kinase resulted in no significant effect on the catalytic rate in steady-state assays using double-stranded poly(dI–dC) as a substrate.

Characterization of the Translational Start Site—Previous studies have suggested alternate transcriptional, translational, or proteolytic products of the MEL DNA methyltransferase (17–21, 41), and functional roles for different sizes of the enzyme have been proposed (20, 21). Reports on the size of the mammalian proteins on SDS-PAGE range from 120 to 190 kDa.
other types of functional assays including modulation of inhi-
putative translational start site (17); **, the formerly predicted start
1–130) based on the gene sequence (Genbank accession X14805). *, the
N-terminal region of the DNA methyltransferase (amino acids
studies of protease digests of the murine DNA methyltransferase. The
catalysis. These
phosphorylated residue is in a domain that is unessential for
The
methyltransferase is understandable in light of the fact that the
transferase is very susceptible to proteolysis and can be
between the 158- and 212-kDa size markers (Fig. 2C) (21, 37).
The original report on the translational product of the MEL
DNA methyltransferase cDNA predicting a 1573-amino acid
protein with a mass of 175 kDa (16) was later revised to predict
DNA methyltransferase cDNA predicting a 1573-amino acid
methyltransferase (28) suggested a new open reading frame upstream of
The preliminary results showing that phosphoryl-
functional significance of phosphorylation of the DNA methyl-
mains a possibility.
Our work will facilitate the future characterization of the
functional significance of phosphorylation of the DNA methyl-
transferase. The preliminary results showing that phosphorylation
	
bution by nucleic acids and interactions with other proteins. Our
ability to express and purify recombinant DNA methyl-
transferase should facilitate in vitro studies of site-directed
Other model systems, such as COS cell ex-
pression (39) or transgenic mice (1) might be useful in assess-
ing the functional significance of phosphorylation of serine 514
in vivo.

Acknowledgments—We kindly thank James Flynn for providing pu-
rified DNA methyltransferase and Timothy Bestor for providing the
pATH52 antibodies.

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FIG. 8. Map of the N-terminal region of the DNA methyltrans-
ferase. The scaled line represents the predicted primary sequence of the
N-terminal region of the DNA methyltransferase (amino acids 1–130) based on the gene sequence (Genbank accession X14805). *, the formerly predicted start
1–130) based on the gene sequence (Genbank accession X14805). Cloning of the human DNA methyltrans-
protein with a mass of 157 kDa (16) was later revised to predict
DNA methyltransferase cDNA predicting a 1573-amino acid
protein with a mass of 175 kDa (16) was later revised to predict