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Mammalian DNA (Cytosine-5-)-methyltransferase Expressed in *Escherichia coli*, Purified and Characterized*

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Besides modulating specific DNA-protein interactions, methylated cytosine, frequently referred to as the fifth base of the genome, also influences DNA structure, recombination, transposition, repair, transcription, imprinting, and mutagenesis. DNA (cytosine-5-)-methyltransferase catalyzes cytosine methylation in eukaryotes. We have cloned and expressed this enzyme in *Escherichia coli*, purified it to apparent homogeneity, characterized its properties, and we have shown that it hemimethylates DNA. The cDNA for murine maintenance methyltransferase was reconstructed and cloned for direct expression in native form. Immunoblotting revealed a unique protein (M_r = 190,000) not present in control cells. The mostly soluble overexpressed protein was purified by DEAE, Sephadex, and DNA cellulose chromatography. Peak methylating activity correlated with methyltransferase immunoblots. The purified enzyme preferentially transferred radioactive methyl moieties to hemimethylated DNA in assays and on autoradiograms. All of the examined properties of the purified recombinant DNA methyltransferase are consistent with the enzyme purified from mammalian cells. Further characterization revealed enhanced *in vitro* methylation of premethylated oligodeoxynucleotides. The cloning of hemimethyltransferase in *E. coli* should allow facilitated structure-function mutational analysis of this enzyme, studies of its biological effects in prokaryotes, and potential large scale methyltransferase production for crystallography, and it may have broad applications in maintaining the native methylated state of cloned DNA.

The chemistry of cytosine-5 methylation consists of transfer of a methyl moiety from *S*-adenosyl-L-methionine (AdoMet)[1] to carbon 5 of the pyrimidine ring of cytosine. This simple one-carbon transfer, catalyzed by DNA (cytosine-5-)-methyltransferase (DNA MTase), is ubiquitous, affecting approximately 5 × 10^7 cytosines/mammalian diploid nucleus (1). Cytosine methylation is the most common modification of DNA found in nature and has been implicated in the control of developmental processes (2), DNA repair (3–5), chromatin organization (6–8), transcription (9–11), X chromosome inactivation (12–13), transposition (14–15), recombination (16), mutagenesis (17–18), replication (19), and genomic imprinting (20). DNA MTase has been shown in mice to be essential for embryonic survival (21) and has been proposed to play a role in general biological processes such as cellular aging (22), carcinogenesis (23), human genetic diseases (24), and evolution (17, 25).

The recognition sequence for DNA MTase is highly specific with almost all cytosine methylation occurring in the duplex palindrome 5′-C-p-G-3′ (CpG). Over half of CpG dinucleotide palindromes are methylated in the mammalian genome (26). After semiconservative replication of DNA, both daughter duplexes are hemimethylated, and DNA MTase, which is localized to replication foci (27), fully methylates the duplex CpG dinucleotides. This process, termed maintenance methylation, restores the parental genomic methylation pattern and is consistent with the *in vitro* propensity of the DNA MTase for hemimethylated sequences (28–29).

DNA MTase can also methylate certain CpGs that are not in a hemimethylated configuration, a process referred to as *de novo* methylation. Although the mechanisms for *de novo* methylation are not completely understood, a number of studies have reported the appearance of newly methylated CpG dinucleotides in the genome (29–32). Only one gene encoding mammalian DNA MTase has been found, and maintenance methylation and *de novo* methylation are generally believed to be catalyzed by a single enzyme (33–34). Several studies have noted the appearance of *de novo* methylated cytosines in genomic regions containing preexisting methylated cytosines (*i.e.* methylation spreading) such as occurs in newly integrated viral DNA in the genome (31, 35–37). Since cytosine methylation can affect the DNA binding of certain transcriptional regulatory factors, the introduction of additional methylated cytosines within gene regulatory sequences may influence gene expression (35). This spreading of cytosine methylation in gene regulatory sequences has been implicated in the gene silencing characteristic of fragile X syndrome (38–39), cellular senescence (22), and X chromosome inactivation (13).

The importance of cytosine methylation in general and the DNA MTase in particular has led us to express this enzyme in *Escherichia coli* and to further study its mechanisms. Although the cloned cDNA for murine DNA MTase (33) has been expressed in mammalian COS cells (40), we report the first successful expression and purification of catalytically active mammalian DNA MTase in *E. coli*, providing a potential means for preserving native methylation patterns of cloned DNA in this widely used and simplified system. The purification to apparent homogeneity of DNA maintenance methyltransferase overexpressed in *E. coli* will facilitate mutational analysis of this enzyme and may allow its large scale production for crystallography. Studies of the effects of the recombinant methyltransferase on the prokaryotic genome and cellular processes will be useful in further elucidating the biological significance of DNA methylation.

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[1] The abbreviations used are: AdoMet, *S*-adenosyl-L-methionine; DNA MTase, DNA (cytosine-5-)-methyltransferase; CpG, 5′-C-p-G-3′; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; IPTG, isopropyl-1-thio-β-D-galactopyranoside.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—For standard transformations *E. coli* Sure cells (*mcrA*$^-$, *mcrCB*$^-$, *mrr*$^-$, *hsd*$^-$, *recB*$^-$, *lacI*$^{q+}$; Stratagene) were used routinely. The prokaryotic expression vector pKK223-3 was obtained from Pharmacia Biotech Inc. The cDNA for murine DNA MTase (EMBL accession 14805 (corrected version)) was kindly provided by Timothy Bestor (Columbia University) as overlapping coding sequences (pMG and pR2K) cloned into pBluescript SK M13$^+$ (33). pMG includes all of the sequence from the *Eco*RI linker at the 5′ terminus of the cDNA clone to a *Bgl*II site near the 3′ end of coding (33). pR2K contains the sequence between the unique *Xho*I site at nucleotide 3138 and an *Eco*47III site just downstream of the AATAAA polyadenylation signal.

Plasmid Construction—The identity of pMG and pR2K was verified with endonuclease digestion. Each plasmid contained an internal *Xho*I site in the DNA MTase coding sequence as well as a 3′ *Xho*I site in the pBluescript sequence (33). Both plasmids were digested with *Xho*I and gel-purified, and the *Xho*I-*Xho*I sequence of pR2K was ligated into the digested and gel-purified pMG plasmid lacking this segment. This fused the coding sequences at the *Xho*I site (nucleotide 3138) without alteration of the original sequence as confirmed with extensive restriction digests. The newly formed plasmid containing the entire DNA MTase coding sequence in pBluescript was used as template for PCR amplification (20 cycles) of the coding sequence and 3′-untranslated region using Vent DNA polymerase (New England Biolabs) and Perfect Match DNA polymerase enhancer (Stratagene) under standard conditions (see "Polymerase Chain Reaction"). The proofreading Vent DNA polymerase was used to assure amplification fidelity of the sequence and the Perfect Match enhancer was used to facilitate PCR of the approximately 5-kilobase pair segment. PCR amplification was chosen as a cloning strategy since the ATG codon should be within 15 base pairs of the unique *Eco*RI site in pKK223-3 for effective subsequent ribosome binding. Since there were no unique sites in the ATG region to allow cloning within this restricted distance from the ribosome binding site, primers were synthesized that created a *Sma*I site just 5′ of the DNA MTase ATG start codon and a *Hin*dIII site just downstream of the 3′ terminus (sense primer, 5′-CCTTACCCGGGATGGCAGACTCAAATAGATC-3′; antisense primer, 5′-CGGTTAAAGCTTTTGTAAAACGACGGCCAGT-3′). The PCR product was digested with *Sma*I and *Hin*dIII, phenol extracted, gel purified, and ligated into pKK223-3 between its unique *Sma*I and *Hin*dIII sites just 3′ of the ribosome binding sequence. Restriction digests with *Sma*I and *Hin*dIII as well as several endonucleases at DNA MTase internal unique sites confirmed successful cloning of the cDNA into the expression vector within appropriate distance of the ribosome binding site (pTOT1, see Fig. 1). Primers unique to the DNA MTase coding sequence and internal to the original set of primers amplified the expected fragment from the pTOT1 construct but not from the control pKK223-3 vector (sense primer, 5′-ATGGCAGACT-CAAATAGATCCCC-3′; antisense primer, 5′-CTGGTGTGACGTCGAA-GACT-3′). The constructed pTOT1 expression plasmid contains a *tac* promoter (hybrid of the strong *trp* and *lac* promoters (41)), ribosome binding site, complete coding region of DNA MTase (4565 base pairs) and 3′-untranslated region, termination signal (*rrnB*), T7 promoter, M13 primer sequence, ampicillin resistance, and a pBR322 origin of replication (see Fig. 1).

Polymerase Chain Reaction—Amplifications were conducted in a Perkin Elmer Cetus GeneAmp PCR System 9600 thermocycler. Standard procedures were used, and cycling consisted of 1 min at 94 °C, 3 min at 55 °C, and 5 min at 72 °C. Aerosol-resistant pipette tips were used for assembling all PCR reactions.

DNA MTase Assays—Mammalian DNA MTase was routinely assayed in a 100-μl volume containing a standard assay mix (10% glycerol, 50 mM Tris acetate, pH 7.8, 10 mM EDTA, 2 mM dithiothreitol, 5 μg/μl RNase A, 0.7 μg/ml pepstatin, 0.5 mM Pefabloc SC, 0.5 μg/ml leupeptin, 2 μg/ml aprotinin (protease inhibitors were from Boehringer Mannheim)), 3 μCi of [methyl-^{3}H]AdoMet (60 Ci/mmol, ICN) at 1.5 μM final concentration, 5 μg of DNA unless otherwise specified, and enzyme sample (27, 29–30, 42). The enzyme does not require magnesium, and 10 mM EDTA is used to prevent any possible nuclease digestion of DNA substrates. Glycerol at 10% is used due to the inherent lability of the DNA MTase. Incubations were at 37 °C for 1 h unless otherwise indicated. After completion of the assay, reactions were terminated by the addition of SDS to 0.6% followed by a 30-min incubation at 60 °C with 400 μg/ml proteinase K (42). Two volumes of 0.5 N NaOH were added, and the samples were incubated at 60 °C for 10 min to hydrolyze any remaining traces of RNA (42). The samples were cooled on ice, and carrier salmon sperm DNA was added (20 μg/assay). DNA was precipitated in 10% trichloroacetic acid, 5 mM sodium pyrophosphate for 15 min at 4 °C and washed 5 times on a Whatman GF/C filter with 5% trichloroacetic acid, 5 mM sodium pyrophosphate, and twice with 100% ethanol (29–30). Washed filters were transferred to 5-ml Scintiverse BD (Fisher) in glass vials and counted on a scintillation counter. One unit of DNA MTase activity is defined as the amount of enzyme required to transfer 1 pmol of tritiated methyl groups to DNA in 1 h (29–30).

DNA Substrates—Polymer poly(dI·dC)·poly(dI·dC) (Pharmacia) was dissolved in 10 mM Tris, pH 7.5, and 100 mM NaCl, heated to 45 °C for 5 min, aliquoted at 1 μg/μl and stored at −20 °C. Oligodeoxynucleotides were synthesized on an Applied Biosystems 380A DNA synthesizer using standard procedures (25). Methylated 5-cytosine (Glen Research) was added as the phosphoramidite where indicated (see Table I). All synthesized oligonucleotides were gel-analyzed and used only if complete synthesis was evident. For annealing oligonucleotides, complementary strands were mixed (500 ng/μl each) and incubated for 10 min at 75 °C in 20 mM Tris-HCl, pH 7.5, and 50 mM NaCl, slowly cooled to room temperature, and analyzed for annealing efficiency on 3% agarose gels stained with ethidium bromide.

SDS-Polyacrylamide Gel Electrophoresis (PAGE)—Polyacrylamide gels (5%) were prepared and run at 50 mA. Electrophoresis was terminated when tracking dye reached the bottom of the gel. Where indicated, gels were stained for protein either with silver nitrate (43) or Coomassie Brilliant Blue (44) using standard procedures.

Immunoblotting—Transfer onto nitrocellulose (0.45 μm; Schleicher and Schuell) from SDS-PAGE gels was performed at 65 mA for 2 h at 4 °C on a TE Series transfer electrophoresis unit (Hoefer Scientific Instruments) in SDS transfer buffer (44) to facilitate transfer of high molecular weight protein. Staining for DNA MTase was performed with the ProtoBlot Western blot AP system as recommended (Promega) using 1:10,000 rabbit DNA MTase polyclonal antibody (anti-pATH52, Ref. 34) (kindly provided by Timothy Bestor, Columbia University), 1:7,500 antirabbit IgG AP conjugate, and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate.

Protein Analysis—Except for column fractions assayed for protein by absorbance at 280 nm, protein concentration was determined using the Bio-Rad Coomassie assay kit. Standard curves were established using γ-globulin.

Cell Cultures—Cultures of *E. coli* Sure cells (transformed either with pTOT1 or control vector pKK223-3) of 5 ml and 50 ml in 2YT medium supplemented with ampicillin (100 μg/ml) were successively grown to saturation from a single colony at 37 °C. The *tac* promoter is not fully suppressed by the *lac* suppressor in this system, and some expression of the DNA MTase occurs in the absence of isopropyl-1-thio-β-D-galactopyranoside (IPTG). Large scale cultures were inoculated with the saturated cell suspension (3.3 ml/liter) and grown at 37 °C until the absorbance at 600 nm was approximately 0.5. At this point, the cells were induced with 1 mM IPTG. After 3 h, the cells were harvested by centrifugation (4,000 × *g* for 15 min, 4 °C), washed once in phosphate-buffered saline, and recentrifuged. Cells were either immediately lysed or stored frozen at −70 °C as a cell pellet. We found no difference in DNA MTase activity between cells immediately lysed and those stored as cell pellets at −70 °C overnight.

Purification—Unless noted, all procedures were carried out at 4 °C. The washed cell pellet was resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 5% (v/v) glycerol, 2 mM EDTA, 1 mM 2-mercaptoethanol, 0.23 M NaCl, 0.1 mM dithiothreitol, 130 μg/ml lysozyme, 0.5 μg/ml leupeptin, 2 μg/ml aprotinin, 0.5 mM Pefabloc SC, and 0.7 μg/ml pepstatin) at 3 ml/g of cells (45). Cells were blended (Waring) at low speed for 3 min, and after 20 min sodium deoxycholate was added with stirring to 0.05%. The mixture was blended for 30 s at low speed and sonicated (5 pulses on ice for 15 s). For more complete DNA sheering, the mixture was blended for 30 s at high speed. The sample was diluted with lysis buffer (4 ml/g of cell pellet) lacking lysozyme, blended at high speed for 30 s, and centrifuged at 20,000 × *g* for 30 min (45). The supernatant (S1) containing soluble protein was removed, and the pellet was resuspended vigorously in 100 ml of high salt (0.4 M NaCl) lysis buffer lacking lysozyme. The resuspended high salt mixture was centrifuged as for the S1 solution. The supernatant (S2) containing protein insoluble in 0.23 M NaCl was stored frozen at −70 °C. Since several preliminary purifications indicated that over 80% of the DNA MTase was present in the soluble S1 fraction and combining S1 and S2 reduced the resolution and yield of DNA MTase from *E. coli*, all subsequent purifications were carried out using only the S1 lysate (see Fig. 5*C* for solubility of DNA MTase).

The S1 lysate was dialyzed 3 h with two changes of 6 liters of dialysis/column buffer (20 mM Tris-HCl, pH 7.8, 5 mM dithiothreitol, 10% glycerol, 5 mM EDTA, and protease inhibitors as for the lysis

buffer). The dialyzed S1 solution was diluted with an equal volume of column buffer and loaded onto a DEAE-Sephacel column (2.5 × 12 cm bed volume). The eluted column was washed with 2 bed volumes of column buffer to remove unbound protein and further eluted with a 200-ml 0–400 mM NaCl gradient. Fractions were collected and assayed for methylating activity as indicated above. Active pooled fractions were stored at −70 °C in 50% glycerol, 5 mM EDTA.

For ammonium sulfate precipitation, pooled active fractions from DEAE chromatography were diluted with 1 volume of column buffer and brought to 30% ammonium sulfate with gentle stirring over 10 min followed by continued stirring over 20 min on ice. The mixture was centrifuged for 20 min at 10,000 × *g*, and the supernatant was brought to 60% ammonium sulfate, stirred, and recentrifuged. The 60% ammonium sulfate pellet containing the DNA MTase (30) was resuspended in 2 ml of column buffer, loaded onto a Sephadex G-150 column (2 × 70 cm bed volume) and eluted. Active fractions were pooled and stored as above. DNA cellulose (4 mg of double-stranded DNA/g of solid, Sigma) chromatography was performed in a 1 × 3-cm bed volume and eluted with a 0–400 mM NaCl gradient. Active fractions were pooled and stored at −70 °C in 50% glycerol, 5 mM EDTA.

Gel Scanning of SDS-PAGE—To estimate percentage of total cellular protein represented by the recombinant DNA MTase, Coomassie-stained 5% SDS-PAGE gels were scanned on an Apple OneScanner, plotted, and integrated for density using the Image 1.49 program on a Macintosh IIfx computer. A total of four different protein concentrations of the S1 and S2 lysates (representing a 4-fold difference in total protein loaded) from two independent DNA MTase purifications were resolved on SDS-PAGE gels, scanned, and plotted in duplicate. The M_r 190,000 protein band (identified with molecular mass markers) was integrated for density in each lane and compared with the total integrated density of all proteins in the same lane to obtain percentage DNA MTase of total *E. coli* protein. For similar determinations in the mammalian system, a photomicrograph (kindly provided in reprint form by Steven Smith, City of Hope National Medical Center, Duarte, CA) of a SDS-PAGE gel resolving the crude lysate fraction of total human placental protein containing the identified DNA MTase (46) was also gel-scanned, plotted, and integrated for density as for the *E. coli* crude lysates.

RESULTS

We chose to express the maintenance DNA MTase in its native form to allow its use in future *in vivo* studies (*e.g.* preserving methylation patterns of cloned DNA) without potential interference with activity or DNA binding from a fusion product. Plasmid pTOT1 was constructed to express the native DNA MTase from the strong inducible *tac* promoter (Fig. 1). Immunoblotting kinetic studies for DNA MTase indicated full expression of this enzyme within 3 h of IPTG induction (data not shown). We cloned pTOT1 into *mcr*$^-$ (modified cytosine restriction) cells to prevent potential DNA degradation by the *mcr* system (47). The lysed *E. coli* cells containing pTOT1 (expression vector) revealed a unique protein (M_r = 190,000) on immunoblots probed with the DNA MTase polyclonal antibody (Fig. 2). This protein was not present in lysates of cells containing pKK223–3 (control vector lacking the DNA MTase insert). The calculated molecular mass of the DNA MTase is 172,238 based on its coding sequence. However, this enzyme has been shown previously to resolve at an apparent relative molecular mass of 190,000 on SDS-PAGE gels, which is thought to be due to posttranslational modifications of the enzyme and/or its molecular shape (33).

E. coli cells expressing mammalian DNA MTase do not appear to grow as well as cells containing the control vector and typically require 3.5 h to reach an A_{600} of 0.5 at 37 °C, whereas control cells reach this stage of growth within 3 h. The pTOT1 cells produce slightly smaller colonies on culture plates and less turbid overnight cultures compared with cells containing the control vector (data not shown). These differences in comparison to control cells became more pronounced as the cells were transferred to successive culture plates over a period of several months. To prevent progressive cellular proliferative retardation, we periodically transformed fresh *mcr*$^-$ cells with the pTOT1 expression vector. We have not yet fully quantified the degree of apparent cellular proliferative and growth impairment. It seems possible that its cause may be related to

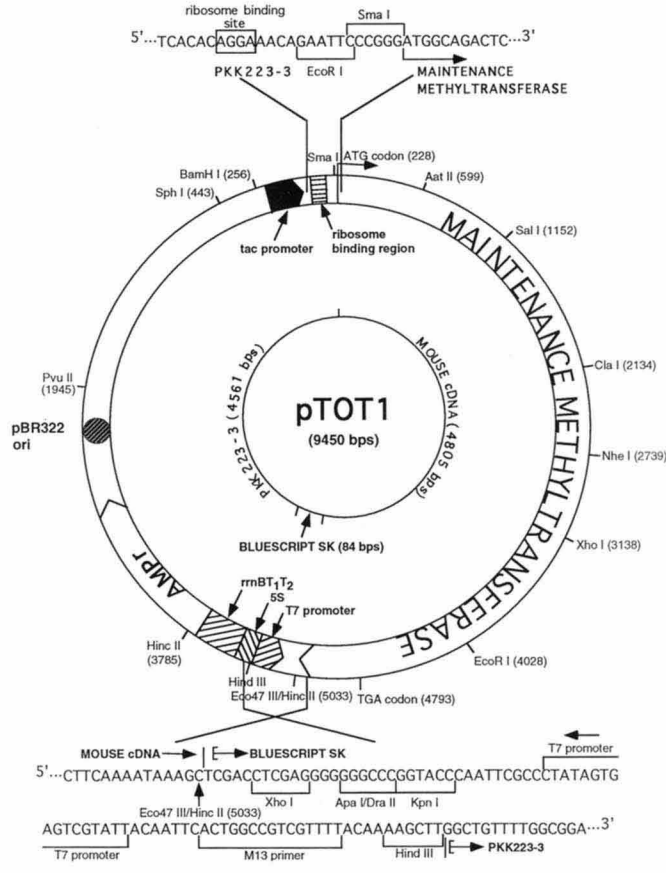

FIG. 1. **Schematic illustration of expression vector pTOT1 containing the cDNA for maintenance DNA MTase.** Overlapping coding sequences for the DNA MTase (pMG and pR2K, kindly provided by Timothy Bestor, Columbia University) were endonuclease digested, ligated, amplified, and cloned into the pKK223–3 prokaryotic expression vector downstream of the *tac* promoter and ribosome binding site (see "Experimental Procedures"). Depicted sequences are the 5′ and 3′ junctions of the DNA MTase insert within the cloning vector. The T7 promoter, M13 primer sequence, and termination region (ribosomal terminator) are also illustrated.

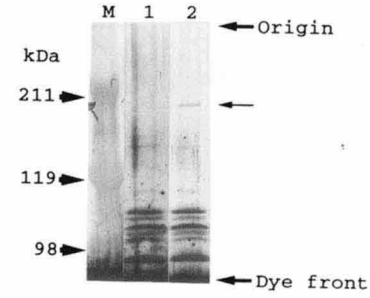

FIG. 2. **Expression of mammalian DNA MTase in *E. coli*.** Lysates from *E. coli* cells transformed with either pKK223–3 (control vector lacking the DNA MTase insert) or pTOT1 (expression vector containing the DNA MTase insert) were resolved by 5% SDS-PAGE, transferred to nitrocellulose, and probed with DNA MTase polyclonal antibody. *Lane M*, prestained high molecular weight protein marker; *lane 1*, control lysate from cells transformed with pKK223–3; *lane 2*, expression lysate from cells transformed with pTOT1. The *unlabeled arrow* indicates the novel protein (M_r = 190,000) not present in control cells. Lysate samples were prepared from 1 ml of a 5-ml saturated cell suspension (transformed either with pTOT1 or pKK223–3 and induced with IPTG (see "Experimental Procedures")), microcentrifuged 1 min at room temperature, resuspended in 100 μl of 1 × SDS gel loading buffer, heated to 100 °C for 3 min, and loaded (10 μl) onto SDS-PAGE (44).

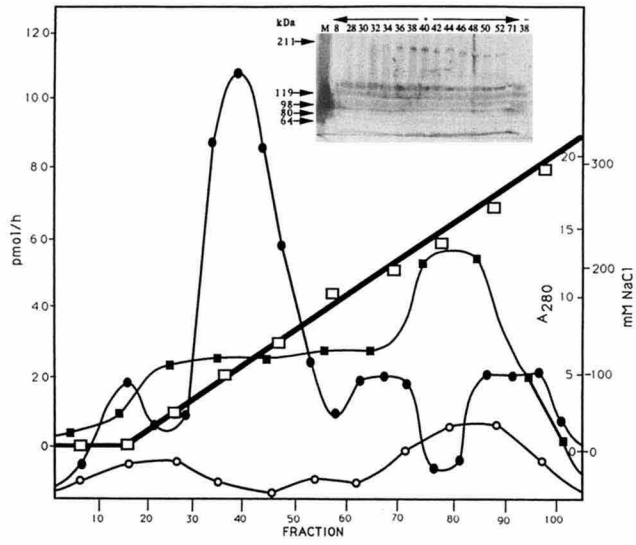

FIG. 3. **Correlation of DNA MTase activity with methyltransferase immunoblotting.** Individual 5-liter cultures of *mcr*$^-$ cells were transformed with pTOT1 (expression vector) or pKK223–3 (control vector) constructs and induced with IPTG. Expression and control cell extracts were chromatographed on two DEAE-Sephacel columns run simultaneously side-by-side. Eluted fractions from each column using 0–400 mM NaCl were assayed for DNA MTase activity with 5 μg of poly(dI·dC)·poly(dI·dC) as substrate (control assays lacked added DNA substrate). Methylating activity (*closed circles*, pTOT1-transformed cells; *open circles*, pKK223–3-transformed cells) is expressed as pmol/h of [^{3}H]CH_3 incorporated by fractions from each of the columns minus pmol/h of the assay control (lacking added DNA) for each fraction from the respective column. (The mean activity value for controls lacking DNA substrate in assays was 1.33 pmol/h (range = 0.44–2.83) for pTOT1 fractions and 0.70 pmol/h (range = 0.44–1.28) for pKK223–3 fractions. The mean activity value for the pKK223–3 (vector only control) fractions containing added DNA substrate in assays was 0.56 pmol/h (range = 0.22–1.33).) The salt gradient (mM NaCl, *open squares*) and protein concentration (A_{280}, *closed squares*) are also shown (same for both columns). The *inset* depicts immunoblotting, using the polyclonal DNA MTase antibody (34), of DEAE-Sephacel eluted fractions (numbered at *top*) from the pTOT1-transformed cells (+) or the pKK223–3-transformed cells (−).

effects of DNA MTase expression on the *E. coli* genome, although the large size of the novel protein product itself may also be a factor. Evidence for methylation of high molecular weight *E. coli* genomic DNA *in vitro* can be seen (see Fig. 6*B*), suggesting that a similar process may occur *in vivo* affecting the growth of these cells.

To assess enzymatic catalysis by the cloned DNA MTase, the DNA methylating activity (as measured by transfer of tritiated methyl groups from AdoMet to DNA) of DEAE-purified fractions was compared for cells transformed with pTOT1 and pKK223–3 (Fig. 3). The DEAE columns were simultaneously chromatographed and eluted with a salt gradient. Peak methylating activity for the fractions from the pTOT1-transformed cell lysates eluted in the range of 100–150 mM NaCl, consistent with results of the DNA MTase purified from mammalian cells (29–30, 48). No obvious methylating peak was seen for the DEAE-chromatographed lysates of pKK223–3-transformed control cells. Immunoblots performed on the pTOT1 DEAE fractions indicated a M_r 190,000 protein correlating with peak methylating activity (fractions 32–52; pTOT1), which was not apparent below 100 mM salt (fractions 8–30; pTOT1) or above 150 mM salt (fraction 71; pTOT1). Fig. 3 also shows that the most intense M_r 190,000 bands (fractions 38–42; pTOT1) correlated with fractions having the highest methylating activity. The control DEAE column showed no evidence of the M_r 190,000 protein as indicated by the absence of this band at peak methylating activity for the pTOT1 column (fraction 38; pKK223–3).

The cloned DNA MTase was purified to apparent homogeneity by assaying for methylating activity in a three-column system based on protein charge (DEAE), size and shape (Sephadex), and DNA-affinity (DNA cellulose) (Fig. 4). Due to the presence of various inhibitory substances in crude fractions and lability of the enzyme (49), meaningful estimates of total purification factor could not be obtained consistent with reports by others (29–30, 50). Gel filtration yielded a single peak of methylating activity in the M_r 180,000–205,000 range, consistent with polyacrylamide gel estimates. While size separation is efficient in this expression system due to the relatively large size of the mammalian DNA MTase compared with most *E. coli* proteins (Fig. 5*C*), some protein impurities remain in the Sephadex fraction, and a final purification based on the affinity of this enzyme for DNA is quite effective in producing a homogeneous purification as assessed by silver staining (Fig. 4*D*). Although improvements of the purification procedure are expected to increase the yield of recombinant DNA MTase, we recovered almost a full milligram (887 μg) of apparently pure enzyme from about 10 liters of *E. coli* cells. The apparently homogeneous protein exhibiting peak methylating activity following DNA cellulose chromatography reacted with the DNA MTase antibody on immunoblots (Fig. 4*E*).

Partially purified recombinant DNA MTase was used for comparison of substrate preference with increasing DNA MTase purity, assessment of relative effectiveness of purification steps, estimates of solubility and degree of expression of the cloned DNA MTase in *E. coli*, and DNA substrate analysis studies (Fig. 5). A hemimethylated oligodeoxynucleotide was synthesized containing methyl moieties at approximately 15-base pair intervals for use as substrate in DNA MTase assays (see "Experimental Procedures" for chemical synthesis and Table I for structure of hemimethylated oligodeoxynucleotide). Preferential transfer of radioactive methyl moieties to the oligodeoxynucleotide substrate containing hemimethylated CpG sites over the control lacking substrate was apparent after DEAE purification (Fig. 5*A*), and this ratio improved with gel filtration (Fig. 5*B*). Ethidium bromide staining of agarose gels indicated minor amounts of large molecular weight *E. coli* genomic DNA present after DEAE purification (data not shown), accounting for the slight activity of control assays lacking oligonucleotide substrate (Fig. 5*A*). The chemically synthesized hemimethylated oligodeoxynucleotide underwent greater methylating activity in DEAE and gel filtration fractions than the highly methylatable *de novo* substrate, poly(dI·dC)·poly(dI·dC), indicating preferential hemimethylation by the recombinant DNA MTase.

The pooled active fractions as well as the crude lysates were assessed on polyacrylamide gels for protein content and purity (Fig. 5*C*). The soluble (S1) and insoluble (S2) SDS-PAGE crude lysate fractions were estimated for percent DNA MTase by scanning stained gels (see "Experimental Procedures"). The DNA MTase comprised approximately 2% (range of 1.0–3.0%) of total *E. coli* protein in the S1 fraction and about 0.3% (range of 0–0.53%) for the insoluble S2 fraction, indicating that approximately 85% of the enzyme is expressed in soluble form (see Fig. 5*C* for comparison of S1 and S2 fractions). The overall expression of DNA MTase in these cells is about 2.5% of total *E. coli* protein. By contrast, mammalian cells contain a mean of 0.05% DNA MTase of total human placental protein (see "Experimental Procedures" under "Gel Scanning of SDS-PAGE" and Ref. 46).

To demonstrate that the recombinant DNA MTase is indeed active with a preference for hemimethylated DNA, we reacted the partially purified enzyme with oligodeoxynucleotides in the

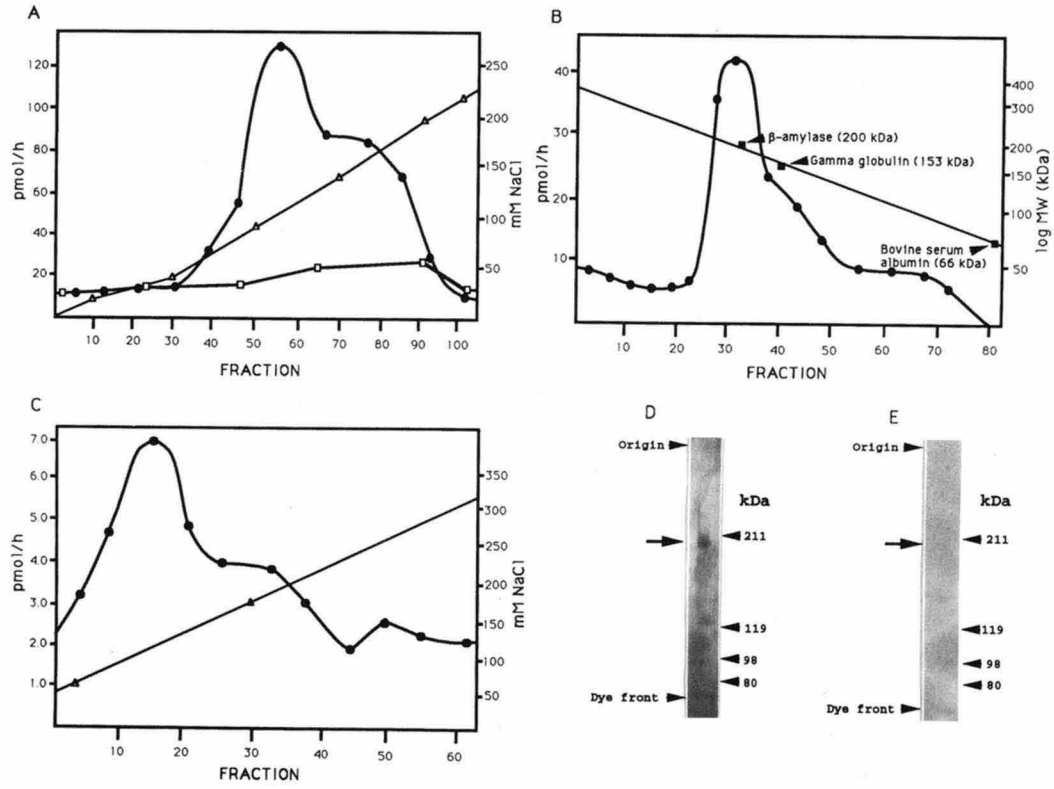

FIG. 4. **Purification of DNA MTase to apparent homogeneity.** Expression of the DNA MTase construct (pTOT1) was induced in 12 liters of *E. coli* culture, pelleted, and lysed, and the DNA MTase was purified on the basis of assaying for methylating activity. *A*, DEAE-Sephacel fractionation (*closed circles*, assayed with nonmethylated duplex 60 mer (see Table I for DNA structure) as substrate; *open circles*, control assay lacking DNA template; *open triangles*, 0–400 mM salt gradient). Active fractions were pooled and ammonium sulfate-precipitated for gel filtration (see "Experimental Procedures"). *B*, Sephadex G-150 chromatography depicting methylating activity (*closed circles*) and calibration with gel filtration protein standards (*closed squares*). *C*, DNA cellulose chromatography of active fractions pooled from gel filtration (*closed circles*, methylating activity of nonmethylated duplex 60-mer; *open triangles*, 0–400 mM salt gradient). Assays were performed as described under "Experimental Procedures" with 5 μg of DNA where indicated. *D*, silver stain of pooled active fractions from DNA cellulose (5 μg of protein loaded). *Arrow* indicates purified M_r 190,000 protein. *E*, immunoblot of pooled DNA cellulose active fractions (5 μg of protein loaded). *Arrow* indicates purified protein reacting to polyclonal DNA MTase antibodies.

TABLE I
Cytosine methylation of oligodeoxynucleotides

DNA structure[a]	Type	Mean[b]
		pmol/h
Complete 60-mer sequence 5′-GTGAATTCACATAGTAC**CG**GATGT**CG**ACTAAT**CG**ATATTG**CG**CATCT**CG**AGTGAATTCTG-3′ 3′-CACTTAAGTGTATCATG**GC**CTACA**GC**TGATTA**GC**TATAAC**GC**GTAGA**GC**TCACTTAAGAC-5′		
Oligonucleotide		
5′ . . . **CG**GATGT**CG**ACTAAT**CG**ATATTG**CG**CATCT**CG** . . . 3′ 3′ . . . **GC**CTACA**GC**TGATTA**GC**TATAAC**GC**GTAGA**GC** . . . 5′	Trihemimethylated duplex	213 ± 29
5′ . . . **CG**GATGT**CG**ACTAAT**CG**ATATTG**CG**CATCT**CG** . . . 3′ 3′ . . . **GC**CTACA**GC**TGATTA**GC**TATAAC**GC**GTAGA**GC** . . . 5′	Trimethylated duplex	92 ± 19
5′ . . . **CG**GATGT**CG**ACTAAT**CG**ATATTG**CG**CATCT**CG** . . . 3′ 3′ . . . **GC**CTACA**GC**TGATTA**GC**TATAAC**GC**GTAGA**GC** . . . 5′	Nonmethylated duplex	73 ± 40
5′ . . . **CG**GATGT**CG**ACTAAT**CG**ATATTG**CG**CATCT**CG** . . . 3′ 3′ . . . **GC**CTACA**GC**TGATTA**GC**TATAAC**GC**GTAGA**GC** . . . 5′	Pentamethylated duplex	16 ± 19

[a] All oligodeoxynucleotides are 60 base pairs in length (full sequence shown at top of table) except where indicated. Asterisks indicate position of methylated cytosines placement during chemical synthesis of oligodeoxynucleotide substrates.

[b] Assays were conducted with excess substrate (5 μg of DNA) for 3 h. Other assay conditions were as indicated under "Experimental Procedures." Each value is the mean ± S.E. of three independent determinations. Control values (*i.e.* samples otherwise identical to and assayed side-by-side with substrate-containing samples but lacking added DNA substrate; mean = 144.1 ± 6 pmol/h) were subtracted from each sample value in each individual experiment before determination of the indicated means and S.E. All assays utilized 40 μg of partially purified DNA MTase (SD fraction, Fig. 5*C*).

presence of radioactive AdoMet, resolved the samples on agarose gels, and subjected the gels to autoradiography (Fig. 6). The gel-isolated hemimethylated oligonucleotide produced the most intense band on autoradiography, demonstrating preferential transfer of methyl moieties to hemimethylated CpGs. Some radioactivity was apparent in the otherwise identical

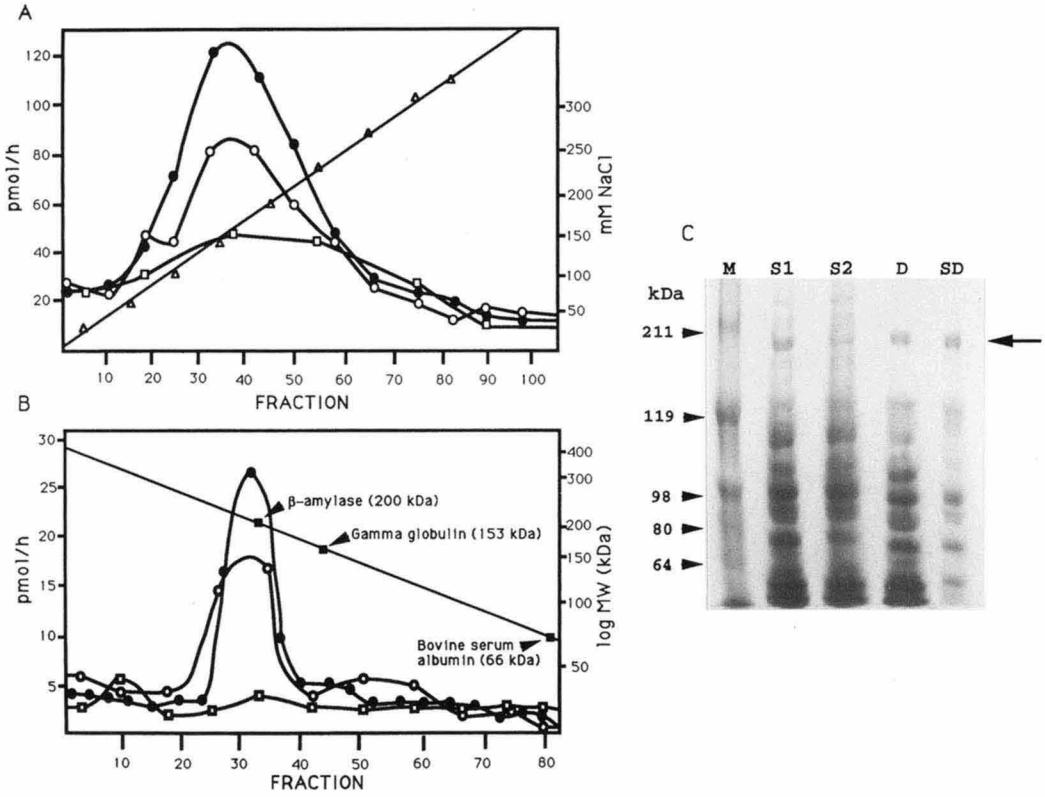

FIG. 5. **Partial purification of DNA MTase using hemimethylated oligodeoxynucleotide substrate.** Approximately 10 liters of *E. coli* cells transformed with pTOT1 were induced and lysed as indicated under "Experimental Procedures." DNA MTase was partially purified by pooling chromatography fractions on the basis of methylating activity (*i.e.* transfer of radioactive methyl moieties to trihemimethylated oligodeoxynucleotide substrate (see Table I for structure)). *A*, DEAE-Sephacel (*closed circles*, 2 μg of trihemimethylated oligonucleotide as substrate; *open circles*, 2 μg of poly(dI·dC)·poly(dI·dC) as substrate; *open squares*, no added DNA substrate in the methylation assay; *open triangles*, 0–400 mM salt gradient). *B*, Sephadex G-150 chromatography of ammonium sulfate-precipitated pooled active fractions from the DEAE column (symbols as for DEAE chromatography (*closed squares*, column calibration using indicated gel filtration protein standards)). *C*, Coomassie stain of 5% SDS-PAGE gel. *Lane M*, molecular weight marker; *lane S1*, soluble crude lysate; *lane S2*, insoluble crude lysate; *lane D*, pooled active fractions from DEAE chromatography; *lane SD*, pooled active fractions from Sephadex G-150 column (approximately 500 μg of protein loaded in each lane). *Arrow* indicates the M_r 190,000 protein (DNA MTase).

nonmethylated oligodeoxynucleotide (*i.e. de novo* methylation), and this activity was greater than that for the identical fully methylated oligonucleotide containing no methylatable CpGs. Thus it is apparent that the recombinant DNA MTase transfers methyl moieties directly to these oligodeoxynucleotides with a preference for hemimethylated CpG sites and with a much lower propensity for nonmethylated CpG sites. Very little methylation appears to occur at sites other than CpG (Fig. 6*B*, *lane 4*).

To further characterize the enzymatic activity of the DNA MTase purified from *E. coli*, we quantitated in assays the methyl receptivity of otherwise identical oligonucleotides differing only in placement of methyl moieties (Table I). These analyses utilized the more purified gel filtration fraction (Fig. 5*C*) containing no evidence of contaminating *E. coli* DNA. Table I shows that the hemimethylated oligodeoxynucleotide substrate received the most radioactive methyl transfer catalyzed by the recombinant DNA MTase consistent with the DNA MTase partially purified from mammalian cells (51). Also similar to the mammalian cell enzyme, nonmethylated oligonucleotides can undergo *de novo* methylation, and sequences containing no methylatable CpGs (*i.e.* premethylated at all CpG sites) are poor templates for the DNA MTase (Table I), demonstrating its strong preference for cytosine methylation specifically in CpG dinucleotides (29–30, 51–52). A duplex trimethylated oligodeoxynucleotide containing only two *de novo* methylatable CpGs on each stand (Table I) is more receptive to *de novo* methylation (22.9 pmol/h/CG) than an otherwise identical nonmethylated oligonucleotide containing five *de novo* methylatable CpGs on each stand (7.3 pmol/h/CG), indicating enhanced *de novo* methylation of a premethylated oligodeoxynucleotide containing methylatable CpGs.

DISCUSSION

The widely-used techniques of DNA cloning and PCR amplification strip mammalian genomic DNA of its original cytosine methylation. DNA that lacks its native cytosine methylation pattern may give different results in mobility shift analysis, endonuclease digestions, and other procedures analyzing its properties and behavior. We developed the idea that the methylation pattern of cloned DNA could be preserved in host bacteria expressing the maintenance DNA MTase. However, the cDNA for this enzyme has previously been expressed only in mammalian cells (COS-1) (40). Whereas this may be of use in studying the effects of variations in DNA MTase levels in mammalian cells, we chose to clone and express DNA MTase in *E. coli*. We developed this system not only for its possible use in maintaining methylation patterns of cloned DNA in bacteria but also because of the widespread use of *E. coli* as a protein expression system, the simplification of cell culture and purification processes, the potential of large scale production of the enzyme for crystallography, and the facilitation of mutagenesis studies of this enzyme.

The known potential for *de novo* methylation and methylation spreading by the DNA MTase (29–30, 35) could be a factor in preserving methylation patterns of genomic DNA in this system; however, both of these processes occur in proportion to greater DNA MTase levels (48, 51) and number of cell genera-

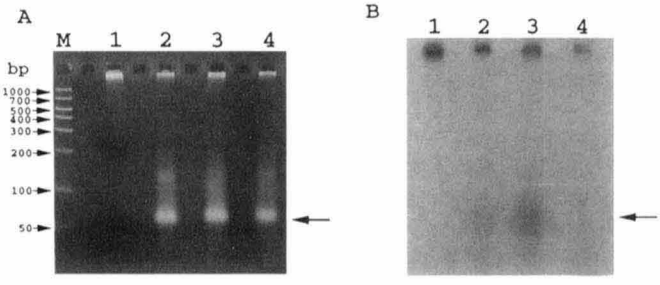

FIG. 6. **Methyl transfer by the recombinant DNA MTase.** *A*, ethidium bromide-stained agarose gel. A 100-μl assay mix containing 1.66 μM [methyl-^{3}H]AdoMet (85 Ci/mmol) and 200 μg of partially purified recombinant DNA MTase (fraction D, Fig. 5*C*) was incubated for 3 h at 37 °C with each separate reaction either containing 5 μg of DNA or lacking added DNA (control). Phenol-extracted reaction mixes were resolved on a 4.5% agarose gel and stained with ethidium bromide. *Lane M*, molecular weight marker; *lane 1*, control lacking DNA; *lane 2*, nonmethylated duplex; *lane 3*, trihemimethylated duplex; *lane 4*, pentamethylated (*i.e.* fully methylated at CpG sites) duplex (see Table I for DNA structures). Each lane consisted of 1 μl of a 10-μl solution (500 ng of DNA where indicated). *B*, autoradiogram of ^{3}H-labeled methyl transfer to DNA. A different freshly prepared 4.5% agarose gel was loaded with 7 μl of the same phenol-extracted samples depicted in *A* above (3.5 μg of DNA where indicated) and impregnated with En^{3}Hance for 3 h, soaked in 5% acetic acid for 1 h, and dried on filter paper. The film for autoradiography was preflashed and exposed to the gel for 14 days at −70 °C before developing. *Lane 1*, control lacking added DNA; *lane 2*, nonmethylated duplex as substrate; *lane 3*, trihemimethylated duplex; *lane 4*, pentamethylated (fully methylated) duplex (see Table I for DNA structures). Minor diffusion of oligodeoxynucleotide bands due to processing in En^{3}Hance and acetic acid is apparent. High molecular weight DNA in wells is considered to be *in vitro* methylated *E. coli* genomic DNA (see also *gel A*). Each oligodeoxynucleotide is identical except for its methylated state.

tions (31, 35). Modulating the DNA MTase expression by limiting IPTG induction and minimizing cell culturing times may be useful approaches for reducing the possibility of *de novo* methylation. Analysis of the cloned product with methylation-sensitive isoschizomers (44) or methylation sequencing (53–54) would be prudent to assess the possibility of ectopic methylation.

Previously it was thought that the mammalian DNA MTase might be toxic to *E. coli* since *de novo* methylation of the *E. coli* genome may activate the *mcr* system leading to DNA degradation (47), even though the mammalian DNA MTase is primarily a maintenance methyltransferase and appears to *de novo* methylate only as a secondary function (29–30). In order to circumvent this potential problem, we cloned the reconstructed murine MTase cDNA in *mcr*$^-$ cells. The *mcr*$^-$ cells expressing DNA MTase are slightly less proliferative than control *mcr*$^-$ cells (*i.e.* containing the cloning vector alone), perhaps relating to *de novo* methylation of the *E. coli* genome. Transformation of the vector into fresh *mcr*$^-$ cells appears to improve cellular proliferation to near control levels. In spite of this minor growth impairment, these cells are able to overexpress the DNA MTase to relatively high levels compared with the levels of this enzyme in mammalian cells.

Studies of the effects of expression of the cloned mammalian DNA MTase on the *E. coli* genome, on the control of cellular processes in *E. coli*, and on replication rates as well as cell viability may contribute to understanding the control mechanisms of this enzyme and its biological significance. A number of prokaryotic cellular control processes could be affected by expression of this recombinant enzyme in these cells such as the transcription of key regulatory genes, DNA repair, replication, and recombination. We have previously reported several theoretical molecular mechanisms of cellular senescence (22), a hallmark of which is reduced replicative capacity, and have suggested that *de novo* methylation by the DNA MTase may contribute to this phenomenon in aging eukaryotic cells (22). Prokaryotic cells do not senesce (22), and studies are in progress analyzing the *E. coli* cells now expressing this protein for evidence suggestive of senescing cells (*e.g.* morphological changes, slowing of cell replication) and the DNA MTase as one of the putative "mortality gene" products.

The maintenance DNA MTase, purified from mammalian cells, is highly susceptible to proteolytic degradation (34) and loss of enzyme activity due to its lability (29–30). Moreover, the DNA MTase is present in very limited quantities in mammalian cells (46). The expression of the DNA MTase in *E. coli* and purification of this enzyme to apparent homogeneity may help overcome some of these problems. It is generally known that the use of *E. coli* allows rapid, easy growth of large numbers of cells with less endogenous protein heterogeneity and bypassing of nuclear isolation protocols. In the case of this specific enzyme, its purification from *E. coli* may also be facilitated by its relatively large size compared with most *E. coli* proteins, allowing more effective size separation and reducing the risk of proteolytic degradation and loss of enzyme activity. Whatever the choice of purification protocol, the expression of mammalian DNA MTase in *E. coli* should allow greater availability of purified enzyme. All of the properties of the purified recombinant DNA MTase examined in this study including relative molecular mass, elution in salt gradients, affinity for DNA, immunoreactivity, and substrate preference are consistent with the known properties of the enzyme purified from mammalian cells (29–30, 33–34, 49, 51–52).

Although it is generally thought that the eukaryotic DNA MTase is capable of maintenance and *de novo* methylation without assistance from associated mammalian proteins or factors, this important question is still not fully resolved (55). Purification of the mammalian enzyme has helped address this issue, but minor contaminants that assist the DNA MTase could still be present in apparently pure fractions. Our studies indicate that the enzyme is indeed capable of both types of DNA methylation. The expressed product in *E. coli* was originally derived from a single mammalian gene (33), and when this cDNA is expressed in *E. coli* and purified, it can perform both maintenance and *de novo* methylation of DNA. Whatever other proteins may be involved in the eukaryotic methylation process, it is clear that the essential features of maintenance and *de novo* methylation are not dependent upon associated proteins unique to the mammalian replication apparatus.

Similar to the mammalian DNA MTase isolated from mammalian cells (56–58), the recombinant enzyme purified from *E. coli* has a preference for hemimethylated CpG dinucleotides, has a tendency to *de novo* methylate DNA, and transfers methyl moieties at very low levels in substrates not containing methylatable CpG dinucleotides. Although some cytosine methylation can occur in other dinucleotides in the mammalian genome containing cytosine in the 5′ position (31, 59–60), and such activity has occasionally been reported to be at relatively high levels (59), our studies with the recombinant enzyme indicate that this occurs only very rarely in oligodeoxynucleotides containing these dinucleotides.

The mechanisms for the propensity of the enzyme to methylate in regions already containing methyl moieties (*i.e.* genomic methylation spreading) are not fully understood (35). These studies indicate enhanced *de novo* methylation of oligodeoxynucleotides containing preexisting methyl moieties, which suggests *in vitro* methylation spreading. A more detailed study of methylation spreading *in vitro* will be reported elsewhere.

Currently, work is aimed toward preserving methylation patterns of cloned DNA using our expression system. Other

intended studies are the effect of expression of the mammalian MTase on control of biological processes in prokaryotic cells, further delineation of the functional domains of the maintenance methyltransferase in mutagenesis studies, and large scale production of this enzyme for crystallography. Finally, studies are in progress focusing on a more extensive analysis of the molecular mechanisms of methylation spreading using the defined *in vitro* oligodeoxynucleotide system reported in this initial study.

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