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Human DNA-Demethylating Activity: A Glycosylase Associated With RNA and PCNA

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Abstract We have partially purified and characterized the 5-methylcytosine removing activity (5-meC-DNA Glycosylase) from HeLa cells with 700-fold enrichment. This activity cleaves DNA specifically at fully methylated CpG sites. The mechanism of 5-meC removal is base excision from fully methylated CpG loci on DNA, producing abasic sites. Hemi-methylated DNA is not a substrate. A prominent 52 KDa protein is present in all partially purified fractions. This activity is tightly associated with other nuclear factors and proteins, which resulted in differential fractionation of this activity on ion exchange columns. One nuclear factor associated with this activity is identified as RNA. Another nuclear protein, proliferating cell nuclear antigen (PCNA) is also associated with this enzyme. Glycosylic removal of 5-meC from DNA by this activity could be involved in the regulation of transcription, replication, differentiation, and development through resultant hypomethylation of DNA. J. Cell. Biochem. 79:249–260, 2000.

Key words: 5-meC-DNA glycosylase; demethylation; PCNA; RNA; base excision repair

The molecular mechanisms that modulate DNA 5-methylcytosine (5-meC) content are incompletely understood, but may act at nodal points of regulation of genetic information. Methylation of DNA cytosines is found almost ubiquitously in nature. These affect DNA-protein interactions, and so may be of consequence in many biological processes including transcription, differentiation, development, and recombination [Adams, 1990]. It also appears that DNA methylation plays a variety of roles at different stages of human carcinogenesis [Laird et al., 1995, 1996; Gonzalgo and Jones, 1995; Schmutte and Jones, 1998]. In addition, DNA 5-meCs are major sites of mutations, especially transitions to thymines [Jones et al., 1992]. Studies of point mutations in p53 and other genes have suggested DNA 5-meC to play a substantial role as an "endogenous mutagen"; substitution of DNA 5-meC by thymine is responsible for 30–40% of all human germ line point mutations [Rideout et al., 1990].

Methylation of cytosine and hypermethylation of promoter regions on DNA has been correlated with gene silencing mechanisms [Nyce, 1991; Volpe et al., 1993]. The process of DNA cytosine methylation on DNA is purely post-replicative and catalyzed by DNA-(cytosine-5)-methyltransferase (MCMT); the mechanism has been recognized as consisting of both maintenance methylation and de novo methylation [Bestor et al., 1988]. An association between reduced DNA 5-meC content, (i.e., hypomethylation) at specific gene regions and transcriptional activity has been demonstrated [Razin and Cedar, 1991]. Cell cycle regulatory genes including Rb, p15, p16, WT1, and cyclin D1, are but a few of the mammalian genes apparently under the control of such hypomethylational regulation [Schmutte and Jones, 1998; Fournel et al., 1999; Kitazawa et al., 1999]. Such hypomethylation of DNA occurs during the processes of eukaryotic cellular differentiation and embryonic development

Abbreviations used: MCMT, DNA-(cytosine-5)-methyltransferase; PCNA, proliferating cell nuclear antigen; 5-meC, 5-methylcytosine; HPLC, high performance liquid chromatography; 5-medC, 5-methyl-2′-deoxy cytidine.

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[Kuykendall and Cox, 1989, Mazin, 1993]. There is also substantial evidence that the reduction of DNA 5-meC content, commonly termed "demethylation", is an active process, with replacement of 5-meC by cytosines [Razin et al., 1986]. 5-meC in DNA appears to be a key recognition signal for regulation of many genes, mediated through various DNA binding proteins, factors, and enzymes. However, the processes governing homeostasis of DNA 5-meC levels, especially the mechanism of active reduction of 5-meC content, have not been definitively elucidated.

Various enzymatic pathways of DNA "demethylation" have been described. One is removal of 5-meC as a free base from DNA by the enzyme 5-meC-DNA glycosylase, found in human HeLa cells, chick embryos, and differentiating mouse myoblasts [Vairapandi and Duker, 1993, Jost et al., 1994, 1995, 1999]. Another involves the excision of nucleotides containing 5-meC from DNA by a cellular fraction containing RNA. While, it was originally reported that this demethylation involved RNA, more recent results indicate that partially purified preparations are insensitive to RNAse treatment [Swisher et al., 1998]. Either mode of removal of 5-meC from DNA would, of necessity, be followed by synthesis of a DNA repair patch containing unmethylated cytosines. A link between these mechanisms, with removal of 5-meC by a DNA glycosylase requiring an RNA cofactor that presumably guides the enzyme to the specific substrate locus, has been demonstrated in the chick embryo system [Frémont et al., 1997; Jost et al., 1997]. A third mechanism involves a demethylase activity that directly transforms methylated cytosines to cytosines has been identified and it's encoding gene has been cloned. Although this activity has not yet been fully characterized, preliminary data indicate that 5-meC may react with water to produce cytosine and methanol [Bhattacharya et al., 1999; Cervoni et al., 1999; Ramchandani et al., 1999]. This may indicate that the cytosine product is generated by direct removal of the methyl group [Cedar and Verdine, 1999]. Very recently, the presence of demethylase activity on this cloned protein (pHis-d-Mtase), has been disputed [Ng et al., 1999; Wade et al., 1999].

The mechanisms of human DNA demethylation remain unclear. Therefore, we investigated the possibility of the involvement of other factors in DNA "demethylation." We partially purified this human DNA-demethylating activity from HeLa cells and demonstrated the action mechanism as base excision by glycosylic removal of 5-meC from DNA while producing base loss (abasic) sites. This activity is very specific for 5-meC sites on fully methylated CpG sites on DNA. While we observed differential fractionation of the demethylating activity, all active fractions have a core 52-kDa protein. RNA molecules and proliferating cell nuclear antigen (PCNA) were found to copurify with the glycosylase and were identified in association with the core protein. Kinetic analyses show, that except for one fraction, all the partially purified activities have similar $S_{0.5}$ values of about 200 pMoles. These factors can be of consequence in the regulation of DNA "demethylation" at specific sites, which in turn may modulate the cell cycle events mediated through DNA methylation.

MATERIALS AND METHODS

Partial Purification of 5-meC-DNA Glycosylase

5-meC-DNA glycosylase activities were assayed by measuring the release of radiolabeled 5-meC from poly(dG-5-medC):poly(dG-5-medC) as previously described [Vairapandi and Duker, 1993, 1994]. The purification methodology closely followed published procedures [Vairapandi and Duker, 1996]. Nucleic acids were removed from HeLaScribe nuclear extract (E3091, Promega, Madison, WI) by PEI treatment. The supernatant was dialyzed against buffer A (40 mM Tris-HCl pH 7.4, 0.2 mM EDTA, 2 mM dithiothreitol [DTT], 5% glycerol) was loaded onto a DEAE-cellulose column (DE-52, Whatman, Clifton, NJ; prepared according to the manufacturer's instructions; 1.5 cm I.D). Bound proteins were eluted with 0–1 M linear KCl gradient in buffer A. Protein content of the fractions was monitored by 280-nm absorbance and salt concentration with a conductivity meter. Fifty μl dialyzed aliquots were used in enzyme assays. DEAE-fractionation resulted in different activity pools. One bound to the DEAE column and eluted at 250–500 mM KCl (DEAE-bound). Purification of the DEAE-bound activity has been described earlier [Vairapandi and Duker, 1996]. Another activity did not bind to the column and eluted in the late wash fractions (DEAE-wash).

Active fractions in the DEAE-wash were pooled and loaded onto a phosphocellulose column (Whatman PC-11 prepared according to the manufacturer's suggestion; 1.5 cm I.D). Bound proteins were eluted with 0–1 M linear KCl gradient. Phosphocellulose column-bound activity eluted at 700 mM KCl, was pooled and passed through heparin-agarose (Promega, A2501) column (0.8 cm I.D); equilibrated and washed with buffer A. A linear 0–1 M KCl gradient, eluted bound proteins. Heparin agarose fractionation of the DEAE washed-out activity had two activity peaks. These were pooled separately as WM and WH, concentrated by dialysis against 50% glycerol in buffer A, and stored at −70°C.

Protein estimation for the concentrated fractions was done by the micro Bradford assay method with the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA). SDS-PAGE analysis and silver staining was performed as described earlier [Laemmli, 1970; Merril et al., 1981].

Syntheses of Other Radiolabeled Substrates

Poly(dG-dC):poly(dG-dC) was labeled by nick translation with 5′,5-^{3}H-dCTP (NET 369, New England Nuclear/ Dupont, Boston, MA) and DNA breaks were closed by ligation by T4 ligase [Vairapandi and Duker, 1996]. The specific activity was 75,000 cpm/μg DNA. ^{14}C-labeled-5-meC was prepared by condensation of ^{14}C-labeled urea (U 8629; Sigma, St. Louis, MO) and 3-bromo-2-methyl-acrylonitrile according to Celewicz and Shetlar [1992]. Purification of ^{14}C-5-meC was by repeated isocratic high performance liquid chromatography (HPLC) and the product identity confirmed by NMR analysis.

Analysis for DNA—Abasic Site Formation by 5meC—DNA Glycosylase Activity

The formation of DNA abasic sites following enzymatic 5-meC excision was directly demonstrated by reductive labeling of these sites by tritiated sodium borohydride. Poly(dG-5-medC):poly(dG-5-medC) DNA substrate was prepared as described before [Vairapandi and Duker, 1993]. Three-hundred ng of substrate was incubated with increasing concentrations of purified enzyme fractions. After an 18 h incubation at 37°C, the DNA product was phenol: chloroform-extracted and ethanol-precipitated. The DNA was air dried, taken up in 50 μl 0.4 M potassium phosphate, pH 6.5, and dissolved after agitation on a vortex mixer. Sodium borohydride (New England Nuclear NET 023×; 50–75 Ci/mMole) was thawed and 1.5 ml of ice cold 0.4 M potassium phosphate, pH 6.5, was added. Fifty μl of the labeled $NaBH_4$ solution was immediately added to each reaction mix and the reaction proceeded at room temperature for 1 h. The unreacted $NaBH_4$ was removed and the DNA desalted by two passages through centricon-10 filters (Amicon Corp., Danvers, MA). DNA retained above the filter was collected and the radioactivity counted in Scintiverse. A parallel set of experiments was conducted with ^{3}H-radiolabeled poly(dG-5-medC):poly(dG-5-medC) to follow the release of radiolabeled 5-meC by the purified enzyme fractions.

Analysis of RNA Association in Enzyme Fractions

RNA extraction from enzyme fractions and radiolabeling were performed according to Fremont et al. [1997]. Purified enzyme fractions (100 μl), were dialyzed against 0.15 M NaCl, 20 mM HEPES pH 7.5, 5 mM EDTA: SDS was added to a final concentration of 0.5%. Proteins were digested for 1 h by 200 mg/ml proteinase K, samples extracted four times with phenol: chloroform, and the RNA ethanol-precipitated. After sedimentation, the RNA was dissolved in 0.2 M NaCl and twice reprecipitated by ethanol. It was dissolved in DNase- and RNase-free water and labeled in a volume of 10 μl containing 30 μCi [γ-^{32}P] ATP, 5 U polynucleotide kinase (GIBCO BRL, Gaithersburg, MD), 5 mM DTT, and 50 U ribonuclease inhibitor in 1× polynucleotide kinase buffer. After a 30 min incubation at 37°C, the reaction mixture was diluted with an equal volume of 2× loading buffer and fractionated on a 6% polyacrylamide urea gel, dried, and analyzed by autoradiography.

Analysis of PCNA Protein in Enzyme Fractions

Fifty μl of the column-purified enzyme fractions were separated by 10% SDS-PAGE electrophoresis and transferred to a HyBond ECL (Amersham. Arlington Heights, IL) nitrocellulose membrane. The membrane-transfered proteins were probed for Proliferating Cell Nuclear Antigen (PCNA), by the monoclonal mouse anti-PCNA antibody (PC10, Santa Cruz

Biotechnologies, Santa Cruz, CA) using standard Western probing protocols. The PCNA-bound antibodies were visualized by anti-mouse horseradish peroxidase conjugated antibody (HRP), and the enhanced chemiluminescence (ECL) Western blot detection system (Amersham).

Identification of 5-meCpG as the Specific Substrate for 5-meC–DNA Glycosylase Action

Synthetic single-stranded DNA (50 μg each) of 37mer; (5′ GGG AGA GAG GGA AGC 5me-CGG AGG GAA C5Me-CG GGA AAG 3′) and 40 mer; (5′ TCC CCT TTC CCG GTT CCT TCC TC5me-C GGC TTC CCT CTC TCC C 3′; prepared in Temple University Core Facility), were hybridized in 6× SSC (0.9 M NaCl, 0.9 M sodium citrate, pH 7.0), for 30 min at 88°C and then at 70°C for 1 h as previously described [Vairapandi and Duker, 1996]. The duplex DNA, with a 3′ recessive end on one strand, was separated from single-stranded DNA on a 20% polyacrylamide gel. After elution, the 3′-end of the hybridized, double stranded DNA (5 μg) was filled in with cold dGTP and [α^{32}P]-dATP by the Klenow fragment of DNA polymerase I and was repurified on 20% polyacrylamide gel [Sambrook et al., 1989].

This end-labeled 40 base pair DNA (50,000 ^{32}P cpm) was used in each reaction with 10 μl of the purified enzyme fractions in a volume of 50 μl for 18 h at 37°C. To test the RNase resistance of the enzyme fractions, one set of reactions were carried out in the presence of 10 μg of RNase A (10 mg/ml Conc.). Enzyme fractions were pre-incubated with RNase A for 10 min at room temperature in the glycosylase assay buffer, before the addition of radiolabeled DNA substrate. After an 18 h incubation at 37°C, the DNA was extracted by phenol:chloroform, ethanol-precipitated, and dissolved in 10 μl of sterile distilled water. An equal volume of formamide was added. The samples were heated at 55°C for 5 min, electrophoresed through a denaturing 20% urea-polyacrylamide gel; the gel was exposed to X-ray film for autoradiography.

Demonstration of PCNA-Bound 5meC-DNA Glycosylase Activity

Two-hundred μg of HeLa Scribe (Promega), nuclear protein was immunoprecipitated with anti-PCNA antibody (Santa Cruz Biotechnologies; PC10) in phosphate buffered saline (PBS) with 3% non-fat milk. The immunocomplex was pulled down with protein A agarose beads (Calbiochem, San Diego, CA) and washed thrice (PBS with 0.1% Tween 20). A final wash was given with 5-meC-DNA glycosylase assay buffer. These beads were used directly as the enzyme source, in the site specific nicking assay. Mouse anti–rabbit IgG (Amersham) was used for non-specific immunocomplex preparation and used as the negative control in the site specific nicking assay. A parallel set of immunocomplexes were also prepared to determine the presence of PCNA by Western probing.

RESULTS

Fractionation of Enzyme Activity During Purification

Sequential chromatography using DEAE-cellulose, phosphocellulose, and heparin-agarose columns was employed to achieve partial purification of 5-meC-DNA glycosylase. The activity reproducibly fractionated into three pools on DEAE-cellulose. One did not bind to the column; the others were eluted at 20 mM and at 250–500 mM KCl, respectively. From these three pools, the DEAE-bound (250–500 mM KCl) peak of activity has been described earlier. The DEAE-bound activity eluted from phosphocellulose column at 100–300 mM KCl. Heparin-agarose column purification of DEAE bound activity yielded two peaks, which eluted at 250 mM KCl and 400 mM KCl. These were designated BM and BH, respectively [Vairapandi and Duker, 1996].

The DEAE-wash peak of activity was further purified. It was eluted from the phosphocellulose column at 700 mM KCl. DEAE-wash and phosphocellulose purified activity eluted from heparin agarose column at 400 mM KCl and 650 mM KCl and were designated as WM and WH, respectively. The WM activity was about 700-fold enriched over the HeLa nucleoprotein.

All Enzyme Fractions Are Specific for Fully Methylated CpG Sites and Produce Abasic Sites on DNA

Non-specific endonuclease and exonuclease activities in the purified enzyme fractions were checked in an overnight incubation assay with radiolabeled unmethylated poly(dG-dC):poly(dG-dC). No radioactivity was released from this

TABLE I. 5-meC-DNA Glycosylase Activity Towards Various Substrates*

Substrate	Co-factor	^{3}H cpm released by enzyme fractions				
		Control (no enzyme)	BM	BH	WM	WH
Un-methylated poly(dG-dC): poly(dG-dC)	Nil	110	95	105	100	105
Methylated poly(dG-5MedC): poly(dG-5MedC)	Nil	8	584	486	6604	444
	10 mM EDTA	0	0	0	0	0
	10 mM NaCl	0	0	0	0	
^{14}C	Recovered as 5MeC	810	668	671	683	807
5Me.Cytosine	Recovered as Thymine	0	0	0	0	0

*The activities of the different enzyme fractions and the effects of various factors are represented in 1A. All reaction mixtures included 10 μl of enzyme. Backgrounds were measured in reactions with no added enzyme and used as controls. In the reactions with poly(dG-dC):poly(dG-dC) or poly(dG-5-medC):poly(dG-5-medC) as substrate, 30,000 cpm of ^{3}H-radioactivity were assayed. Reactions with free 5-meC included 1,000 cpm of ^{14}C radioactivity which were assayed by thin layer chromatography in 10:3 isopropanol:water on silica gel plates. The Rf values were 0.5 for 5-meC and 0.8 for thymine in this system.

DNA over than that of the control reaction by any of the partially purified fractions. All these activities specifically released ^{3}H-radiolabeled 5-meC only from poly(dG-5-medC):poly(dG-5-medC; Table I). Therefore, the release of radiolabeled 5-meC was not due to non-specific DNA degradation. Both HPLC and TLC analyses of released radioactivity revealed their identities to be a mixture of 5-meC, thymine, and some deoxyribonucleotides. Reactions of ^{14}C-labeled 5-meC with the purified enzyme fractions for 18 h, followed by TLC analysis of the products, showed no deamination of 5-meC to thymine, with all radioactivity recovered in 5-meC. Either 10 mM NaCl or 10 mM EDTA abolished the enzyme activity (Table I). Time-dependent and protein concentration-dependent enzyme kinetic assays for the partially purified 5-meC-DNA glycosylase activities were performed. All four partially purified activities had linear reaction rates, up to 24 h of incubation at 37°C. Enzyme concentration vs. reaction velocities for all fractions did not reach saturation, even at high protein concentrations (data not shown).

Formation of abasic sites on DNA by the purified fractions was demonstrated. Unlabeled poly(dG-5-medC):poly(dG-5-medC) was reacted with increasing amount of enzyme for 18 h and the DNA was extracted and precipitated. This enzyme-reacted DNA product was mixed with tritium-labeled $NaBH_4$, which reduces the aldehydic moiety of DNA abasic sites with an efficiency that exceeds 90% [Costaing et al., 1992]. Unreacted $NaBH_4$ was removed and the DNA bound radioactivity determined by liquid scintillation counting. The results are shown in Figure 1.

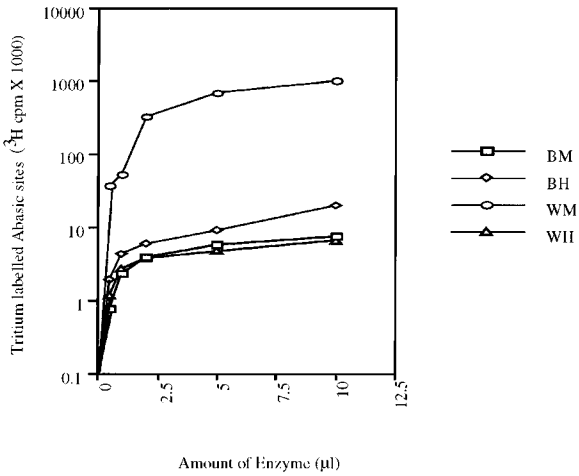

Fig. 1. Demonstration of DNA abasic site formation after glycosylase action. Formation of abasic sites on the DNA backbone are demonstrated in Figure 1. Unlabeled poly(dG-5-medC) poly(dG-5-medC) was reacted with increasing amounts of the purified enzyme fractions overnight. The reacted high molecular weight DNA backbone was extracted and precipitated from the reaction mixes and ^{3}H radiolabeled $NaBH_4$ was added to label the abasic sites. After removing the unreacted $NaBH_4$ by centricon—10 filtration, the DNA was taken for scintillation counting and the results are presented.

All the enzyme fractions' action resulted in DNA products with radiolabeled abasic sites. The formation of abasic sites increased directly

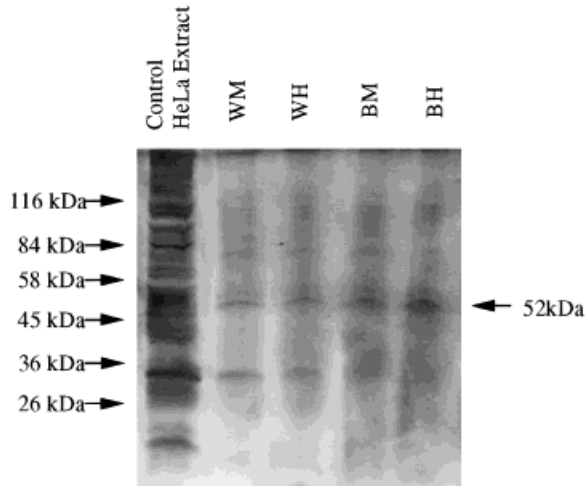

Fig. 2. SDS-polyacrylamide gel analysis of 5-MeC-DNA glycosylase activities. Protein gel electrophoresis was done on a 10% SDS-polyacrylamide gel and the separated protein bands were labeled by silver staining as described. Prestained SDS-PAGE standard markers in the 26–120 kDa range (Sigma, P1677), were used. DEAE-washed, purified fractions are designated as WM and WH. DEAE-bound, purified fractions are designated as BM and BH. The 52 kDa band is marked by an arrow.

with the amount of enzyme in the reaction mixtures. The abasic sites formation also followed the increasing release of radiolabeled 5meC, in another parallel set of experiments with ^{3}H-radiolabeled poly(dG-5-medC):poly(dG-5-medC) substrate. This clearly demonstrated the 5meC-specific enzyme activity in these fractions to be glycosylic, which introduces base loss sites into DNA.

All Partially Purified Activities Have A Core 52 kDa Protein

SDS-PAGE analysis and silver staining of all four purified fractions showed enrichment of one 52 kDa protein band (Fig. 2). The DEAE-wash fractions (WM and WH) each had an additional band of about 36 kDa, which was absent from all DEAE-bound fractions. There were other minor protein bands of equal intensity in all fractions. While there was substantial purification of 5-meC-DNA glycosylase, with an enrichment of a major 52 kDa protein band, a homogenous preparation has not been achieved.

Kinetic Analyses of the Partially Purified Activities

Substrate concentration dependent enzyme kinetic assays for the partially purified 5-meC-DNA glycosylase activities were performed. While the substrate concentration dependant release of 5-meC from poly(dG-5-medC):poly(dG-5-medC) did reach saturation, the double reciprocal plots of the same data were not linear. The enzyme kinetics indicated a complex reaction mechanism, resulting in an allosteric sigmoidal curve rather than a straight line (data not shown). This is a co-operative multimeric enzymatic process, where the formation of one product triggers the formation of a second product with a different reaction rate. With 5-meC-DNA glycosylase, 5-meC on DNA is the first substrate and free 5-meC is first product, while the apyrimidinic site is the second substrate for other DNA-incising activities. Thus, the 5-meC-DNA glycosylase actvity appears to either possess, or be associated with, a number of other nuclear factors and proteins.

Co-operativity in the reaction mechanism was further analyzed by the Hill equation. The Hill coefficients (n), were derived from the slopes of the Hill plots (Data not shown). Based on the Hill coefficient, a non-linear regression analysis for the V vs. S_n was done. The k apparent and V_{Max} values were derived from the equation: $V_{app} = VS_n/[K+S_n]$. The $S_{0.5}$ values (half-saturating concentration), are calculated from the k_{app} values and are presented in Table II. All purified fractions except WM had approximately the same $S_{0.5}$ values, but with variable, V_{Max} values. These different V_{Max} and similar $S_{0.5}$ values may indicate interactions of other nuclear factors, with possible tight associations at sites other than the active site of 5-meC-DNA glycosylase. Though WM had a smaller $S_{0.5}$ value than the other fractions, the K_{app} value was very close to the WH K_{app} value.

Identification of RNA in All Enzyme Fractions and PCNA Only in DEAE Wash Enzyme Fractions

Kinetic analyses revealed a cooperative model of reaction, with a possible association of other nuclear factors and proteins. The involvement and presence of RNA and mammalian DEAD box protein p68 in demethylation activity had been reported in the chick embryo [Frémont et al., 1997; Jost et al., 1997, 1999, 1999a]. We therefore investigated the presence of other factors in 5-meC-DNA glycosylase fractions. Association of RNA was checked by first

TABLE II. Kinetic Parameters of 5-meC-DNA Glycosylase*

Enzyme fraction	K apparent (pMole)	$S_{0.5}$ (pMole)	V_{max} (pMole)	Hill's coefficient
BM	43 ± 9	252 ± 27	46.2 ± 4.4	0.68
BH	85 ± 36	175 ± 64	14.6 ± 2.3	0.86
WM	28 ± 8	64 ± 14	82.0 ± 8.0	0.80
WH	32 ± 7	225 ± 20	28.0 ± 2.7	0.64

*Kinetic parameters were calculated from the non-linear regression analyses of substrate concentration dependent activities of the purified 5-meC-DNA glycosylase fractions over a V vs. S_n plot (n = Hill coefficient values).

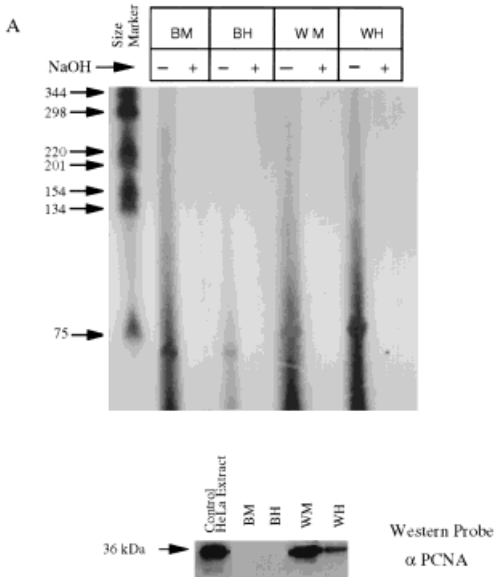

Fig. 3. Demonstration of RNA in all enzyme fractions and PCNA only in DEAE-wash enzyme fractions. One-hundred μl of the partially pure enzyme fractions were deproteinized. RNA from the samples was extracted, labeled with radioactivity for analysis, as described in Materials and Methods section. 1 kb DNA ladder (GIBCO, BRL; Cat.# 15615-016) was also labeled in the same way and run as size markers alongside the labeled RNA molecules (**A**). Fifty μl of the purified enzyme fractions were run and separated on 10% SDS-PAGE gels. The separated protein bands were transferred to a nitrocellulose membrane and probed with an anti-PCNA antibody as described (**B**).

extracting RNA from the enzyme preparations and radiolabeling the RNA at the 5′ ends; the results are shown in Figure 3A. All enzyme fractions have an associated RNA species of 60–90 nucleotides. The identity of the RNA molecules was confirmed by alkaline-lability, as seen from the degradation and absence of the labeled RNA species in the NaOH-treated lanes.

Silver-staining of the enzyme fractions after SDS-PAGE electrophoresis identified the presence of a 52 kDa protein as the major component of 5-meC-DNA glycosylase in all active fractions. Another prominent band was noted near the 36 kDa region in the WM and WH fractions. The nuclear protein PCNA migrates above the 30 kDa range. Enzyme fractions were separated on 10% SDS-PAGE and transferred to nitrocellulose membrane which was probed with anti-PCNA antibody PC-10; the results are shown in Figure 3B. The DEAE-wash fractions WM and WH are positive for the presence of PCNA, while the DEAE-bound BM and BH fractions are not apparently associated with PCNA. Therefore, PCNA is one of the protein factors co-purified with the DEAE-wash 5-meC-DNA glycosylase activity.

Demonstration of 5-meC-DNA Glycosylase's Specificity Towards Fully Methylated CpG Sites on DNA and Resistance to Prior RNAse Treatment

Specificity of enzyme activity towards fully methylated and hemimethylated DNA CpG sites were investigated using a synthetic duplex DNA substrate with two CCGG sites. One site is fully methylated (25th base from the 3′-end); the other, hemi-methylated (12th base from the 3′-end). These sites are MspI-sensitive and HpaII-resistant. MspI digestion generates 25mer and 12mer DNA fragments, whereas there is no cleavage of this DNA by HpaII. Digestion by MspI or HpaII was used to generate marker fragments; these were electrophoresed on denaturing gels alongside the enzyme-reacted DNAs. The MspI-digestion was used to identify the positions of DNA fragments cleaved at fully methylated or hemimethylated CpG and HpaII-treated DNA was used to identify the position of uncut synthetic DNA.

This synthetic DNA was reacted with purified enzyme preparations. Another set of enzyme preparations was pre-treated with RNase A for 10 min and the reactions were carried out. All the reaction products generated by

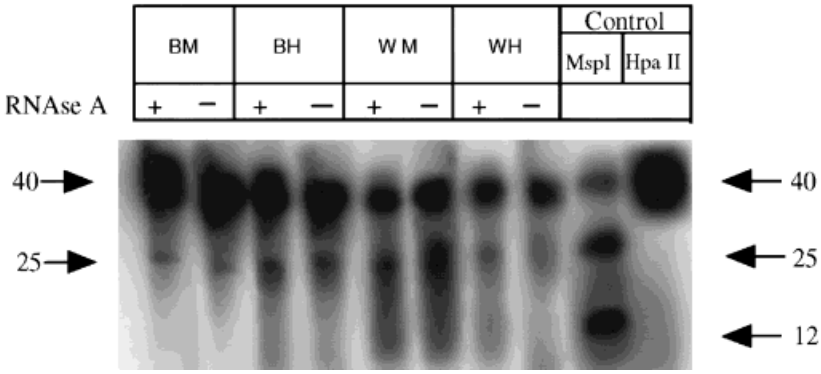

Fig. 4. Demonstration of 5-meC specific and RNase resistant 5-meC-DNA glycosylase activities. 5me-CpG specific glycosylase action of the purified fractions is shown. The site-specific nicking assays were performed with the radiolabeled synthetic double-strand 40mer DNA (50,000 ^{32}P cpm; equivalent to 45 ng). The reaction volume was 50 μl and 10 μl of enzyme fractions were used in each assay. One set of enzyme fractions were pre-treated with 10 μg of RNase A and another set of reactions were carried out without RNase treatment for comparison. MspI-digested control DNA are used in the marker lanes to show the corresponding position of cleavage at fully methylated (25mer), and hemimethylated (12mer) CpG sites on the 40 bp DNA. HpaII-digested control DNA are used to show the position of uncut 40 bp DNA. The corresponding purified fraction is marked on top of the each lane. The reaction products were analysed on a 20% urea-polyacrylamide gel.

both the untreated and RNAse treated enzyme preparations were analysed on the same denaturing gel. The results are shown in Figure 4. A 25mer band, corresponding to cleavage at the fully methylated site on DNA, was generated by all enzyme fractions. The absence of an enzyme-generated 12mer shows that the hemimethylated CpG site is not a substrate. RNase pre-treatment of the purified enzyme fractions, did not abolish the glycosylase activity in site specific nicking assays. The single 25 base band formation confirms the presence of fully methylated CpG specific glycosylase activity with an additional abasic site cleaving activity without other nuclease activities in the purified fractions.

Therefore, 5-meC-DNA glycosylase is specific for 5-meC at fully methylated CpG sites and the glycosylase activity of these enzyme fractions is RNase resistant.

Association of PCNA With 5meC-DNA Glycosylase Activity

To confirm the association of PCNA with 5-meC-DNA glycosylase activity, the PCNA immunocomplex was prepared as described and used in the site specific nicking assay as the enzyme source. The results are shown in Figure 5. The non-specific IgG immunocomplex did not have any enzyme activity. Whereas the PCNA immunocomplex had 5meCpG specific nicking activity and resulted in the formation of 25mer band. (Fig. 5A). Another set of immunocomplexes were prepared for western probing to analyse the presence of PCNA in the PCNA specific immunocomplexes (Fig. 5B). PCNA protein was identified only in the PCNA specific immunocomplex and not in the non-specific immunocomplex. This confirmed that 5-meC-DNA glycosylase activity is positively associated with the nuclear protein PCNA.

DISCUSSION

Our earlier studies demonstrated the glycosylic nature of the 5methyl cytosine removing activity from HeLa cells [Vairapandi and Duker, 1993]. However, it was claimed that this activity is solely a product of non-specific DNA digestions with subsequent pyrimidine metabolism [Steinberg, 1995]. Therefore, varied characterization experiments were carried out to exclude this possibility. We show that there was no degradation of unmethylated poly(dG-dC):poly(dG-dC) by any of the partially purified enzyme fractions. This demonstrates the separation of 5-meC-DNA glycosylase from non-specific nucleases during purification. All partially purified activities specifically released ^{3}H-radiolabeled 5-meC only from poly(dG-5-medC):poly(dG-5-medC) DNA. Therefore, the glycosylase is specific for methylated DNA.

In enzyme kinetic analysis we had observed the release of ^{3}H radiolabeled 5-meC from as

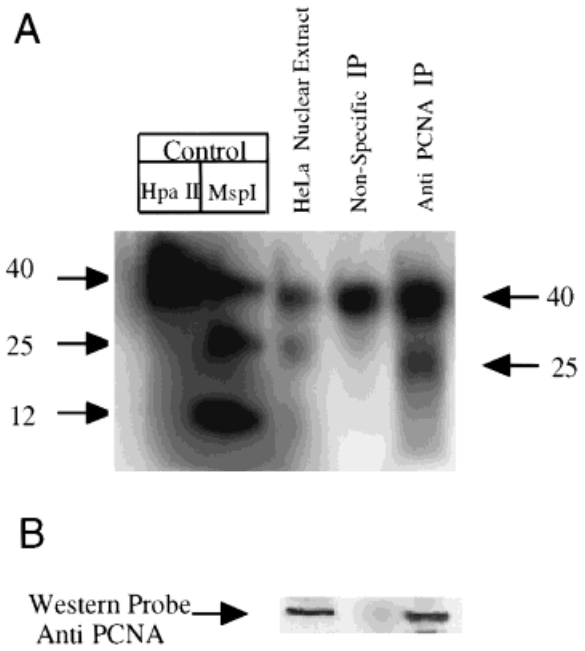

Fig. 5. Demonstration of PCNA immuno-complex associated 5meC-DNA glycosylase activity. Two-hundred μg of HeLa-Scribe nuclear protein was immunoprecipitated with anti-PCNA antibody and the immunocomplex was pulled down with protein A-agarose beads. These PCNA immunocomplex beads were used as enzyme source in the site-specific nicking assay for 5meC-DNA glycosylase assay with the radiolabeled 40 bp synthetic DNA substrate. A non-specific immunocomplex was prepared with mouse anti-rabbit IgG and was used as the negative control in this assay. The reaction products were analysed on 20% urea-polyacrylamide gel and the results are shown in **A**. Another set of immunocomplexes were parallely prepared for western probing to confirm the precipitation of PCNA in the immuno-complexes. HeLaScribe nuclear extract was used as positive control and the Western probing results are shown in **B**.

early as 1 h of incubation. So 5meC release from DNA was not due to non-specific degradation during prolonged incubations. We directly demonstrated the production of DNA base loss (abasic) sites by reductive labeling of DNA-deoxyriboses following the glycosylase reaction. This demonstrated the glycosylic nature of the partially purified 5-meC-DNA glycosylase activity. Finally, the specificity of the partially purified enzyme fractions towards fully methylated and hemimethylated substrates were examined using synthetic DNA substrate with defined 5-meCpG sites; the enzyme activities cleave DNA specifically at fully methylated CpG sites. This confirmed the enzyme preparations specificity for DNA 5-meC sites within fully methylated CpG sequences for their activity. Therefore, the substrate for the enzyme is 5-meC within DNA. The partially purified preparations have both glycosylase and abasic site-nicking activities without any other non-specific nicking activities.

Partial purification of 5-meC-DNA glycosylase showed the presence and enrichment of a major 52 kDa band. This is consistent with the results obtained in the purification of 5-meC-DNA glycosylase from chick embryos, where separation of the glycosylase from an endonuclease acting at base loss sites was attained only at the final step of the purification. The chick embryo 5-meC-DNA glycosylase activity was identified as a 52.5 kDa peptide and isolated as a single band from preparative SDS-polyacrylamide gels which co-purified with the mismatch-specific thymine-DNA glycosylase activity [Jost et al., 1995]. The cloning and expression of human thymine-DNA glycosylase gene described the gene product as a 60 kDa protein [Neddermann et al., 1996]. The human mismatch specific thymine-DNA glycosylase and 5-meC-DNA glycosylase activities do not reside on the same molecule [Neddermann and Jiricny, 1993; Frémont et al., 1997].

There are differences between the human and chick embryo 5-meC-DNA glycosylase activities. The human activity specifically acts at fully methylated CpG sites and is inhibited by as low as 10 mM NaCl or 10 mM EDTA and is dependent on the presence of Mg^{++} ions (Table I). The chick embryo glycosylase has a 10-fold preference for hemi-methylated DNA sites over fully methylated loci and is insensitive to the presence of either EDTA or NaCl in the enzyme assay reactions [Jost et al., 1995]. It has not yet been determined if these differences are due to the other proteins present in the partially purified human preparation, or attributable to the physiological functions of the chicken activity during development and differentiation. There is a possibility that the specificity of 5-meC-DNA glycosylase may resemble the dual action of MCMT specificity for the hemimethylated DNA during maintenance methylation and for unmethylated DNA during de novo methylation. 5-meC-DNA glycosylase activity towards fully methylated DNA would result in formation of hemi-methylated DNA, preserving the methylation pattern. By contrast, glycosylic removal of 5-meC from hemimethylated DNA would result in a totally unmethylated DNA CpG site, with loss of the

methylation pattern. The substrate specificity of these glycosylases for hemi-methylated or fully methylated DNAs may reflect either the events of cellular development or the stage of the cell cycle.

Kinetic calculations for the partially purified human 5-meC-DNA glycosylase fractions showed approximate $S_{0.5}$ value of about 200 pMoles. Though this value is very high, the K_{app} values for the purified enzyme fractions are three- to 10-fold less than the $S_{0.5}$ values, possibly because of their cooperative nature. This high $S_{0.5}$ may reflect 5-meC congregation in normal embryonic cells and tissues, where most CpG loci are fully methylated [Mazin, 1993]. Such 5-meC aggregation is rare in most cells, except for the retrotransposons, especially the human young Alu sub family [Schmid et al., 1991]. The human genome contains approximately 10^6 Alu elements; on average, one Alu element per 3 kb, or 10 per gene. Transposons are rich in CpG dinucleotides, abundant, and heavily methylated; the large majority of genomic 5-meC are within these elements [Schmid et al., 1996]. Although possible host benefits from proliferation of transposable elements have been hypothesized, they are generally considered as highly specialized intragenomic parasites [Yoder et al., 1997]. However, no physiological role for partial demethylation of fully methylated transposon CpG sites has been posited. In view of the kinetics of 5-meC-DNA glycosylase, these transposable elements may suggest a cooperative role for this enzyme. The finding of CpG-rich RNA association with the chick embryo activity is consistent with this role [Jost et al., 1997]. We have also identified RNA moieties with purified enzyme fractions; removal of these moieties did not have any effect on the 5meC-DNA glycosylase activity under our assay conditions.

The identification of the association of PCNA with the DEAE-wash activities suggest a possible 5meC-DNA glycosylase:PCNA complex. This association of PCNA was consistent throughout three different column purifications. PCNA is a very acidic protein [Kelman and O'Donnell, 1995]. It's association may have mediated differential fractionation of the glycosylase into DEAE-wash and DEAE-bound activities. We have confirmed the association of PCNA with 5meC-DNA glycosylase activity. The multiple roles of PCNA in DNA replication include processivity of DNA polymerase δ and a stimulation of DNA polymerase ϵ [Stillman, 1996]. PCNA has a substantial role in the nucleotide excision repair and mismatch repair pathways [Pan et al., 1995; Umar et al., 1996]. Recent studies demonstrated PCNA to interact with proteins involved in cell cycle progression, which are not a part of the DNA replication apparatus; these include p21, cyclin D, Gadd45, MyD118, and Fen1 [Kelman, 1997; Vairapandi et al., 1996a]. Since PCNA can traverse duplex DNA, it could be an ideal platform for many DNA-scanning proteins [Kelman and Hurwitz, 1998]. It may also act as a communication point, or signal processing center, for a variety of important cellular processes. These would include DNA replication, nucleotide and base excision repair, post-replication mismatch repair, and at least one apoptotic pathway [Jonsson and Hubscher, 1997]. The human MCMT, forms a complex with PCNA and it is a target for $p21^{WAF1}$. This complex formation occurs at foci of newly replicated DNA and does not alter MCMT activity. A p21-derived peptide disrupts this interaction, suggesting that $p21^{WAF1}$ regulated DNA methylation by blocking access of MCMT to PCNA [Chuang et al., 1997]. We have shown here the association and co purification of PCNA with purified human 5-meC-DNA glycosylase as well as the co-immunoprecipitation of PCNA with this activity. Thus, PCNA may form a complex with both methylating and demethylating activities. This underscores the importance of tight modulation of dynamic methylation status of DNA, possibly reflecting its importance in many cell cycle processes and pathways.

While the physiological functions of 5-meC-DNA glycosylase are unknown, it could be involved in cellular regulation, differentiation and proliferation. The importance of DNA methylation is emphasized by the lethality of inactivation of the murine MCMT gene [Li et al., 1992]. The methylation status of specific CpG loci may be determined by relative levels of MCMT and 5-meC-DNA glycosylase as well as of other DNA-binding proteins [Vairapandi and Duker, 1993, 1996; Frémont et al., 1997; Jost et al., 1993, 1997; Szyf, 1994]. At least three different components may be necessary for establishment of a specific methylation pattern: DNA methyltransferases, the demethylation systems of DNA (passive and/or active), and various other cis or trans determination

factors. DNA methylation is performed by MCMT, active demethylation is carried out by either base excision or by other mechanisms [Bestor et al., 1988; Vairapandi and Duker, 1993; Jost et al., 1997; Bhattacharya et al., 1999]. SP1 binding sites and B1 like repetitive elements can act in cis either to repel or to attract DNA methylation [Turker and Bestor, 1997]. Sp1 transcription proteins and the demethylating activities can act as trans-acting elements, promoting the hemimethylated DNA pattern formation. It is also possible that RNA and PCNA-like proteins may be involved as trans-acting elements influencing DNA methylation status. Cloning of the encoding gene of this demethylating activity, with identification and definitive characterization of both Cis- and Trans-acting factors, are essential for elucidation of its physiological functions. Such characterization of 5-meC-DNA glycosylase, in conjunction with studies of cellular differentiation, development, and transcription, will reveal the roles of DNA 5-meC in the internal regulatory networks of the eukaryotic cell.

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