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Effects of chromatin structure on the enzymatic and DNA binding functions of DNA methyltransferases DNMT1 and Dnmt3a in vitro

Andrea K. Robertson[a], Theresa M. Geiman[a], Umesh T. Sankpal[a], Gordon L. Hager[b], Keith D. Robertson[a,*]

[a] *Epigenetic Gene Regulation and Cancer Section, LRBGE/NCI/NIH, Bldg. 41, Rm. C306, 41 Library Dr., Bethesda, MD 20892, USA*
[b] *HAO/LRBGE/NCI/NIH, Bldg. 41, Rm. B602, 41 Library Dr., Bethesda, MD 20892, USA*

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Abstract

DNA methylation is an epigenetic modification of the genome critical for numerous processes, including transcriptional repression and maintenance of chromatin structure. Recent studies have revealed connections between DNA methylation and other epigenetic modifications such as ATP-dependent chromatin remodeling. It remains unclear, however, exactly how chromatin and epigenetic chromatin modifications affect the biological properties of the DNA methyltransferases (DNMT1, DNMT3A, and DNMT3B). Using a highly purified system and the 5S rDNA gene as free DNA or assembled into a mononucleosome, we have compared the effects of chromatin structure on DNMT1 and Dnmt3a. The catalytic efficiency for both enzymes decreased on the mononucleosome, ~8-fold for DNMT1 and 17-fold for Dnmt3a. DNMT1 and Dnmt3a bound to DNA and mononucleosomal substrates in gel shift experiments with approximately equal affinity and in a cooperative manner. We also show that DNMT1 interacts with hSNF2H chromatin remodeling enzyme and that DNMT1 binds mononucleosomes with higher affinity in the presence of hSNF2H. These findings raise interesting implications about the interactions of mammalian DNA methyltransferases with chromatin and provide the first evidence that a chromatin remodeling enzyme can alter the biological properties of a DNMT.
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The methylation of cytosine residues within the CpG dinucleotide in mammalian cells is an important mediator of gene expression, genome stability, X chromosome inactivation, genomic imprinting, chromatin structure, and embryonic development [1,2]. The majority of CpG sites in mammalian cells are methylated in a non-random fashion. Specifically, repetitive and parasitic elements tend to be hypermethylated while CpG island-associated promoters tend to be hypomethylated [3,4]. DNA methylation in cells is mediated by three DNA methyltransferases, DNMT1, DNMT3A, and DNMT3B [1,5]. Studies have shown that all three DNMTs interact with each other and furthermore that they function in concert to establish and maintain genome-wide DNA methylation patterns [6,7]. Numerous studies have demonstrated that DNMT1 is specialized to perform maintenance DNA methylation following DNA replication since it is targeted to replication foci via interaction with PCNA [8–10]. DNMT3A has a small but significant preference for unmethylated DNA and DNMT3B methylates unmethylated and hemimethylated DNA with equal efficiency, leading to the classification of these latter two DNMTs as de novo methyltransferases [10–12].

While much is known about how the DNMTs function in vitro on naked DNA, far less is known about

* Corresponding author. Present address: Department of Biochemistry and Molecular Biology, University of Florida, Box 100245, Gainesville, FL 32610, USA. Fax: +1 352 392 2953.
E-mail address: keithr@ufl.edu (K.D. Robertson).

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how they function in a chromatin environment. Recent exciting findings have revealed numerous connections between DNA methylation and other classes of epigenetic chromatin modifications, such as ATP-dependent chromatin remodeling [13]. In vivo, DNA is wrapped around the surface of a histone octamer, which is composed of two molecules each of histones H2A, H2B, H3, and H4 [14]. The N-terminal 'tails' of the core histones, particularly of histones H3 and H4, are extensively modified by acetylation, methylation, and phosphorylation [15]. Chromatin structure is also dynamically modified by SNF2 family ATP-dependent chromatin remodeling proteins. Mutations in certain members of this family have been shown to result in the disruption of DNA methylation patterns in mammalian cells [13,16,17]. SNF2 family proteins often exist as components of larger chromatin remodeling complexes and utilize the energy of ATP hydrolysis to mobilize or reorganize nucleosomal structure [18]. Such remodeling activity may activate or repress transcription. Genetic disruption of the murine *lsh* gene, an SNF2-like family member, results in massive hypomethylation in both repetitive and single copy loci [16]. Naturally occurring mutations of the *ATRX* gene in humans with ATRX (α-thalassemia, mental retardation, X-linked) syndrome also result in subtle DNA methylation abnormalities [17]. Therefore, these studies reveal that ATP-dependent chromatin remodeling is intimately linked to the control of DNA methylation in vivo. It remains unclear at this time, however, exactly how chromatin modifications or the enzymes responsible for these modifications 'target' DNA methylation to certain regions and/or restrict it from others.

In the present study we sought to better define the role of chromatin and chromatin remodeling in regulating the activity of DNA methyltransferases. To accomplish this we utilized a highly defined in vitro system to compare the effects of chromatin structure on the activity of DNMT1 compared to Dnmt3a. By comparing activity on naked DNA with activity on the same sequence assembled into a highly purified and homogeneous mononucleosome preparation, any effects can be directly attributed to chromatin structure. In enzyme kinetic studies we found that the catalytic efficiency of DNMT1 and Dnmt3a decreased on the mononucleosome substrate, approximately 8-fold for DNMT1 and 17-fold for Dnmt3a, while the ability of both DNMTs to bind the substrates was not significantly altered by chromatin structure. We also demonstrate directly that DNMT1 interacts with the SNF2 family member ATP-dependent chromatin remodeling enzyme hSNF2H by co-immunoprecipitation and show that a significant fraction of DNMT1 and hSNF2H proteins co-localize in transfected HeLa cells. Interestingly, hSNF2H enhances the binding of DNMT1 to the mononucleosome substrate (approximately 3-fold) in an ATP-independent manner. These studies therefore reveal that chromatin structure differentially influences the catalytic activity and substrate binding of the different classes of DNMT (de novo versus maintenance) and that the interaction of a DNMT with a chromatin remodeling enzyme is capable of altering its biological function (DNA binding affinity). Such effects may help to explain why chromatin remodeling enzymes are essential for the establishment of normal cellular DNA methylation patterns in vivo.

Materials and methods

Preparation and purification of recombinant proteins and DNA substrates. Recombinant proteins were expressed by first infecting Sf9 insect cells with baculovirus stocks for hexahistidine-tagged DNMT1, Dnmt3a, hSNF2H, and RbAp48. Proteins were subsequently purified from infected Sf9 whole cell extracts with nickel agarose resin as described previously [10]. Glutathione-*S*-transferase (GST) was purified from *Escherichia coli* as described [19]. The 208 bp sea urchin 5S ribosomal DNA fragment was isolated by digestion of plasmid pPolI with *Ava*I. The 208 bp fragment was run on a 1.2% agarose gel, excised, and the DNA was extracted from the gel slice using the Qiaquick gel extraction kit (Qiagen). The DNA was quantitated by absorption at 260 nm. Native core histones were purified from HeLa cells according to published procedures [20]. Mononucleosomes were assembled by step salt dialysis according to standard procedures [20]. The reconstituted mixture was then loaded onto a 10–40% glycerol gradient and the mononucleosomes were separated from free DNA by ultracentrifugation followed by fractionation. The peak containing purified mononucleosomes was pooled and concentrated using a Centricon 10 spin concentrator according to manufacturer's instructions (Amicon). Mononucleosome was quantitated by dilution into 2 M NaCl and absorption spectroscopy at 260 nm. In this paper we refer to the 208 bp naked DNA substrate as 208_{DNA} and the 208 bp fragment assembled into mononucleosomal form as 208_{Mono}. ATPase activity of hSNF2H was measured as described previously [21].

Preparation of nuclear extract and immunoprecipitations. Nuclear extract was prepared from P19 embryonic carcinoma cells by a modified method [22]. Briefly, cells were washed once with 1× PBS (pH 7.5) and resuspended in 100 μl lysis buffer/10^7 cells (10 mM Hepes (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 0.025% NP-40, 0.5 mM DTT, 0.2 mM PMSF, and 1 μg/ml aprotinin, leupeptin, and pepstatin A). Cells were incubated on ice for 20 min and the nuclei were pelleted at 7500 rpm. Nuclei were incubated with 50 μl/10^7 cells of storage buffer (20 mM Hepes (pH 7.5), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 25% glycerol, 0.2 mM PMSF, and 1 μg/ml aprotinin, leupeptin, and pepstatin A) on ice for 30 min. Nuclear extract was dialyzed against buffer C (20 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 0.3 mM EDTA, 2 mM EGTA, 1 mM DTT, 0.2 mM PMSF, 25% glycerol, and 1 μg/ml protease inhibitors) to 100 mM NaCl. Immunoprecipitations were performed using standard methods, in which the nuclear extract buffer was adjusted to 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, and 0.5% NP-40. In each case, 200 μl (800 μg) of nuclear extract was first pre-cleared with protein A/G plus agarose beads (Santa Cruz Biotechnology), incubated with 20 μl antibody (or species matched normal IgG) for 2 h at 4 °C with rotation, followed by incubation with 20 μl protein A/G plus agarose for 2 h at 4 °C with rotation. Washing was performed five times in 500 μl wash buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, and 0.5% NP-40) at 4 °C, 5 min each on a rotator. For immunoprecipitation and Western blotting, a DNMT1 rabbit polyclonal antiserum was generated from a recombinant bacterially expressed fragment of human DNMT1 (#1020,amino acids 914–1087) and the anti-hSNF2H antibody H-300 was purchased from Santa Cruz Biotechnology.

DNA methyltransferase assays. DNA methyltransferase assays were performed as previously described with minor modifications [23]. DNA or mononucleosome saturation curves were performed with 0.81 μM tritium-labeled *S*-adenosyl-L-methionine ([^{3}H]SAM) (NEN) and either 96 nM DNMT1 or 45 nM Dnmt3a. Reactions were incubated at 37 °C for 1–2 h, with duplicate reactions for each time point. Reactions were carried out in a 50 μl volume in buffer containing 50 mM Tris (pH 8.0), 5 mM EDTA, 10% glycerol, and 1 mM DTT. After scintillation counting, the counts per minute (cpm) were converted to nM CH_3 by a conversion factor generated from a standard curve of known concentrations of [^{3}H]SAM plotted against their scintillation counts. Assay data were plotted and fitted to a hyperbolic equation using Sigma Plot 8.0 software.

End-labeling of DNA and mononucleosomes. The 208_{DNA} and 208_{Mono} fragments were radioactively end-labeled with [γ-^{32}P]ATP (Amersham–Pharmacia) and T4 polynucleotide kinase (New England Biolabs). Approximately 1.25 μg of DNA or 1.0 μg of mononucleosome was mixed in a 20 μl volume with 1× T4 PNK buffer, 20 U T4 polynucleotide kinase, and 2 μl [γ-^{32}P]ATP. Reactions were incubated at 37 °C for 1 h and then passed through G-50 spin columns (Roche Applied Science) according to the manufacturer's instructions to remove unincorporated radioactivity. Labeled samples were recovered and stored at 4 °C for use within 1–3 days.

Electrophoretic gel mobility shift assays (EMSAs) and quantitation. Binding reactions were carried out in 20 μl volumes in the following buffer: 25 mM Tris–HCl (pH 8.0), 1 mM EDTA, 3 mM $MgCl_2$, 10% glycerol, 0.1% Triton X-100, and 1 mM DTT. Labeled probe, 75 ng poly d(I–C) (Roche Applied Science), and the appropriate proteins were then added. Reactions were assembled on ice and then incubated for 1 h on ice or, in the case of experiments evaluating effects of hSNF2H, at room temperature (25 °C). A 0.1 volume of 50% glycerol was then added to each sample. Samples were loaded onto 4% (3.5% for experiments with hSNF2H), 1.0 mm native polyacrylamide gels and then electrophoresed in 0.5× TBE buffer at 100 V for 1.5 h (until the bromophenol blue marker had run off the bottom of the gel, bromophenol blue was not added to reactions containing 208_{Mono}). Gels were dried on blotting paper and exposed to X-ray film or a PhosphorImager screen. For quantitation, exposed PhosphorImaging screens were scanned and the data were processed by ImageQuant software. The percent shifted species was determined as follows: all experiments contained a control reaction lacking DNMT1 or Dnmt3a. The migration of the labeled mononucleosome or DNA in this reaction was defined as zero percent shifted and the ratio of the PhosphorImager counts in the area of the lane above this band to the total counts in the lane was defined as background and subtracted from all other lanes. Subsequent lanes containing protein-bound probe were partitioned identically, and the ratio of shifted counts to total counts minus the background counts was calculated to give a percent shift. Values were then plotted and fitted to a sigmoidal equation with Sigma Plot 8.0.

Immunofluorescence co-localization. An expression plasmid for green fluorescent protein (GFP)-tagged DNMT1 was transfected into HeLa cells using lipofectamine according to the manufacturer's instructions (Invitrogen) and endogenous hSNF2H was detected by immunofluorescence staining with a mouse monoclonal anti-hSNF2H antibody (Upstate Biotechnology). Cells were processed, stained, and imaged exactly as described previously [19].

Results

Kinetic parameters of DNMT1 and Dnmt3a on naked DNA versus chromatin substrates

In this study we sought to investigate the effects of chromatin structure on the activities of mammalian DNA methyltransferases. By comparing the DNA methyltransferase activity and binding properties of purified DNA methyltransferases upon a single DNA sequence existing either in its free form or assembled into the basic repeat unit of chromatin—the mononucleosome, we could begin to examine structural effects of chromatin on DNA methyltransferase activity. Any such effects would result not from a particular DNA sequence but from the altered topology of the DNA helix within the mononucleosome and any consequent change in its interactions with the enzyme. The DNA substrate utilized in this study is a 208 bp *Ava*I fragment containing the sea urchin 5S rDNA sequence. This sequence was selected because of its well-documented nucleosome positioning properties, because it could be used to efficiently produce large amounts of highly purified and homogeneous mononucleosome, and because it is one of the most widely utilized and well-characterized systems for studying aspects of chromatin structure and remodeling [24–27]. In addition, the 5S rDNA is a physiologically relevant methylation target sequence because it has been shown that a significant fraction of rDNA repeats are methylated in vivo [28–30]. The 208 bp rDNA fragment was mixed with purified core histones to assemble mononucleosomes, which were then purified to remove any contaminating free DNA (Fig. 1). Throughout this report the DNA and mononucleosomal forms of the 208 bp rDNA substrates are designated '208_{DNA}'

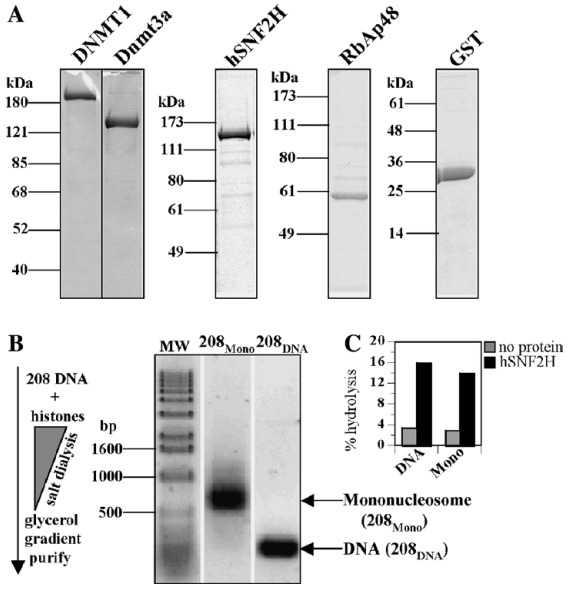


Fig. 1. Recombinant proteins and DNA substrates (208_{DNA} and 208_{Mono}) used for these studies. (A) Hexahistidine-tagged DNMT1, Dnmt3a, hSNF2H, and RbAp48 were expressed in Sf9 cells via baculovirus infection and purified with nickel agarose resin. GST was expressed in *E. coli* and purified with glutathione–Sepharose resin. (B) Scheme for preparation and purification of mononucleosome (left) and an ethidium bromide-stained agarose gel showing the purified 208_{DNA} and 208_{Mono} substrates (right). (C) The 208_{DNA} and 208_{Mono} substrates stimulate ATP hydrolysis by recombinant hSNF2H.

and '208_{Mono},' respectively. Two mammalian DNA methyltransferases were assayed for this study, both in purified, recombinant form (Fig. 1). Together they represent both known sub-types of mammalian DNA methyltransferases: the so-called maintenance methyltransferase, DNMT1, and the de novo methyltransferases, of which Dnmt3a is a member.

Assays of DNMT1 and Dnmt3a activity on a range of 208_{DNA} and 208_{Mono} concentrations yielded saturation curves for the alternate substrates (Figs. 2A and B). These data yielded comparative kinetic constants (Table 1, Fig. 2C). Both enzymes showed increased K_M^{CG} values on 208_{Mono} relative to 208_{DNA}: 12-fold for DNMT1 and 15-fold for Dnmt3a. At saturating mononucleosome concentrations, both enzymes had approximately the same maximal velocity as they had on DNA. In fact, DNMT1 activity was slightly greater on mononucleosome under saturating conditions than on DNA. Overall, the two enzymes were less catalytically efficient on mononucleosome than on DNA, approximately 8-fold less for DNMT1 and 17-fold less for Dnmt3a

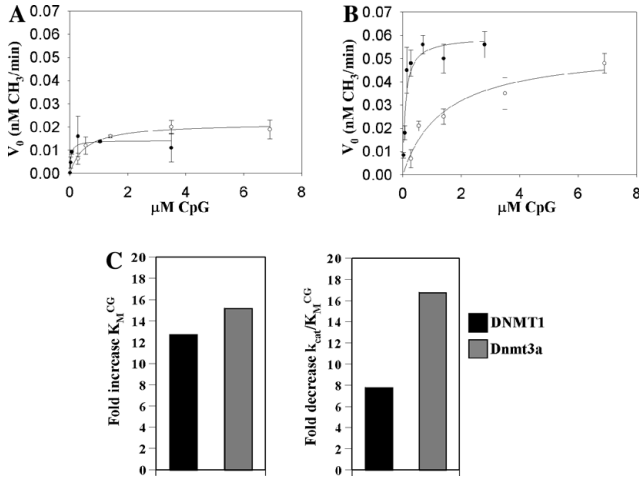


Fig. 2. Activity of DNMT1 and Dnmt3a on 208_{DNA} and 208_{Mono}. (A) Saturation curves of DNMT1 enzymatic activity on the 208_{DNA} (filled circles) and the 208_{Mono} (open circles) substrates. (B) Saturation curves of Dnmt3a activity on the 208_{DNA} (filled circles) and the 208_{Mono} (open circles) substrates. Reactions contained 96 nM DNMT1 or 45 nM Dnmt3a, respectively. (C) Graphs summarizing the fold-change in kinetic parameters of DNMT1 and Dnmt3a on mononucleosome relative to DNA. Data are derived from Table 1.

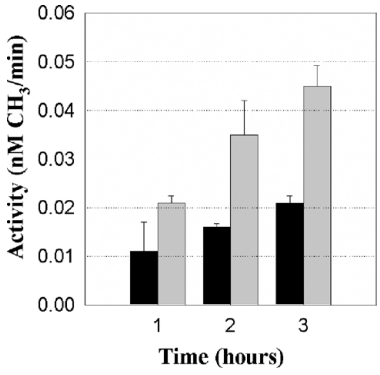


Fig. 3. Activation of DNMT1 activity over longer time periods. DNMT1 activity is non-linear with time with extended reaction times, especially on mononucleosome. Assays of DNMT1 activity using 1 μg 208_{DNA} (black bars) or 208_{Mono} (gray bars) for increments of 1–3 h show a greater rate of increase in DNMT1 activity on mononucleosome (~2.4-fold) than on DNA. This behavior was not observed with Dnmt3a (not shown).

(Fig. 2C). Interestingly, DNMT1 exhibited activity that was non-linear with time at longer reaction times. At incubation times greater than 1 h, DNMT1 activity (expressed as nM CH_3/min) increased measurably at a steady rate, continuing until about 3 h when the reaction rate leveled off (Fig. 3). This behavior was not observed with Dnmt3a, which yielded activity that was consistently linear with time (data not shown). The rate of DNMT1 activity increased approximately 2.4-fold faster on 208_{Mono} than on 208_{DNA}.

Effects of chromatin on the DNA binding activity of DNMT1 and Dnmt3a

The binding affinities of DNMT1 and Dnmt3a for the 208_{DNA} and 208_{Mono} substrates were then examined by EMSA. Each enzyme bound the DNA and mononucleosomal forms with similar affinity (Fig. 4). DNMT1 bound the 208 bp templates half-maximally at 100 nM DNMT1, with a slightly higher affinity for DNA than for mononucleosome (Fig. 4C). Dnmt3a bound the 208 bp templates half-maximally at 150 nM Dnmt3a and bound mononucleosome with slightly higher affinity than DNA (Fig. 4F). A striking feature of all the binding experiments was the narrowness of the range of protein concentrations within which the probe shifted from

Table 1
Kinetic constants for DNMT1 and Dnmt3a activity on the 5S 208 DNA and mononucleosome substrates

Substrate	Enzyme: DNMT1		Dnmt3a	
	DNA	Mononucleosome	DNA	Mononucleosome
K_M^{CG} (μM)	0.041 ± 0.025	0.52 ± 0.12	0.092 ± 0.033	1.40 ± 0.57
V_{max} (nM CH_3/min/μM enzyme)	0.14 ± 0.02	0.23 ± 0.03	1.30 ± 0.20	1.20 ± 0.30
k_{cat}/K_M^{CG} ($M^{-1}h^{-1}$)	210,000 ± 90,000	27,000 ± 6000	860,000 ± 200,000	51,000 ± 2000

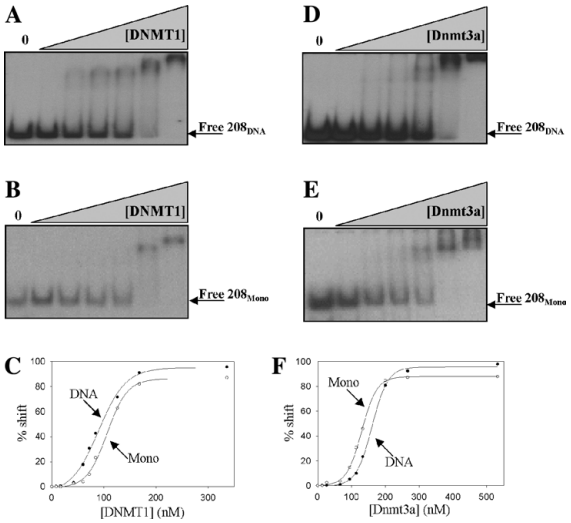


Fig. 4. Binding of DNMT1 and Dnmt3a to DNA and mononucleosome. EMSAs were performed to determine the relative binding affinities of the two DNMTs for 208_{DNA} and 208_{Mono}. (A) Binding of DNMT1 to 4 nM ^{32}P-end-labeled 208_{DNA} or (B) 4 nM labeled 208_{Mono} probes was measured, quantitated, and (C) the data are plotted (DNA—closed circles and mononucleosome—open circles). Similar analyses were performed with Dnmt3a (D, 208_{DNA}; E, 208_{Mono}; F, plot). In the experiments shown in (A,B), binding reactions contained 0, 42, 60, 72, 84, 126, or 168 nM DNMT1. Experiments shown in panels (D,E) contained 0, 66.5, 95, 114, 133, 200, or 266 nM Dnmt3a. The gels were scanned and quantitated using a PhosphorImager as described in Materials and methods. The plots also contain values from additional gel shift experiments (not shown) using a broader range of enzyme concentrations. Experiments were performed in duplicate and the results shown are representative of both repetitions. DNMT1 bound the 208_{DNA} and 208_{Mono} similarly, with half-maximal binding occurring at approximately 100 nM DNMT1, and bound with greater affinity to DNA than to mononucleosome. Dnmt3a also bound the DNA and mononucleosome similarly, with half-maximal binding occurring at approximately 150 nM Dnmt3a, but bound with greater affinity to mononucleosome than to DNA.

0% to 100% bound. Protein concentrations higher than the level at which 100% shifting was achieved produced super-shifted bands with successively slower migration patterns (Fig. 4 and data not shown). Additionally, Hill plots of the binding data yielded straight lines with slopes greater than one. The data indicate that DNMT1 and Dnmt3a each binds cooperatively to both the DNA and mononucleosomal substrates (Fig. 5).

DNMT1 interacts with chromatin remodeling enzyme hSNF2H and this interaction alters the DNA binding properties of DNMT1

These studies investigated the enzymatic and binding activities of DNMT1 on DNA and chromatin in a purified, in vitro environment. However, the situation in living cells is certain to be more complex because DNA methyltransferases are known to interact with a number of other chromatin-associated proteins [1,23,31–33]. To begin to clarify these complex interactions and their

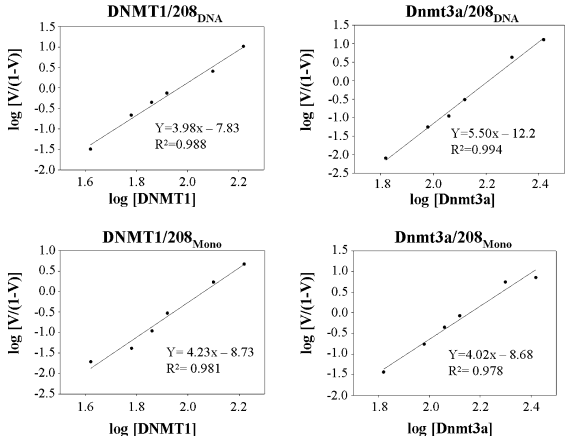
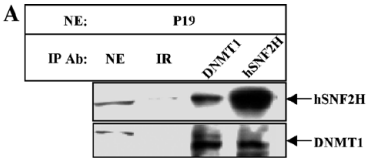


Fig. 5. Hill plots for DNMT1 and Dnmt3a binding to DNA and mononucleosome yield lines with slopes greater than one. These indicate that DNA or mononucleosome cooperatively binds DNA methyltransferases. V, fraction of DNA or mononucleosome bound.

effects on DNA methyltransferase behavior, native DNMT1 was purified from P19 cells, and DNMT1-containing fractions were screened for various chromatin-associated proteins. One such factor that co-purified with DNMT1 was the ATP-dependent chromatin remodeling enzyme hSNF2H (data not shown). These findings were confirmed by reciprocal co-immunoprecipitation experiments in which an anti-DNMT1 antibody successfully immunoprecipitated endogenous hSNF2H from nuclear extract, and an anti-hSNF2H antibody successfully immunoprecipitated DNMT1 (Fig. 6A).



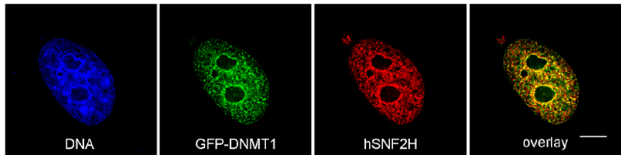


Fig. 6. DNMT1 interacts with hSNF2H. (A) Endogenous DNMT1 and hSNF2H can be reciprocally co-immunoprecipitated from nuclear extract (NE) derived from murine P19 embryonic carcinoma cells. The antibody used for immunoprecipitation is shown along the top and the antibody used for the subsequent Western blot is indicated on the right. 'IR'' is species matched normal IgG used as a negative control. (B) Co-localization of DNMT1 and hSNF2H in transiently transfected HeLa cells. HeLa cells were transfected with a plasmid expressing GFP-tagged DNMT1, fixed, and then stained for endogenous hSNF2H. A representative interphase cell is shown. In the left-most panel, cells are stained with Hoechst 33342 to visualize DNA, the left-middle panel shows the localization of GFP-DNMT1, and the right-middle panel shows the localization of hSNF2H. The two images are merged in the right-most panel where yellow color represents co-localization. The scale bar corresponds to 5 μm.

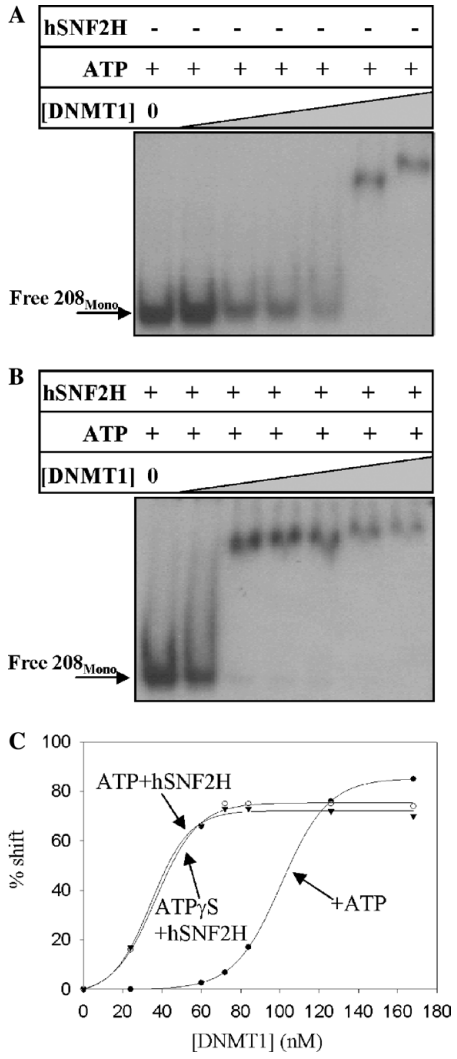


Fig. 7. Effect of hSNF2H on DNMT1 binding to mononucleosomes. DNMT1 binding to 208_{Mono} was titrated in the absence (A) or presence (B) of purified hSNF2H (360 nM) and analyzed by EMSA. From left to right, lanes contained 0, 24, 60, 72, 84, 126, or 168 nM DNMT1. All reactions contained 1.0 μM ATP, 4.0 nM ^{32}P-labeled 208_{Mono} probe, and 75 ng poly d(I–C). Identical reactions were also carried with DNMT1, hSNF2H, and 1.0 μM ATPγS (data not shown). (C) The percent shifted species in each lane was quantified on a PhosphorImager, plotted, and fitted to a sigmoidal curve equation. Filled circles are reactions with DNMT1 and ATP; open circles are reactions with DNMT1, hSNF2H, and ATP; triangles are reactions containing DNMT1, hSNF2H, and ATPγS. In the presence of hSNF2H, the concentration of DNMT1 that bound 50% of the mononucleosome was approximately 3-fold lower than in the absence of hSNF2H. In control experiments in which equal mass amounts of irrelevant proteins (GST or RbAp48) were substituted for hSNF2H, the concentration of DNMT1 needed for half-maximal binding to mononucleosome was the same as for DNMT1 alone (data not shown).

Immunofluorescence microscopy supports the interaction of DNMT1 and hSNF2H because a significant fraction of both proteins co-localize in HeLa cells. DNMT1 and hSNF2H co-localized extensively in DAPI-dense heterochromatin regions of interphase cells (Fig. 6B). Together, these data indicate that DNMT1 and hSNF2H bind stably to each other.

To examine the potential functional effects of the hSNF2H interaction with DNMT1, EMSA binding experiments of DNMT1 to 208_{Mono} were performed to which was added purified, recombinant hSNF2H (Fig. 1A). The recombinant hSNF2H demonstrated both 208_{DNA} and 208_{Mono}-stimulated ATPase activity (Fig. 1C), however, we focused our studies on 208_{Mono} because it was the most relevant substrate for a chromatin remodeling enzyme and for studying the effects of chromatin on DNMT1 activity. Addition of hSNF2H increased the binding affinity of DNMT1 for 208_{Mono} by approximately 3-fold (Fig. 7). We confirmed that the shifted band resulted from DNMT1 since addition of DNMT1 antibody resulted in a supershift (data not shown). Purified hSNF2H alone did not produce a discrete shifted species (Fig. 7B, lane 1). Interestingly, this effect did not require ATP hydrolysis since DNMT1 binding affinity increased whether or not the binding reaction contained ATP, the non-hydrolyzable ATP analog ATPγS, or no ATP (data not shown). To demonstrate the specificity of this effect, control experiments were performed in which other irrelevant purified proteins (GST and RbAp48) were added to the DNMT1 binding reaction instead of hSNF2H. These irrelevant proteins had no effect on DNMT1 binding to the mononucleosome (data not shown), demonstrating that the hSNF2H effect was specific. Although the DNA binding activity of DNMT1 was altered by hSNF2H, we did not detect significant changes in its enzymatic activity on the 208_{Mono} substrate in the presence of hSNF2H (data not shown), indicating that changes in binding affinity do not necessarily translate directly into changes in enzymatic activity in our purified in vitro system.

Discussion

In the present manuscript we have examined the relative enzymatic and DNA binding activities of two mammalian DNA methyltransferases, DNMT1 and Dnmt3a, on a single substrate existing in two forms—as free DNA or packaged into a mononucleosome. Both enzymes yielded significantly higher K_M^{CG} values on mononucleosome compared to DNA, approximately unchanged maximal velocities, and significantly lower catalytic efficiency on mononucleosome relative to DNA. DNMT1 and Dnmt3a bound to both forms of the 208 bp fragment with similar affinity, and in a cooperative manner. We also show that the chromatin remodeling enzyme hSNF2H interacts and co-localizes with DNMT1. Interestingly, although the catalytic activity of DNMT1 was not significantly altered in the presence of hSNF2H on the mononucleosome, its binding to mononucleosome was enhanced. This effect was

specific for hSNF2H but did not depend on its ATPase activity. Thus, these studies demonstrate that assembly of DNA into the basic repeat unit of chromatin has markedly inhibitory effects on the activity of both maintenance and de novo methyltransferases but both enzymes still retain the capacity to methylate a chromatin substrate. The ability of hSNF2H to enhance the binding of DNMT1 to the mononucleosomal substrate also provides the first mechanistic insight into why a DNA methyltransferase may need to interact with a chromatin remodeling enzyme and may also help to explain why mutations in some chromatin remodeling enzymes [16,17] result in aberrant hypomethylation of the genome.

The higher K_M^{CG} values for DNMT1 and Dnmt3a on mononucleosome are likely the result of a combination of factors. Although EMSA studies indicated that the enzymes bound the mononucleosomal form virtually as well as the DNA form, the Michaelis constant reflects the interplay of multiple kinetic rate constants. Although the DNA methyltransferases may non-specifically bind the surface of the mononucleosome equally as well as they bind to free DNA, their ability to access and methylate the fraction of cytosine residues that are partially occluded by histones would likely be hindered, resulting in slower kinetics and a higher K_M value. However, at saturating mononucleosome concentrations, the enzyme would be presented with enough accessible cytosine residues that it could achieve a maximal velocity comparable to that on DNA. The significant reduction in enzymatic activity of DNMT1 on a chromatin substrate that we observed here is consistent with a recent report [34]. Okuwaki and Verreault [34] showed that the ability of DNMT1 to methylate DNA was reduced by assembly of DNA into chromatin and the degree of repression was somewhat dependent on DNA sequence. This study did not, however, describe kinetic parameters nor compare the effects of chromatin on the two classes of DNMT (maintenance and de novo). Other enzymes that must operate on DNA, such as the nucleotide excision repair (NER) machinery, have also been shown to be dramatically repressed by the presence of chromatin [35]. Interestingly NER activity could be partially restored by addition of recombinant ACF, a chromatin remodeling complex containing ACF1 and ISWI, the latter being the *Drosophila* homolog of hSNF2H [35,36].

DNMT1 enzymatic activity on the 208 substrates increased non-linearly with time for approximately three hours under the conditions used, then decreased. This behavior can be reasonably explained by previous findings that DNMT1 is subject to both allosteric activation and competitive product inhibition by methylated DNA [10,37,38]. It has been reported that the methylated cytosine product is a more potent activator than inhibitor [37]. Thus, over time as product accumulated, it stimulated the enzyme until a sufficiently high level of end product had accumulated that its inhibitory effect was dominant. Interestingly, DNMT1 activity was stimulated at a greater rate (approximately 2.4-fold) on the 208_{Mono} substrate than on the 208_{DNA} substrate. This result may indicate that the allosteric domain known to bind methylated DNA functions more efficiently on a mononucleosomal surface than on free DNA. Structural studies of DNA in mononucleosomes have found numerous differences between free DNA and mononucleosomes including the conformation of the DNA helix, the torsional angles of its bases, and of course its constraint in a coiled shape [14]. It is possible that one or more of these factors may facilitate the binding of methylated DNA, whether in *cis* or in *trans*, to the allosteric domain, thereby enhancing its ability to activate the enzymatic activity of DNMT1. The effect of mononucleosome structure on the allosteric domain of DNMT1 may explain why DNMT1 has a higher V_{max} on mononucleosome than on DNA (unlike Dnmt3a, which lacks this domain). This effect, while relatively subtle with mononucleosomal particles, may be amplified in vivo where chromatin fibers are much longer and tightly packaged into multi-layered suprastructures.

The fact that both DNMT1 and Dnmt3a bound mononucleosome with approximately the same affinity as DNA is perhaps not surprising. Although mammalian DNA methyltransferases selectively methylate cytosines of CpG pairs, they are thought to bind DNA non-specifically in a random fashion then scan along the molecule until they encounter a CpG substrate [10,39]. Therefore, although the mononucleosomal form of DNA was partially occluded by histones, it still presented free DNA surfaces to which the DNMTs could bind with equal affinity. Although the differences were small, DNMT1 bound 208_{DNA} with higher affinity than 208_{Mono}, while Dnmt3a bound 208_{Mono} with higher affinity than 208_{DNA}. Such preferential differences between the two enzymes may be even more pronounced in living cells on far longer pieces of chromatin and may reflect the different localizations of the two DNA methyltransferases (with DNMT1 targeting replication foci throughout S phase) [40,41]. Hill plots with positive coefficients, as well as the formation of incrementally super-shifting bands with increasing protein concentration, indicate that DNMT molecules bind cooperatively to the 208 bp substrate. Other chromatin-associated proteins, such as MENT, have also been shown to bind cooperatively to DNA [42]. This finding may help to account for such phenomena as methylation spreading or even the maintenance of heterochromatic regions. Several studies have shown that the aberrant de novo methylation of CpG islands in tumor cells, such as the one in the e-cadherin promoter region, is a gradual process and that methylation from regions adjacent to the CpG island may spread into the island, eventually resulting in silencing of the gene [43]. The cooperative binding of DNMT1 may

facilitate this process, especially if DNMT1 is over-expressed or improperly targeted in tumor cells.

The finding that DNMT1 co-purifies and co-immunoprecipitates with the chromatin remodeler hSNF2H indicates a direct coupling between these two epigenetic processes. Associations between the DNMTs and enzymes involved in chromatin remodeling, such as histone deacetylases and histone methylases, have been reported for DNMT1 and other DNA methyltransferases [23,31–33,44]. Other indirect connections come from findings that mutations of certain ATP-dependent chromatin remodeling enzymes of the SNF2 family, such as lsh and ATRX, result in disrupted DNA methylation patterns [16,17]. DNMT1 and DNMT3B also co-immunoprecipitate with Tip5, a component of the NoRC complex, which is a chromatin remodeling complex involved in rDNA gene silencing that also contains hSNF2H [45]. It is likely that in cells, copying of the methylated DNA template onto daughter DNA strands and remodeling of the chromatin fibers occur concomitantly, perhaps so concomitantly that the enzymatic components are joined into a single complex to ensure an efficient process. DNMT1 may require hSNF2H and/or other chromatin remodeling enzymes in order to gain access to its target sites in chromatin.

We further report that addition of purified hSNF2H enhances the binding of DNMT1 to the mononucleosome by at least 3-fold in an ATP-independent manner. This influence of hSNF2H upon DNMT1 may be increased in vivo with lengthy chromatin arrays or chromatin that is packaged into a more complicated or higher-order structure. In vitro the enhanced DNMT1 binding does not depend upon ATP hydrolysis, which indicates that it is unlikely to be a consequence of chromatin remodeling. Instead, it may be that the interaction between hSNF2H and DNMT1 stabilizes or otherwise conformationally alters the structure of DNMT1 and thereby increases its ability to bind its template. It may also reflect one of the limitations of a highly purified in vitro assay system, that is, other factors may be missing from the reaction which influence the properties of hSNF2H and DNMT1. Addition of other protein co-factors, as well as the use of other chromatin templates, will be the subject of future studies. This is, however, the first direct evidence that interaction of a DNA methyltransferase with a chromatin remodeling enzyme does produce a measurable change in its biological properties. Such a change, the increased DNMT binding to mononucleosome, is consistent with other reports that SNF2 family members are required for proper DNA methylation in mammalian cells.

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