The enzyme responsible for maintenance methylation of CpG dinucleotides in vertebrates is DNMT1. The presence of DNMT1 in DNA replication foci raises the issue of whether this enzyme needs to gain access to nascent DNA before its packaging into nucleosomes, which occurs very rapidly behind the replication fork. Using nucleosomes positioned along the 5 S rDNA gene, we find that DNMT1 is able to methylate a number of CpG sites even when the DNA major groove is oriented toward the histone surface. However, we also find that the ability of DNMT1 to methylate nucleosomal sites is highly dependent on the nature of the DNA substrate. Although nucleosomes containing the Air promoter are refractory to methylation irrespective of target cytosine location, nucleosomes reconstituted onto the H19 imprinting control region are more accessible. These results argue that although DNMT1 is intrinsically capable of methylating some DNA sequences even after their packaging into nucleosomes, this is not the case for at least a fraction of DNA sequences whose function is regulated by DNA methylation.

DNA methylation is an important epigenetic mark involved in gene silencing (1), X chromosome and transposon inactivation (2, 3), genomic imprinting (4), and chromosome condensation (5). Cytosine methylation of CpG dinucleotides is catalyzed by DNA methyltransferases (DNMTs), which belong to two distinct classes of enzymes. The first class consists of de novo methyltransferases (DNMT3a and DNMT3b) that methylate DNA irrespective of whether the template is hemi-methylated or not (6, 7). These enzymes are involved in the establishment of new DNA methylation patterns during development (8). The maintenance DNA methyltransferase DNMT1 belongs to the second class of enzymes. Disruption of the mouse Dnmt1 gene results in genome-wide demethylation and developmental arrest (9). Thus, the role of DNMT1 in propagating parental DNA methylation during replication cannot be substituted by other DNMTs.

The basic repeating unit of chromatin is the nucleosome core particle, which consists of 147 base pairs of DNA wrapped around the surface of an octamer formed by two molecules each of histones H2A, H2B, H3, and H4 (10). During DNA replication, the segregation of pre-existing histones and the deposition of histones H2A, H2B, H3, and H4 (11). Deposition of newly synthesized histones H3/H4 is mediated, at least in part, by a conserved protein known as chromatin assembly factor 1 (CAF-1), which brings histones H3/H4 to the replication fork via an interaction with the proliferating cell nuclear antigen (PCNA) (12–15). PCNA is a DNA polymerase processivity factor that forms a sliding clamp around DNA and interacts with a number of DNA replication and DNA repair proteins, many of which are constitutively present at the replication fork (16).

Interestingly, DNMT1 also interacts with PCNA at the replication fork (17–19) and a fraction of CpGs are subject to maintenance methylation prior to maturation of Okazaki fragments on the lagging strand (20, 21). This suggests that DNMT1 may need to be present immediately behind the replication fork in order to promote efficient methylation of nascent DNA prior to its packaging into nucleosomes. However, it is also known that a significant fraction of CpG dinucleotides escape this first wave of maintenance DNA methylation behind the replication fork (22). Packaging of these hemi-methylated CpGs into nucleosomes and chromatin higher order structures may prevent their subsequent methylation by DNMT1.

In this study, we addressed whether the first level of DNA compaction in eukaryotic chromosomes, the nucleosome core particle, represents a barrier to maintenance DNA methylation. We find that DNMT1 can methylate a number of distinct CpG sites even when the target cytosine in the major groove is facing the histone surface. However, at least with some DNA sequences, sites located in the central region of nucleosome core particles, where histones H3/H4 contact the DNA, are refractory to DNA methylation. Interestingly, we also find that DNA sequences derived from two loci in which DNA methylation is involved in imprinting (the Air promoter and H19 imprinting control region) exhibit differences in their accessibility to DNMT1. Although CpG sites packaged into nucleosomes reconstituted onto DNA derived from the H19 locus are efficiently methylated by DNMT1, nucleosomes assembled onto Air promoter DNA strongly block DNA methylation. Our results therefore argue that, although DNMT1 clearly possesses an intrinsic ability to methylate many CpG sites packaged into nucleosomes, there are striking differences in accessibility among naturally occurring DNA sequences in which function is modulated by DNA methylation.

**EXPERIMENTAL PROCEDURES**

**Preparation of Hemi-methylated DNA Substrates—Control and hemi-methylated 5 S DNA substrates were prepared in PCR reactions where one of the two primers contained 5-methylcytosine residues (Mircosynth, Balgach, Switzerland). PCR reactions were carried out as described previously (23). Amplified DNA was isolated from a 2% agarose gel and purified using MinElute gel extraction kit (Qiagen). For preparation of 5'–32P-labeled DNA, one of the PCR primers was phosphorylated with T4 polynucleotide kinase (New England Biolabs) and [γ-32P]ATP (Amersham Biosciences). DNA substrates containing the 5′-adenosylmethionine.
Nucleosomal DNA Methylation by DNMT1

S rRNA gene were obtained by PCR using an EcoRI fragment derived from plasmid pULESS as template. The DNA fragment encodes a 196-bp *Lytechinus variegatus* 5′ S rRNA gene fragment extending from nt −90 relative to the transcription start site (†). The 146-bp DNA substrates used in Fig. 4 were prepared using 146-mer synthetic oligonucleotides (GenScript Corp.) containing a single 5-methylcytosine residue either in the sense (CpG5) or the antisense strand (CpG96) as template for second strand synthesis primed by shorter oligonucleotides. The long template oligonucleotides spanning nucleotides −74 to +72 relative to the transcription start site. The 147-bp DNA substrate with the mouse *H19* CTCF binding site (nucleotides 3278–3424, GenBank™ accession number AF049091) was amplified by PCR from plasmid H18, which includes the *H19* imprinting control region from nucleotides 134–1490. The nucleosome reconstitution was confirmed by nucleoprotein gel electrophoresis, DNase I, and exonuclease III (ExoIII) digestion. Nucleoprotein gel analysis was performed using 6% polyacrylamide gels (acrylamide:N,N′-bisacrylamide ratio = 29:1) in 0.5× TBE buffer (Tris borate-EDTA) at 150 V for 2–3 h. DNase I digestion was carried out at 37 °C for 10–60 s in DNMT1 reaction buffer (see below) containing 5 mM MgCl₂. The reaction was stopped by the addition of 0.5% SDS, DNA was extracted with phenol:chloroform, precipitated with ethanol, and resolved in 6% polyacrylamide gels containing 7 M urea in 1× TBE for 3 h. DNase I digestion was carried out at 37 °C for 60 s, and the reaction was stopped by the addition of 0.5% SDS. DNA was extracted and analyzed in a 6% polyacrylamide gel containing 7 M urea in 1× TBE.

DNA Methylation Assays—DNA methylation reactions were carried out with 0.4 pmol of total DNA (free DNA + nucleosomal DNA) in 10 μl of 50 mM Tris-HCl, pH 7.8, 1 mM EDTA, 5% glycerol, 1 mM dithiothreitol, 100 μg/ml bovine serum albumin, 1 μM [methyl-3H]-S-adenosylmethionine ([3H]AdoMet) (NCI, 0.55 mCi/μM), and 25 ng (0.5 unit or 0.14 pmol) of recombinant human DNMT1 (New England BioLabs, 50 ng/μl or 1 unit/μl) in 50 mM NaCl in buffer A. Active DNMT1 fractions were applied to a Mono Q column (0.1 ml, Amersham Biosciences), which was eluted with a linear gradient from 0.05 to 0.6 M NaCl. Active fractions were pooled and further fractionated using either a Superox 6 (2.4 ml, Amersham Biosciences) gel filtration column or a Mono S column (0.1 ml, Amersham Biosciences) as described in Fig. 2A. A rabbit antisera against the N-terminal region of human DNMT1 (New England BioLabs) was used to follow DNMT1 by Western blotting. Column fractions were assayed for DNA methylation activity using 500 ng of a 196-bp 5′ S rDNA gene fragment containing three hemi-methylated CpGs.

**RESULTS**

Core Particle Reconstitution and Nucleosome Positioning—To address whether the DNA methylation activity of DNMT1 is influenced by nucleosomes, NCPs were assembled using purified histone octamers and DNA fragments derived from the *L. variegatus* sca urchins 5′ S rRNA gene. This sequence was chosen because of its known ability to position nucleosomes with remarkable precision (30). Hemi-methylated DNA substrates were produced by PCR (see “Experimental Procedures”). Histone octamers were purified from chicken erythrocytes (Fig. 1A) and assembled onto a 196-bp DNA fragment by stepwise salt dilution (26). Nucleoprotein gel analysis showed that, after nucleosome assembly, most of the DNA was found in a slower migrating form corresponding to NCPs (Fig. 1B). DNase I digestion revealed a ladder of prominent cutting sites with the 10-bp periodicity characteristic of NCPs, whereas naked DNA was cut randomly (Fig. 1C). We did not detect any differences in the nucleoprotein gel mobility or DNase I digestion pattern of NCPs reconstituted onto either non-methylated or hemi-methylated DNA (Fig. 1C, lanes 2 and 4). Three mononucleosome bands were detected in nucleoprotein gels (Fig. 1B). This was due to the presence of a mixture of NCPs in which histone octamers occupy distinct positions along the 196-bp DNA fragment. The slower and faster migrating species were designated N1 and N2, respectively. In some of our nucleoprotein gels, the faster migrating N2 nucleosome band clearly appeared as a doublet (Figs. 1B, lane 2), suggesting that N2 contained two octamer positions. To map octamer positions in these distinct species, NCPs containing a 5′-22p-labeled DNA fragment were resolved in nucleoprotein gels. Each species was extracted separately from the gel and subjected to ExoIII digestion. In this assay, ExoIII processively digests the 5′-labeled strand from the 3′-end until it reaches the 3′-border of the histone octamer where the enzyme exhibits a strong pause. Although the gel-purified N1 nucleosomes were contaminated by some N2 nucleosomes (Fig. 1D, lane 2), we detected a strong N1-specific ExoIII pause site (asterisk in Fig. 1D, lane 5). The length of the protected fragment (about 165 bp) argued that N1 consisted of an octamer located about 20–165 bp from the 5′-end, as depicted in Fig. 1F. Consistent with the presence of a doublet in nucleoprotein gels, ExoIII digestion of gel-purified N2 nucleosomes generated two protected fragments of 175 and 185 bp (asterisks in Fig. 1D, lane 6). N2 nucleosomes therefore consisted of two octamer positions, 30–175 bp and 40–185 bp from the 5′-end (Fig. 1E). ExoIII also produced a number of bands shorter than 147 bp (Fig. 1D). The most prominent bands were produced upon ExoIII digestion of both N1 and N2 nucleosomes isolated from nucleoprotein gels, arguing that they did not arise from alternative histone octamer positions.

**Methylation of Nucleosomal DNA by DNMT1**—Recombinant human DNMT1 purified from insect cells has been characterized extensively (31, 32). Recombinant DNMT1 was used to methylate 196-bp 5′ S rRNA gene fragments containing three hemi-methylated sites at positions 136, 158, and 181 of the antisense strand. Positions 136 and 158 are located inside in
each of the three octamer positions identified in N1 and N2, whereas position 181 is outside of N1 and at the very edge of N2 NCPs (Fig. 2C). NCPs were assembled onto cold DNA at increasing histone/DNA molar ratio and reconstitution was confirmed using nucleoprotein gels stained with ethidium bromide (Fig. 2A, lanes 1–3). With a histone/DNA molar ratio of 0.8, most of the input DNA was packaged into NCPs (Fig. 2A, lane 3); subsequent experiments did not exceed this ratio to avoid aggregation of histones and DNA at higher molar ratio of octamers and DNA. Under these conditions, species that migrated above NCPs (due to more than one octamer per 196-bp DNA fragment) or DNA aggregation were not detected (Fig. 2A). Free DNA or NCPs were incubated with DNMT1 and [3H]AdoMet and incorporation of [3H]methyl into the DNA substrate quantitated using a filter binding assay. Remarkably, a very similar kinetics of DNA methylation was obtained with free DNA or NCP reconstitution mixtures containing histone:DNA molar ratio of 0.4 and 0.8. This result suggested that the hemi-methylated sites in the 196-bp DNA substrate were equally accessible to DNMT1 in both free DNA and NCPs. Free DNA did not accumulate during the course of DNMT1 reactions with NCPs (Fig. 2A and B). This showed that NCPs containing hemi-methylated DNA were stable and that DNMT1 did not disrupt NCPs under our assay conditions.

To demonstrate that DNA methylation did occur within intact NCPs, the reaction products containing [3H]methylated DNA were resolved in nucleoprotein gels. The gels were stained with ethidium bromide, and DNA methylation was detected by fluorography. DNA or NCP substrates lacking methylation were not methylated (Fig. 2B, lanes 1 and 3). Therefore the de novo methyltransferase activity that has been reported for recombinant DNMT1 (32) was negligible under our assay conditions. In contrast, when the DNA substrate was hemi-methylated, DNA methylation was detected by fluorography in both the free DNA and NCPs present in the same reaction. There was no striking preference for methylation of free DNA, and both N1 and N2 nucleosomes were methylated (Fig. 2B, lanes 2 and 4).

Because the DNA substrate used in these experiments contained three hemi-methylated CpGs and position 181 was located outside of the histone octamer in the N1 nucleosome and at the border of N2 nucleosomes (Fig. 2C), it was possible that all of the DNA methylation observed with NCPs occurred at CpG181. To exclude this possibility, we examined whether CpG136 and CpG158, both located inside the N1 and N2 nucleosomes (Fig. 2C), were methylated. To map the sites of methylation, [3H]methylated DNA was extracted from nucleoprotein gels and digested with Scal or Fnu4II, and methylation of each restriction fragment was detected by polyacrylamide gel electrophoresis (PAGE) and fluorography (Fig. 2D). Scal digestion produced a short 30-bp fragment that contained only CpG181 and a long 166-bp fragment that contained both CpG136 and -158. This digest revealed that both CpG136 and/or -158 were methylated in both free DNA and NCPs (Fig. 2D, lanes 2 and 5). Recovery of the short 30-bp Scal fragment (which contained CpG181 only) after DNA extraction and gel electrophoresis was inefficient. However, a comparison of the ethidium bromide stain and the fluorogram revealed that equal
amounts of undigested (196-bp) and 166-bp Scal fragments were methylated to similar extents (Fig. 2D, lanes 2 and 5), arguing that DNMT1 did not methylate CpG181 efficiently even in free DNA. Given that DNMT1 is relatively non-sequence-specific, this may reflect an effect of DNA conformation, rather than DNA sequence context. Fnu4HI digestion produced a long 148-bp DNA fragment, which contained only CpG136, and a short 48-bp fragment, which included both CpG158 and -181. Methylation of CpG136 was observed in both free DNA and NCPs (Fig. 2D, lanes 3 and 6). Similarly, [3H]methyl incorporation into the 48-bp fragment, which primarily reflected methylation of CpG158 (given the lack of methylation of CpG181 revealed by Scal digestion), also occurred in both free DNA and NCPs (Fig. 2D, lanes 3 and 6). CpG158 was located near the edge of N1/N2 NCPs (about 7 bp in N1 and either 17 or 27 bp in the two N2 nucleosomes). In each case, CpG158 was part of the DNA segments that contact histones H2A/H2B. In contrast, CpG136 was located about two helical turns (22 bp) further from the edge of the nucleosome in DNA segments that contacted histones H3/H4. Methylation of CpG136 suggested that other more centrally located CpG dinucleotides may also be methylated by DNMT1.

To examine the influence of CpG position on the efficiency of DNA methylation in more detail, we assembled NCPs onto fragments of the L. variegatus 5 S rRNA gene containing single hemi-methylated sites at CpG96 and -138. Unlike the 196-bp DNA substrate used in Fig. 2, nucleoprotein gel analysis of NCPs reconstituted onto 162-bp DNA substrates gave rise to a single nucleosome band, suggesting that histone octamers occupied one major position (Fig. 3B). ExoIII mapping revealed nucleosome-specific pause sites in the sense and antisense strands (Fig. 3A). These ExoIII pause sites were consistent with the presence of one predominant octamer position where the target cytosine residues at CpG96 and CpG138 were respectively located about 55 and 15 bp from the edge of the histone octamer (Fig. 3A). As illustrated in Fig. 3B, these DNA segments contacted histones H2A/H2B (CpG138) and histones H3/H4 (CpG96). The efficiency of methylation of CpG138 was only slightly reduced upon packaging into NCPs (50% compared with free DNA), whereas methylation of CpG96 was more severely impaired (15%, Fig. 3B). Thus, DNMT1 preferentially methylated target cytosine residues located near the edge of the nucleosome. Experiments carried out with a number of other DNA substrates based on the 5 S rRNA gene also reached the same conclusion (data not shown).

Maintenance methylation requires recognition of hemi-methylated target sites in which the 5-methyl group of cytosine protrudes from the major groove of DNA. Following this recognition step, the phosphodiester backbone surrounding the cytosine to be methylated needs to be rotated to bring the target cytosine into the catalytic site of the enzyme. This DNA conformational change, often referred to as base flipping even though no direct contact with the base is actually involved, is also catalyzed by several DNA repair enzymes (33). Histone-DNA interactions could potentially influence both recognition of hemi-methylated CpGs and base flipping. To test whether the orientation of the target cytosine relative to the histone octamer influences the efficiency of DNA methylation, we pre-
pared two NCPs that contain single hemi-methylated CpGs separated by 6 bp (CpG35 and CpG41, Fig. 4A). DNase I preferentially cleaves nucleosomal DNA where the minor groove is wider, i.e. sites where the minor groove faces away from the histone surface. Based on DNase I footprinting, the target C at CpG35 is located halfway between two strong DNase I cleavage sites (Fig. 1C). At this site, the minor groove is facing the histone surface, and the major groove containing the target C is therefore exposed to the solvent. In contrast, the target C at CpG41 is only 1 bp away from a prominent DNase I cleavage site (Fig. 1C). The target C at CpG41 must therefore be located in a compressed major groove that is facing toward the histone surface. Despite their different orientation relative to the histone surface, CpG35 and -41 were methylated with similar efficiency by DNMT1 (Fig. 4A). The recent high-resolution structure of the nucleosome core particle provides valuable information about conformational flexibility of the DNA phosphodiester backbone (assessed from real-space R-factors (10)).

A few sites located 15 and 25 bp either side of the dyad axis (where the major groove is facing the histone octamer) exhibit striking differences in conformational flexibility of the two DNA strands (10). Because base flipping requires a conformational change in the DNA phosphodiester backbone (33), we reasoned that methylation of CpG dinucleotides positioned at or near these sites might occur more efficiently when the target C is in the strand that exhibits the highest conformational flexibility. To test this possibility, we prepared two NCPs containing a target C at CpG95 in the sense strand or CpG96 in the antisense strand. At this site, DNMT1 methylated NCP DNA slightly less efficiently than free DNA (~40%), but there was no striking difference in the methylation of target cytosine residues located in either the sense or the antisense strand (Fig. 4B). Interestingly, methylation of CpG96 occurred more efficiently within this 146-bp nucleosomal substrate (Fig. 4B) than when the same CpG and flanking DNA sequences were located in a mononucleosome reconstituted onto a 162-bp DNA substrate (Fig. 3B). CpG96 may be present in slightly different histone environments in the two nucleosome substrates. Alternatively, crystallographic studies revealed that nucleosomes containing 146 bp of DNA, as opposed to the optimal length of 147 bp, exhibit a considerable degree of DNA conformational distortion and flexibility (10). It is possible that greater DNA
flexibility facilitates CpG96 methylation by DNMT1 in the nucleosome core particle containing only 146 bp of DNA.

CpG Methylation of Nucleosomes Containing H19 and Air DNA—Paternal chromosome-specific expression of the Igf2 gene is achieved by differential methylation of the upstream region of the H19 gene (24), which is itself located downstream of Igf2. Expression of Igf2 is ensured by the presence of methylation in the upstream region of H19 (34), which prevents binding of the CTCF protein and allows a powerful enhancer located downstream of H19 to trans-activate the Igf2 promoter (35, 36). The absence of methylation from the H19 upstream region on the maternal chromosome allows CTCF to bind chromosome is due to the expression and

nal alleles (37). In this case, silencing of the paternal Stream genes, known as H19 (35, 36). The absence of methylation from the paternal and mat-

ion in proliferating cells.

Air methylation of the H19 upstream region (34) and a CpG

that is part of an MluI site in the Air promoter (37). We reconstituted 147-bp DNA fragments containing these hemi-methylated CpGs into NCPs such that the target cytosines were located in the central region of the nucleosome, where histones H3/H4 contact the DNA and conformational flexibility is minimal (10). Surprisingly, methylation of CpG68 in H19 occurred with equal efficiency in both free DNA and NCPs (Fig. 5B, columns 1 and 2). Nucleoprotein gel analysis did not reveal any appearance of free DNA, suggesting that nucleosomes were stable during the course of the methylation reaction (Fig. 5B, agarose gel). This result effectively ruled out the possibility that the accessibility of the CpG68 site to DNMT1 was merely due to inherent instability of NCPs reconstituted onto H19 DNA. In striking contrast to H19 CpG68, we found that nu-

clesomes severely impeded DNMT1-dependent methylation of a site located in a very similar location (CpG67) of a DNA fragment derived from the Air promoter (Fig. 5B, columns 3 and 4). Remarkably, the same target site remained inaccessible to DNMT1 even when placed much closer to the edge of the nucleosome (20 bp) by reconstitution onto a different DNA fragment derived from the Air promoter (Fig. 5B, columns 5 and 6). Thus, the Air promoter site analyzed here was methyl-

lated poorly, irrespective of whether it was present near the edge or within the central region of the nucleosome. Competitive nucleosome reconstitution experiments (39) did not reveal any significant difference in the affinity of histone octamers for the H19 and Air DNA substrates (data not shown). Thus, the differential methylation of H19 and Air DNA did not simply reflect a higher stability of the NCPs containing Air DNA.

To test the possibility that NCPs containing Air DNA may be generally inaccessible to DNA methylation, the same preparation of NCPs used for the DNMT1 experiments were incubated with the bacterial de novo methyltransferase, Sss1. The H19 and Air DNA substrates, respectively, contained 10 and 11 CpG dinucleotides, several of which were located near the ends of each DNA (Fig. 5A). Surprisingly, de novo methylation was severely inhibited in both the H19 and Air NCPs, arguing that many sites in each nucleosome were protected from de novo methylation (Fig. 5C). Unlike DNMT1, the Ssdl de novo methyltransferase was therefore strongly inhibited by nucleosomes irrespective of the DNA substrate used. This result also reinforced our conclusion that NCPs containing H19 DNA were not inherently unstable.

Nucleosome Methylation by DNMT1 Purified from Human Cells—It seemed possible that DNMT1 purified from human cells may be distinct from recombinant DNMT1 in its ability to methylate nucleosomal DNA. To address this possibility, we purified active DNMT1 from HeLa cell nuclear extracts. DNMT1 purification over a series of conventional chromatography steps was followed by Western blotting and DNA methylation assays (Fig. 6). A Coomassie Blue-stained gel following the last chromatographic step revealed the presence of a major 190-kDa polypeptide that co-fractionated with DNMT1 activity (Fig. 6B). This polypeptide was identified as DNMT1 by Western blotting and mass spectrometry. Our most purified DNMT1 fractions did not contain any polypeptides that were stoichiometric with DNMT1. However, we could not rule out the possibility that DNMT1 complexes may have been disrupted during the course of purification. To test this possibility, we determined the native size of DNMT1 purified from human cells by gel filtration and compared it with that of DNMT1 present in crude nuclear extracts. DNMT1 from crude extracts eluted as a broad peak. HDAC1 was previously identified as a component of a multi-protein complex containing DNMT1 (40). The higher molecular mass fractions containing DNMT1 coincided with one of two peaks of HDAC1 (Fig. 6C, fractions...
However, substantial amounts of DNMT1 were also present in lower molecular mass fractions where HDAC1 was not detectable (Fig. 6C, fractions 19–22). The form of DNMT1 we purified (Mono Q pool in Fig. 6A) was mainly present in fractions 18–20 of the gel filtration column (Fig. 6C, bottom). This result showed that the form of DNMT1 that we had purified was not associated with other proteins and represented a major fraction of the enzyme present in crude extracts. DNMT1 purified from human cells (Mono S pool) had a 2–3-fold higher specific activity than recombinant DNMT1, and both enzymes were capable of methylating accessible nucleosomal sites (not shown). In addition, nucleosomal CpGs that were poorly methylated by recombinant DNMT1 were also refractory to methylation by the enzyme purified from human cells.

Fig. 5. Methylation of NCPs containing the H19 and Air DNAs by DNMT1 and SssI. A, NCPs were reconstituted onto 147-bp DNA substrates derived from the H19 imprinting control region or the Air promoter. The position of the hemi-methylated CpG in each NCP is indicated by a flag. The locations of all CpG dinucleotides in the DNA substrates are shown by tick marks. B, free DNA and NCPs were resolved in 6% polyacrylamide nucleoprotein gels that were stained with ethidium bromide. DNA methylation was carried out with free DNA or NCP substrates in the presence of DNMT1 (B) or SssI (C) and [3H]AdoMet. DNA methylation was measured using a filter binding assay and liquid scintillation counting. The bar graphs show the percentage of methylation of each NCP compared with free DNA (an average of three independent experiments).

Fig. 6. Purification of DNMT1 from human cells. A, purification scheme. B, fractions from the final Mono S column were analyzed in an SDS-8% polyacrylamide gel (upper panel, lane M, protein size markers) and assayed for DNMT1 activity using [3H]AdoMet and a 196-bp 5S rRNA gene fragment containing three hemi-methylated sites as substrate (bottom panel). C, fractionation of crude nuclear extracts and partially purified DNMT1 by gel filtration. A crude HeLa cell nuclear extract (top and middle panels) and a partially purified DNMT1 fraction (bottom panel) were fractionated using a Superose 6 gel filtration column. Proteins in each column fraction were resolved in an SDS-8% polyacrylamide gel. DNMT1 and HDAC1 in the crude nuclear extract were detected by Western blotting, and partially purified DNMT1 (Mono Q fraction) was visualized by Coomassie Blue staining (bottom panel). Lane M, protein size markers. The elution position of protein gel filtration markers is shown at the top.
Inhibition of methylation at these sites was not significantly relieved by the addition of increasing amounts of cellular DNMT1 (Fig. 7A) or prolonged incubation with the enzyme (Fig. 7B).

**DISCUSSION**

In this study, we sought to determine whether DNA wrapped around the histone octamer is accessible to the maintenance DNA methyltransferase DNMT1. We found that, although DNMT1 clearly possesses the intrinsic ability to modify CpG dinucleotides on the surface of nucleosomes, its activity is highly dependent on the nature of the DNA substrate used. Although a number of CpG sites on the surface of nucleosomes reconstituted on the 5 S rRNA gene or the H19 promoter were methylated efficiently, nucleosomes containing the Air promoter were refractory to methylation by DNMT1.

**Nucleosome Structure and Maintenance Methylation**—Three distinct mechanisms could potentially influence the ability of DNA methyltransferases to gain access to CpG dinucleotides within nucleosomes: direct recognition and spontaneous site exposure via nucleosome sliding or transient dissociation of short stretches of DNA. Widom and co-workers (41) demonstrated that short-range nucleosome sliding along the 5 S rRNA gene is negligible and therefore does not significantly contribute to site exposure under ionic strength and temperature conditions very similar to those used in our assays. Instead, they proposed that site exposure occurs through the spontaneous transient dissociation of short stretches of DNA, beginning at one end of the core particle and extending progressively inwards (41, 42). The dissociation of each 10-bp segment of DNA requires breaking additional histone-DNA interactions in the minor groove. As a result, the probability of exposure is greater for sites located near the edge than those within the central portion of the nucleosomal DNA. This mechanism may well explain why DNMT1 preferentially methylated CpG dinucleotides located near the edge of NCPs reconstituted onto the 5 S rRNA gene. However, our experiments with H19 and Air NCPs showed that sites located near the edge of NCPs were blocked from de novo methylation by SsSl (Fig. 5C) or maintenance methylation by DNMT1 (Fig. 5B). This argues that spontaneous site exposure is not sufficient to facilitate methylation of these NCPs. This is most likely because of the fact that spontaneous site exposure is a very transient process (41). Consequently, perhaps the affinity and/or concentration of SsSl and DNMT1 are not sufficient to methylate transiently exposed sites under our experimental conditions.

Many parameters are likely to modulate the direct recognition and methylation of CpG dinucleotides on the surface of nucleosomes. The recognition of the 5-methyl group of cytosines, which protrudes from the major groove, is facilitated by the fact that histone-DNA interactions occur exclusively via the minor groove (10). Thus, CpG dinucleotides are likely to be relatively accessible even where the major groove faces toward the histone surface. However, it is not known whether DNA contacts outside of the target CpG facilitate DNMT1 binding or activity. Our efforts to footprint DNMT1 onto hemi-methylated DNA or nucleosomes did not succeed. The importance of knowing whether DNMT1 needs to contact the DNA beyond the target CpG is apparent from the fact that the MeCP2 protein, which recognizes a single CpG dinucleotide methylated on both strands, is incapable of binding many of its target sites in nucleosomes (43). This is presumably because MeCP2 needs to contact up to 5 bp either side of the target CpG (44). It seems likely that DNA conformational flexibility, which varies considerably along the nucleosomal DNA (10), will also have an impact on base flipping of the target cytosine. This is an important issue because, in addition to DNMT1, a large number of DNA repair enzymes are also known to utilize this mechanism to gain access to lesions in DNA bases. These include several DNA glycosylases (33). Interestingly, two distinct uracil DNA glycosylases are substantially inhibited by NCPs (23, 45). It is not known whether inhibition of these enzymes by nucleosomes occurs at the level of base flipping. It is also clear that the path of the DNA helix (smooth bending versus kinked DNA) around the histone octamer is dependent upon the local DNA sequence (46). The fact that equivalent positions in the H19 and Air NCPs exhibit striking differences in their methylation by DNMT1 (Fig. 5B) may be due to such sequence-dependent variations in local DNA structure and/or conformational flexibility.

**Maintenance Methylation, Nucleosome Assembly, and DNA Replication**—Although a large number of CpGs are rapidly methylated behind the replication fork, a sizeable fraction also exhibit delayed DNA methylation (22, 47). Our results argue that a fraction of the CpGs that escape maintenance methylation during replication can be modified via the intrinsic ability of DNMT1 to methylate at least some CpGs on the surface of nucleosomes. However, other factors such as the presence of histone H1 or folding into chromatin higher order structures (e.g. CpGs packaged into heterochromatin) may further restrict access to certain CpGs in vivo. Methylation of those CpGs may
necessitate the additional participation of ATP-dependent nucleosome remodeling factors. For instance, the nucleolar remodeling factor, NoRC, plays a role in promoting the methylation and silencing of a fraction of rRNA gene repeats and associates with both DNMT1 and DNMT3b in vivo (48). In addition, mice lacking the putative ATP-dependent remodeling factor, Lsh, exhibit perinatal lethality and substantial losses of DNA methylation (as much as 70–90%) throughout the genome in several tissues (49). However, it is not clear whether Lsh exerts this function through de novo methylation during embryonic development, rather than maintenance DNA methylation during replication. In this respect, given the profound effect of nucleosomes on DNA methylation by SasI, it will be interesting to determine whether the de novo methyltransferases DNMT3a/3b are also inhibited by nucleosomes. Clearly, de novo methyltransferases must be prevented from methylating CpG islands associated with housekeeping genes (50, 51). It seems likely that chromatid structure does play a role in preventing inappropriate promoter inactivation via de novo DNA methylation. This issue is interesting because tumor suppressor genes are often silenced by promoter methylation (52).

Faithful maintenance methylation over many cell divisions is critical for at least a subset of key CpG dinucleotides such as those involved in genomic imprinting. Seen from this perspective, even a mildly inhibitory effect of nucleosomes on DNMT1 would result in substantial losses in DNA methylation over the course of many cell divisions. This argument may be sufficient to justify the presence of DNMT1 at the replication fork where it binds to the PCNA clamp, an interaction that has been reported to stimulate the methylation of naked DNA (53). CAF-1-dependent deposition of histones H3/H4 is known to occur almost as soon as sufficient DNA is present behind the replication fork. Given that both CAF-1 and DNMT1 interact with PCNA via similar motifs (15, 18), it will be important to determine how these two proteins can function on nascent DNA without mutually interfering with each other at the replication fork.

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