Interaction between DNMT1 and DNA replication reactions in the SV40 *in vitro* replication system

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In contrast to normal cells, cancer cells exhibit both genetic and epigenetic instability. These unique properties give rise to genetic and epigenetic heterogeneity in a given population of cancer cells and provide a means for the population to undergo phenotypic progression by clonal selection. DNA methylation at CpG dinucleotides is one of the epigenetic marks that are frequently disturbed in cancer cells. To understand how the CpG methylation pattern is changeable in cancer cells, it is necessary to know how it is faithfully maintained in normal cell proliferation. Toward this goal, we have developed a novel *in vitro* system that is based on the well-established SV40 *in vitro* replication system and functions to reconstitute concurrent DNA replication and DNA maintenance methylation reactions. We found that DNA methylation was maintained only when exogenous DNA methyltransferase 1 (DNMT1) and *S*-adenosyl methionine (SAM) were added to the reaction. We demonstrated that DNMT1 associates with replicating and/or replicated chromatin irrespective of the DNA methylation status of template DNA. Moreover, the PCNA-binding domain (PBD) of DNMT1 is not required for the association. Taken together, we suggest that DNMT1 is recruited to replicating and/or replicated chromatin in a constitutive manner independent of the DNA methylation reaction. The *in vitro* system described in this report is very useful for analyzing the molecular mechanism underlying the DNA maintenance methylation reaction. (*Cancer Sci* 2008; 99: 1960–1966)

Genomic information is encoded not only by DNA sequence but also by epigenetic modifications. DNA methylation is one of the epigenetic modifications in higher eukaryotes (reviewed in Klose and Bird[1]). In mammalian cells, DNA methylation occurs at 5′-carbon of cytosines in 5′-CpG-3′ dinucleotide (CpG site), and 60–90% of total CpG sites and 4% of total cytosines are methylated. DNA methylation in the promoter region of genes represses the transcription.

The distribution of methylated CpG (mCpG) is frequently disturbed in cancer cells compared to normal counterparts (reviewed in Ushijima and Baylin[2,3]). Cancer cells often show DNA hypomethylation of the genome on average, which may contribute to genomic instability. At the same time, it is often observed in cancer cells that cytosine methylation is dense at specific loci including tumor suppressor genes (TSGs), thereby repressing TSGs and contributing to the progression of the disease. However, it is not known how cancer cells accumulate such adaptive changes in the mCpG pattern.

In normal cells, once an mCpG pattern is established during development and differentiation, it should be faithfully maintained in cell proliferation throughout life. When mCpG is replicated by the conventional DNA replication mechanism, not methylated cytosine but unmethylated cytosine is incorporated to the base pair with the template guanine. Then, enzymes called DNA methyltransferases (DNMTs) methylate the hemi-methylated CpG to produce fully methylated CpG. This reaction is called the maintenance methylation because it ensures the transmission of the mCpG pattern to daughter cells. Three DNMTs, DNMT1, DNMT3a, and DNMT3b, are known, and DNMT1 is primarily responsible for maintenance methylation, while DNMT3a and DNMT3b are responsible for methylating cytosine of unmethylated CpG sites (de novo methylation) (reviewed in Goll and Bestor[4]).

It has been argued that DNMT1 and DNA replication reactions are intimately coordinated *in vivo*. Cytological approaches indicate that DNMT1 is colocalized with replication foci during the S phase.[5,6] Inhibition of DNMT1 negatively affects DNA replication efficiency in human cells.[7] It was suggested that maintenance methylation of the nascent strand occurs immediately after its synthesis.[8–10] Fractionation of human cell extracts showed that the peak fraction of DNA methylation activity coincided with that of DNA replication activity.[11]

Several hypotheses, although not mutually exclusive, have been proposed to explain how DNMT1 reaction is coupled with DNA replication. First, DNMT1 has the intrinsic property of preferentially binding and methylating hemi-methylated CpG.[12] Second, it was shown that DNMT1 binds to proliferating cell nuclear antigen (PCNA), a replication protein serving as a sliding clamp to ensure processive DNA synthesis. This interaction provides a means to establish a direct link between DNMT1 and the replication machineries.[13,14] Third, very recently, DNMT1 was found to be recruited to hemi-methylated DNA at the replication focus via a protein called UHRF1/NP95/ICBP90.[15,16] Clearly, numerous protein–protein interactions have been reported for DNMT1. However, the relative significance of these interactions in the coupling of DNMT1 with replication reactions remains elusive. A biochemical approach that analyzes the DNA replication and DNA methylation reactions simultaneously may provide useful information to answer the question.

SV40 (Simian virus 40), which infects humans and monkeys, replicates its circular DNA genome using the host's replication machineries and large T antigen (T-ag) as the sole virus-encoded protein required for the replication. An *in vitro* system consisting of cytoplasmic S100 extracts derived from 293 cells and recombinant T-ag was established to reconstitute the replication of SV40 DNA (reviewed in Kelly[17]). The DNA in the reaction is replicated bidirectionally starting from the replication origin. Although the system has been widely used to dissect the mammalian replication reaction, it has not been used to study the replication-coupled DNMT1 reaction *in vitro*. In this study, we developed an SV40 system that reconstitutes DNA replication and maintenance methylation reactions concurrently. Using this system, we investigated how DNMT1 and replication reactions interact.

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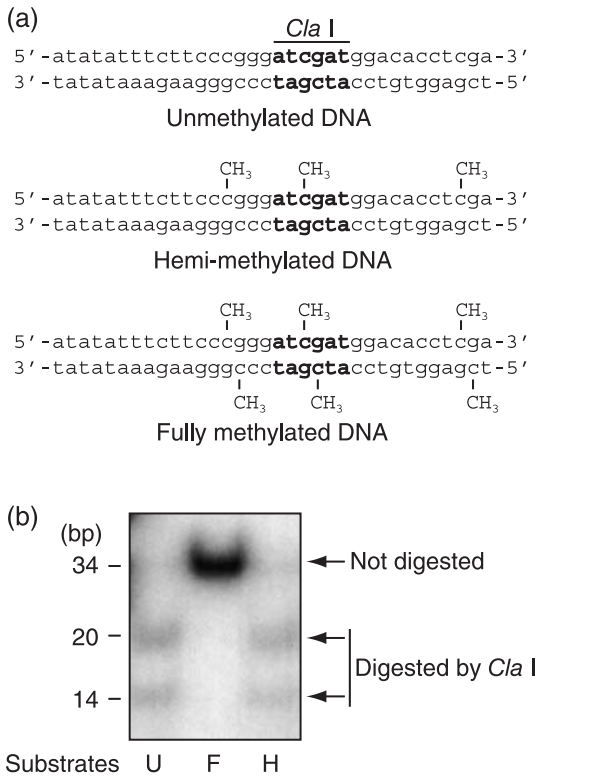

Fig. 1. *Cla* I digestion of methylated DNAs. (a) Un-, hemi-, and fully methylated oligonucleotide substrates for *Cla* I digestion. *Cla* I recognition sites are indicated in bold letters. (b) Oligonucleotides were digested with *Cla* I, electrophoresed on 20% polyacrylamide gel, and analyzed by autoradiography. U, unmethylated; H, hemi-methylated; and F, fully methylated oligonucleotides. The signal intensities of the 20-bp and 14-bp DNA fragments appeared weaker than expected (the half of that of the 34-bp fragment). It was possible that the signal-radioactivity dose relationship was not within the range of linearity.

Materials and Methods

***Cla* I digestion of methylated oligonucleotides.** Double-stranded oligonucleotides for *Cla* I digestion were prepared by annealing the following oligonucleotides: 5′-ATATATTTCTTCC*CG*G-G**AT*CG*AT**GGACACCTCGA-3′ (upper strand) and 5′-TCGAG-GTGTCCATCGATCCCGGGAAGAAATATAT-3′ (lower strand).

Cytosines within the underlined CpG sites were methylated as shown in Fig. 1a. Bold letters indicate *Cla* I sites. The 5′ end of oligonucleotides was labeled with [γ-^{32}P] dATP. 2.8 ng of oligonucleotide DNAs was digested with 67 U of *Cla* I in 10 μL of 1 × M buffer (10 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 1 mM DTT, and 50 mM NaCl) at 30°C for 2 h.

Preparation of 293 cell S100 extracts and T-ag. Cytoplasmic extracts from human 293 cells (S100 extracts) were prepared as described previously.[18] Recombinant T-ag was expressed in Sf9 insect cells infected with T-ag-expressing baculovirus and purified by affinity chromatography, as described previously.[18]

Preparation of templates in the SV40 *in vitro* replication system. pSVO11C was constructed by inserting a *Cla* I site at the *Ssp* I site of pSVO11.[19] Plasmids were amplified in *E. coli* strain JC8679 (*E. coli* Genetic Stock Center, USA) defective for *dam* and *dcm* methylase genes.[20] The absence of methylated DNAs in thus prepared plasmid DNAs was confirmed by *Mbo* I and *Eco*R II digestions (data not shown). Closed circular forms of plasmid DNAs were purified by centrifugation in CsCl-ethidium bromide gradients. Plasmid DNAs were methylated at CpG sites using *Sss* I methylase (New England Biolabs).

Preparation of recombinant DNMT1 (rDNMT1). Mouse DNMT1s were produced in Sf9 insect cells as recombinant proteins C-terminally tagged with an 8xHis sequence (rDNMT1-H) or N-terminally tagged with a 6xHis-3xHA-3xFlag sequence (HHF-rDNMT1) using the Bac-to-Bac baculovirus expression systems (Invitrogen, USA). The rDNMT1-H and HHF-rDNMT1 cDNAs were cloned into pFastBac1 and pFastBacHTb vectors (Invitrogen), respectively. The T164A mutant DNMT1 cDNA was constructed by the site-directed mutagenesis. The vectors were used to obtain the baculoviruses expressing the proteins. The proteins were produced in Sf9 insect cells infected with the baculoviruses. The proteins were purified using HiTrap Chelating HP columns (GE Healthcare).

SV40 *in vitro* replication system. *In vitro* replication of SV40 DNAs was conducted as described previously.[19] Briefly, 150 ng of plasmid DNAs was subjected to the reaction containing 1 μg of T-ag and 180 μg of S100 extracts in 25 μL for 1 h at 37°C. For the analyses of replicated DNA by autoradiography, nascent strand DNAs were labeled with [α-^{32}P] dATP present in the replication reaction. 640-μM *S*-adenosyl methionine (SAM) (New England Biolabs) and 0.32–2 μg of rDNMT1 were added where appropriate. The reactions were terminated by chilling the mixture on ice or by adding 2.5 μg of aphidicolin to the reaction (100 ng/μL).

Analyses of maintenance methylation by TLC. Nascent strand DNAs were labeled with [α-^{32}P] dGTP present in the replication reaction. Nuclease digestion and TLC were conducted as described previously.[21] Briefly, DNA was digested with micrococcal nuclease (Sigma) and spleen phosphodiesterase II (Sigma) to generate 3′-monophosphate nucleotides. The mononucleotides were separated on TLC plates (Funakoshi, Japan) and analyzed by autoradiography. Separation in the first and second dimensions was done in buffers containing isobutyric acid, H_2O, and NH_4OH (66:20:1, v/v), and saturated $(NH_4)_2SO_4$, isopropanol, and 1 M NaOAc (80:2:18, v/v), respectively.

Chromatin immunoprecipitation experiments. pSVO11C was replicated in the presence of 2 μg of rDNMT1-H. DNA-protein complexes were semipurified using SizeSeptember 400 Spun Columns (GE Healthcare), cross-linked with 1% formaldehyde, and sonicated with Sonifier 250 (Branson). rDNMT1-H and RPA were immunoprecipitated using anti-His antibody (sc-803; Santa Cruz, USA) and anti-RPA32 antibody (ab16850; Abcam, England), respectively. Coimmunoprecipitated DNA fragments were analyzed by real-time polymerase chain reaction (PCR) using SYBR Green PCR Master Mix (Applied Biosystems) and the Sequence Detection System 7000 (Applied Biosystems). The following primers were used in real-time PCR: GCACG-AGTGGGTTACATCGAA (nucleotides 1230–1250 of pSVO11C) and TCTCAAGGATCTTACCGCTGTTG (nucleotides 1259–1281 of pSVO11C).

Identification of DNMT1 bound to biotin-labeled replicated DNA. pSVO11C was replicated in the presence of 1.5 μg of rDNMT1-H; 100 μM each of dATP, dCTP, dGTP, and dTTP; and 10 μM biotin-dUTP. This concentration of biotin-dUTP does not inhibit the replication reaction (data not shown). After DNA replication, free nucleotides were removed from the reaction using ProbeQuant G-50 Micro Columns (GE Healthcare). Biotin-labeled replicated chromatin was isolated using avidin-coated beads (MagneSphere Magnetic Paramagnetic Particles; Promega, USA). It was confirmed that more than 90% of replicated DNAs were captured onto the beads (data not shown). rDNMT1 and DNA polymerase δ coprecipitated with DNAs were analyzed by immunoblotting analyses using anti-His antibody and anti-DNA polymerase δ catalytic subunit (p125) antibody (sc-8797; Santa Cruz), respectively.

Immunoprecipitation-immunoblotting experiments pSVO11C were replicated in the presence of 2 μg of HHF-rDNMT1. After

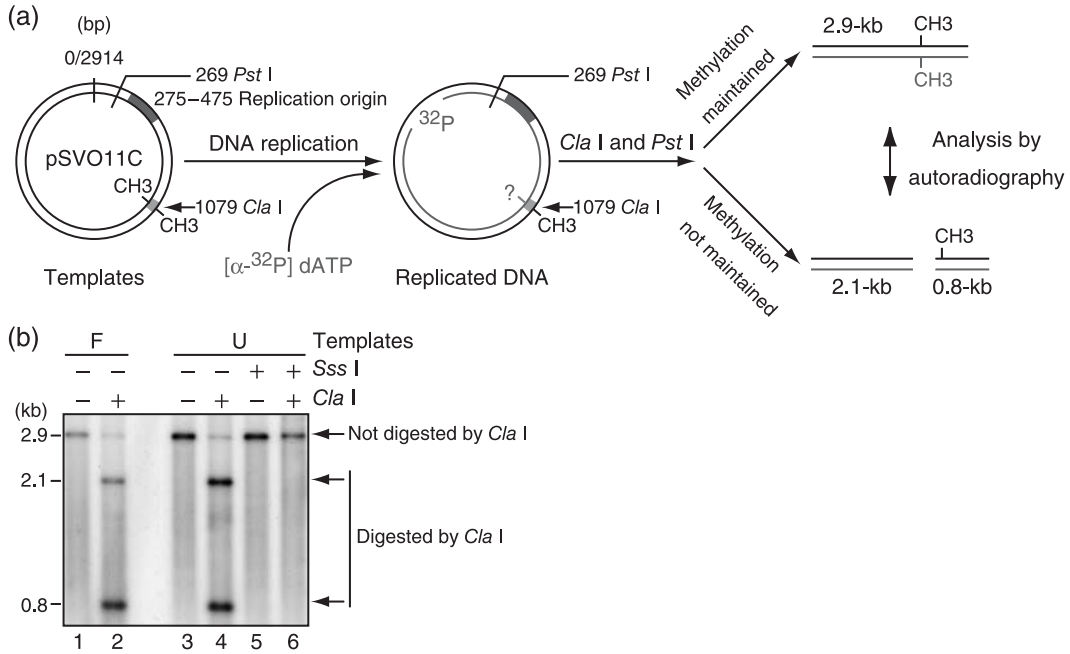

Fig. 2. DNA methylation is not maintained in the conventional SV40 *in vitro* replication system. (a) Experimental procedure. pSVO11C was replicated in the presence of [α-^{32}P] dATP. Replicated DNA was linearized by *Pst* I and *Cla* I digestions. Digested DNAs were electrophoresed on 1.0% agarose gel and analyzed by autoradiography. When DNA methylation is maintained, replicated DNA is resistant to *Cla* I digestion and a 2.9-kb *Pst* I fragment is observed. When DNA methylation is not maintained, 2.1-kb and 0.8-kb *Cla* I-*Pst* I digests are observed. Positions of relevant sites in pSVO11C are shown in nucleotide number. (b) Un- or fully methylated pSVO11C DNAs were replicated and analyzed as described in (a). In lanes 5 and 6, replicated DNAs were fully methylated by *Sss* I methylase prior to restriction digestion. U, unmethylated and F, fully methylated templates. The signal intensity of the 2.1-kb band was expected to be approximately three times stronger than that of the 0.8-kb band, which we did not observe in this experiment. We do not know the reason for this observation.

DNA replication, HHF-rDNMT1 was immunoprecipitated using anti-Flag antibody (A2220; Sigma). Immunoprecipitated HHF-rDNMT1 and coimmunoprecipitated PCNA were analyzed by immunoblotting analyses using anti-HA-tag antibody (16B12; BAbCO, USA) and anti-PCNA antibody (sc-56; Santa Cruz), respectively.

Results

***Cla* I digestion distinguishes fully methylated DNA from hemi-methylated DNA.** To examine whether DNA methylation is maintained in the SV40 *in vitro* replication system or not, it was necessary to establish a method to distinguish fully methylated DNAs from hemi-methylated ones. It is known that some restriction enzymes are sensitive to DNA methylation of the target sequence. Most of the methylation-sensitive restriction enzymes digest neither fully nor hemi-methylated DNAs. However, some do digest not fully methylated DNAs but hemi-methylated DNAs, albeit at relatively reduced efficiencies compared to the digestion of unmethylated DNAs. Specifically, *Cla* I, which digests 5′-ATCGAT-3′, cleaves not the fully methylated 5′-ATmCGAT-3′ but the hemi-methylated target sequence when an excess amount of the enzyme is included in the reaction.[22]

To confirm the previous observations, we prepared three versions of the 34-nt double-stranded oligonucleotide DNAs, as shown in Fig. 1a. The oligonucleotides contain a non-palindromic sequence with three 5′-CG-3′ sites. The middle 5′-CG-3′ is contained in a *Cla* I target sequence. The three CpG dinucleotides of the un-, hemi-, and fully methylated oligonucleotides contain methyl cytosine in none of the strands, one strand, and both strands, respectively. 5′-Labeled oligonucleotide DNAs (2.8 ng) were incubated with 67 U *Cla* I at 30°C for 2 h. This amount of *Cla* I represents a 250-fold molar excess relative to the total number of the *Cla* I sites. After digestion, DNA was purified, electrophoresed on 20% polyacrylamide gel, and analyzed by autoradiography. We found that in this condition, *Cla* I efficiently digested the un- and hemi-methylated oligonucleotide DNAs, but not the fully methylated DNA (Fig. 1b). This result indicates that an excess amount of *Cla* I can distinguish fully methylated DNAs from hemi-methylated ones.

DNA methylation is not maintained in the conventional SV40 *in vitro* replication system. pSVO11 contains the SV40 replication origin and has been widely used as template DNA in the SV40 *in vitro* replication system. Because this plasmid does not contain a *Cla* I site, a *Cla* I target sequence was inserted, giving give rise to pSVO11C (Fig. 2a). We amplified pSVO11C in a *dam*$^-$ *dcm*$^-$ *E. coli* strain, producing plasmid DNAs devoid of any methylated nucleotides including those at 5′-GATC-3′ (the underlined adenine is methylated by Dam) or 5′-CCWGG-3′ (the underlined cytosine is methylated by Dcm, and W is A or T). Purified pSVO11C (unmethylated pSVO11C) was treated with *Sss* I methylase to methylate all CpG sites (fully methylated pSVO11C).

The plasmids were *in vitro* replicated in a reaction mixture containing 293 cell S100 extracts and recombinant T-ag as described in 'Materials and Methods' (the conventional SV40 *in vitro* replication system). [α-^{32}P] dATP was included in the reaction to label nascent strand DNAs. The reaction was terminated after 1 h and DNAs were purified, and linearized with *Pst* I (pSVO11C contains a single *Pst* I site) with or without *Cla* I digestion. The product was electrophoresed on 1.0% agarose gel and analyzed by autoradiography (Fig. 2). Successful *Cla* I digestion of 2.9-kb *Pst* I-digested linearized DNA produces two DNA fragments of 2.1-kb and 0.8-kb. Replication products derived from unmethylated pSVO11C were digested with *Cla* I, indicating that the de novo methylation of unmethylated DNA producing fully methylated DNA does not occur in the system

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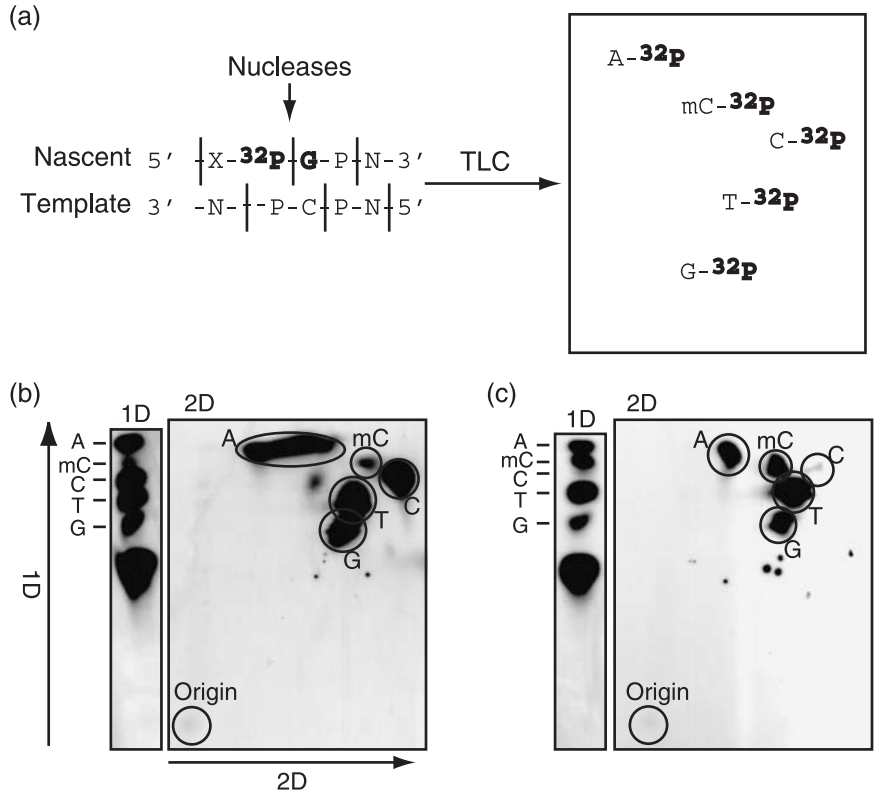

Fig. 3. Detection of CpG and mCpG in a nascent strand of replicated DNA using TLC. (a) Experimental procedure. pSVO11C was replicated in the presence of [α-^{32}P] dGTP. Replicated DNA was digested with micrococcal nuclease and spleen phosphodiesterase II to give rise to 3′-monophosphate nucleotides. 3′-Labeled mononucleotides (Np) derived from NpG were fractionated by two-dimensional TLC as described in 'Materials and Methods'. The relative positions of Ap, Cp, mCp, Tp, and Gp are deduced from a previous study.[21] (b) Fully methylated pSVO11 was replicated and analyzed as described in (a). 3′-Labeled mononucleotides were detected by autoradiography after TLC in the first dimension (1D, left) and the second dimension (2D, right). Origin indicates the position of the origin of TLC. (c) Replicated DNA was fully methylated by *Sss* I methylase prior to nuclease digestion and analyzed as in (b).

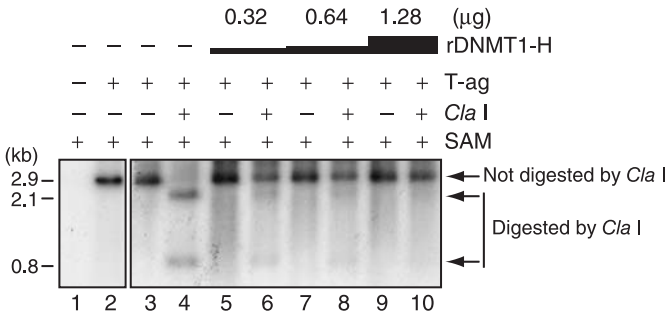

Fig. 4. Recombinant DNA methyltransferase 1 (rDNMT1) and *S*-adenosyl methionine (SAM) support maintenance methylation. Fully methylated pSVO11C was replicated in the presence of rDNMT1-H and 640-μM SAM as indicated. Maintenance methylation was analyzed as in Fig. 2a.

(Fig. 2b, lane 4). When the replication product was fully methylated by *Sss* I methylase prior to *Cla* I treatment, the DNA was resistant to the digestion, as expected (lane 6). We found that replication products derived from fully methylated pSVO11C were digested by *Cla* I (lane 2). These results suggest that the replication products derived from fully methylated DNA template are not significantly maintained for CpG methylation in the conventional SV40 *in vitro* replication system.

CpG methylation of the replication products was also examined by thin layer chromatography (TLC), as shown in Fig. 3a.[21] pSVO11C was replicated in the presence of [α-^{32}P] dGTP. The replication products were treated with micrococcal nuclease and spleen phosphodiesterase II. This treatment digests DNAs into 3′-monophosphate nucleotides and transfers incorporated ^{32}P to the 3′ position of mononucleotide that was originally positioned at the 5′ side of [α-^{32}P] dGTP (N of NpG). When these mononucleotides are subjected to two-dimensional TLC, the N of NpG in nascent strands is fractionated at different positions according to the identity of the nucleotide of A, T, G, C, or methylated cytosine (mC). Because the label was introduced by [α-^{32}P] dGTP, the detected C and mC are invariably derived from CpG and mCpG, respectively. Therefore, it is possible to directly measure the relative amounts of CpG and mCpG in the nascent DNA strand.

Mononucleotides obtained from replication products were subjected to two-dimensional TLC. We found that nascent strand DNAs replicated from fully methylated template DNA contained mCpG at much smaller amounts than CpG (Fig. 3b). When the replicated DNA was fully methylated by *Sss* I methylase prior to nuclease digestion, the CpG signal disappeared and the mCpG signal became significantly strong, confirming the identity of mCpG and CpG signals (Fig. 3c). Together with the results obtained by *Cla* I digestion experiments, we conclude that the DNA maintenance methylation reaction does not significantly occur in the conventional SV40 *in vitro* replication system.

Exogenous recombinant DNMT1 supports maintenance methylation reaction in the SV40 *in vitro* replication system. DNMT1 is the enzyme primarily responsible for the maintenance methylation reaction in mammals. DNMT1 utilizes SAM as a methyl group donor. Lack of the maintenance methylation reaction in the SV40 *in vitro* replication system can be ascribed to the scarcity of SAM or DNMT1. We prepared and purified recombinant mouse DNMT1 (rDNMT1) protein using DNMT1-expressing baculovirus. When 640-μM SAM was added to the reaction, the replication products derived from fully methylated pSVO11C were not maintained for DNA methylation (Fig. 4, lane 4). However, when C-terminally His-tagged rDNMT1 (rDNMT1-H) was added together with SAM, the maintenance methylation reaction occurred in a dose-dependent manner of the added rDNMT1-H (Fig. 4, lanes 5–10). At the largest amounts of supplemented rDNMT1-H (1.28 μg per reaction), virtually all replicated DNAs possessed fully methylated *Cla* I sites. These results indicate that the SV40 *in vitro* replication system does not contain DNMT1 in an amount sufficient to reconstitute

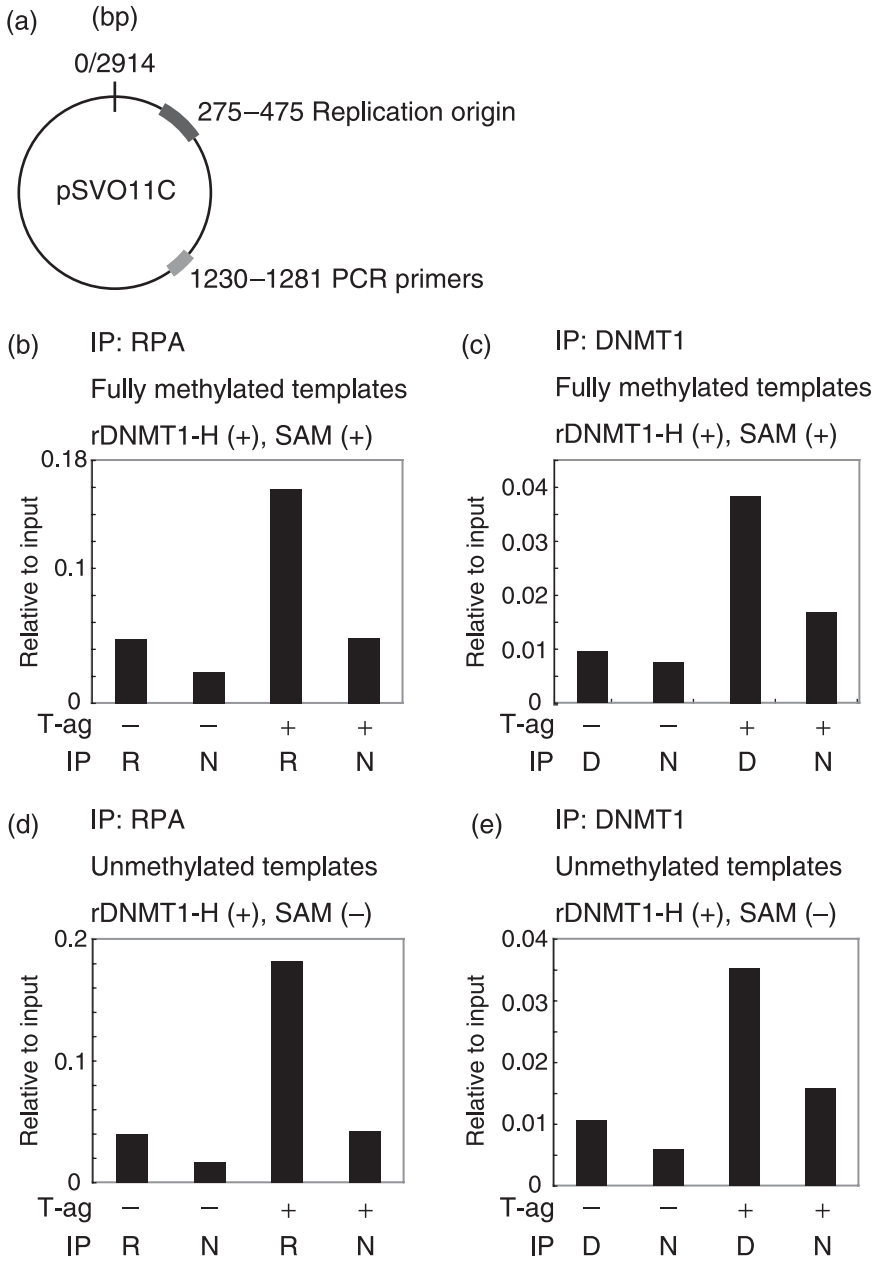

Fig. 5. Chromatin immunoprecipitation experiments to detect DNA methyltransferase 1 (DNMT1) binding to replicating and/or replicated chromatin. pSVO11C-protein complex was analyzed by chromatin immunoprecipitation experiments, as described in 'Materials and Methods'. (a) Positions of polymerase chain reaction primer set. (b–e) pSVO11C was replicated in the presence of 2 μg of rDNMT1-H. DNA-protein complex derived from the reaction with (+) or without (–) T-ag was immunoprecipitated (IP) with anti-RPA32 antibody (R), anti-His antibody (D) or normal IgG (N). Fully methylated (b,c) and unmethylated (d,e) pSVO11C was replicated in the presence and absence of *S*-adenosyl methionine (SAM), respectively. Two independent experiments gave similar results and one representative experiment is shown here.

the maintenance methylation reaction *in vitro*, and more importantly, the reaction occurs when exogenous rDNMT1 and SAM are added.

DNMT1 is recruited to replicating and/or replicated chromatin. Numerous proteins are known to physically interact with DNMT1. However, it is not known how DNMT1 binds to and reacts with replicated DNAs. Since we found that rDNMT1 reacts with replicated DNAs to maintain DNA methylation in the SV40 *in vitro* replication system, we biochemically analyzed how DNMT1 interacts with the replicated chromatin.

We first conducted chromatin immunoprecipitation experiments. pSVO11C was replicated in the presence of rDNMT1-H. After the reaction was terminated, DNA-protein complexes were cross-linked and the 2.9-kb pSVO11C DNA was sonicated to produce 300–500-bp fragments. rDNMT1-H was immunoprecipitated using anti-His antibody that recognizes the His-tag at the C-terminus of rDNMT1-H. For the precipitated fractions, real-time PCR was conducted using a primer set at nucleotides 1230–1281 (Fig. 5a). Antibodies recognizing RPA32, a subunit of RPA (replication protein A), were used as positive control to detect the interaction between replication proteins and replicating DNA. As expected, specific physical interaction between RPA32 and pSVO11C was observed in a replication-dependent manner (T-ag is required for DNA replication) in both cases where fully or un-methylated pSVO11C was used as template (Fig. 5b,d). We found specific interaction between rDNMT1-H and pSVO11C in a replication-dependent manner (Fig. 5c,e). Interestingly, the interaction was observed not only when fully methylated pSVO11C was used as template, but also when unmethylated pSVO11C was replicated in the presence of rDNMT1-H but not SAM (Fig. 5c,e). DNMT1 binds hemi-methylated CpG on its own *in vitro*.[12] However, in Fig. 5e, it was expected that rDNMT1-H did not catalyze the methylation reaction due to the lack of SAM and thus replicated DNA from unmethylated template pSVO11C contained neither fully nor hemi-methylated CpG. This reasoning leads to the suggestion that DNMT1 associates with replicating and/or replicated chromatin even in the absence of methylated CpG.

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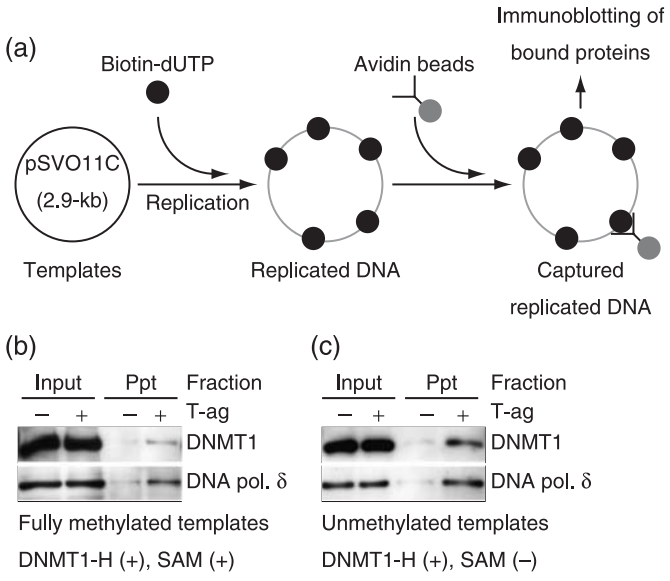

Fig. 6. DNA methyltransferase 1 (DNMT1) associates with replicating and/or replicated chromatin. (a) Experimental procedure. pSVO11C was replicated in the presence of biotin-dUTP. Replicated DNA was isolated by avidin beads. Proteins bound to replicating and/or replicated chromatin were analyzed by immunoblotting. (b,c) pSVO11C was replicated in the presence of 1.5 μg of rDNMT1-H and biotin-dUTP. Replicating and/or replicated pSVO11C derived from the reaction with (+) or without (–) T-ag was analyzed for accompanying DNA polymerase δ and rDNMT1-H using anti-DNA polymerase δ and anti-His antibodies, respectively. Fully methylated (b) and unmethylated (c) pSVO11C was replicated in the presence and absence of *S*-adenosyl methionine (SAM), respectively.

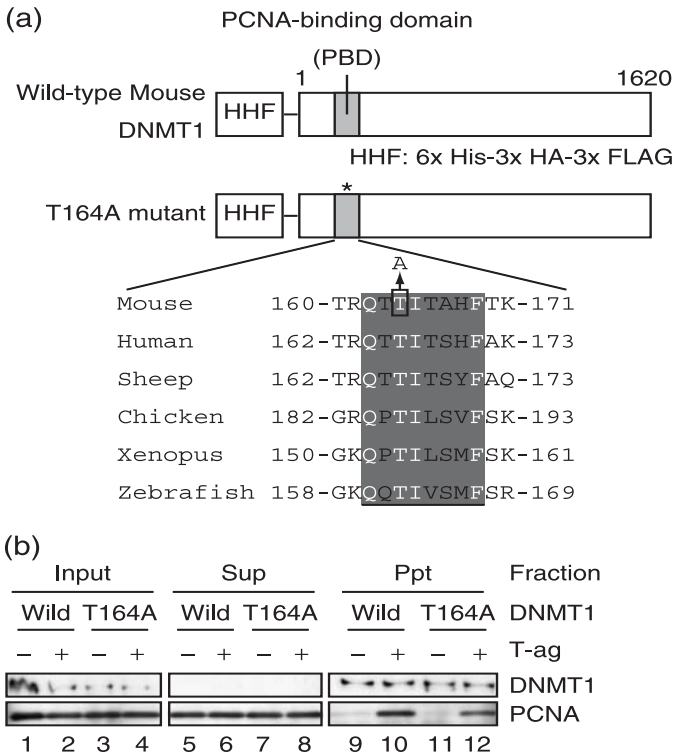

Fig. 7. DNA methyltransferase 1 (DNMT1) associates with replicating and/or replicated chromatin in a proliferating cell nuclear antigen (PCNA)–binding domain (PBD)–independent manner. (a) Schematic representation of wild-type and T164A mutant HHF-rDNMT1. PBD sequences contained in DNMT1 of various species are shown below. Most conserved amino acids are highlighted and one of such residues (T at amino acid 164 in mouse DNMT1) was replaced with alanine in T164A mutant. (b) Fully methylated pSVO11C was replicated in the presence of 2 μg of wild-type or T164A mutant HHF-rDNMT1 and *S*-adenosyl methionine (SAM). After the reaction was terminated, HHF-rDNMT1 was immunoprecipitated using anti-Flag antibody, and coprecipitated PCNA was analyzed by immunoblotting. HHF-rDNMT1 and PCNA in the immunoprecipitates were detected using anti-HA antibody and anti-PCNA antibody, respectively. Although the PCNA signal for T164A (lane 12) appeared weaker than that for wild-type (lane 10) in this particular experiment, this result was not reproductively observed in other independent experiments.

To analyze proteins bound to replicating and/or replicated chromatin specifically, biotin-dUTP was included in the replication reaction. pSVO11C DNAs that are undergoing or have completed DNA replication, but not unreplicated ones, incorporate biotin-dUTP and can be precipitated using avidin-coated beads. pSVO11C was replicated in the presence of biotin-dUTP and rDNMT1-H. Proteins associated with bead-precipitated chromatin were analyzed by immunoblotting using anti-His and anti-DNA polymerase δ antibodies (Fig. 6a). As expected, DNA polymerase δ was detected in the precipitated fraction in a replication (T-ag)-dependent manner in both cases using fully or unmethylated template DNAs (Fig. 6b,c). Interestingly, rDNMT1-H behaved similarly to DNA polymerase δ. It associated with replicating and/or replicated chromatin not only in the case where replication products were actively methylated by DNMT1 (Fig. 6b), but also in the case where DNMT1 did not catalyze DNA methylation of replicated DNA derived from unmethylated template in the absence of SAM (Fig. 6c).

Taken together, we conclude that DNMT1 is recruited to replicating and/or replicated chromatin independently of the methylation status of template DNA and the ongoing reaction catalyzed by DNMT1. One possible explanation is that a constitutive component of the replicating machineries recruits DNMT1 to DNAs.

DNMT1 mutant defective for PCNA-binding associates with replicating and/or replicated chromatin. It is known that DNMT1 directly associates with PCNA via its N-terminally positioned PCNA-binding domain (PBD).[13] PBD is a well-conserved motif responsible for binding to PCNA in a wide variety of PCNA-binding proteins including DNMT1, $p21^{Waf1}$, and Fen1 (Fig. 7a and reviewed in Warbrick[23]). Several DNMT1 mutants that fail to bind to PCNA due to missense mutations at highly conserved PBD amino acids have been reported.[13,24] Given that PCNA is a constitutive replication component, we examined whether the physical interaction between PBD of DNMT1 and PCNA was required for the observed interaction between DNMT1 and replicating and/or replicated pSVO11C chromatin. Wild-type and T164A mutant mouse rDNMT1s were prepared from Sf9 insect cells using baculovirus. It was reported that the substitution of alanine for threonine at amino acid 164 abolished the direct physical interaction between DNMT1 and PCNA.[13] The recombinant DNMT1s are tagged by His, Flag, and HA sequences at their N-termini (HHF-rDNMT1) and can be immunoprecipitated with anti-Flag antibody (Fig. 7a).

Fully methylated pSVO11C was replicated in the presence of wild-type or T164A HHF-rDNMT1 and SAM. After the replication reaction, HHF-rDNMT1 was immunoprecipitated using anti-Flag antibody and PCNA was examined for coimmunoprecipitation using anti-PCNA antibody. As shown in Fig. 7b, PCNA was robustly detected in the immunoprecipitates of both wild-type and T164 mutant rDNMT1s, indicating that DNMT1 and PCNA associate either directly or indirectly in a PBD-independent manner. Importantly, the association was T-ag-dependent, indicating that only replicating and/or replicated chromatin binds to DNMT1 in an PBD-independent manner.

Discussion

Epigenetic modification of chromatin includes DNA methylation and various histone modifications that together regulate gene activity. Epigenetic pattern in a given type of normal cells should be faithfully maintained during cell proliferation to inherit the gene expression pattern characterizing that particular type of cells. In contrast, cancer cells show epigenetic instability that leads to increased heterogeneity of the epigenetic pattern. Such epigenetic heterogeneity in a clonal population of cancer cells affords an opportunity for the population to be selected for ever-malignant clones via, for example, epigenetically inactivating TSGs. It is for this reason that elucidation of the mechanism of epigenetic instability and development of anticancer drugs targeting epigenetics are gaining much attention. However, it is largely unknown how epigenetics are maintained faithfully in normal cells.

In this study, we focused on the question of how DNA methylation is maintained during replication. A number of cytological and biochemical studies have suggested an intimate relationship between replication and maintenance methylation reactions. Although several molecular links between DNMT1 and chromatin proteins have been identified, the relative significance of such interactions in the coordinated reactions remains elusive because no *in vitro* system that can be experimentally manipulated has been established so far. We developed a novel *in vitro* system in which genetics (DNA replication) and epigenetics (maintenance of DNA methylation) concurrently occur. Because the reaction is an essentially open system, we can easily add recombinant proteins, either wild-type or mutant forms, and small molecules such as SAM to the reaction, and monitor the effects of such manipulations. We found that the conventional SV40 *in vitro* replication system does not support maintenance methylation of nascent DNA strands. However, by adding recombinant DNMT1 and SAM, we succeeded in reconstituting the reaction.

It has been suggested that DNMT1 is directly recruited to the replication fork, for example, via PCNA or hemi-methylated DNA-binding proteins, such as UHRF1/NP95/ICBP90. In this study, we found that DNMT1 associates with replicating and/or replicated chromatin derived from unmethylated template DNAs (Figs 5 and 6). Because hemi-methylated DNAs are not expected to be generated in the reaction conditions, it appears that hemi-methylated DNA-binding proteins are not required for the observed association. Moreover, it was demonstrated that the canonical PBD in DNMT1 is not required for the association either (Fig. 7). These results suggest that DNMT1 is recruited to replicating and/or replicated chromatin via a still unknown mechanism. Because the association occurs even when template DNA is unmethylated, DNMT1 may be constitutively recruited to the replication site even when its activity is not immediately required (idling state). When the replication fork encounters methylated DNA to produce hemi-methylated daughter DNAs, DNMT1 may be recruited from its idling state to its active position via, for example, UHRF1/NP95/ICBP90, to perform its catalytic activity. Such close apposition of DNMT1 at the replicating chromatin may facilitate timely recruitment of DNMT1 and increase the kinetics of the maintenance methylation reaction.

Taken together, we found that the *in vitro* maintenance methylation system described in this report is useful for detailed analysis of the reaction, and will provide an insight into the molecular mechanism underlying the faithful maintenance of epigenetics in the future.

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