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Mammalian DNA Methyltransferases Show Different Subnuclear Distributions

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Abstract In mammalian cells, DNA methylation patterns are precisely maintained after DNA replication with defined changes occurring during development. The major DNA methyltransferase (Dnmt1) is associated with nuclear replication sites during S-phase, which is consistent with a role in maintenance methylation. The subcellular distribution of the recently discovered de novo DNA methyltransferases, Dnmt3a and Dnmt3b, was investigated by immunofluorescence and by epitope tagging. We now show that both Dnmt3a and Dnmt3b are distributed throughout the nucleoplasm but are not associated with nuclear DNA replication sites during S-phase. These results suggest that de novo methylation by Dnmt3a and Dnmt3b occurs independently of the replication process and might involve an alternative mechanism for accessing the target DNA. The different subcellular distribution of mammalian DNA methyltransferases might thus contribute to the regulation of DNA methylation. J. Cell. Biochem. 83: 373–379, 2001.

Key words: DNA methyltransferase; DNA replication; DNA methylation; replication foci; Dnmt1; Dnmt3; PCNA

DNA methylation is a postreplicative modification found in most prokaryotic and eukaryotic genomes with a variety of different biological functions [Colot and Rossignol, 1999]. In mammalian cells, this modification occurs mostly at cytosine residues of CpG dinucleotides and, while the pattern of methylated and unmethylated sites is stably maintained over many cell division cycles, it also undergoes defined changes throughout development.

Until now three different DNA methyltransferases have been identified in mammalian cells. Dnmt1 is the major and ubiquitously expressed DNA methyltransferase. It has a clear preference for hemimethylated sites and is mainly involved in the maintenance of a given DNA methylation pattern after DNA replication [Bestor et al., 1988; Margot et al., 2000]. The other two DNA methyltransferases, Dnmt3a and Dnmt3b, are tissue-specific and can de novo methylate DNA sites [Okano et al., 1998]. The targeted disruption of *Dnmt1* showed that DNA methylation is crucial for mammalian development and is involved in parental imprinting and X-chromosome inactivation [Li et al., 1992, Li et al., 1993b; Panning and Jaenisch, 1996]. Likewise, both de novo methyltransferases were shown to be required for mammalian development [Okano et al., 1999] and their relevance was strengthened by the discovery that the human ICF syndrome is linked to mutations in *Dnmt3a* [Okano et al., 1999; Xu et al., 1999]. Studies on the connection between DNA replication and DNA methylation showed that Dnmt1 was, like other DNA replication enzymes, localized at subnuclear replication foci [Leonhardt et al., 1992]. Three independent sequences located in the N-terminal, regulatory domain of Dnmt1, have been identified that are each sufficient to target Dnmt1 to nuclear replication sites. The first sequence is a PCNA binding domain (PBD) [Chuang et al., 1997] followed by the initially identified replication foci targeting sequence (RFTS) [Leonhardt et al., 1992] and the polybromo homology domain (PBHD) [Liu et al., 1998]. This direct coupling of Dnmt1 to the replication machinery could increase the precision of the maintenance methylation and would

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also give the DNA methyltransferase a direct access to 'naked' DNA stripped of most of its chromatin structure.

These results raise the question whether Dnmt3a and Dnmt3b are localized at replication foci like Dnmt1 or whether de novo methylation by these enzymes occurs independently of DNA replication. This question gains further relevance from the recent observation that Dnmt1 deficient somatic cell lines largely maintain their methylation pattern [Rhee et al., 2000], suggesting that under certain circumstances Dnmt3a or Dnmt3b might be able to substitute for Dnmt1.

We have investigated the potential connection of Dnmt3a and Dnmt3b with DNA replication by epitope tagging and immunofluorescent staining and failed to detect any localization at replication foci. Our results indicate that de novo methylation by Dnmt3a and Dnmt3b does not seem to be directly coupled to DNA replication and suggest a fundamental difference in the regulation and enzymatic mechanism between Dnmt1 and Dnmt3 methyltransferases.

MATERIALS AND METHODS

Generation of Flag-Tagged Dnmt3 Expression Constructs

The Dnmt3a and Dnmt3b1 cDNAs [Okano et al., 1998] were amplified by the expand long template PCR system (Roche, Mannheim, Germany) using the conditions given by the supplier and the primer pairs indicated in Table I. This method allowed for the introduction of a Flag tag (DYKDDDDK) either at the N- or C-terminus of both proteins. Furthermore, the primers for the N-tagged constructs provide an optimized translation initiation signal [Kozak, 1986], whereas the endogenous sequence, that was in part used for the C-tagged constructs, lacks the critical G at +4 position (Fig. 1). The PCR products were cloned into the TA eukaryotic expression vector pCR3.1 (Invitrogen, Groningen, The Netherlands) and the resulting plasmids purified by column (Qiagen, Hilden, Germany) according to the instructions of the manufacturer.

Cell Culture and Western Blot Analysis

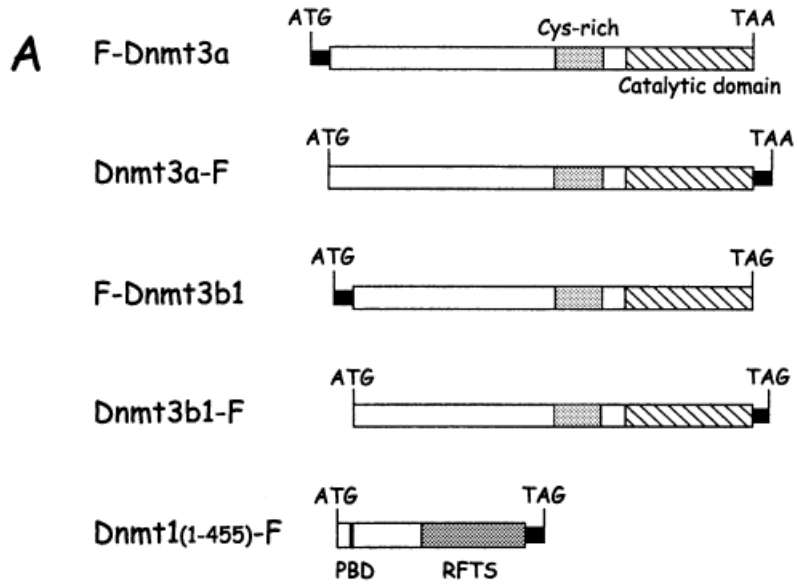
COS-7 cells (African green monkey kidney fibroblast-like cells, transformed with SV40T antigen) were grown in a humidified incubator at 37°C and 5% CO_2 in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum, 4.5 g/L glucose, 3.4 g/L $NaHCO_3$, 1 mM sodium pyruvate and 50 μg/ml gentamycin (GIBCO BRL, Karlsruhe, Germany). Cells were transfected using the DEAE dextran pretreatment method and analyzed as described previously [Leonhardt et al., 1992]. Protein expression was assayed by western blotting using whole cell extracts. Briefly, after several washes with cold phosphate buffered saline (PBS), the cells were scraped and collected by centrifugation. They were resuspended in 150 μl of XTB buffer [Leonhardt et al., 1992] containing protease inhibitors and incubated on ice for 5 min. The lysate was spun at 10,000*g* for 10 min and the supernatant was analyzed by SDS–PAGE electrophoresis under reducing conditions. The proteins weretransferred to PVDF membranes and probed with anti-Flag (M2, Sigma, Taufkirchen, Germany) followed by HRP-conjugated secondary antibodies. The blots were developed

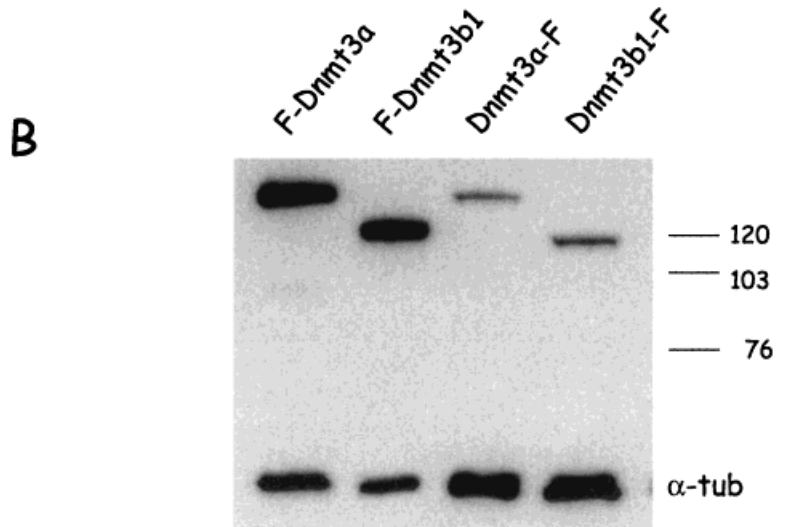
TABLE I. Nucleotide Sequence of DNA

	F-Dnmt3a
Forward	5′GCCACC**ATG**GACTACAAAGACGATGACG-ATAAA**ATG**CCCTCCAGCGGCCCCG3′[a]
Reverse	5′TTACACACAAGCAAAATATTCC3′
	Dnmt3a-F
Forward	5′GCCACC**ATG**CCCTCCAGCGGCCCCG3′
Reverse	5′TTATTTATCGTCATCGTCTTTGTAGTCCA-CACAAGCAAAAT ATTCC3′
	F-Dnmt3b1
Forward	5′GCCACC**ATG**GACTACAAAGACGATGACG-ATAAA**ATG**AAGGGAGACAGCAGAC3′
Reverse	5′CTATTCACAGGCAAAGTAGTCC3′
	Dnmt3b1-F
Forward	5′GCCACC**ATG**AAGGGAGACAGCAGAC3′
Reverse	5′CTATTTATCGTCATCGTCTTTGTAGTCTT-CACAGGCAAAGT AGTCC3′

[a]Flag sequence is underlined; the ATG start codon of the construct and of the native protein is indicated by bold letters.

Fig. 1. Generation and characterization of epitope tagged expression constructs of Dnmt3a and Dnmt3b1. **A:** The structure of the expression constructs together with relevant regulatory sequences is schematically shown with the Flag epitope displayed as black squares. PBD (thick black line in the truncated Dnmt1 construct) stands for PCNA binding domain and RFTS for replication foci targeting sequence. **B:** Equivalent amounts of protein from extracts of transfected COS cells were loaded on an 8% gel. After electrophoresis and transfer, the Western blot was probed with anti-Flag and anti-tubulin antibodies. Molecular weight markers are indicated in kDa. **C:** The surrounding sequence of the translation start site (boxed) of *Dnmt3a* and *Dnmt3b* is compared with the Kozak consensus [Kozak, 1986] and the synthetic sequence used in the F-Dnmt3 constructs. The conserved guanosine at position +4 is shown in bold.





C

Kozak's	G	C	C	A/G	C	C	A T G	G
Dnmt3a	C	C	A	G	C	A	A T G	C
Dnmt3a-F	G	C	C	A	C	C	A T G	C
F-Dnmt3's	G	C	C	A	C	C	A T G	G
Dnmt3b1	G	A	A	A	C	A	A T G	A
Dnmt3b1-F	G	C	C	A	C	C	A T G	A

using the ECL$^+$ detection procedure (Amersham, Freiburg, Germany) and were recorded with a LAS-1000 Luminescence image reader (Fuji, Raytest, Berlin, Germany). Signals were quantified with the Image Gauge software (Fuji). The transfection rate of the different constructs was found to be similar whether the tag was at the N- or C-terminal end of the protein. The blots were probed with anti-tubulin antibodies (DM1A, Sigma, Taufkirchen, Germany) to assess for loading differences.

Immunofluorescence Microscopy

Mouse C2C12 were grown as above except that the medium was supplemented with 20% fetal calf serum. On the day before transfection, 10^5 cells were seeded in 100 mm tissue culture dishes containing glass coverslips. The cells were transfected by the calcium phosphate-DNA coprecipitation method, followed by glycerol shock treatment 6 h later. The cells were fixed 24–36 h after transfection by incubation for 10 min in 3.7% formaldehyde in PBS or for 5 min in cold methanol. All subsequent incubations were done at room temperature and in solutions containing PBS. The cells were washed several times with PBS, blocked for 30 min in 0.2% fish skin gelatin and incubated for 1 h with the first antibody. Rabbit antisera were used against Dnmt1 [Cardoso and Leonhardt, 1999], Dnmt3a and Dnmt3b (generous gift by Dr. En Li), whereas PCNA was stained with a FITC-conjugated mouse monoclonal antibody (PC10, Pharmingen, Hamburg, Germany). After extensive washes with 0.1% NP-40, the cells were incubated for 1 h with the fluorescently conjugated secondary antibody, washed again several times with 0.1% NP-40 and counterstained with Hoechst 33258. The cells were mounted in mowiol with 2.5% DABCO [Cardoso and Leonhardt, 1995] and examined with an Axioplan 2 microscope (Zeiss) equipped with phase contrast and epifluorescence optics. Images were collected with a cooled CCD camera (SensiCam) using the Axiovision software (Zeiss) and assembled with Photoshop and Illustrator software (Adobe).

RESULTS

Expression of Dnmt3a and Dnmt3b1

Biochemical and genetic data indicate that Dnmt3a and Dnmt3b are involved in de novo rather than maintenance methylation [Li et al., 1992, 1993b), but little is known about their subcellular distribution and their relationship with subnuclear replication sites. We set out to determine their localization by immunofluorescent stainings of the endogenous and epitope-tagged proteins. To control for potential fusion artefacts we generated Dnmt3a and Dnmt3b1 constructs with Flag tags either at the N- or C-terminus. In case of Dnmt3b, which exists in three splice variants [Okano et al., 1998], the longest isoform (b1) was chosen to include all potential regulatory sequences (Fig. 1).

These constructs were assayed for expression of full length, stable, and soluble proteins by transfection of COS-7 cells. Extracts were prepared and assayed by western blot analysis using a monoclonal antibody against the Flag tag. In all four cases, stable and soluble proteins were detected (Fig. 1B). Interestingly, the expression level of N- and C-terminally tagged constructs differed dramatically, although their transfection efficiency was equivalent. Quantification of the blots (see methods) indicates roughly equivalent levels of expression of the Dnmt3a and 3b1 proteins, but a more than 20-fold stronger signal of the N- versus C-tagged proteins. This difference in expression was reproducibly observed in protein extracts from several independent transfection experiments. The most obvious difference between these constructs is that the C-tagged constructs depend in part on the endogenous signals of Dnmt3a and Dnmt3b1 for translation initiation, while the N-terminal constructs contain an optimal synthetic sequence (Fig. 1C). Comparison of the relevant sequence surrounding the start codon shows that both Dnmt3a and Dnmt3b poorly align with the Kozak consensus sequence, which could explain their low expression level in vivo.

Dnmt3a and Dnmt3b1 Do Not Associate With Subnuclear Replication Sites

In mammalian cells, DNA replication occurs in microscopically visible, subnuclear foci containing proteins directly and indirectly involved in the precise duplication of the genome during S-phase. Since Dnmt1 is localized at these replication foci, we determined the localization of the newly discovered Dnmt3a and Dnmt3b enzymes. The four different epitope-tagged constructs of Dnmt3a and Dnmt3b were expressed in C2C12 mouse myoblasts and their cellular localization determined by immuno-

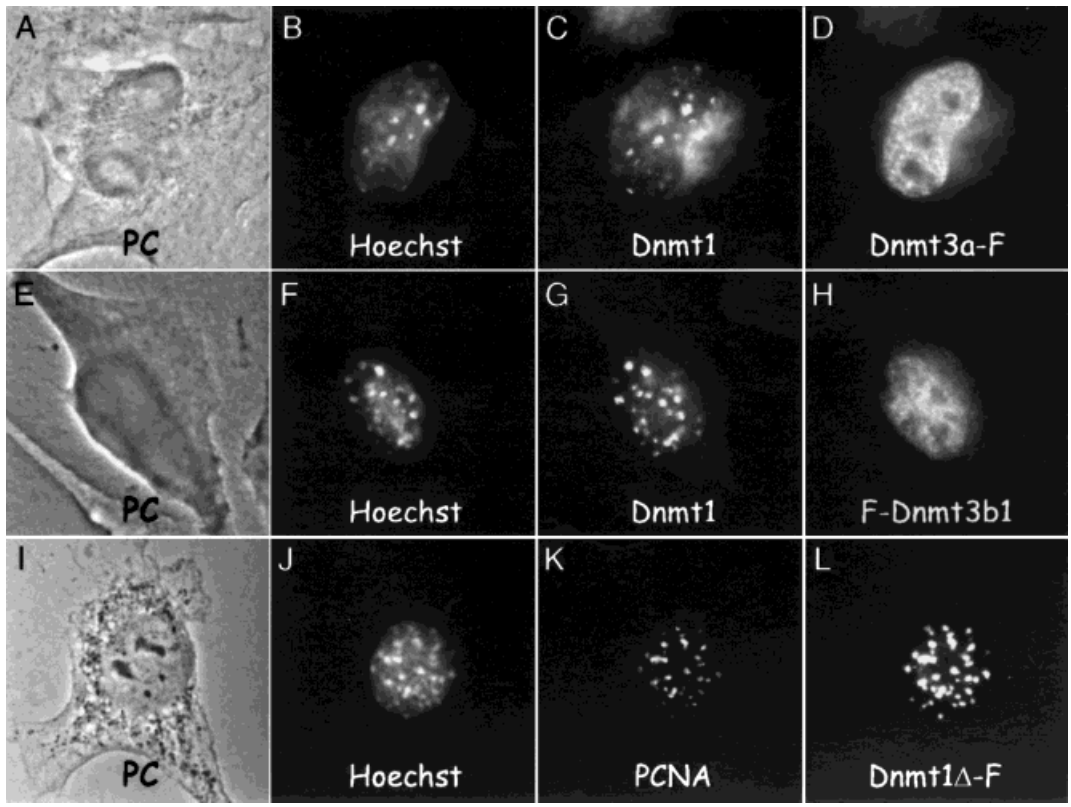


Fig. 2. Subnuclear localization of Dnmt3 constructs during S-phase. C2C12 myoblasts were transiently transfected and analyzed by immunofluorescence. Comparable examples of transfected cells are shown for Dnmt3a (**A-D**), Dnmt3b1 (**E-H**) and Dnmt1 (**I-L**) along with their corresponding phase contrast (PC) and Hoechst staining. Nuclear replication sites are visualized by staining for either the endogenous Dnmt1 (C and G) or PCNA (K).

fluorescence. A Flag-tagged construct of Dnmt1 containing the PBD and the RFTS was used as control. In interphase cells, all three methyltransferase constructs were distributed throughout the entire nucleus with the exception of nucleoli (data not shown).

The subnuclear localization of Dnmt3a and Dnmt3b was investigated by double staining of transfected C2C12 cells for the expressed Dnmt3 and for the endogenous Dnmt1 protein (Fig. 2). During S-phase Dnmt1 associates with DNA replication sites and takes on a characteristic, punctate staining (Fig. 2C and G). Dnmt1 was chosen as the positive control in this case since the protein is well characterized and a reliable S-phase cell marker [Leonhardt et al., 1992; Chuang et al., 1997; Liu et al., 1998). In contrast to endogenous Dnmt1, the Flag-tagged Dnmt3a (Fig. 2D) and Dnmt3b (Fig. 2H) show a disperse distribution and do not associate with replication sites. Identical results were obtained with constructs containing the Flag sequence either at the N- or at the C-terminus. The level of expression of the transfected genes shows some intercellular variability, but the lack of an association of Dnmt3s with replication foci has been consistently observed in many independent experiments. A truncated Dnmt1 construct (aa 1-455 of the short isoform) containing PBD and RFTS tagged at the C-terminus with the Flag epitope was clearly localized at replication sites (Fig. 2I–L) ruling out that the epitope might disturb the subcellular localization.

The analysis of the subcellular distribution of the endogenous Dnmt3a and Dnmt3b is hindered by their very low expression level yielding only weak signals and relatively high background. The direct staining for the endogenous protein with anti-Dnmt3a and anti-Dnmt3b antibodies also showed a disperse nuclear distribution during S-phase, but we failed to detect any localization at replication sites (data not shown). Taken together, our results show a clear difference in the subnuclear distribution of the maintenance (Dnmt1) and de novo

methyltransferases (Dnmt3a and Dnmt3b) during S-phase suggesting that de novo methylation occurs independently of DNA replication.

DISCUSSION

The regulation of DNA methylation in mammalian cells involves an interplay of maintenance and de novo methyltransferases. The maintenance enzyme, Dnmt1, was found to be subjected to a complex regulation of its subcellular localization during development and during the cell cycle. It is localized in the cytoplasm in oocytes and in preimplantation embryos [Carlson et al., 1992; Cardoso and Leonhardt, 1999] and is associated with replication foci during S-phase in somatic cells [Leonhardt et al., 1992]. On the other hand, little is known about the biochemistry of de novo methylation, and in particular whether the methylation step also needs to be associated with the replication apparatus. In this study, we could show that the de novo methyltransferases, Dnmt3a and Dnmt3b, are not associated with nuclear replication foci suggesting that de novo methylation occurs independently of DNA replication (Fig. 2).

The targeted inactivation of these three methyltransferase genes has shown that all of them are required for viability and that they cannot substitute for one another [Li et al., 1992; Okano et al., 1999]. The fact that these enzymes are not redundant and do not seem to have overlapping functions [Hsieh, 1999; Okano et al., 1999] fits with their different biochemical properties, their different expression pattern, and the different subcellular localization described in this study. These clear differences between Dnmt3a, Dnmt3b1, and Dnmt1 are difficult to reconcile with the recent report that Dnmt1 deficient cells largely maintain their methylation level [Rhee et al., 2000] unless other, still unknown methyltransferases are involved. Alternatively, the somatic *Dnmt1* inactivation might have left some residual activity as has been reported for the embryonic stem cell targeting of the same region of *Dnmt1* [Li et al., 1993a].

In contrast to Dnmt1, the de novo methyltransferases, Dnmt3a and Dnmt3b, show a very low and tissue-specific expression [Okano et al., 1998, 1999]. These results match the poor expression encountered with the constructs that were based on translation initiation signals of endogenous *Dnmt3a* and *Dnmt3b* genes (Dnmt3a-F and Dnmt3b-F, Fig. 1B). Comparison of sequences around the initiation site with the Kozak consensus suggests that the low expression level of *Dnmt3* genes may largely be due to poor translational efficiency which fits with the observation that the simple addition of an improved translation initiation signal increased the expression level over 20-fold (Fig. 1). This may either reflect the need to tightly control these de novo methyltransferases in order to prevent hypermethylation of the genome and/or may indicate some additional translational control mechanisms that allow selective expression in some tissues. Alternatively, some additional upstream translational start site might exist as was the case with Dnmt1 [Tucker et al., 1996; Yoder et al., 1996].

The fact that Dnmt3a and Dnmt3b are not, like Dnmt1, associated with the replication machinery fits with their different biological functions but points also to some mechanistic differences. The group of cytosine methyltransferases that Dnmt1 and Dnmt3 belong to do not simply bind to DNA like, e.g., transcription factors, but perform a complicated multistep enzymatic reaction with their target DNA site. The methylation reaction involves sequence specific DNA binding, flipping the target cytosine out of the double helix, formation of a transient covalent complex with the cytosine residue, and the transfer of the methyl group. This complex mechanism is hard to imagine in the context of densely packed chromatin. The tight association of Dnmt1 with the replication machinery gives it direct access to newly replicated and still 'naked' DNA. The absence of Dnmt3a and Dnmt3b from replication foci suggests that these methyltransferases utilize a different mechanism for accessing DNA and for interacting with their target sites. Sequence comparison of all three mammalian DNA methyltransferases shows that they differ mainly in their regulatory domain. These different regulatory domains might reflect different mechanisms to access DNA, which might involve auxiliary factors like chromatin remodelling complexes.

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