J. Biochem. 128, 315–321 (2000)

Maintenance-Type DNA Methyltransferase Is Highly Expressed in Post-Mitotic Neurons and Localized in the Cytoplasmic Compartment[1]

Koichi Inano,* Isao Suetake,† Takayuki Ueda,‡ Yasuko Miyake,‡ Masahiko Nakamura,† Masahiko Okada,* and Shoji Tajima†,[2]

Department of Laboratory Medicine, Faculty of Medicine, and ‡Research Laboratory for Molecular Genetics, Niigata University, Niigata 951-8510; †Institute for Protein Research, Osaka University, Suita, Osaka 565-0871; and §National Cardiovascular Center Research Institute, Suita, Osaka 565-8565

Received April 19, 2000; accepted June 1, 2000

Maintenance-type DNA methyltransferase (Dnmt1) is usually down-regulated in non-proliferating cells. In the present study, we detected significant expression of Dnmt1 protein in adult mouse brain where the majority of the cells are in a post-mitotic state. A significant amount of Dnmt1 protein was fractionated into the post-nuclear fraction for both cerebrum and cerebellum. The Dnmt1 in this fraction was enzymatically active. An immunofluorescence study revealed that Dnmt1 protein was mainly expressed in neurons and seemed to be localized in the cytoplasmic compartment. Primary culturing of neurons confirmed the expression and localization of Dnmt1 in the cytoplasmic compartment. The findings that the Dnmt1 transcript in the brain utilized the somatic-type exon and that the apparent size of the Dnmt1 protein in the cytoplasm was identical to that in proliferating culture cells indicate that the cytoplasmic Dnmt1 in neurons was of the somatic-type.

Key words: Dnmt1, neurons, subcellular localization.

In vertebrates, the 5th positions of the cytosine residues in CpG sequences in genomic DNA are often methylated (*1*). Dynamic regulation of DNA methylation is known to contribute to physiological phenomena such as tissue-specific gene expression (*2–4*), genomic imprinting (*5*), X chromosome inactivation (*6*), and carcinogenesis (*7*). In vertebrates, two types of DNA methyltransferase activity have been reported, *i.e., de novo*- and maintenance-type DNA methyltransferase activities. In mouse, *de novo*-type DNA methyltransferase activity contributes to the creation of tissue-specific DNA methylation patterns at the implantation stage of embryogenesis (*8*, *9*), and maintenance-type DNA methyltransferase activity ensures clonal transmission of lineage-specific methylation patterns in somatic cells. When the maintenance-type DNA methyltransferase, designated as Dnmt1, is destroyed in mouse, homozygous mutant embryos cannot survive past midgestation (*10*), their genomic imprinting being canceled (*11*).

In mouse somatic cells, Dnmt1 is localized in nuclei and specifically accumulates at the replication foci in late S-phase nuclei (*12*). The expression of Dnmt1 is under the control of the cell cycle. In somatic cells, Dnmt1 protein and activity are highest in the S-phase and lowest in the G_1-phase. When the cell cycle is arrested due to low serum conditions or differentiation is induced, Dnmt1 decreases (*13*, *14*). This regulation of Dnmt1 is a posttranscriptional event, as the arrest of the cell cycle does not affect the transcription in isolated nuclei (*13–15*), and shortens the half lives of the Dnmt1 mRNA and protein through an unknown mechanism (*14*, *16*).

Unlike in somatic cells, in germlines the expression and localization of Dnmt1 are specifically regulated. During spermatogenesis in mouse, transcription starts from a testis-specific site at the pachytene spermatocyte stage. The transcript does not produce functional Dnmt1 (*17*). In growing oocytes, Dnmt1 is first localized in the nuclei. As oogenesis proceeds, Dnmt1 is accumulated in the cytoplasm, and at ovulation Dnmt1 is excluded from the nuclei (germinal vesicles) (*17*, *18*). Unlike in mouse, in *Xenopus* oocytes, about half of the Dnmt1 is transported into nuclei, while the rest remains in the cytoplasmic compartment (*19*). Recently, the domain of Dnmt1 responsible for the localization of the molecules in the cytoplasm in mouse oocytes, which was mapped to a rather wide region of the amino-terminal regulatory domain including the cysteine-rich region, has been reported (*20*). Furthermore, the amount of accumulated Dnmt1 in mouse mature oocytes and one-cell embryos is 3,000 times higher than that in murine erythroleukemia (MEL) cells on a per-cell basis (*18*). Similar accumulation of Dnmt1 is also observed in *Xenopus* oocytes (*19*). During oogenesis and early development, transcription starts from a specific site in the mouse Dnmt1 gene, of which the mRNA encodes an oocyte-specific Dnmt1 lacking 118 amino acids of the amino-terminus of the somatic-type (*17*).

Interestingly, the nerve cells in the brain, especially

[1] Supported by the Program for the Promotion of Fundamental Studies in Health Sciences of the Organization for Pharmaceutical Safety and Research of Japan, and the Ministry of Education, Science, Sports and Culture of Japan.

[2] To whom correspondence should be addressed. Phone: +81-6-6879-8627, Fax: +81-6-6879-8629, E-mail: tajima@protein.osaka-u.ac.jp

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; Dnmt1, maintenance-type DNA methyltransferase; MAP2, microtubule-associate protein 2; PBS, Dulbecco's phosphate–buffered saline; PCNA, proliferating cell nuclear antigen.

those in the cerebellum, most of which are in a post-mitotic state, have been reported to express a high level of the Dnmt1 transcript (*21*). It has also been reported that DNA methylation plays important roles in neuronal differentiation and brain development: *e.g.*, nerve growth factor-dependent neurite outgrowth in PC12 cells requires DNA methylation (*22*), and the *Otx2* gene, which encodes a transcription factor that plays a crucial role in brain morphogenesis, is under the control of Dnmt1 (*23*). In the present study, we examined the expression and distribution of the Dnmt1 protein in the brain. As expected from the high transcript level of Dnmt1 (*21*), the Dnmt1 protein was significantly expressed in brain cells, especially in neurons. Unexpectedly, the majority of the Dnmt1 protein was localized in the cytoplasmic compartment of neurons. The Dnmt1 in the cytoplasmic fraction was enzymatically active. The high expression level and unusual localization of Dnmt1 in neurons may provide us with clues to the unknown function of Dnmt1.

MATERIALS AND METHODS

Cells—Mouse embryonic fibroblasts, C3H10T1/2 cells, were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum in a humidified incubator at 37°C under a 5% CO_2 atmosphere.

Immunoprecipitation and DNA Methylation Activity—Cultured cells or excised tissues from adult C57BL6 mice (10–15 weeks after birth) were homogenized with a Dounce homogenizer in 5 volumes of ice-cold 50 mM Tris-HCl buffer, pH 7.5, containing 0.25 M sucrose, 25 mM KCl, 5 mM $MgCl_2$, and a protease inhibitor cocktail, Complete (Boehringer). The nuclei in the homogenates were isolated from the rest of the components (post-nuclear fractions) by centrifugation at 1,500 $\times g$ for 10 min at 4°C. The nuclear extracts and post-nuclear fractions were used as enzyme sources as described (*24*). The DNA methylation activities were measured as described (*19*), except that 3 μCi of [^{3}H]-*S*-adenosyl-L-methionine (80.0 Ci/mmol; Amersham Pharmacia Biotech-UK) per assay was used. The activity in each preparation was determined by three measurements with different concentrations of the enzyme source. The specific activities were determined for three different mice and cell cultures, and their means and standard deviations were calculated. The activities were normalized to the DNA content of each tissue (or cells). DNA was determined as described (*25*).

The subfractionated samples were immunoprecipitated with the immunoselected anti-Dnmt1 antibodies (*24*) raised against the amino acid sequences 1,037–1,386 (*26*, *27*), electrophoresed on 7% SDS–polyacrylamide gels, and then detected by Western blotting by means of alkaline phosphatase-coupled second antibodies. The detected bands were quantified with an image analyzer, MCID (Imaging Research, Canada). Monoclonal antibodies against post-nuclear and nuclear fraction markers, *i.e.*, Hsp90 and histones, were purchased from Santa Cruz Biotechnology and Chemicon, respectively. The bands were detected with horseradish peroxidase-coupled second antibodies and an ECL detection kit (Amersham Pharmacia Biotech-UK).

Immunocytochemistry—Isolated mouse brain was fixed and cryosectioned as described by Nagai *et al.* (*28*). In brief, mouse was perfused through the ascending aorta with 30 ml of ice-cold 0.1% $NaNO_2$ and then 30 ml of ice cold 4% paraformaldehyde in Dulbecco's phosphate buffered saline (PBS) for fixation. After perfusion, brain was dissected out and further fixed by immersion in 4% paraformaldehyde in PBS for one day at 4°C. The brain thus fixed was cryosectioned. For immunofluorescent staining, sections were incubated overnight at 4°C with immunoselected anti-mouse Dnmt1 antibodies, 1/1,000 diluted with PBS containing 0.4% SDS, 2% Triton X-100, and 1% bovine serum albumin. After incubation, the specimens were incubated with anti–rabbit IgG conjugated to ALEXA 488 (Molecular Probes), and observed as described by Kimura *et al.* (*19*). For all the samples the exposure and printing were performed under identical conditions.

Primary Culture of Neurons—Brains were excised from 17-day rat embryos and homogenized, then cells were cultivated on polyethyleneimine-coated coverslips in Neurobasal medium (GIBCO) supplemented with B-27 (GIBCO) and nerve growth factor as described (*29*). As a marker for neurons, monoclonal antibody against microtubule-associated protein 2 (MAP2) was used (Sigma). The cells were grown on coverslips for a week, then fixed with 3.7% formalin in PBS for 10 min at room temperature. After the fixation, specimens were treated with 0.2% Triton X-100 in PBS for 30 min at room temperature, incubated with methanol for 30 min at –20°C, and washed two times for 5 min each with 0.2% Triton-X-100 in PBS. The double staining was performed basically as described (*19*). The fixed cells were incubated with 1/1,000 diluted anti–mouse Dnmt1 antibodies in PBS containing 0.4% SDS, 2% Triton X-100, and 1% BSA for 1 h at room temperature. After incubation, the specimens were washed four times with PBS containing 0.1% Triton X-100 (washing buffer), incubated with anti–rabbit IgG conjugated to ALEXA 488 in PBS containing 0.1% TritonX-100 and 1% bovine serum albumin for 1 h, then washed four times with washing buffer. The ALEXA stained cells were further incubated with anti-MAP2 monoclonal antibody in PBS containing 0.1% Triton X-100 and 1% bovine serum albumin for 1 h at room temperature. The specimens were then washed and incubated with anti-mouse IgG conjugated to TRITC (Chemicon). After washing four times, the cells were stained with Hoechst 33258, then immersed in PBS containing 50% glycerol. The stained samples were observed as described elsewhere (*19*, *30*). Exposure and printing of all samples were performed under identical conditions.

5′ RACE and RT-PCR—Total RNAs were prepared with TRIzol (Gibco-BRL) according to the manufacturer's instructions. A 5′ RACE system (Gibco-BRL) was used with total RNAs treated with RNAase-free DNAase I as templates and an oligonucleotide primer, 5′-TCCCGTTGGCGGGACAACCG-3′, located in the fourth exon (*31*). The synthesized cDNAs tailed with poly(dC) were amplified by PCR with a poly[d(GI)]-anchor primer and a nested primer, 5′-GTTTGCAGGAATTCATGCA-3′, located in the third exon. The amplified products were analyzed by agarose gel electrophoresis, subcloned into the pGEM-T easy vector (Promega), then sequenced.

To amplify the pachytene spermatocyte-specific transcript, an exon 1p–specific sense primer, 5′-GCCCTCTGAAATAGAACAAGCTTGGGG-3′ (*17*), and an exon 4–specific primer, 5′-TCCCTCACACACTCCTTTTG-3′, were used. The total RNAs were reverse-transcribed with a random-

hexamer primer and SuperScript II at 42°C (Gibco-BRL). The amplification reaction comprised 40 cycles of incubation of the reaction mixture for denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 73°C for 1 min.

RESULTS

Dnmt1 Protein Is Significantly Expressed in the Post-Nuclear Fraction of Brain—Goto *et al.* reported that mature nerve cells in the brains of adult mice are rich in the Dnmt1 transcript (*21*). In general, Dnmt1, both the transcript and protein, rapidly decreases when cell proliferation is arrested (*13*). Since nerve cells in the brain are in a postmitotic state, the high expression level of the Dnmt1 transcript seems to be unusual. To determine whether the high level of the Dnmt1 transcript expressed is translated into the functional Dnmt1 protein, we tried to detect the Dnmt1 protein in the central nervous system.

The cerebrum and cerebellum were excised and fractionated into nuclear and post-nuclear fractions. Dnmt1 in each fraction was determined by immunoprecipitation followed by Western blotting. A significant amount of Dnmt1 protein was detected in both the cerebrum and cerebellum, the amounts being comparable to that in proliferating culture cells, C3H10T1/2 cells (Fig. 1A), indicating that the high level of the Dnmt1 transcript in the brain was functionally translated into the protein. However, unexpectedly, a considerable amount of Dnmt1 protein was detected in the post-nuclear fractions of both cerebrum and cerebellum under fractionation conditions under which Hsp90, a cytoplasmic marker, and core histones, nuclear markers, were in the corresponding fractions (Fig. 1A). The mobilities of the Dnmt1 protein in both the post-nuclear and nuclear fractions of the cerebrum and cerebellum on SDS-PAGE

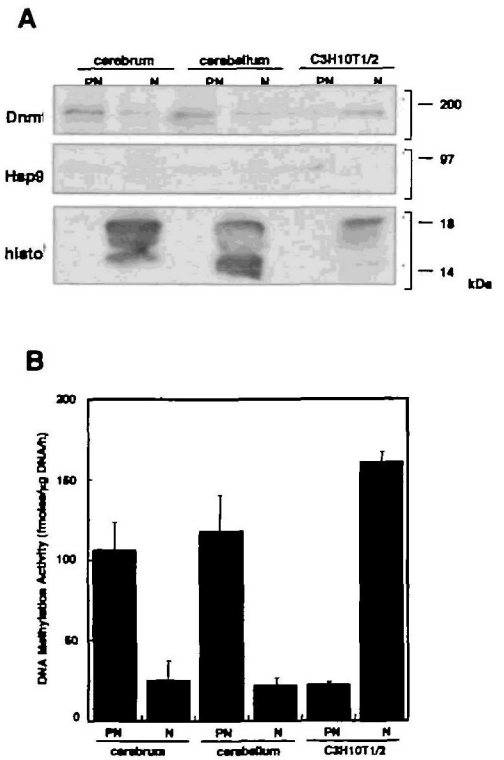

Fig. 1. **Dnmt1 protein expression in the cerebrum and cerebellum.** (A) Cerebrum, cerebellum, and proliferating C3H10T1/2 cells were subfractionated into post-nuclear (PN) and nuclear (N) fractions. Post-nuclear and nuclear fractions prepared from the tissues or C3H10T1/2 cells containing 15 or 5 μg DNA, respectively, were immunoprecipitated with anti-Dnmt1 and then analyzed by Western blotting. Markers for post-nuclear (Hsp-90) and nuclear (histones) fractions were detected directly by Western blotting. (B) The DNA methylation activities in post-nuclear and nuclear fractions were determined using poly (dI-dC) as a methyl acceptor. Bars indicate the means of activities determined for three different mice or cell cultures (±standard deviations). The specific activity in each preparation was determined from the activities obtained with three different concentrations of the enzyme source.

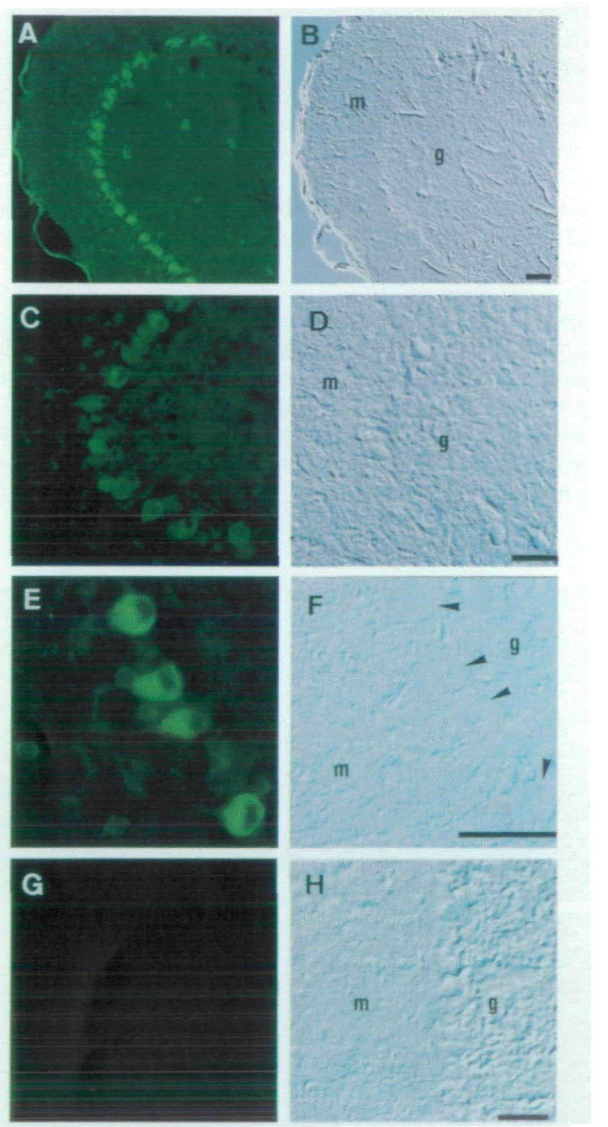

Fig. 2. **Dnmt1 protein expressed in the cerebellum.** The brain from an 8-week mouse was fixed and then cryosectioned at 14 μm. The sections were reacted with anti-Dnmt1 antibodies (A, C, and E) or anti-Dnmt1 antibodies pre-absorbed with excess of the antigen against which they were raised (G), and then with secondary antibodies coupled with ALEXA-488. The fields in panels A, C, E, and G were also observed under Nomarski optics (B, D, F, and H). Arrowheads in panel F indicate Purkinje's cells. g, granulocyte layer; and m, molecular layer. Bar, 50 μm.

were identical to that in proliferating C3H10T1/2 cells (Fig. 1A) or murine erythroleukemia cells (data not shown). Coinciding with this distribution of the Dnmt1 protein in the post-nuclear fractions of the cerebrum and cerebellum, significant levels of DNA methylation activities were detected in the post-nuclear fractions (Fig. 1B). Under the fractionation conditions used, the majority of both the protein and activity in proliferating C3H10T1/2 cells was distributed in the nuclear fractions. The densitometrically determined contents of Dnmt1 protein in the post-nuclear fractions of the cerebrum and cerebellum per tissue DNA content were respectively 80 and 86% of that in the nuclear fraction of C3H10T1/2 cells (Fig. 1A). The specific DNA methylation activities per tissue (or cell) DNA (approximately a per-cell basis) in the post-nuclear fractions of the cerebrum and cerebellum, and in the nuclear fraction of C3H10T1/2 cells were 107 ± 20, 118 ± 27, and 161 ± 6 fmol/μg DNA/h, respectively (Fig. 1B). If the DNA methylation activities detected in the brain in the present study were solely due to Dnmt1, the specific activities of Dnmt1 in the post-nuclear fractions of cerebrum and cerebellum would be about 83 and 85%, respectively, of that in the nuclear fraction of C3H10T1/2 cells. Dnmt1 in the post-nuclear fractions of the brain thus seemed to be almost fully active.

Immunochemical Study of the Expression of Dnmt1 in Neurons—Brain cells mainly comprise slowly proliferating glial cells and post-mitotic nerve cells. To determine which type of cells contributed to the Dnmt1 protein expressed in the brain, we next stained sections of mouse brains with anti-Dnmt1 antibodies. The cytoplasmic region of relatively large cells, which were a sign of neurons, were positively stained compared to the rest of the cells (Fig. 2, and data not shown for cerebrum). In particular, Purkinje's cells in the cerebellum were strongly stained (Fig. 2). The cytoplasmic region of the cells was stained more strongly than that of nuclei (Fig. 2E). The antibodies pre-absorbed with excess

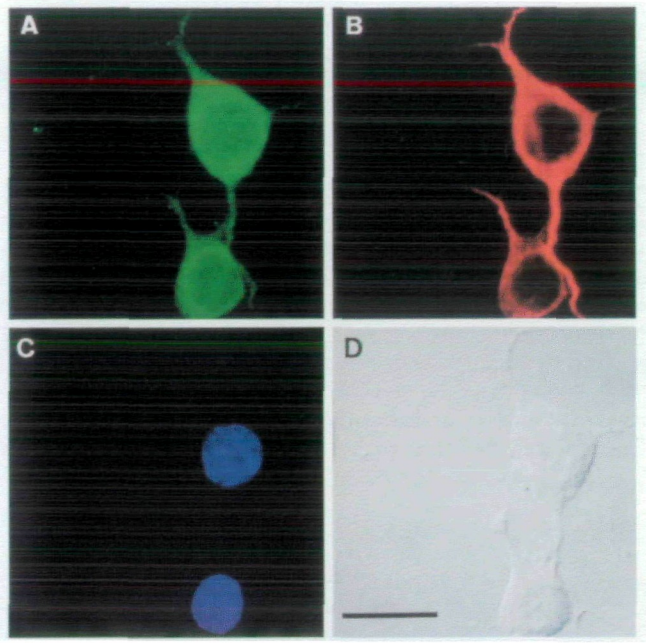

Fig. 4. **Dnmt1 protein mainly existed in the cytoplasmic compartment in neurons.** Higher magnification of the immunostaining in Fig. 3 is shown. The samples were stained with anti-Dnmt1 (A), anti-MAP2 (B), and Hoechst 33258 (C), and the same field was observed under Nomarski optics (D). Bar, 20 μm.

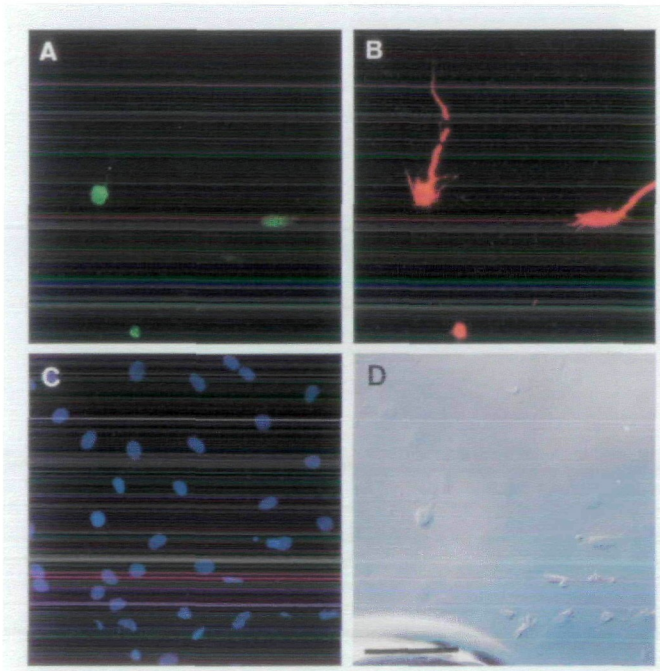

Fig. 3. **Dnmt1 protein specifically expressed in MAP2-positive neurons.** A primary culture of cells prepared from a fetal rat brain was fixed and stained with anti-Dnmt1 (A), anti-MAP2 (B), and Hoechst 33258 (C), and the same field was observed under Nomarski optics (D). Bar, 100 μm.

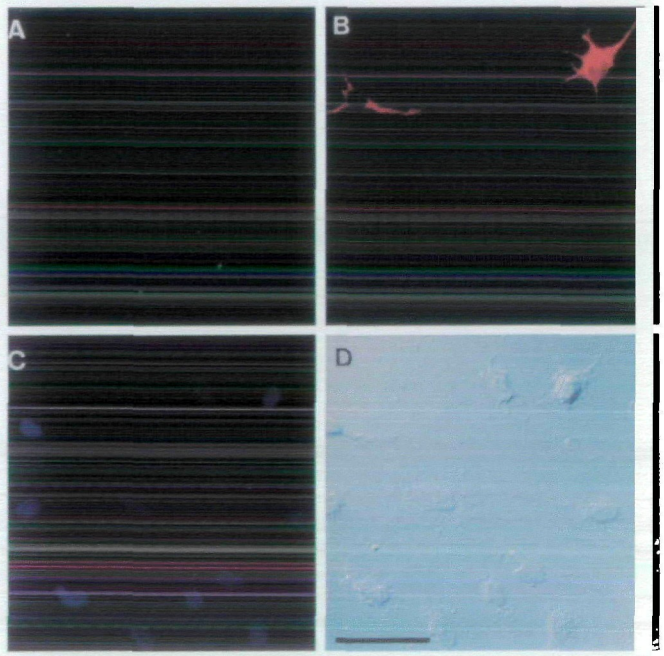

Fig. 5. **Specificity of anti-Dnmt1.** The primary culture of neuronal cells was fixed and immunostained as in Figs. 3 and 4 with anti-Dnmt1 antibodies pre-absorbed with excess antigen (A), anti-MAP2 (B), and Hoechst 33258 (C), and the same field was observed under Nomarski optics (D). Bar, 50 μm.

of the antigen against which they were raised completely chased the staining out of the neurons (Fig. 2G). This cytoplasmic staining of Purkinje's cells with an immunofluorescent probe was confirmed by the staining with horseradish peroxidase-conjugated antibodies (data not shown).

These results suggested that neurons, which are in a post-mitotic state, significantly expressed the translated product of Dnmt1, and, interestingly, that the Dnmt1 protein was present mainly in the cytoplasmic compartment. To confirm these points, we next prepared a primary culture of neurons and identified the expressed Dnmt1 protein. Neurons in the culture were identified with anti-MAP2 monoclonal antibody (Fig. 3B). As shown in Fig. 3A, Dnmt1 was strongly expressed in the MAP2-positive cells. The MAP2-negative cells did not express a detectable amount of Dnmt1 (compare panels A–D). To determine the localization of Dnmt1 inside the cells, the cells were observed under higher magnification (Fig. 4, A–D). The MAP2 protein was distributed exclusively in the cytoplasmic compartment. On the other hand, although the Dnmt1 protein seemed to exist inside the nuclei, the nuclei were stained more weakly than the cytoplasm. As the nuclei in specimens were thicker than the cytoplasmic regions, it can be concluded that the majority of Dnmt1 was distributed in the cytoplasm (Fig. 4A). As in the brain section (Fig. 2), the antibodies pre-absorbed with excess of the antigen against which they were raised chased the staining out of the neurons (Fig. 5).

Exon Utilization of Dnmt1 in Brain—The amino-terminal portion of Dnmt1 in oocytes is 118 amino acid residues shorter than that of somatic cells, and is excluded from nuclei (*17*). The oocyte-type is produced through alternative exon usage. The exclusion of this oocyte-type Dnmt1 from nuclei, however, is not due to deletion of the amino-terminal sequence of somatic-type molecules, since the somatic-type Dnmt1 ectopically expressed in oocytes is also retained in the cytoplasm (*20*). In addition, the Dnmt1 in the cytoplasm of the brain may not be of this oocyte-type, as its mobility on SDS-PAGE was identical to that in somatic cells such as C3H10T1/2 cells (Fig. 1A) or murine erythroleukemia cells (data not shown).

To examine the possibility that the Dnmt1 mRNA in the brain utilized an alternative 5′ exon, *i.e.* one other than the oocyte- or somatic-type, we performed 5′ RACE and analyzed the 5′ sequence of the transcript in the brain. As shown in Fig. 6A, the major product of 5′ RACE was about 300 bp, which was the expected size of the somatic-type transcript (*17*). The sequencing of the major 5′ RACE products after subcloning revealed that the exon utilized was identical to that of the somatic-type transcript (data not shown). We could not detect the oocyte-type sequence in either the cerebrum or cerebellum. However, both the cerebrum and cerebellum gave faint bands corresponding to PCR products larger than the somatic-type transcript (Fig. 6A). Interestingly, the sequencing of some of the materials (Fig. 6, asterisks) revealed that these transcripts were of the pachytene-spermatocyte type (data not shown). The expression of the pachytene-spermatocyte specific transcript in the cerebrum and cerebellum was further confirmed by RT-PCR using a specific PCR primer set for the transcript. The templates prepared, not from liver and oocytes, but from cerebrum, cerebellum and testis gave bands of the expected size of about 650 bp (Fig. 6B). This

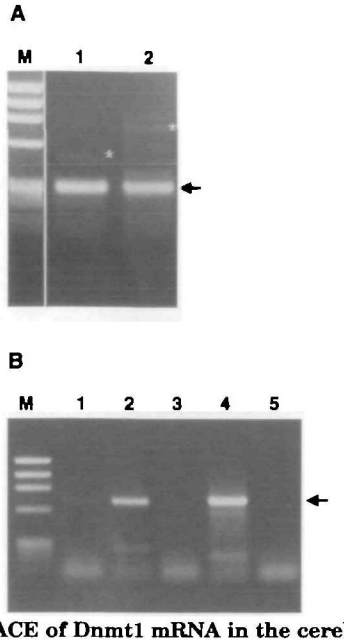

Fig. 6. **(A) 5′ RACE of Dnmt1 mRNA in the cerebrum (lane 1) and cerebellum (lane 2).** The arrow indicates the major band that was PCR-amplified from the somatic-type transcript, and asterisks indicate the bands from the pachytene-spermatocyte specific transcript. **(B) RT-PCR products amplified with a specific primer set for pachytene spermatocyte-specific transcript in the cerebrum (lane 1), cerebellum (lane 2), liver (lane 3), testis (lane 4), and ovary (lane 5).** The arrow indicates the band that was PCR-amplified from the pachytene-spermatocyte specific transcript. The PCR products were electrophoresed in an 1.2% agarose gel, then stained with ethidium bromide. M, *Hae*III digest of ϕX174 DNA.

pachytene-spermatocyte specific transcript does not produce Dnmt1 protein (*32*, *33*). Therefore, the transcript may not contribute to the Dnmt1 protein and activity in brain.

DISCUSSION

In the present study, we characterized the Dnmt1 protein expressed in the brain. We found that neurons in the brain express a significant amount of Dnmt1 compared to non-neuronal cells. The amount of immunoprecipitatable Dnmt1 protein in the brain (Fig. 1A), and the immunofluorescence observations of brain sections (Fig. 2) and primary culturing of neurons (Fig. 3) support this conclusion. The majority of the Dnmt1 protein in neurons, surprisingly, was localized in the cytoplasm. This was concluded from the findings that the Dnmt1 in the brain was specifically fractionated into the post-nuclear fraction (Fig. 1A), and that the Dnmt1 protein was detected mainly in the cytoplasmic compartment (Figs. 2 and 4). The Dnmt1 in the cytoplasmic compartment of neurons is enzymatically active, as the fraction contained a significant amount of DNA methylation activity (Fig. 1B). The Dnmt1 in neurons was translated from the somatic-type transcript. This was supported by the fact that the mobility of the Dnmt1 protein in the post-nuclear fraction on SDS-PAGE was identical to that in the nuclear fractions of brain cells and proliferating culture cells (Fig. 1A), and the majority of the transcript of Dnmt1 in the brain contained a somatic-type exon 1 (Fig. 6A). The pachytene-spermatocyte specific transcript, which was also

detected in brain (Fig. 6B), may not contribute to the Dnmt1 protein and activity in brain, since the transcript is reported to be not translated, thus does not produce Dnmt1 protein (*32*, *33*).

How does the highly expressed Dnmt1 in neurons reside in the cytoplasm? There are three possibilities: (i) the translocation of Dnmt1 into nuclei is the rate-limiting step as Dnmt1 is overexpressed in neurons, (ii) Dnmt1 is translocated into nuclei but then recycled to the cytoplasm, as the anchoring sites for Dnmt1 in nuclei are missing in neurons, and (iii) a neuron-specific factor in the cytoplasm causes Dnmt1 to stay in the cytoplasm. The high expression level of Dnmt1 in neurons, which is different from in other post-mitotic cells, is one of the important factors that explain the mechanism. However, the high expression level by itself cannot explain the cytoplasmic localization, since transiently expressed Dnmt1, which was expressed at extremely high level, was exclusively translocated into nuclei on transfection into cultured cells (data not shown). A recycling mechanism is an attractive hypothesis but needs to be examined. Considering that Dnmt1 in nuclei is under the control of the cell cycle, and that it has a shorter half-life in the G_1/G_0 phase than in the S phase (*14*, *16*), it is reasonable to assume that the Dnmt1 protein translocated into nuclei in neurons may also quickly be degraded.

A likely explanation for the cytoplasmic localization of Dnmt1 in neurons is as follows. High levels of Dnmt1 proteins in neurons are the result of high level of the transcript, and the proteins in the cytoplasmic compartment are anchored through a specific factor in neurons. After neurons enter the post-mitotic phase, the Dnmt1 proteins translocated into nuclei are eventually degraded due to their relatively short half-life in post-mitotic cells, and thus only the molecules in the cytoplasm escape degradation.

What is the meaning of the high level of Dnmt1 in the cytoplasm in neurons? Post-mitotic neurons do not regenerate, and thus the cells have to survive till the end of the life of the whole body. For this, the expression of the genes must be precisely regulated, *i.e.*, a limited number of genes must be active and the rest should be completely silent. DNA methylation is known to inactivate a gene (*34*). Actually, the total methylated deoxycytidine level in the genome has been reported to decrease in the senescence period in mouse liver but not in the brain (*35*). On the other hand, Dnmt1 not only plays a role in maintaining the DNA methylation patterns during the DNA replication (*12*), but may also act to maintain the methylation patterns just after DNA repair in non-proliferating cells (*36*). The direct interaction of Dnmt1 with proliferating cell nuclear antigen (PCNA) (*37*) may also support this, as PCNA is a prerequisite component for DNA repair as well as DNA replication. Thus, it can be estimated that the high level of Dnmt1 in neurons may guarantee that the silent genes in neurons are not demethylated, and the high level of Dnmt1 in the cytoplasm of neurons may be a reservoir of functional Dnmt1 for a time of necessity. In addition, the translocation of all the Dnmt1 may be dangerous or toxic for cells since Dnmt1 also exhibits *de novo* methylation activity (*24*, *38*). This unusual expression and localization of Dnmt1 in neurons could provide a clue for understanding the unknown function of Dnmt1 in neurons.

We wish to thank Drs. Kimiko Shimizu, Takaki Shima, Takashi Hirose, and Katsuya Nagai of our Institute for their technical support in preparing the frozen brain sections and primary cultures of neuronal cells.

REFERENCES

1. Antequera, F. and Bird, A. (1993) CpG islands in DNA methylation in *Molecular Biology and Biological Significance* (Jost, J.P. and Saluz, H.P., eds.) pp. 169–185, Birkähuser Verlag, Basel
2. Shen, C.-K. J. and Maniatis, T. (1980) Tissue-specific DNA methylation in a cluster of rabbit β-like globin genes. *Proc. Natl. Acad. Sci. USA* **77**, 6634–6638
3. Razin, A. and Cedar, H. (1991) DNA methylation and gene expression. *Microbiol. Rev.* **55**, 451–458
4. Tajima, S. and Suetake, I. (1998) Regulation and function of DNA methylation in vertebrates. *J. Biochem.* **123**, 993–999
5. Jaenisch, R. (1997) DNA methylation and imprinting: Why bother? *Trends Genet.* **13**, 323–329
6. Riggs, A.D. and Porter, T.N. (1996) X-Chromosome inactivation and epigenetic mechanisms in *Epigenetic Mechanisms of Gene Regulation* (Russo, V.E.A, Martinssen, R.A., and Riggs, A.D., eds.) pp. 231–248, Cold Spring Harbor Press, Cold Spring Harbor, NY
7. Laird, P.W. and Jaenisch, R. (1996) The role of DNA methylation in cancer genetics and epigenetics. *Annu. Rev. Genet.* **30**, 441–464
8. Monk, M. (1990) Changes in DNA methylation during mouse embryonic development in relation to X-chromosome activity and imprinting. *Phil. Trans. R. Soc. Lond.* **326**, 299–312
9. Okano, M., Bell, D.W., Haber, D.A., and Li, E. (1999) DNA methyltransferases Dnmt3a and Dnmt3b are essential for *de novo* methylation and mammalian development. *Cell* **99**, 247–257
10. Li, E., Bestor, T.H., and Jaenisch, R. (1992) Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* **69**, 915–926
11. Li, E., Beard, C., and Jaenisch, R. (1993) Role for DNA methylation in genomic imprinting. *Nature* **366**, 362–365
12. Leonhardt, H., Page, A.W., Weier, H.-U., and Bestor, T.H. (1992) A targeting sequence directs DNA methyltransferase to sites of DNA replication in mammalian nuclei. *Cell* **71**, 865–873
13. Szyf, M., Bozovic, V., and Tanigawa, G. (1991) Growth regulation of mouse DNA methyltransferase gene expression. *J. Biol. Chem.* **266**, 10027–10030
14. Liu, Y., Sun, L., and Jost, J.P. (1996) In differentiating mouse myoblasts DNA methyltransferase is posttranscriptionally and posttranslationally regulated. *Nucleic Acids Res.* **24**, 2718–2722
15. Teubner, B. and Schulz, W.A. (1995) Regulation of DNA methyltransferase during differentiation of F9 mouse embryonal carcinoma cells. *J. Cell Physiol.* **165**, 284–290
16. Suetake, I., Kanou, Y., and Tajima, S. (1998) Effect of aphidicolin on DNA methyltransferase in the nucleus. *Cell Struct. Funct.* **23**, 137–142
17. Mertineit, C., Yoder, J.A., Taketo, T., Laird, D.W., Trasler, J.M., and Bestor, T.H. (1998) Sex-specific exons control DNA methyltransferase in mammalian germ cells. *Development* **125**, 889–897
18. Carlson, L.L., Page, A.W., and Bestor, T.H. (1992) Properties and localization of DNA methyltransferase in preimplantation mouse embryos: implications for genomic imprinting. *Genes Dev.* **6**, 2536–2541
19. Kimura, H., Suetake, I., and Tajima, S. (1999) *Xenopus* maintenance-type DNA methyltransferase is accumulated and translocated into germinal vesicles of oocytes. *J. Biochem.* **125**, 1175–1182
20. Cardoso, M.C. and Leonhardt, H. (1999) DNA methyltransferase is actively retained in the cytoplasm during early development. *J. Cell Biol.* **147**, 25–32
21. Goto, K., Numata, M., Komura, J., Ono, T., Bestor, T.H., and Kondo, H. (1994) Expression of the DNA methyltransferase gene in mature and immature neurons as well as proliferating cells in mice. *Differentiation* **56**, 39–44

22. Persengiev, S.P. and Kilpatrick, D.L. (1996) Nerve growth factor induced differentiation of neuronal cells requires gene methylation. *Neuro Report* **8**, 227–231
23. Stancheva, I. and Meehan, R.R. (2000) Transient depletion of xDnmt1 leads to premature gene activation in *Xenopus* embryos. *Genes Dev.* **14**, 313–327
24. Takagi, H., Tajima, S., and Asano, A. (1995) Overexpression of DNA methyltransferase in myoblast cells accelerates myotube formation. *Eur. J. Biochem.* **231**, 282–291
25. Labarca, C. and Paigen, K. (1980) A simple, rapid, and sensitive DNA assay procedure. *Anal. Biochem.* **102**, 344–352
26. Bestor, T., Laudano, A., Mattaliano, R., and Ingram, V. (1988) Cloning and sequencing of a cDNA encoding DNA methyltransferase of mouse cells. The carboxyl-terminal domain of the mammalian enzymes is related to bacterial restriction methyltransferases. *J. Mol. Biol.* **203**, 971–983
27. Yoder J.A., Yen, R.-W.C., Vertino, P.M., Bestor, T.H., and Baylin, S.B. (1996) New 5′ regions of the murine and human genes for DNA (cytosine-5)-methyltransferase. *J. Biol. Chem.* **271**, 31092–31097
28. Nagai, N., Nagai, K., and Nakagawa, H. (1992) Effects of orbital enucleation on glucose homeostasis and morphology of the suprachiasmatic nucleus. *Brain Res.* **589**, 243–252
29. Brewer, G.J., Torricelli, J.R., Evege, E.K., and Price, P.J. (1993) Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. *J. Neurosci. Res.* **35**, 567–576
30. Miyagawa, J., Muguruma, M., Aoto, H., Suetake, I., Nakamura, M., and Tajima, S. (1999) Isolation of the novel cDNA of a gene of which expression is induced by a demethylating stimulus. *Gene* **240**, 289–295
31. Tucker, K.L., Talbot, D., Lee, M.A., Leonhardt, H., and Jaenisch, R. (1996) Complementation of methylation deficiency in embryonic stem cells by a DNA methyltransferase minigene. *Proc. Natl. Acad. Sci. USA* **93**, 12920–12925
32. Trasler, J.M., Alcivar, A.A., Hake, L.E., Bestor, T., and Hecht, N.B. (1992) DNA methyltransferase is developmentally expressed in replicating and non-replicating male germ cells. *Nucl. Acids Res.* **20**, 2541–2545
33. Jue, K., Bestor, T.H., and Trasler, J.M. (1995) Regulated synthesis and localization of DNA methyltransferase during spermatogenesis. *Biol. Rep.* **53**, 561–569
34. Bird, A.P. (1995) Gene number, noise reduction and biological complexity. *Trends Genet.* **11**, 94–100
35. Ono, T., Uehara, Y., Kurishita, A., Tawa, R., and Sakura, H. (1993) Biological significance of DNA methylation in the aging process. *Age Ageing* **22**, S34–43
36. Brooks, P.J., Marietta, C., and Goldman, D. (1996) DNA mismatch repair and DNA methylation in adult brain neurons. *J. Neurosci.* **16**, 939–945
37. Chuang, L.S.-H., Ian, H.-I., Koh, T.-W., Ng, H.-H., Xu, G., and Li, B.F.L. (1997) Human DNA-(cytosine-5) methyltransferase-PCNA complex as a target for $p21^{WAF1}$. *Science* **277**, 1996–2000
38. Tajima, S., Tsuda, H., Wakabayashi, N., Asano, A., Mizuno, S., and Nishimori, K. (1995) Isolation and expression of a chicken DNA methyltransferase cDNA. *J. Biochem.* **117**, 1050–1057