4454–4461 *Nucleic Acids Research, 1998, Vol. 26, No. 19*

Modulation of plasmid DNA methylation and expression in zebrafish embryos

Philippe Collas*

Norwegian College of Veterinary Medicine, Department of Biochemistry, PO Box 8146 Dep., 0033 Oslo, Norway

Received May 26, 1998; Revised and Accepted July 24, 1998

ABSTRACT

Gene expression is under the influence of DNA methylation and assembly of chromatin structure. This paper reports the modulation of transgene expression in zebrafish embryos by altering DNA methylation with 5-azacytidine and heterochromatin formation with sodium butyrate, an inhibitor of histone deacetylation. A CMV promoter–luciferase fusion gene construct (pCMVL) microinjected into zebrafish eggs becomes gradually methylated during development, starting at ~12 h post-injection. When methylated *in vitro* by *Hpa*II methylase prior to injection, the construct is rapidly demethylated *in vivo* before being *de novo* methylated. Demethylation is independent of DNA replication, indicating that it is an active DNA repair process. Demethylating activity has been characterized in zebrafish embryo nuclear extracts, in which this activity is heat-labile, sensitive to protease and RNase and requires ATP hydrolysis. Demethylating activity *in vitro* is dependent on the developmental stage of the embryo from which extracts are prepared. *In vivo*, luciferase transcripts are detected prior to *de novo* plasmid methylation. Furthermore, incubation of pCMVL-injected embryos with 5-azacytidine or butyrate immediately after injection inhibits plasmid methylation and extends the period of luciferase expression. When applied after *de novo* methylation has occurred, both inhibitors prevent methylation of newly replicated DNA and promote transgene expression. These data suggest that methylation of the injected construct during early development induces repression of the transgene, perhaps by converting the construct to a repressive chromatin structure.

INTRODUCTION

Epigenetic modification of DNA by methylation of cytosine in CpG dinucleotides represents a mechanism of regulation of gene expression. Only a subset of these sites are methylated in a given cell type (1) and this distinct pattern of methylation is preserved by a maintenance methylase which modifies hemimethylated residues after each round of DNA replication (2). Methylation generally correlates with transcriptional inactivation of chromosomes, transgenes, disease genes and certain developmentally regulated genes (3). Three possible mechanims by which methylation affects gene expression have emerged: methylation may interfere directly with binding of ubiquitous transcription factors in cells where the gene is not expressed (4); binding of specific factors to methylated DNA may promote gene repression (5); methylation may induce gene inactivation by altering chromatin structure (6).

Gene-specific methylation patterns are established during embryonic development. In the early mouse embryo, the gametic methylation pattern is erased by genome-wide demethylation (7,8). Nevertheless, methylation states are continuously changing in the early embryo (9) and sites of methylation display tremendous variation (2,10,11). There is increasing evidence that demethylation occurs through an active (DNA replication-independent), rather than a passive (replication-dependent) mechanism. In cultured mouse cells and chicken embryos, demethylation can be induced independently of DNA replication (12,13). Transient transfection of the α-actin gene into rat myoblasts has shown that unintegrated plasmids are fully and actively demethylated (14), as is plasmid DNA integrated into early mouse embryos (15). Demethylation in extracts of chicken nuclei (16) and mammalian cells (17) has been shown to involve removal of the entire 5-methylcytosine by a DNA glycosylase (18) and its replacement by a cytosine (16). The reaction is not sequence-specific and favours hemimethylated substrates (16). In whole cell extracts, demethylation is protease-resistant but RNase-sensitive, suggesting a role of RNA in this process (17).

There is evidence to suggest that expression of a transgene is under the influence of methylation and of the assembly of a repressive chromatin structure. Similarities between position effect variegation in *Drosophila* and variegated transgene expression in mice (19) and zebrafish (20) suggest that transgene expression is affected by heterochromatin formation. The histone deacetylation inhibitor butyrate (21) has been suggested to prevent the formation of heterochromatin (22) and to alter transgene methylation in zebrafish (23). In the latter study, however, no correlation was established between transgene methylation and expression. However, more recently, microinjection of methylated DNA templates into *Xenopus* oocyte nuclei has shown that methylation by itself does not immediately block transcription, but promotes the formation of a repressive chromatin structure (6).

Transgenic lines of zebrafish have been produced in several laboratories (20,24,25). Common features of these studies are the

*Tel: +47 22 96 45 69; Fax: +47 22 60 09 85; Email: philippe.collas@veths.no

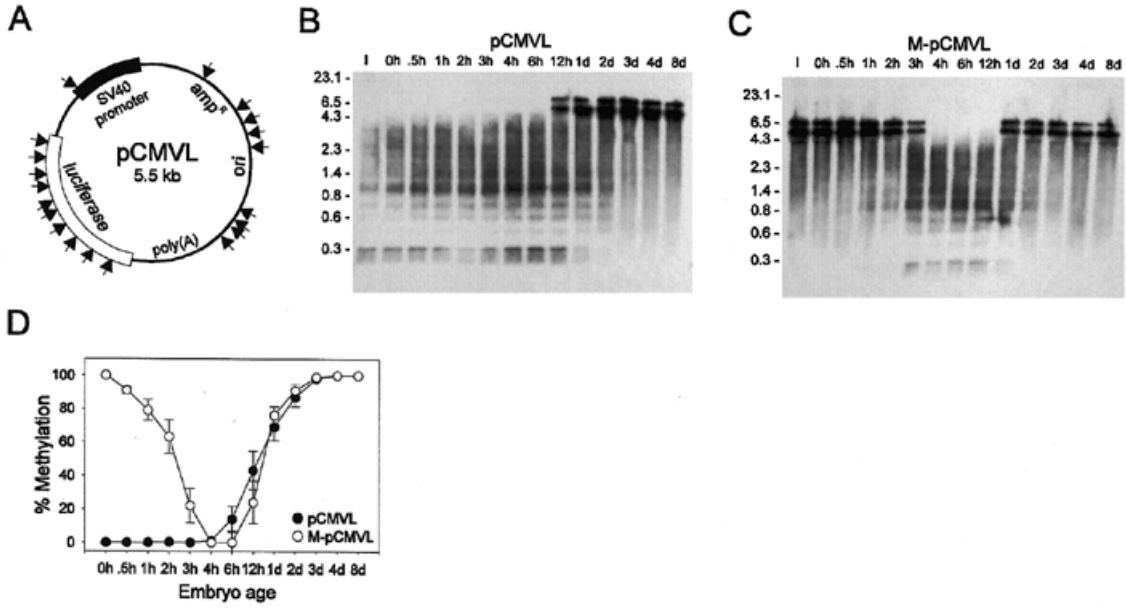

Figure 1. Methylation pattern of plasmid DNA microinjected into zebrafish embryos. (**A**) Map of the pCMVL plasmid; arrows indicate *Hpa*II methylation sites (not drawn to scale). (**B–D**) pCMVL or pCMVL methylated *in vitro* by *Hpa*II methylase (M-pCMVL) was injected into fertilized eggs. At the indicated time points, DNA was isolated, digested with *Hpa*II and methylation of injected substrates analysed by Southern blotting using probe CMVL. Methylation pattern of pCMVL (B) and M-pCMVL (C). Size markers are indicated in kb on the left. (D) Mean proportion (± SD) of methylated plasmid DNA recovered from embryos after injection of pCMVL and M-pCMVL. Data were quantified from duplicate blots.

low frequency of transgene expression in transgenic individuals and variegation of expression in these individuals (20,25). Methylation has been suggested to account for variegated transgene expression in zebrafish (23,26), however, convincing evidence is lacking and attempts to alter transgene methylation and correlate the extent of methylation with expression have been limited. In this study, we used zebrafish embryos microinjected with unmethylated and methylated constructs, together with alterations of plasmid methylation during development, to establish a correlation between transgene methylation and transcriptional and translational activities. The data demonstrate that transgene expression can be modulated by altering transgene methylation with demethylating and chromatin remodelling agents.

MATERIALS AND METHODS

Nuclear extracts

Zebrafish embryos were chilled to 0°C, washed in buffer N (10 mM HEPES, pH 7.5, 250 mM sucrose, 50 mM NaCl, 5 mM $MgCl_2$, 10 µg/ml cytochalasin B, 1 mM dithiothreitol and protease inhibitors) and homogenized in 10 vol buffer N with three strokes of a loose fitting pestle and two strokes of a tight fitting pestle in a glass homogenizer. Lysates were decanted for 20 min on ice and the supernatant collected and centrifuged through 1 M sucrose at 1000 *g* for 10 min. Pelleted nuclei were re-washed through sucrose and resuspended in buffer A (10 mM HEPES, pH 7.5, 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 10% v/v glycerol, 0.5 mM spermidine, 0.15 mM spermine, 1 mM dithiothreitol and protease inhibitors) (16). Nuclei were lysed by slow addition of 4 M ammonium sulphate to 0.4 M, the lysate incubated for 30 min on ice and the viscous substance sedimented at 150 000 *g* for 1.5 h at 4°C. Protein concentration of the supernatant (nuclear extract) was adjusted to 15 µg/µl with buffer A and extracts frozen at –80°C. Prior to use, extracts were diluted 1:1 with 20 mM HEPES (pH 7.5), 100 mM NaCl.

Demethylation and methylation substrates

Two demethylation substrates were used: (i) a 5.5 kb plasmid (pCMVL) containing the cytomegalovirus (CMV) promoter driving the firefly luciferase (*luc*) gene (26; Fig. 1A); (ii) a 733 bp fragment of the *luc* coding region of pCMVL (27) containing four *Hpa*II methylation sites (see Fig. 3A). *In vitro* methylation of the 733 bp fragment and pCMVL was performed on 10–50 µg DNA by incubation with 5 U *Hpa*II methylase (New England Biolabs, Beverly, MA) per µg DNA for 1 h at 37°C. The reaction was terminated by incubation with 200 µg/ml proteinase K for 1 h at 55°C. *In vitro* methylated DNA (referred to as M-733 and M-pCMVL) was extracted with phenol:chloroform (1:1) and precipitated with ethanol. Methylation was verified by cleavage by *Msp*I but not by its methylation-sensitive isoschizomer *Hpa*II. For analysis of methylation *in vivo*, unmethylated pCMVL was used.

Methylation and demethylation assays

In vitro demethylation. A demethylation reaction mix consisted of 40 µl nuclear extract (300 µg total protein) containing 0.75 µl ATP-generating system (1 mM ATP, 10 mM creatine phosphate, 25 µg/ml creatine kinase) and 1 µl (300 ng) M-733 DNA. After incubation at 28°C for 3 h, the reaction was terminated by addition of 60 µl 20 mM EDTA. The reaction mixture was digested with 200 µg/ml proteinase K at 55°C for 1 h, the DNA extracted with phenol, phenol:chloroform (1:1) and chloroform

and ethanol precipitated. Samples were sedimented at 12 000 *g* for 30 min and sediments dissolved in H_2O. The DNA was digested for 1 h at 37°C with *Hpa*II or *Msp*I. Digestion products were ethanol precipitated, dissolved in H_2O and equal amounts of DNA loaded onto agarose gels.

In vivo methylation and demethylation analysis. Newly fertilized zebrafish eggs were microinjected with 10^6 copies of unmethylated or *in vitro* methylated substrate (pCMVL and M-pCMVL, respectively) and embryos cultured at 28°C as described (28). At the indicated time points, groups of 20 embryos were homogenized, digested with proteinase K and DNA isolated as above. The DNA was digested with *Hpa*II or *Msp*I before loading equal amounts onto agarose gels.

Gel electrophoresis, probe synthesis, Southern and northern blotting

DNA samples were electrophoresed in 1% agarose in 1× TBE and directly electroblotted onto a nylon GeneScreen membrane (DuPont NEN, Brussels, Belgium) in 0.5× TBE using a BioRad (Hercules, CA) semi-dry blotter at 3.6 mA/cm^2 membrane for 12 min. Membranes were rinsed in 5× SSC and DNA crosslinked by a 2 min UV exposure. Fluoresceinated DNA probes were synthesized using the Amersham (Little Chalfont, UK) Gene Images labelling kit as described by the manufacturer. Template DNA consisted of the 733 bp fragment described above or pCMVL, to produce probe 733 and probe CMVL, respectively. Reactions were terminated by addition of 20 mM EDTA and probes stored at –20°C. Southern blots were performed using the Amersham Gene Images hybridization kit. Membranes were hybridized with probe 733 or CMVL, as indicated, overnight at 62°C. Stringency washes were 20 min in 1× SSC, 0.1% SDS and 20 min in 0.5× SSC, 0.1% SDS, each at 62°C. Membranes were blocked, incubated in a 1:7500 dilution of alkaline phosphatase-conjugated anti-fluorescein antibodies for 1 h, washed and exposed on Hyperfilm MP (Amersham). Proportion of methylated substrate was quantified by measuring area and intensity of bands corresponding to methylated (*Hpa*II-resistant) substrate after background subtraction using the OptilabPro software (Graftek, Mirmande, France; 29). For Northern blot analysis, mRNA was extracted from pools of 10 embryos using the mRNA Direct kit (Dynal, Oslo, Norway), denatured, electrophoresed through 1% agarose in MOPS/formaldehyde buffer and transferred to a nylon GeneScreen membrane. Transcripts were detected with probe 733 using the Amersham Genes Image kit according to the manufacturer's instructions.

Mitotic index determination

Blastomeres were isolated from 1–6 h embryos by gently pipetting in Ca^{2+}-free 10% Hank's salts containing 2 mM EDTA. Blastomeres were slightly swollen in H_2O for 5 min, fixed in 3% paraformaldehyde for 10 min and DNA stained with 0.1 µg/ml propidium iodide. Mitotic index was determined by fluorescence microscopy as the percentage of cells in prometaphase or metaphase. Over 100 cells from 10 embryos were examined at each time point in each of three replicates.

Luciferase assay

Groups of five zebrafish embryos were lysed by repeated pipetting or gentle homogenization in 10% Hank's salts containing protease inhibitors on ice, protein concentration of the lysate determined and 250 µg total protein diluted to 40 µl in 10% Hank's salts. Ten microlitres of luciferin substrate were added to the lysate and the mixture exposed on film as described (26).

RESULTS

Substrate methylation and demethylation in developing zebrafish embryos

During embryonic development, genes are selectively methylated or demethylated according to a specific developmental programme. To determine whether plasmid DNA was also subjected to methylation after injection into eggs, a CMV promoter–*luc* reporter gene construct (Fig. 1A; pCMVL) was injected into newly fertilized zebrafish eggs. Plasmid methylation was examined during development after DNA extraction, digestion with *Hpa*II and Southern blotting using probe CMVL. The construct remained unmethylated for the first 6 h of development, after which it became methylated, as shown by the gradual disappearance of *Hpa*II cleavage products (Fig. 1B and D; pCMVL). At day 3 and beyond, only methylated plasmid DNA was detected (Fig. 1B). These results indicate that plasmid DNA is methylated within hours of microinjection into zebrafish eggs.

To determine whether a demethylating activity reminiscent of that characterizing early mammalian embryo development could be detected in developing zebrafish embryos, the methylation profile of a methylated substrate microinjected into eggs was examined. Demethylation substrate consisted of pCMVL methylated *in vitro* by *Hpa*II methylase (M-pCMVL) and methylation analysed by Southern blotting as above. M-pCMVL was gradually demethylated after injection such that at 4 h only demethylated vectors were detected (Fig. 1C and D; M-pCMVL). However, the substrate was consistently remethylated at 12–24 h, indicating that demethylation was only transient (Fig. 1C and D). At all stages of development, undigested reaction products did not produce fragments <5.5 kb (the size of pCMVL; data not shown), confirming that fragments of <5.5 kb did not represent degradation products of M-pCMVL. These results argue that *in vitro* methylated plasmid DNA injected into zebrafish eggs is transiently demethylated before being *de novo* methylated. In addition, examination of the methylation patterns of pCMVL and M-pCMVL shows that with both substrates ~30% of *de novo* methylation occurred at 12 h of development (Fig. 1D). This suggests that *de novo* plasmid methylation is developmentally regulated.

Plasmid demethylation *in vivo* is independent of DNA replication

Repair of methylated DNA can occur through a passive (replication-dependent) or active (replication-independent) mechanism (15). To determine by which mechanism demethylation occurred in zebrafish embryos, M-pCMVL demethylation was examined in non-replicating embryos. The DNA synthesis inhibitor aphidicolin is ineffective in arresting zebrafish embryos before the mid-blastula transition (MBT), thus DNA replication was inhibited with the topoisomerase I inhibitor camptothecin (30). Culture of embryos for 2 h between 1 and 3 h of development in 25 µM camptothecin did not cause any nuclear defects, as judged by

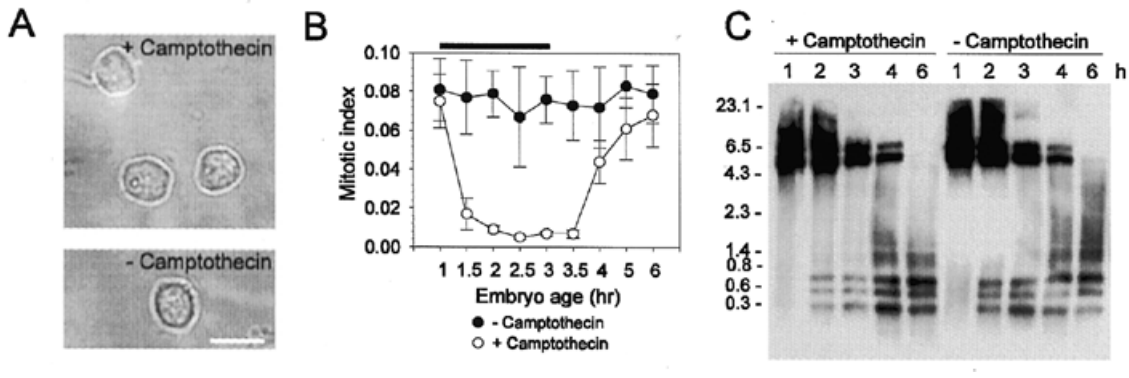

Figure 2. DNA demethylation *in vivo* is independent of DNA replication. (**A**) Phase contrast view of nuclei isolated from 3 h zebrafish incubated for 2 h in 0 (bottom) or 25 µM (top) topoisomerase I inhibitor camptothecin. Bar, 5 µm. (**B**) Mitotic index of blastomeres isolated from embryos cultured in 0 (●) or 25 (○) (µM camptothecin between 1 and 3 h development (bar). (**C**) Southern blot analysis of M-pCMVL demethylation in embryos cultured in 0 or 25 µM camptothecin as in (B), using probe CMVL.

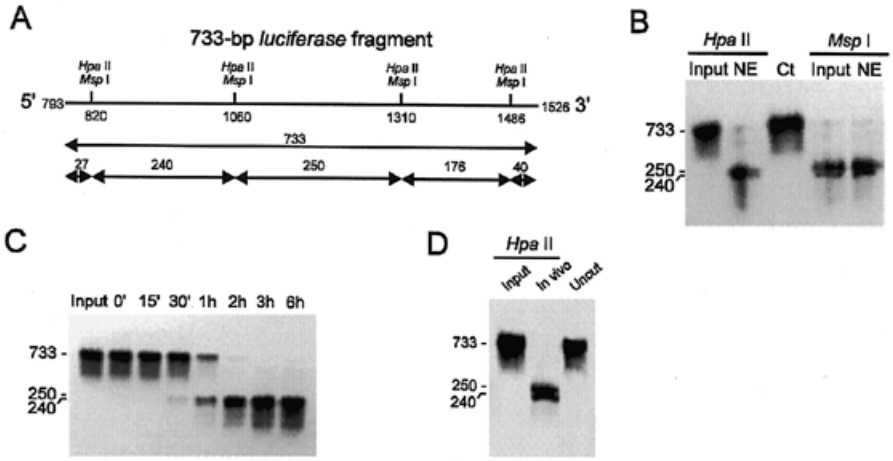

Figure 3. Active DNA demethylation in nuclear extracts of zebrafish embryos. (**A**) Map of the 733 bp DNA fragment of the *luc* gene used as demethylation substrate *in vitro*. Sites of methylation by *Hpa*II methylase are shown. (**B**) Demethylation of M-733 DNA in nuclear extract of 4 h embryos. M-733 (300 ng) (Input) was incubated in nuclear extract (NE) for 6 h at 28°C. The DNA was isolated and either left undigested (control, Ct) or digested with *Hpa*II or *Msp*I and analysed by Southern blotting using probe 733. (**C**) Time course of demethylation in nuclear extract. M-733 DNA was incubated in nuclear extracts of 4 h embryos and demethylation monitored by Southern blotting analysis at indicated time points as in (B). (**D**) M-733 demethylation *in vivo*. M-733 DNA (Input) was injected into fertilized eggs and, after 4 h, isolated DNA was digested with *Hpa*II and analysed by Southern blotting as in (B). Size of detected reaction products is indicated in bp.

phase contrast microscopy of nuclei (Fig. 2A). Reversible camptothecin-induced cell cycle arrest was shown by a rapid decline in the mitotic index of blastomeres isolated from these embryos and return to the control level after removal of the inhibitor (Fig. 2B).

M-pCMVL demethylation was subsequently examined in embryos arrested in interphase between 1 and 3 h of development with 25 µM camptothecin. Southern blotting analysis showed that the inhibitor did not affect the time course of M-pCMVL demethylation (Fig. 2C). Thus, substrate demethylation *in vivo* is not dependent on DNA replication, arguing that it is an active process. Since camptothecin has been shown to inhibit transcriptional elongation (31), the data also suggest that M-pCMVL demethylation is not dependent on transcription.

DNA demethylating activity in nuclear extract of zebrafish embryos

To characterize this *in vivo* demethylating activity, a cell-free system capable of demethylating exogenous DNA was developed. In *in vitro* experiments, a 733 bp demethylation substrate was used (M-733) that was methylated at four sites by *Hpa*II methylase (Fig. 3A). Cleavage of M-733 by *Msp*I but not by *Hpa*II set the basis for analysis of substrate demethylation. Since a previous result showed that the M-pCMVL substrate was demethylated in 4 h old embryos (Fig. 1C), M-733 demethylation was examined in nuclear extracts prepared from 4 h embryos. M-733 (300 ng; Fig. 3B, Input) was incubated for 6 h in extract (300 µg protein) containing an ATP-generating system. After

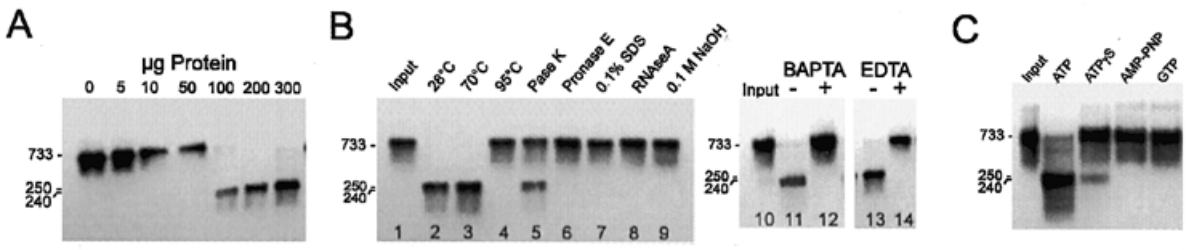

Figure 4. Demethylating activity in nuclear extract is heat-labile, protease- and RNase-sensitive and requires Ca^{2+} and ATP hydrolysis. M-733 demethylation substrate was incubated in (**A**) dilutions of nuclear extract (undiluted = 300 μg protein) or (**B**) heated extract (lanes 2–4), extract treated with 400 μg/ml proteinase K, 100 μg/ml pronase E, 0.1% SDS, 100 μg/ml RNase A, 0.1 M NaOH (pH 11), 5 mM BAPTA or 10 mM EDTA (lanes 5–14). (**C**) M-733 was incubated in extract containing either 1 mM ATP, 1 mM ATPγS, 1 mM AMP-PNP or 100 μM GTP. M-733 demethylation was analysed by Southern blotting using probe 733.

DNA isolation and digestion with *Hpa*II or *Msp*I, products were analysed by Southern blotting using probe 733. M-733 was demethylated in nuclear extract, as shown by the appearance of *Hpa*II 250 and 240 bp cleavage products (Fig. 3B, *Hpa*II, NE). Control M-733 exposed to nuclear extract but not digested with *Hpa*II did not display any cleavage products (Fig. 3B, Ct), indicating that the specific 250 and 240 bp products seen in Figure 3B were not due to unrepaired nicks. Monitoring the time course of M-733 demethylation in nuclear extract showed that essentially complete demethylation occurred in 2 h (Fig. 3C).

To verify that this *in vitro* demethylation reflected *in vivo* activity, demethylation of the M-733 substrate was examined in live 4 h old embryos. M-733 (40 pg) was injected into fertilized eggs and, after 4 h, DNA was isolated from pooled embryos, digested with *Hpa*II and products analysed by Southern blotting using probe 733. As shown in Figure 3D, substrate DNA recovered from embryos was cleaved by *Hpa*II (*In vivo*), indicating substrate demethylation. Control undigested DNA migrated exclusively at 733 bp (Uncut), indicating that the 250 and 240 bp bands represented *Hpa*II digestion products rather than a degraded substrate. These results indicate that nuclear extracts prepared from 4 h zebrafish embryos contain demethylating activity reminiscent of that detected *in vivo*.

Factors affecting demethylation in nuclear extracts

Having established that DNA demethylation *in vitro* reflected *in vivo* activity, factors capable of affecting *in vitro* demethylation were investigated. M-733 was incubated for 3 h in nuclear extracts from 4 h embryos and demethylation analysed by Southern blotting using probe 733. As shown in Figure 4A, substrate demethylation was affected by the amount of protein in the extract. Demethylating activity was heat-labile, since it was inhibited by heating the extract for 5 min at 95 but not 70°C (Fig. 4B, lanes 2–4). Treatment of extract with 400 μg/ml proteinase K partially impaired the activity, whereas 100 μg/ml pronase E at 37°C for 30 min completely inhibited the activity (lanes 5 and 6). Demethylation was abolished by denaturation of extract proteins with 0.1% SDS (lane 7), but not by solubilization with 0.1% of the non-ionic detergent Triton X-100 (not shown). Demethylation was RNA-dependent, as shown by inhibition of the activity by alkaline treatment of the extract (pH 11) or pre-incubation of the extract with 100 μg/ml RNase A for 1 h at 37°C (lanes 8 and 9). Demethylation was also inhibited by 10 mM EDTA or 5 mM Ca^{2+} chelator BAPTA in the reaction (lanes 10–14), indicating a requirement for Ca^{2+} and Mg^{2+}.

To determine the energy requirement for substrate demethylation, M-733 was incubated in nuclear extract containing either 1 mM ATP, 1 mM slowly hydrolysable non-phosphorylating ATP analogue ATPγS or 1 mM non-hydrolysable ATP analogue AMP-PNP, each with 10 mM creatine phosphate and 25 μg/ml creatine kinase. Demethylation was inhibited by ATPγS and AMP-PNP, indicating a requirement for ATP hydrolysis (Fig. 4C). Replacing ATP by 100 μM GTP (Fig. 4C), omitting ATP or depleting the extract of endogenous ATP with 50 U/ml hexokinase and 20 mM glucose also inhibited demethylation (not shown).

Previous results argued that the extent of plasmid demethylation *in vivo* was influenced by the developmental stage of the embryo (Fig. 1). To determine whether this variation in demethylating activity was duplicated in the cell-free system, M-733 demethylation in nuclear extracts prepared from embryos at increasing stages of development was examined by Southern blotting using probe 733. Figure 5 shows that demethylation occurred in extracts from 2 h to 4 day embryos only (*Hpa*II), indicating that these embryos contain DNA demethylating activity, whereas early (1 h) and late (≥8 day) embryos do not. These data suggest that DNA demethylating activity *in vitro* and *in vivo* is dependent on developmental stage of the embryo. This activity persists well after tissue differentiation has taken place.

Plasmid methylation and transient transgene expression during development

Although DNA demethylation is necessary for gene expression in general, expression of a microinjected transgene may not be strictly dependent on its methylation state. To establish a relationship between methylation and transgene expression, transcriptional and translational activities of the *luc* transgene were monitored in embryos injected with pCMVL or M-pCMVL. After pCMVL injection, the first luciferase transcript was detected at 2 h by northern blotting analysis (Fig. 6A, top panel) and reverse transcription–PCR (not shown). Luciferase assays revealed that maximum luciferase expression occurred on day 1 (Fig. 6B, pCMVL). In contrast, after injection of M-pCMVL transcription was delayed (12 h; Fig. 6A, bottom) and luciferase expression was severely impaired (Fig. 6B, M-pCMVL). Moreover, retrospective comparison of transgene methylation and expression profiles (Figs 1 and 6) indicates that onset of pCMVL or

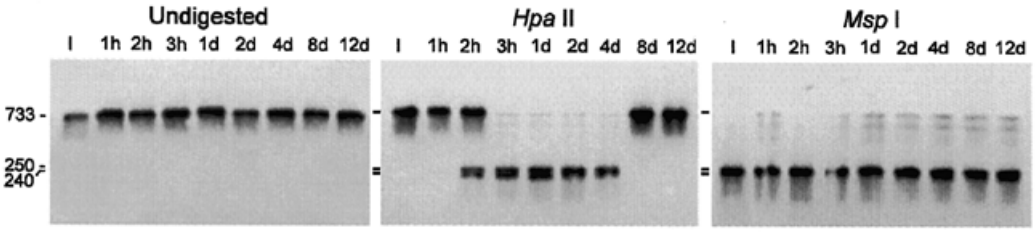

Figure 5. Demethylation *in vitro* is dependent on developmental stage of the embryo from which the extract is prepared. Nuclear extracts were made from embryos at the indicated time points after fertilization. M-733 was incubated in extracts for 3 h and demethylation analysed by Southern blotting using probe 733 after digestion of reaction products with *Hpa*II or *Msp*I. Undigested reaction products are shown in the left panel.

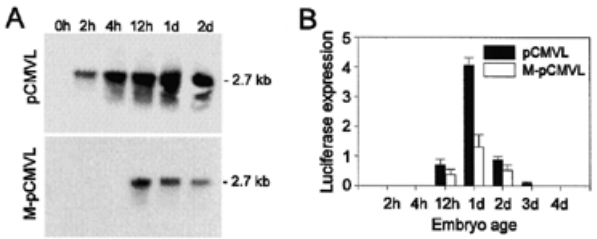

Figure 6. Transcriptional and translational activity of the *luc* gene of pCMVL and M-pCMVL. (**A**) Northern blot analysis of *luc* transcription from pCMVL (top) and M-pCMVL (bottom) in injected zebrafish embryos. (**B**) Mean (± SD) luciferase expression in embryos injected with pCMVL or M-pCMVL.

M-pCMVL transcription occurred prior to *de novo* methylation of the plasmids.

Alteration of transgene methylation and expression with butyrate and 5-azacytidine

Butyrate is a histone deacetylation inhibitor that is known to suppress variegation in *Drosophila*, presumably by preventing heterochromatin formation (22). In addition, the cytostatic compound 5-azacytidine (5-AC) is a specific inhibitor of cytosine methyltransferase that blocks methylation by being incorporated into newly synthesized DNA (32). Whether methylation of pCMVL could be manipulated *in vivo* by these inhibitors was investigated. Eggs were injected with pCMVL and immediately cultured with either no inhibitor (control), 5 mM butyrate for 48 h or 50 µM 5-AC for 6 h, after which inhibitors were removed and embryos cultured further. Plasmid methylation was assessed by Southern blotting using probe CMVL. Both butyrate and 5-AC affected pCMVL methylation. As shown in Figure 7A, butyrate reversibly inhibited pCMVL methylation during the treatment period. Furthermore, a 6 h incubation in 5-AC also inhibited pCMVL methylation for ~20 h (Fig. 7A). Higher concentrations of or longer incubations with either inhibitor also altered plasmid methylation, but had detrimental effects on embryo development (not shown).

Whether butyrate and 5-AC induced demethylation of *de novo* methylated DNA was then determined. Eggs were injected with pCMVL, cultured for 2 days to allow plasmid methylation (Fig. 7A) and incubated in 5 mM butyrate for 48 h or 50 µM 5-AC for 6 h. As shown in Figure 7B, while pCMVL remained methylated in untreated controls, both inhibitors induced a significant and reversible plasmid demethylation. Together with the previous results, this indicates that butyrate and 5-AC delay or inhibit plasmid methylation in injected embryos and promote demethylation of *in vivo* methylated DNA.

To investigate the effect of altering pCMVL methylation on transient transgene expression, pCMVL-injected embryos were incubated with butyrate (for 48 h) or 5-AC (for 6 h) immediately after injection and luciferase expression measured during and after inhibitor treatment. Intensity of expression was not significantly affected by 5-AC (Fig. 7C), however, butyrate dramatically enhanced expression over several days (Fig. 7C). Furthermore, when applied from day 2 of development, both butyrate and 5-AC induced expression (albeit at moderate levels) during the period of transient plasmid demethylation, whereas untreated embryos did not express luciferase after day 2 (Fig. 7D). Taken together, these results argue that inhibition of DNA methylation and histone deacetylation prevents plasmid methylation or induces plasmid demethylation during embryo development and thereby alters expression of the transgene.

DISCUSSION

Developmental regulation of exogenous DNA demethylation and *de novo* methylation

The profiles of plasmid methylation i*n vitro* and *in vivo* provide an insight into the balance of demethylation and methylation activities in the zebrafish embryo. *In vitro* substrate demethylation is detected in nuclear extracts from 2 h to 4 day embryos. This suggests that zebrafish embryos contain substantial levels of demethylating activity up to at least 4 (but not 8) days of development, which is well after tissue differentiation has taken place. Furthermore, demethylating activity appears 1–2 h before initiation of embryonic genome transcription at the MBT (3 h) (33), perhaps to prepare the rapidly dividing blastomeres for massive transcription. Timing of appearance of demethylating activity with respect to the MBT may be species-specific, since it occurs much earlier in zebrafish than in other rapidly dividing embryos (16). In contrast to *in vitro* data, however, demethylation *in vivo* is detected for only 6 h, after which the substrate is *de novo* methylated. Likely, demethylating activity is overridden by increasing levels of methyltransferase activity allowing *de novo* methylation of newly replicating DNA. In addition, remethylation of demethylated M-pCMVL occurs simultaneously with methylation of the unmethylated plasmid. Thus, the data suggest that the

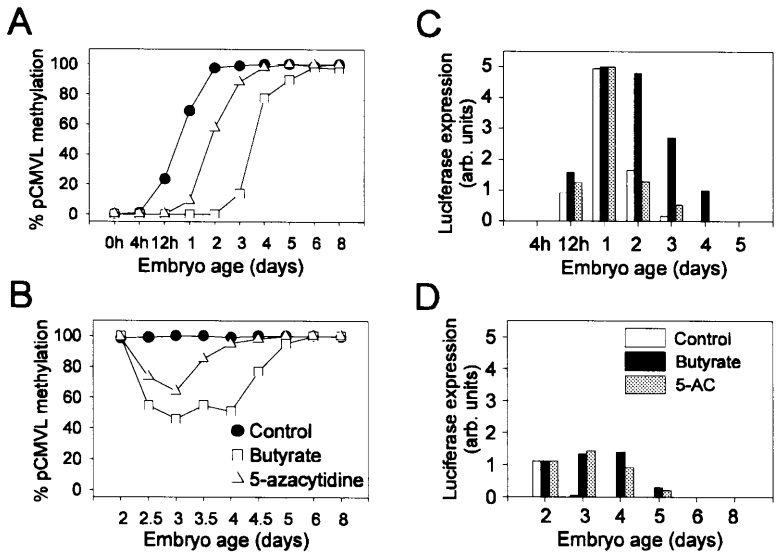

Figure 7. Alteration of DNA demethylation and reporter gene expression with butyrate and 5-azacytidine. pCMVL-injected embryos were cultured with either no inhibitor (Control), 5 mM butyrate for 48 h or 50 µM 5-azacytidine for 6 h, starting immediately (**A**) or 2 days (**B**) after injection. pCMVL methylation was assessed at the indicated time points by Southern blotting using probe CMVL and mean proportion of methylated plasmid quantified on duplicate blots. Standard deviations (not shown) ranged from 5.2 to 15.1%. (**C** and **D**) Luciferase expression of embryos injected with pCMVL and (C) cultured with inhibitors immediately after injection as in (A) or (D) starting 2 days post-injection as in (B). Standard deviations (not shown) ranged from 0.1 to 0.8 units.

methylation state of the injected construct is controlled by a balance between developmentally regulated antagonistic activities.

Methylation of the DNA construct during development is likely to result in permanent repression of the transgene. This contention is supported by the observation that the CMV–*luc* transgene is methylated in non-expressing transgenic zebrafish (unpublished data). In vertebrates, which contain a large number of tissue-specific genes, DNA methylation provides a means of permanently silencing genes whose activity is not required in a given cell type. Although gene expression is known to be affected by methylation in the promoter region, whether methylation of the coding sequence itself is also inhibitory remains unclear. In our construct, *Hpa*II methylase *in vitro* methylates a single site in the CMV promoter region (position 744) and 10 sites in the *luc* coding region (Fig. 1A). Whether methylation in either or both of these regions is inhibitory is at present undetermined. Nonetheless, the CMV promoter alone contains 46 CpG dinucleotides which are all potential sites of methylation by a eukaryotic methylase. Thus it is conceivable that a transgene under the control of a CMV promoter may be permanently repressed by methylation.

Demethylating activity is protein- and RNA-dependent

Demethylation of exogenous DNA has been duplicated in a nuclear extract of zebrafish embryos in the absence of replication. The reaction mimics *in vivo* demethylation and is likely to involve both protein and RNA components. Evidence for a role of RNA in demethylation was provided in a mammalian cell extract by inhibition of the reaction with RNase and conversion of the methyl-containing excised nucleotide into an alkaline- and RNase-sensitive form (17). This suggested the transfer of methylated nucleotides from DNA to RNA during demethylation, however, to my knowledge, no RNA acceptor has been reported to date. *In vitro* demethylation has been shown to occur through nucleotide excision repair (16–18), raising the possibility that the reaction is mediated, at least in part, by a ribozyme or an enzymatic protein–RNA complex. To support this view, ribonuclease P (34) and group I ribozymes (35) can cleave single-stranded DNA *in vitro*. Group II intron RNA is also a catalytic component of a DNA endonuclease that cleaves a single strand of DNA at specific sites (36). Demethylation in chicken nuclear extracts has also been shown to be initiated by a DNA glycosylase which excises the methylated nucleotide (16,18), in agreement with a role of proteins in the demethylation reaction. Nevertheless, although demethylation *in vitro* mimics *in vivo* activity, the nature and exact role of the putative catalytic protein or protein–RNA complex *in vivo* remain to be determined.

Relationship between transgene methylation, transcriptional and translational activity

Previous studies have suggested that expression of a transgene in transgenic mice (19), and to some extent in zebrafish (37), is inversely proportional to the degree of methylation of the transgene. The present study complements these findings by demonstrating a correlation between the extent of DNA methylation and transgene expression in zebrafish embryos. The data show that inhibiting DNA methylation *in vivo* with 5-AC promotes transgene expression during the demethylation period. These results suggest a direct inversely proportional relationship between transgene methylation and expression in developing zebrafish. It should be noted, however, that in mammals centromeric heterochromatin is enriched in 5-methylcytosine, which plays a role in heterochromatin formation (38,39). Thus, induction of transgene expression with 5-AC may reflect an

inhibition of heterochromatin assembly rather than a direct inhibition by methylation.

The results of this study also suggest that altering chromatin structure with an inhibitor of histone deacetylation, butyrate, affects transgene expression. Histone hyperacetylation correlates with transcriptional activity, or the potential for activity, whereas hypoacetylation is associated with transcriptionally silent chromatin and heterochromatin (40). Thus, by preventing histone deacetylation, butyrate is believed to prevent the formation of heterochromatin (22) and may promote transcription of an otherwise repressed gene. To support this view, histone acetylation, by reducing the positive charges of histones and weakening their interaction with DNA, prevents the assembly of repressive nucleosomal arrays (41). The exact pathway(s) used by sodium butyrate to regulate gene expression is not entirely elucidated, but may involve a serine-threonine phosphatase activity (42).

The state of methylation of the injected construct influences the timing of onset of transgene transcription in embryos. In spite of transient demethylation, the methylated plasmid M-pCMVL is transcribed later and at relatively lower levels than its unmethylated counterpart. Furthermore, whereas transcription from pCMVL is initiated prior to methylation of the plasmid, transcription from M-pCMVL starts at the time extensive plasmid methylation occurs. It is possible that transcription occurs from remaining unmethylated templates or, alternatively, that methylation alone does not immediately inhibit transcription. To support this view, injection of methylated and unmethylated templates into *Xenopus* oocyte nuclei recently showed that both methylated and unmethylated promoters are transcribed identically early after injection (6). Later, however, only the unmethylated construct is transcribed whereas the methylated template is silenced (6). Further experiments led to the conclusion that methylation-dependent gene inactivation promotes time-dependent assembly of a repressive chromatin structure involving a higher order nucleosomal structure (6). Taken together, these observations suggest that microinjected constructs are converted or integrated into a repressive (hetero)chromatin structure following methylation.

The present findings have several implications for transgenesis in zebrafish and other species. For example, it will be of interest to correlate methylation and expression patterns of retroviral vectors in transgenic individuals (25). In addition, the data suggest that it should be possible to induce temporary transgene expression in non-expressing transgenics by transiently altering DNA methylation and/or chromatin organization. Furthermore, to improve the efficiency of transgenesis, more sophisticated methods will need to be designed to target a transgene to active chromatin regions early in development.

ACKNOWLEDGEMENTS

The author is grateful to Ms M.-R. Liang for assistance in the laboratory and Dr P. Aleström for valuable discussions. This work was supported by European Union grant BIO4-CT97-0554.

REFERENCES

1 Razin,A. and Riggs,A.D. (1980) *Science*, **210**, 604–610.
2 Brandeis,M., Ariel,M. and Cedar,H. (1993) *BioEssays*, **15**, 709–713.
3 Kass,S., Pruss,D. and Wolffe,A. (1997) *Trends Genet.*, **13**, 444–449.
4 Becker,P.B., Ruppert,S. and Schutz,G. (1987) *Cell*, **51**, 435–443.
5 Nan,X., Campoy,J. and Bird,A. (1997) *Cell*, **88**, 471–481.
6 Kass,S., Landsberger,N. and Wolffe,A. (1997) *Curr. Biol.*, **7**, 157–165.
7 Kafri,T., Ariel,M., Brandeis,M., Shemer,R., Urven,L., McCarrey,J., Cedar,H. and Razin,A. (1992) *Genes Dev.*, **6**, 705–714.
8 Monk,M., Boubelik,M. and Lehnert,S. (1987) *Development*, **99**, 371–382.
9 Monk,M. (1990) *Phil. Trans. R. Soc. Lond. B*, **326**, 179–187.
10 Feil,R., Walter,J., Allen,N.D. and Reik,W. (1994) *Development*, **120**, 2933–2943.
11 Stoger,R., Kubicka,P., Liu,C.G., Kafri,T., Razin,A., Cedar,H. and Barlow,D.P. (1993) *Cell*, **73**, 61–71.
12 Razin,A., Szyf,M., Kafri,T., Roll,M., Giloh,H., Scarpa,S., Carotti,D. and Cantoni,G.L. (1986) *Proc. Natl Acad. Sci. USA*, **83**, 2827–2831.
13 Saluz,H.P., Jiricny,J. and Jost,J.P. (1986) *Proc. Natl Acad. Sci. USA*, **83**, 7167–7171.
14 Paroush,Z., Zeshet,I., Yisraeli,J. and Cedar,H. (1990) *Cell*, **63**, 1229–1237.
15 Kafri,T., Gao,X. and Razin,A. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 10558–10562.
16 Jost,J.-P. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 4684–4688.
17 Weiss,A., Keshet,I., Razin,A. and Cedar,H. (1996) *Cell*, **86**, 709–718.
18 Jost,J.-P., Siegmann,M., Sun,L. and Leung,R. (1995) *J. Biol. Chem.*, **270**, 9734–9739.
19 McGowan,R., Campbell,R., Peterson,A. and Sapienza,C. (1989) *Genes Dev.*, **3**, 1669–1676.
20 Stuart,G.W., Vielkind,J.R., McMurray,J.V. and Westerfield,M. (1990) *Development*, **109**, 577–584.
21 Peter,E., Candido,M., Reeves,R. and Davies,J.R. (1978) *Cell*, **14**, 105–113.
22 Mottus,R., Reeves,R. and Grigliatti,T.A. (1980) *Mol. Gen. Genet.*, **178**, 465–469.
23 Martin,C.C. and McGowan,R. (1995) *Genet. Res. Camb.*, **65**, 21–28.
24 Culp,P., Nüsslein-Vohlhard,C. and Hopkins,N. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 7953–7957.
25 Lin,S., Gaiano,N., Culp,P., Burns,J.C., Friedmann,T., Yee,J.-K. and Hopkins,N. (1994) *Science*, **265**, 666–669.
26 Gibbs,P., Peek,A. and Thorgaard,G. (1994) *Mol. Mar. Biol. Biotechnol.*, **3**, 307–316.
27 Collas,P. and Aleström,P. (1997) *Mol. Mar. Biol. Biotechnol.*, **6**, 48–58.
28 Collas,P., Husebye,H. and Aleström,P. (1997) In Houdebine,L.M. (ed.), *Transgenic Animals: Generation and Use*. Harwood Academic Publishers, Amsterdam, The Netherlands, pp. 119–122.
29 Collas,P., Thompson,L., Fields,A.P., Poccia,D.L. and Courvalin,J.-C. (1997) *J. Biol. Chem.*, **272**, 21274–21280.
30 Ikegami,R., Rivera-Bennets,A.K., Brooker,D.L. and Yager,T.D. (1997) *Zygote*, **5**, 153–175.
31 Ljungman,M. and Hanawalt,P.C. (1996) *Carcinogenesis*, **17**, 31–35.
32 Jones,P.A. (1984) In Razin,A., Cedar,H. and Riggs,A.D. (eds), *DNA Methylation; Biochemistry and Biological Significance*. Springer-Verlag, New York, NY, pp. 165–187.
33 Kane,D.A. and Kimmel,C.B. (1993) *Development*, **119**, 447–456.
34 Smith,D. and Pace,N.R. (1993) *Biochemistry*, **32**, 5273–5281.
35 Robertson,D.L. and Joyce,G.F. (1990) *Nature*, **344**, 467–468.
36 Zimmerly,S., Guo,H., Eskes,R., Yang,J., Perlman,P.S. and Lambowitz,A.M. (1995) *Cell*, **83**, 529–538.
37 Martin,L., Crimaudo,C. and Gerace,L. (1995) *J. Biol. Chem.*, **270**, 8822–8828.
38 Miller,O.J., Schedl,W., Allen,J. and Erlanger,B.F. (1974) *Nature*, **251**, 636–637.
39 Schmid,M., Hoff,T. and Grunert,D. (1984) *Hum. Genet.*, **64**, 257–263.
40 Wu,C. (1997) *J. Biol. Chem.*, **272**, 28171–28174.
41 Hansen,J.C. and Wolffe,A.P. (1992) *Biochemistry*, **31**, 7977–7988.
42 Cuisset,L., Tichonicky,L., Jaffray,P. and Delpech,M. (1997) *J. Biol. Chem.*, **272**, 24148–24153.