Epigenetic modifications affect *Dnmt3L* expression

Ulla AAPOLA^{*1}, Katja MÄENPÄÄ^{*}, Antti KAIPIA[†] and Pärt PETERSON^{*‡}

^{*}Department of Pathology, Institute of Medical Technology, Tampere University Hospital, University of Tampere, 33014 Tampere, Finland, [†]Department of Surgery, Tampere University Hospital, Teiskontie 35, 33521 Tampere, Finland, and [‡]Molecular Pathology, University of Tartu, Tartu, Estonia

Imprinted genes are expressed from a single allele due to differential methylation of maternal or paternal alleles during gametogenesis. *Dnmt3L* (DNA cytosine-5-methyltransferase 3 like), a member of *de novo* methyltransferase *Dnmt3* protein family, is a regulator of maternal imprinting. In the present study, we have characterized the promoter region of the mouse *Dnmt3L* gene. Transient transfection assays performed with 5'-deletion promoter constructs indicated a minimal promoter area within 440 bp upstream from the translational start site. Longer promoter constructs showed decreased activity, suggesting the presence of repressor elements within the upstream sequences. According to electrophoretic mobility-shift assays and mutation analysis, the minimal promoter region contained four functional binding sites for the Sp1 (specificity protein 1) family of transcription factors, Sp1 and Sp3. *In vitro* methylation of *Dnmt3L* promoter constructs decreased the transcriptional activity significantly, demonstrating down-regulation by cytosine methylation.

This was supported by the results from bisulphite sequencing and real-time quantitative reverse transcriptase-PCR analysis of different mouse cell lines and tissues. In testis and embryonic stem cells showing strong *Dnmt3L* expression, all CpG sites studied were fully unmethylated, whereas non-expressive cell lines and tissues with lesser *Dnmt3L* expression showed complete or diverse CpG methylation levels. Treatment of *Dnmt3L* non-expressive cell lines with deacetylase inhibitor trichostatin A and methyltransferase inhibitor 5-aza-2'-deoxycytidine induced the expression of *Dnmt3L* mRNA. Furthermore, we show that the repressional effect of longer promoter fragments was also relieved by these inhibitors, altogether indicating an epigenetic control for *Dnmt3L* gene regulation.

Key words: DNA cytosine-5-methyltransferase 3 like (*Dnmt3L*), epigenetic, genomic imprinting, promoter, specificity protein 1 (Sp1).

INTRODUCTION

In genomic imprinting, one of the autosomal gene alleles is methylated in parent-of-origin specific manner resulting in mono-allelic expression of the gene. Imprints are established during gametogenesis and maintained throughout development; however the exact mechanism of this process is unknown. To date, over 70 imprinted genes have been identified in mammals and many of them are associated with developmental diseases and cancer [1].

Three functional *Dnmts* (DNA methyltransferases) have been described in mammalian cells, *Dnmt1* [2], *Dnmt3a* and *Dnmt3b* [3,4]. *Dnmt1* functions mainly as maintenance methyltransferase, whereas *Dnmt3a* and *Dnmt3b* are *de novo* methyltransferases creating novel methylation patterns during development, but are also needed to maintain the global methylation patterns in ES cells [5]. Previously, we have identified *Dnmt3L* (DNA cytosine-5-methyltransferase 3 like) gene [6,7], a member of the DNA cytosine-5-methyltransferase 3 family based on the homology with *Dnmt3a* and *Dnmt3b*. *Dnmt3L*-deficient mouse studies indicated that the gene is involved in the establishment of maternal imprints in oocytes [8,9]. *Dnmt3L* activity as a functional *Dnmt* has not been proved, most probably because the protein lacks the critical amino acids needed for enzyme activity. However, *Dnmt3L* interacts directly with *Dnmt3a*/*Dnmt3b* [9,10] and stimulates *de novo* methylation by *Dnmt3a* [11], suggesting that *Dnmt3L* may regulate imprinting through these protein interactions. Indeed, similar to *Dnmt3L*, *Dnmt3a* mutant mice also have defective maternal imprinting [9]. All *Dnmt3* family members share similarity with

ATRX (α -thalassaemia/mental retardation syndrome, X-linked) protein [12,13] in its cysteine-rich zinc-finger motif region. *Dnmt3* proteins use this ATRX domain for interaction with histone deacetylase 1 to repress transcription [14–17]. It remains to be studied if in addition to *Dnmt3L*–*Dnmt3a* interaction, deacetylase activity recruited by these proteins is also involved in the establishment of imprints.

Studies with *DNMT3* family members, *DNMT3A* and *DNMT3B*, showed that the 5'-ends of the genes contain several promoters with alternative transcription start sites [18]. The promoters are either CpG-rich or CpG-poor in sequence and typically lack TATA consensus sequences. In the present study, we have cloned and characterized the TATA-less and CpG-rich *Dnmt3L* promoter from mouse by identifying the minimal promoter area and transcriptional regulation elements within it. Furthermore, we provide evidence that the chromatin epigenetic status of the *Dnmt3L* promoter can influence the gene expression level.

MATERIALS AND METHODS

DNA cloning and promoter constructs

Primer sequences for cloning the *Dnmt3L* promoter constructs by PCR were designed according to the mouse chromosome 10 genomic sequence (NT_039506) available in the GenBank[®] database. For the luciferase assay, a genomic fragment containing DNA sequence up to position – 1345 bp relative to the *Dnmt3L* translation-initiation site was amplified by PCR from mouse genomic DNA and cloned upstream to the firefly (*Photinus pyralis*)

Abbreviations used: ATRX, α -thalassaemia/mental retardation syndrome, X-linked; 5-azaCdR, 5-aza-2'-deoxycytidine; *Dnmt*, DNA methyltransferase; *Dnmt3L*, DNA cytosine-5-methyltransferase 3 like; EMSA, electrophoretic mobility-shift assay; ES, embryonic stem (cell); EST, expressed sequence tag; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDAC, histone deacetylase; RT, reverse transcriptase; QPCR, real-time quantitative RT-PCR; Sp1, specificity protein 1; TSA, trichostatin A.

¹ To whom correspondence should be addressed (e-mail ulla.aapola@uta.fi).

luciferase gene in the pLTR-Luc vector, resulting in plasmid pLuc1345. A series of 5'-deletion constructs (pLuc900, pLuc440 and pLuc226) were created using pLuc1345 as a template for PCR amplifications. All PCR cloned constructs were verified by sequencing. Primer sequences for cloning the *Dnmt3L* promoter constructs were (1345F) 5'-TTTGCGGCCGCCCATGG-GTTTCTAACCCGTA-3', (900F) 5'-TTTGCGGCCGCCGA-ACACTGGGCTGAGGTG-3', (440F) 5'-TTTGCGGCCGCC-TACCCCTACCCATTTC-3' and (226F) 5'-TTTGCGGCCGC-ACCCCATCATCCAGGCC-3'; the reverse primer for all cloning reactions was (3LpromR) 5'-TTTGGATCCAATCGG-CTAGAAGGGTCTGC-3'.

Site-directed mutagenesis of the pLuc440 construct was performed using two sets of primers (440F/mutated reverse primer and mutated forward primer/3LpromR) and hybridization of the products in second round PCR was performed with 440F/3LpromR primers. Mutations were introduced into four putative DNA-binding sites for the Sp1 (specificity protein 1) transcription factor, resulting in vectors pLucmut262, pLucmut290, pLucmut300 and pLucmut318. pLucmut262 and pLucmut318 were cloned with the same mutated oligonucleotides that were used in EMSA (electrophoretic mobility-shift assay; see below). Oligonucleotide sequences (5' → 3') for cloning of pLucmut290 and pLucmut300 were (mutSp1-290) CCACCCCCACTCTT-TTCCTCAGTGCTGGG and (mutSp1-300) GCCCCATCC-ACCTATATCCCACTCCTCCCT. Mutated nucleotides at Sp1 core recognition sites are underlined. Mutations were verified by sequencing.

Dnmt3L promoter sequence was analysed *in silico* by using the MatInspector program of the Genomatix software ([19]; <http://www.genomatix.de>).

Cell culture, transfections and luciferase assays

Mouse NIH3T3 fibroblasts and Cos-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) foetal calf serum and antibiotics. Mouse thymic epithelial cells Tec427.1 [20] were cultured in minimum essential Eagle's medium, supplemented with 10 % foetal calf serum, 2 mM L-glutamine and antibiotics. Media and supplements were obtained from BioWhittaker Europe (Verviers, Belgium).

NIH3T3, Tec427.1 or Cos-7 cells (60 000–100 000/35 mm well) were plated a day before transfection. Cells were transfected with 1–2 µg of different luciferase constructs together with 500 ng of pLacZ plasmid using ExGen500 transfection reagent (Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. Promotorless luciferase vector pBL-KS was included and used as a control in luciferase assays. Cells were washed, 24 or 48 h after transfection, twice with ice-cold PBS and lysed by freezing and thawing in Promega's Reporter lysis buffer. Luciferase activities were measured with Luciferase Assay System (Promega) using luminometer Luminoskan Ascent (Thermo Labsystems, Franklin, MA, U.S.A.). All transfections were performed as triplicates at least twice and results were normalized against β-galactosidase activity.

EMSA

Preparation of nuclear extracts was performed as described elsewhere [21]. Nuclear extracts (10 µg) were preincubated with 1 µg of poly(dI-dC) (Roche Applied Science) in 10 µl of binding buffer [10 mM Tris/HCl, pH 7.5/25 mM NaCl/0.5 mM dithiothreitol/1 mM MgCl₂/0.5 mM EDTA/4 % (v/v) glycerol]. After 30 min preincubation on ice, 40 000 c.p.m. of [γ -³²P]ATP-radiolabelled double-stranded oligonucleotide was added and the

mixture was incubated for 30 min on ice. Protein–DNA complexes were electrophoresed on a 4.5 % non-denaturing polyacrylamide gel for 3 h, and the dried gel was exposed to Kodak Biomax MS film at –70 °C. For competition experiments, 100-fold excess of the corresponding unlabelled double-stranded oligonucleotides were added, and for supershift antibody reactions, 2 µg of indicated antibodies were added to the preincubation mixture. Rabbit polyclonal anti-Sp1 (sc-59) and anti-Sp3 (sc-644) antibodies were purchased from Santa Cruz Biotechnology. The sequences of oligonucleotides (5' → 3') used in EMSA were as follows: (Sp1-374) CTCCCTCCCCACCCCTTTCCCGAA; (mutSp1-374) CTCCCTCCCCATTTCTTTCCCGAA; (Sp1-318) CCCTCAGCCCCGCCCCATCCA; (mutSp1-318) CCCTCAGCCCTGTTCCCATCCA; (2xSp1-303) CCACCCCA-CCCCCACTCTCCCTC; (2xmutSp1-303) CCACCCATTTCCCACTCTTTCTCCTC; (Sp1-262) CCTTAAGACCCGCCCC-TGAGTG; (mutSp1-262) CCTTAAGACCTGTTCTTGAGTG; and (consensus Sp1) ATTCGATCGGGCGGGCGGAGC. Numbers indicate the relative positions of Sp1-binding sites with respect to the *Dnmt3L* translation-initiation site (ATG). Mutated nucleotides at Sp1 core recognition sites are underlined.

Oligonucleotides Sp1-318, Sp1-262 and consensus Sp1, containing CpG dinucleotides in Sp1 core recognition sequence, were methylated *in vitro* with CpG methylase *M.SssI* (New England Biolabs, Beverly, MA, U.S.A.) according to the manufacturer's instructions. Methylated oligonucleotides were labelled and incubated with NIH3T3 nuclear extract together with corresponding unmethylated oligonucleotides as explained above.

In vitro methylation

Plasmids pLuc226, pLuc440, pLuc900 and pLuc1345 were methylated by CpG methylase *M.SssI* (New England Biolabs). *In vitro* methylation was performed for 2 h at 37 °C in 10 mM Tris/HCl (pH 7.9), 50 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol and 160 µM *S*-adenosylmethionine. The reaction was terminated by heating at 65 °C for 15 min and the DNA was extracted with phenol/chloroform and precipitated with ethanol. *In vitro* methylated luciferase constructs were transfected into Cos-7, NIH3T3 and Tec427.1 cells as explained above and compared with luciferase activities of mock-methylated constructs.

Genomic DNA and RNA preparations

For bisulphite sequencing performed with different mouse cell lines (ES, NIH3T3 and Tec427.1), genomic DNA was isolated according to the standard procedures using proteinase K treatment, phenol/chloroform extraction and ethanol precipitation. Mouse embryonic stem cells [22] were kindly provided by Dr Z. Shah (IMT, University of Tampere, Finland): 10 cm pools of 5 mm mouse seminiferous tubule segments representing stages II–VI, VII–VIII and IX–I of the mouse seminiferous epithelial cycle were isolated by using transillumination-assisted microdissection [23]. After isolation, all mouse tissues (samples from the mouse seminiferous epithelial cycle, testis, ovary, thymus, spleen, liver, heart and lung) were immediately frozen in liquid nitrogen and stored at –80 °C until total RNA and genomic DNA extractions were performed with TRIzol[®] reagent (Life Technologies) according to the manufacturer's instructions.

Bisulphite sequencing

Sheared genomic DNA (2 µg) in a volume of 50 µl was denatured by adding 5.5 µl of freshly prepared 3 M NaOH and incubating for 15 min at 42 °C. For deamination, 30 µl of 10 mM quinol

and 520 μ l of 3 M sodium bisulphite (pH 5.0) were added and incubated in the dark for 16 h at 50 °C. The DNA was desalted using the Wizard DNA Clean-up System (Promega) and the eluted DNA in 50 μ l was desulphonated with 5.5 μ l of 3 M NaOH for 15 min at 37 °C. The DNA was neutralized with 2 M ammonium acetate and precipitated with 220 μ l of ethanol for 30 min at –70 °C. After washing with 70 % (v/v) ethanol, the precipitated DNA was resuspended in 15–100 μ l TE (10 mM Tris/HCl, pH 8.0/1 mM EDTA).

Bisulphite-treated DNA was amplified by PCR in 25 μ l reaction volume containing buffer (75 mM Tris/HCl, pH 8.8/20 mM $(\text{NH}_4)_2\text{SO}_4$ /2 mM MgCl_2 /0.01 % Tween 20), 200 μ M dNTP, 0.2 μ M primers and 1 unit recombinant *Taq* DNA polymerase (Fermentas). PCR amplification was started with 2 cycles at 94 °C for 4 min, 55 °C for 2 min and 72 °C for 2 min, followed by 35 cycles at 94 °C for 1 min, 55 °C for 2 min and 72 °C for 2 min. Primer sequences for the first-round PCR were (BISBF1) 5'-TTAATGTGTGAGGTTTGTAGAGTTT-3' and (BISBR1) 5'-TCAAACTAAACTATCAAAAATCT-3'. If further amplification was needed, nested PCR was started with denaturation at 94 °C for 2 min, followed by 35 cycles at 94 °C for 1 min, 55 °C for 2 min and 72 °C for 2 min. Primer sequences for nested PCR were (BISBF2) 5'-GTTTTGAGTTTTATAGAA-TTTTATAAT-3' and (BISBR2) 5'-ATCCAAAATTTCAAAA-ATCTTAAACA-3'. The PCR products were gel-purified, subcloned into a pCR2.1-TOPO vector (Invitrogen), sequenced using a Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (PerkinElmer) and then analysed by an automatic DNA sequencer (310; Applied Biosystems).

5-aza-2'-deoxycytidine (5-azaCdR) and trichostatin A (TSA) treatments

Tec427.1 and NIH3T3 cells were plated 24 h before adding the chemicals. For luciferase assays, NIH3T3 cells were transfected with pLuc440 and pLuc1345 constructs as indicated above. The cells were treated for 48 h with different concentrations (from 0.2 to 10 μ M) of Dnmt inhibitor 5-azaCdR (Sigma). The deacetylase inhibitor, TSA (100 nM; Sigma), was added for 24 h together with 5-azaCdR where indicated. TSA alone (100 nM) was added to Tec427.1 and NIH3T3 cells for 12 and 24 h respectively. On each day, cells were treated with fresh medium supplemented with the corresponding chemicals. Subsequently, the cells were used for total RNA isolation using TRIzol[®] reagent (Life Technologies) according to the manufacturer's instructions.

Real-time quantitative RT (reverse transcriptase)-PCR (QPCR)

Total RNA (5 μ g) from different mouse tissues, from mouse seminiferous tubule samples and from ES cells, primary thymic epithelial cells, NIH3T3 and Tec427.1 cells was reverse-transcribed using oligo(dT)₁₈ primer and First Strand cDNA Synthesis kit (Fermentas) according to the manufacturer's instructions in a reaction volume of 20 μ l. RT-PCR amplification was performed in a 15 μ l reaction volume containing QuantiTect SYBR Green PCR Master Mix, 3 mM MgCl_2 , 1.5 μ l cDNA and 500 nM primers 3L4F (5'-AACGCTGAAGTACGTGGA-3') and 3L3R2 (5'-CGGTGGAAGTGAACAT-3'). The PCR was performed with a LightCycler (Roche Applied Science) instrument starting with enzyme activation at 94 °C for 15 min, followed by 50 cycles of 15 s at 94 °C, 20 s at 55 °C and 10 s at 72 °C, with fluorescence detection at 79 °C after each cycle. To control the specificity of the reaction, melting-curve analysis was performed after amplification. Relative amounts of *Dnmt3L* transcripts were normal-

Figure 1 *Dnmt3L* minimal promoter area contains 440 bp upstream from the translational start

(A) Schematic representation of transient transfections with sequentially deleted *Dnmt3L* luciferase promoter constructs into Cos-7 cells. The left side of the Figure illustrates the constructs used in the experiment. Numbers indicate the relative positions with respect to the start of translation. The bars represent the means $\pm$ S.D. for at least two separate experiments, each performed in triplicate and normalized against β -galactosidase activity. Results are shown as fold activations compared with luciferase value of promoterless vector, pBL-KS, designated as 1. (B) Schematic representation of transient transfections with sequentially deleted *Dnmt3L* promoter constructs into Tec427.1 cells (black bars) and NIH3T3 cells (white bars). Details are as in (A).

ized against the amount of mouse GAPDH (glyceraldehyde-3-phosphate dehydrogenase) RNA in each cDNA sample. Standard curves were generated using cloned *Dnmt3L* and GAPDH cDNAs.

RESULTS

Identification of the *Dnmt3L* minimal promoter

We first isolated a genomic DNA fragment containing 1345 bp upstream from *Dnmt3L* translation start site. To test the *Dnmt3L* promoter activity, we cloned the fragment upstream from the luciferase gene and transiently transfected the pLuc1345 construct into Cos-7 cells. pLuc1345 showed 97 times higher luciferase activity when compared with promoterless pBL-KS luciferase vector, indicating that we had isolated an active *Dnmt3L* promoter sequence (Figure 1A). When activity of pLuc1345 was further tested using mouse fibroblasts (NIH3T3) and mouse thymic epithelial cells (Tec427.1), 30-fold activations were seen (Figure 1B). Next, 5'-deletion constructs pLuc900, pLuc440 and pLuc226 were created to identify the minimal promoter area needed for activation. Interestingly, the luciferase activity of pLuc900 was 15 % higher in NIH3T3 and Tec427.1 cells and 55 % higher in Cos-7 cells than the activity of pLuc1345 (Figure 1). Maximum promoter activity was reached with deletion construct pLuc440, showing 50- and 68-fold activations in NIH3T3 and Tec427.1 cells respectively, and over 300-fold activation in Cos-7 cells. pLuc226 lost most of the promoter activity, down to 11–25 % of pLuc440 activity, locating the minimal promoter area within the 440 bp upstream from the translation start site.

Figure 2 *Dnmt3L* minimal promoter area contains five Sp1-binding sites predicted by MatInspector program

Dnmt3L minimal promoter DNA sequence 447 bp upstream from translational start, indicated here as +1. Predicted Sp1-binding sites are underlined and marked below the corresponding sequence with numbers indicating the relative positions with respect to the start of translation. CpG dinucleotides are shown in boldface. Different transcription start sites are indicated as follows: --- --, AJ404467;, NM_019448; and start sites according to EST sequences CA544089, CD565557, D28634, BY709867 and CF892969 are boxed.

Dnmt3L promoter contains several Sp1-binding sites

Minimal promoter sequence was analysed *in silico* by MatInspector program [19] that predicts possible transcription factor binding sites in the sequence. According to the prediction results, the *Dnmt3L* promoter is TATA-less and contains five putative GC boxes known as transcription factor Sp1-binding sites (Figure 2). To investigate the relevance of these binding sites, the corresponding oligonucleotides [Sp1-374, Sp1-318, Sp1-303 (the latter contains two binding sites in the same oligo) and Sp1-262] were tested in EMSA using Cos-7 and NIH3T3 nuclear extracts. As shown in Figure 3, three separate band-shifts appear when oligonucleotides Sp1-262 (Figure 3A), Sp1-303 (Figure 3B) and Sp1-318 (Figure 3C) were used as probes. DNA–protein complexes were specific, since they disappeared when 100-fold excess of competing unlabelled oligonucleotides were added and when labelled oligonucleotides with mutated binding sites were used instead of wild-type oligonucleotides (Figure 3). Since both Sp1 and Sp3 transcription factors recognize the same binding site [24], we performed a supershift assay using Sp1- and Sp3-specific antibodies to characterize formed complexes. Anti-Sp1 antibody supershifted the uppermost complex, whereas two lower complexes were supershifted by anti-Sp3 antibody (Figure 3). All three complexes were lost when both antibodies were used in the supershift assay. The same results were seen in the mobility-shift assay when the consensus Sp1 oligonucleotide was used as a control (Figure 3D). EMSAs performed with the oligonucleotide Sp1-374 did not give a clear result and therefore Sp1-374 was omitted from further studies.

Previously, it has been shown that Sp1 can bind both methylated and unmethylated CpG sites [25]. However, Boyes and Bird [26] demonstrated that proteins that bind methylated DNA could block Sp1 binding. As shown in Figure 2, oligonucleotides Sp1-318 and Sp1-262 contain a CpG dinucleotide in the core recognition sequence. To test whether the methylation affects Sp1 binding to *Dnmt3L* promoter, we *in vitro* methylated these two oligonucleotides and performed EMSA using NIH3T3 nuclear extract. Bandshift analysis indicated that methylation does not interfere with Sp1 binding to *Dnmt3L* promoter (Figure 3E).

Sp1 sites in *Dnmt3L* promoter are functional

To study the functional significance of Sp1/Sp3-binding sites for *Dnmt3L* promoter activity, we mutated all binding sites separately and tested them in a luciferase assay. We transiently transfected

Cos-7 and NIH3T3 cells using the pLuc440 construct with mutations in different Sp1-binding sites and compared the activities of normal and mutated pLuc440 constructs. The most significant decrease in activity was seen with the mutated Sp1-262 construct. Mutation repressed activity down to 21 and 48 % in NIH3T3 and Cos-7 cells respectively (Figure 4). Mutations in Sp1-290-, Sp1-300- and Sp1-318-binding sites caused approx. 30 % repression in Cos-7 cells, and 33–67 % down-regulations were seen with NIH3T3 cells (Figure 4). Results indicated that all four identified Sp1-binding sites had an impact on *Dnmt3L* expression.

In vitro methylation inactivates *Dnmt3L* promoter

The *Dnmt3L* 1345 bp-containing promoter area has 32 CpG dinucleotide sites, suggesting that *Dnmt3L* might be regulated by methylation. To test this, we *in vitro* methylated 14 CpGs containing minimal promoter construct pLuc440 by *SssI* methylase and compared its activity with mock-methylated pLuc440 activity. Both constructs were transiently transfected into Cos-7, NIH3T3 and Tec427.1 cells. In all cell lines, *in vitro* methylation completely blocked promoter activity. With pLuc440 luciferase activity designated as 100 %, the activity of *in vitro* methylated pLuc440 was <2 %. Methylation also decreased luciferase activity in assays performed with *in vitro* methylated pLuc226, pLuc900 and pLuc1345 in NIH3T3, Cos-7 and Tec427.1 cells.

Dnmt3L expression and promoter methylation

The *in vitro* methylation studies have indicated that the *Dnmt3L* gene could be regulated by methylation, and hence we analysed the *Dnmt3L* promoter methylation status in more detail using DNA bisulphite sequencing analysis. Bisulphite treatment converts cytosines into uracils, leaving methylated cytosines unaffected [27]. We studied the methylation levels of 15 CpG dinucleotides within 363 bp upstream from translation start site (Figure 2). As *Dnmt3L* is highly expressed in mouse ES cells [9], we first studied bisulphite-treated genomic DNA extracted from ES cells and found that the promoter was completely unmethylated (Figure 5A). However, bisulphite-treated genomic DNAs of NIH3T3 and Tec427.1 cells showed extensive methylation (approx. 80 %; Figure 5A). Next, we set up a QPCR assay for *Dnmt3L* to test whether the methylation status of the promoter affected the expression level of *Dnmt3L* mRNA. QPCR performed with mRNA extracted from the above-mentioned cells showed very strong *Dnmt3L* expression in ES cells and virtually no expression

Figure 3 Transcription factors Sp1 and Sp3 bind to *Dnmt3L* promoter

(A–D) NIH3T3 and Cos-7 nuclear extracts were incubated with 32 P-labelled oligonucleotides corresponding to four putative Sp1-binding sites predicted within the promoter sequence, and the complexes formed were analysed by EMSA. Competing unlabelled oligonucleotides (100-fold) and antibodies against Sp1 or/and Sp3 were included in the reactions as indicated with +/– symbols on top of the Figures. In reactions with mutated probe, wild-type oligonucleotides were replaced by labelled oligonucleotides with mutated binding sites. Complexes containing Sp1 or Sp3 transcription factors are indicated on the left by arrows. EMSA with (A) Sp1-262 oligonucleotide, (B) Sp1-303 oligonucleotide, (C) Sp1-318 oligonucleotide and (D) consensus Sp1 oligonucleotide. (E) NIH3T3 nuclear extract was incubated with 32 P-labelled *in vitro* methylated (+) and unmethylated (–) Sp1-262, Sp1-318 or consensus Sp1 oligonucleotides. Complexes containing Sp1 or Sp3 transcription factors are indicated on the left by arrows. Numbers indicate the relative positions of Sp1-binding sites with respect to the *Dnmt3L* translation-initiation site.

Figure 4 *Dnmt3L* promoter activity is down-regulated by mutated Sp1-binding sites

Schematic representation of transient transfections into Cos-7 and NIH3T3 cells using pLuc440 constructs with different Sp1-binding site mutations. The bars represent the means $\pm$ S.D. for at least two separate experiments, each performed in triplicate and normalized against β -galactosidase activity. Results are shown as percentage with pLuc440 luciferase activity designated as 100 %. Numbers indicate the relative positions with respect to the start of translation.

in NIH3T3 or Tec427.1 cells (Figure 5B), suggesting that there is a correlation between expression and promoter methylation.

Dnmt3L-deficient mouse studies showed that *Dnmt3L* is needed for mouse spermatogenesis [8,9]. Germ cells mature within the seminiferous epithelium in synchronized cycles. In the mouse, each cycle can be divided into 12 stages where every stage contains a fixed set of developing cell types. We isolated genomic DNA and total RNA from adult mouse seminiferous tubules at the defined stages (II–VI, VII–VIII and IX–I) of the mouse seminiferous epithelial cycle, and studied the *Dnmt3L* promoter methylation status and expression levels in these samples. According the QPCR results (Figure 5B), *Dnmt3L* is expressed in all stages of spermatogenesis with highest levels of mRNA seen during stages VII–VIII when B spermatogonia enter the preleptotene stage of the meiosis and the more mature spermatocytes are at the pachytene stage of meiosis [28]. Bisulphite sequencing of the genomic DNA from the same samples showed that the *Dnmt3L* promoter is unmethylated during all stages of spermatogenesis (Figure 5A). To confirm the *Dnmt3L* expression pattern during germ cell development, we analysed its expression in developing mouse testis at 1, 2 and 3 weeks, as the first wave of spermatogenesis appears. One-week-old testis

Figure 5 Promoter methylation influences the expression level of *Dnmt3L*

(A) Bisulphite sequencing analysis of 15 CpG sites in *Dnmt3L* minimal promoter area (see Figure 2) with samples extracted from different cell lines, developmental stages of the mouse seminiferous epithelial cycle and mouse tissues. Each line represents a separate clone. ○, unmethylated CpGs; ●, methylated CpGs. If sequencing data were not available, the CpG site was left empty. Sample names are indicated on the left and methylation percentage on the right side of the Figure. (B) QPCR analysis of *Dnmt3L* mRNA extracted from samples indicated at the bottom of the Figure. Results are shown as the amount of *Dnmt3L* mRNA (fg) in each sample. The bars represent the means $\pm$ S.D. for two separate experiments, each performed in duplicate and normalized against the amount of GAPDH (G3PDH) mRNA in each sample.

contains mostly spermatogonia and preleptotene spermatocytes. At 2 weeks, pachytene spermatocytes have emerged, and at 3 weeks, meiosis is completed and round spermatids are seen. Consistent with previous expression results, *Dnmt3L* was expressed at all stages with the highest expression level at 2 weeks, at the beginning of meiosis (results not shown).

Subsequently, we collected several adult mouse tissues and purified total RNA and genomic DNA from the same tissue samples. Previously, we have shown that *Dnmt3L* is predominantly expressed in testis and at lower levels in ovary, thymus, heart and foetal tissues [7]. In QPCR assay, *Dnmt3L* mRNA was detected in testis and ovary RNA samples, as expected, where the relative amount of *Dnmt3L* was higher in testis when compared with ovary (Figure 5B). Bisulphite sequencing of the promoter showed no genomic methylation in testis (5%) and a diverse methylation pattern in ovary (60%; Figure 5A). In ovary, *Dnmt3L* expression is limited to growing oocytes [8,9] with the rest of the tissue lacking *Dnmt3L* expression. In thymus, very weak *Dnmt3L* expression by QPCR was seen with a corresponding promoter methylation level of 78% (Figure 5). However, it should be noted

that *Dnmt3L* expression was detected at high levels by QPCR in RNA samples extracted from cultured primary thymic epithelial cells (results not shown), indicating that *Dnmt3L* expression is limited to specific cell types. We also studied methylation levels in lungs, heart, spleen and liver. All the tissues, except lungs, expressed weak or non-existent levels of *Dnmt3L* (Figure 5B). The methylation level in lungs was lower (31%) when compared with three other tissues with methylation levels from 53 to 69% and low *Dnmt3L* expression levels (Figure 5). Taken together, these results indicate that the *Dnmt3L* expression correlates with the genomic methylation status of its promoter.

5-azaCdR and TSA increase *Dnmt3L* mRNA level in NIH3T3 and Tec427.1 cells

We further tested whether by changing the epigenetic status of the chromatin, we could rescue *Dnmt3L* expression in NIH3T3 or Tec427.1 cells. Both cell lines were treated with various concentrations (0.2–10 μ M) of Dnmt inhibitor 5-azaCdR, and subsequently, mRNA was extracted and amplified in QPCR. As

Figure 6 5-azaCdR and TSA up-regulate the *Dnmt3L* mRNA level in NIH3T3 and Tec427.1 cells

(A) NIH3T3 cells were treated or left untreated for 48 h with various concentrations (1–10 μ M) of Dnmt inhibitor 5-azaCdR and/or for 24 h with 100 nM deacetylase inhibitor TSA. Subsequently, mRNA was extracted and amplified in quantitative RT-PCR specific for *Dnmt3L*. Results are shown as fold inductions compared with expression level in non-treated cells, designated as 1. Numbers below the Figure demonstrate different 5-aza-CdR concentrations (μ M) and 100 nM TSA was included as indicated with +/– symbols. Relative amounts of *Dnmt3L* transcripts were normalized against the amount of mouse GAPDH RNA in each cDNA sample. (B) Tec427.1 cells were treated for 48 h with various concentrations (0.2–1 μ M) of Dnmt inhibitor 5-azaCdR and/or for 12–24 h with 100 nM deacetylase inhibitor TSA. Details are as in (A).

expected, *Dnmt3L* expression was raised to a detectable level when the methylation activity was blocked by 5-azaCdR (Figure 6). Previous studies have shown that usually inactive chromatin is not only methylated but, in addition, also deacetylated at the N-termini of histones [29]. Therefore, we incubated cells with the deacetylase inhibitor, TSA, and found that similar to 5-azaCdR, TSA also up-regulates *Dnmt3L* expression in NIH3T3 and Tec427.1 cells (Figure 6). The expression level was found to be higher with TSA when compared with 5-azaCdR. When the cells were treated with both inhibitors together, expression level of *Dnmt3L* was even higher (Figure 6). These results suggest that the *Dnmt3L* promoter is inactivated by methylation and histone deacetylation in non-expressive cell lines.

Luciferase assays with TSA- and 5-azaCdR-treated cells

Figure 1 indicated the repression effect with *Dnmt3L* upstream promoter constructs. Since deacetylase and methylation inhibitors affected *Dnmt3L* expression from genomic DNA, we wanted to test whether the same result could be obtained with episomal plasmid DNA containing *Dnmt3L* promoter. We transiently transfected NIH3T3 cells with pLuc440 and pLuc1345 constructs,

Figure 7 Dnmt and histone deacetylase inhibitors release the repressed state of *Dnmt3L* promoter sequences

Relative luciferase activity after transient transfections with pLuc440 and pLuc1345 constructs into NIH3T3 cells. Transfected cells were treated or left untreated for 48 h with 5 μ M Dnmt inhibitor 5-azaCdR or for 24 h with 100 nM deacetylase inhibitor TSA. Results are shown as fold activations compared with the luciferase value of the promoterless vector, pBL-KS, designated as 1. The bars represent the means $\pm$ S.D. for three separate experiments, each performed in triplicate and normalized against β -galactosidase activity.

treated cells with TSA and 5-aza-CdR, and compared luciferase activity levels of treated cells with that of untreated cells. As seen in Figure 7, both inhibitors not only released the repressed state of luciferase activity of pLuc1345 but also up-regulated the activity of pLuc440. Similar to genomic DNA, TSA had more effect on expression than 5-aza-CdR.

DISCUSSION

In the present study, we have characterized the mouse *Dnmt3L* promoter. We identified the minimal promoter area (Figure 1) and showed that it contains four Sp1/Sp3 transcription factor binding sites that each contribute to the regulation of *Dnmt3L* (Figures 2–4). Sp1 and Sp3 are ubiquitous transcription factors, which bind to the same target sequence. Sp1 usually activates transcription but Sp3 can either activate or repress the transcription through a special repressor domain that is not found in Sp1 protein [30]. Since Sp1 binds to GC-rich target sequences, the role of methylation in Sp1-dependent regulation has been discussed in many studies. It has been shown that (i) methylation does not affect the Sp1 binding [25], (ii) proteins binding to methylated DNA can block Sp1 binding [26], (iii) Sp1 binding can protect promoter from *de novo* methylation [31,32], and (iv) multiple Sp1 sites can target local demethylation in embryonal cells but not in non-embryonal cells [33]. Our bandshift analysis showed that for a *Dnmt3L* promoter, the methylation of Sp1 sites did not affect the Sp1 binding (Figure 3E).

Although the Sp1 binding was not blocked by methylation, we found that the *in vitro* methylation assay demonstrated complete repression of transcript if methylated. Bisulphite sequencing results further demonstrated that the *Dnmt3L* promoter is heavily methylated in non-expressive somatic cell lines and unmethylated in ES cells that express *Dnmt3L* mRNA extensively (Figure 5). In addition, the treatment of non-expressive somatic cells with methyltransferase and deacetylase inhibitors demonstrated clear up-regulation of *Dnmt3L* mRNA. All this indicated that *Dnmt3L* expression is controlled through genomic DNA methylation and histone tail modifications.

We have previously shown that *Dnmt3L* is mostly expressed in testis [7]. Expression is seen in gonocytes during the perinatal

period and in differentiating spermatocytes of newborn and adult mice [8,9]. Our results demonstrated that *Dnmt3L* promoter is totally unmethylated in testis and during all developmental phases of spermatogenesis (Figure 5). In accordance with our methylation results, the gene is expressed at all stages of spermatogenesis with highest mRNA levels seen at the beginning of meiosis during stages VII–VIII of the mouse seminiferous epithelial cycle (Figure 5B). In addition to testis, *Dnmt3L* is highly expressed in ovaries where the heterogeneous methylation pattern of the promoter region can be explained by the over-representation of the other cell types in the tissue. Surprisingly, a high *Dnmt3L* expression was repeatedly observed in lungs with relatively high methylation levels (31%). Indirect support to this finding comes from the database searches where we found a lung-derived EST (expressed sequence tag) sequence (BY13862), which was identical with the 5' region of the *Dnmt3L* gene. One reason for the higher methylation level in the lungs may be the heterogeneous tissue material used in our analysis being more diverse than the results obtained using cell lines or primary cultures that give more uniform results but do not represent the actual *in vivo* situation. We conclude from our results that the promoter methylation has a major influence on *Dnmt3L* expression. Recently, similar connection between promoter methylation and cell type-specific gene expression was reported for *SERPIN5* [34]. However, further studies are needed to elucidate whether the methylation is causing the decrease in *Dnmt3L* mRNA level or is just a consequence of other initiative silencing mechanisms.

Previous studies indicated that *Dnmt3* genes are regulated through several promoters and isoforms. *Dnmt3a* and its isoform *Dnmt3a2* are transcribed from separate promoters and show different cellular localization patterns. *Dnmt3a* is expressed ubiquitously and *Dnmt3a2* is a predominant form in ES cells [35]. *Dnmt3b* has six isoforms that result from alternative splicing of exons 10, 21 and 22 [3,36–38]. In addition, there are several reports describing alternative transcriptional start sites and promoter sequences for *Dnmt3a* and *Dnmt3b* [18,35,39,40]. Mouse *Dnmt3L* has two different transcriptional start sites in database (AJ404467 and NM_019448). Both start sites are supported by mouse EST sequences (CA544089, CD565557, D28634, BY709867 and CF892969; Figure 2). In addition, there is one EST sequence (CA559020) in GenBank®, which shows identity with *Dnmt3L* and contains two novel 5'-exons with the first exon located approx. 7.6 kb upstream from AJ404467 in the middle of the autoimmunity regulator, *Aire* gene [41]. Two extra exons in CA559020 do not change the *Dnmt3L* open reading frame but suggest the possibility of regulation by separate proximal promoters. It seems probable that transcriptional start sites in AJ404467 and NM_019448 are using the same promoter sequence, since they are separated only by 133 bp (Figure 2). Further studies are needed to demonstrate the role of the different transcription start sites of *Dnmt3L*.

Ishida et al. [40] studied mouse *Dnmt3b* promoter and found two novel transcription start sites. They identified the minimal promoter region of *Dnmt3b* and showed that in somatic cells the addition of the promoter upstream regions to the reporter constructs caused a repression effect that was not seen in ES cells. The same repressional effect by a promoter upstream region was seen with human *DNMT3A* and *DNMT3B* promoters [18]. Our studies add the third DNMT3 protein family member, *Dnmt3L*, to this list of CpG-rich TATA-less promoters, implying a common regulatory mechanism of *Dnmt3* family genes. Furthermore, similar to *DNMT3A* and *DNMT3B* promoters, our results show that the *Dnmt3L* upstream promoter sequences repressed the transcription (Figure 1). This is in agreement with the results demonstrating that the repressional effect could partly be released

by *Dnmt* and deacetylase inhibitors (Figure 7). On the basis of the results with *Dnmt3b* [40] it would be interesting to see if the same effect is observed in ES cells.

In conclusion, we have identified and characterized the *Dnmt3L* promoter showing similar features to the other *Dnmt3* promoters. We demonstrated that the *Dnmt3L* expression is regulated by Sp1 and Sp3 transcription factors and through reversible epigenetic modifications, DNA methylation and histone deacetylation.

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