Cytosine methylation of an Sp1 site contributes to organ-specific and cell-specific regulation of expression of the lung epithelial gene *T1α*

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Several recent observations have suggested that cytosine methylation has a role in the *in vivo* transcriptional regulation of cell-specific genes in normal cells. We hypothesized that methylation regulates *T1α*, a gene expressed primarily in lung in adult rodents. In fetuses *T1α* is expressed in several organs, including the entire nervous system, but during development its expression is progressively restricted to lung alveolar type I epithelial cells, some osteoblasts and choroid plexus. Here we report that *T1α* is methylated at a key Sp1 site in the proximal promoter in cells and organs, including brain, where no gene expression is detectable. Conversely, in *T1α*-expressing cells, these sites are not methylated. In embryonic brain *T1α* is unmethylated and expressed; in adult brain the gene is methylated

and not expressed. In lung epithelial cell lines, methylation of the *T1α* promoter *in vitro* decreases expression by approx. 50% (the maximum suppression being 100%). Analysis of mutated promoter constructs indicates that a single Sp1 site in the proximal promoter provides all or most of the methylation-sensitive gene silencing. We conclude that, in addition to regulation by transcription factors, cytosine methylation has a role in the complex expression patterns of this gene in intact animals and primary cells.

Key words: 5-aza-2'-deoxycytidine, lung and brain gene, lung cell lines, promoter methylation.

INTRODUCTION

Methylation of deoxycytidine residues in DNA is one of several mechanisms that regulate gene expression. Although it was disputed for some time [1], the fundamental importance of cytosine methylation as a negative transcriptional regulator has been illustrated by disrupted development in animals with null mutations in genes encoding methylcytosine-binding protein 2 (MeCP2) [2] or one of the methyltransferase family of enzymes [3]. Studies showing the silencing of tumour suppressor genes by methylation [4,5] and identifying a human syndrome linked to a mutation in the MeCP2 [6] have stimulated a renewed interest in the role of methylation as a transcriptional regulator.

The possibility that DNA methylation could mediate dynamic gene regulation has been questioned, in part because no demethylating enzyme had been identified that could remove methyl groups quickly. The recent characterization of a widely expressed demethylase [7], although controversial, and the cloning of novel methyltransferases with some tissue and cellular specificity [8] support the possibility that methylation and demethylation might regulate dynamic changes in gene expression.

Methylation is well known to be important in gene imprinting [9,10] but only recently has it been shown to regulate some normal differentiation genes. For example, the gene for embryonic globin is silenced by methylation in adult primary erythroid cells [11]; the genes encoding leukosialin (CD43) [12,13] and cyclin D1 [14] are silenced by methylation of Sp1 sites in the proximal promoter in non-expressing cells. These findings raise the possibility that the methylation of Sp1 sites might be a

relatively common, and physiological, mechanism of gene repression.

CpG methylation interferes with transcription by at least two mechanisms. Methylation might block the binding of transcription factors to *cis* elements [15]. In addition, other nuclear proteins (MeCPs, certain histones) [16] that bind to methylated elements can indirectly block the access of transcription factors to DNA. That MeCP2 forms a complex with histone deacetylase [17,18] has led to the thinking that gene silencing by methylation requires histone deacetylation, probably resulting in nucleosome formation or altered DNA structure.

We have been studying the regulation of the lung gene *T1α* as being representative of differentiation genes in the lung and of the type I cell phenotype. By using promoter-reporter deletion constructs, gel retardation assays and mutational approaches in lung epithelial cell lines we have shown [19] that *T1α* transcription can be regulated *in vitro* by Sp1/Sp3, TGT3-binding (*forkhead* homologues) and thyroid transcription factor 1 (TTF-1) proteins, findings confirmed subsequently by others [20].

The regulation of the type I cell phenotype is particularly interesting. In adult animals, type I cells arise by the differentiation of nearby type II cells, probably post-mitotic, with which they form the extensive gas-exchange surface of the lung. Although type I and type II cells are therefore closely related, they have different molecular phenotypes, functions and morphology, as described in many studies [21]. Lung injuries often cause the death and desquamation of type I cells, leading to an accelerated transformation of post-mitotic type II cells into type I cells. Although the morphological changes that accompany the formation of new type I cells have been well studied, little is

Abbreviations used: AQP-5, aquaporin 5; 5-azaC, 5-aza-2'-deoxycytidine; Dnmt, DNA methyltransferase; KGF, keratinocyte growth factor; MeCP2, methylcytosine-binding protein 2; SP-C, surfactant protein C; SV40, simian virus 40; TTF-1, thyroid transcription factor 1.

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known about the molecular regulatory events that drive the expression of the type I cell differentiation programme when it is re-expressed during this switch in cellular phenotype.

In vitro either the type I or the type II cell phenotype can be expressed by the same primary, non-mitotic type II cells, depending on culture conditions. If treated with keratinocyte growth factor (KGF or FGF-7) or homologous serum, or maintained in a cuboidal shape, type II cell genes are expressed [22,23]. The same cells, cultured such that they have a flattened morphology, express type I cell proteins [$T1\alpha$, aquaporin 5 (AQP-5), caveolin-1 [24], carboxypeptidase M]; KGF or homologous serum repress the expression of these genes [25–27]. These two phenotypes can be rapidly reversed *in vitro* by altering culture conditions, suggesting that some changes in gene regulation, perhaps simple ones, occur rapidly and affect populations of genes. The initiating molecular event that switches the cells between the two phenotypes is unknown.

We reasoned that methylation might be important in regulating $T1\alpha$ expression because, so far, the transcription factors known to regulate type II cell genes also regulate type I cell genes. Thus gene transactivation via these known protein factors is unlikely to account for the molecular differences between the two cellular phenotypes. In addition, 10 kb $T1\alpha$ 5' flanking sequence does not restrict expression to cells that express the endogenous gene, even though there is an increased specificity for epithelial cells compared with fibroblasts [28]. This suggests the involvement of other types of transcriptional regulation and/or the importance of additional upstream elements. Furthermore, adult lung from mouse [8] or human [29] expresses DNA methyltransferases (Dnmt) 3 α and 3 β ; our preliminary observations suggest that these enzymes are expressed by different populations of lung cells (Y.-X. Cao, E. Li and M. C. Williams, results not shown).

We used various tissues and cells to study the role of methylation in $T1\alpha$ expression. In non-expressing lung cells and cell lines and in normal non-expressing adult rat organs, $T1\alpha$ is methylated at specific sites in the proximal promoter. In non-expressing cell lines, expression can be initiated by the addition of drugs that interfere with methyltransferase activity. Furthermore, methylation of the $T1\alpha$ promoter *in vitro* decreases the expression of a reporter protein, and mutation of two key cytosine residues in the proximal promoter partly reverses this inhibition. Methylation seems to have a role in the normal suppression of $T1\alpha$ expression that occurs as the nervous system develops. In day 14 fetal brain, the promoter lacks methylation and the gene is expressed; in adult brain the promoter is methylated and the gene is not expressed. Taken together, these observations indicate that the normal expression patterns of this differentiation gene are partly established by promoter methylation.

MATERIALS AND METHODS

Tissue samples

Various organs of adult Sprague–Dawley rats (200–250 g), anaesthetized with sodium methohexital, were removed for DNA isolation. Brain tissue was dissected free of the choroid plexus that expresses endogenous $T1\alpha$. Fetal animals were killed on day 14; 'brain' tissue was obtained by decapitation of the animals above the level of the eye.

Cell lines and culture

Cell lines studied were the following: simian virus 40 (SV40) T-type II cells derived from neonatal rat type II cells immortalized *in vitro* with the SV40 viral oncogene large T antigen [30]; MLE

15 cells immortalized *in vivo* with a surfactant protein C (SP-C)-driven SV40 T antigen [31]; and C6 cells, a rat glial tumour cell line (A.T.C.C. 107-CCL). Of these, only SV40T type II cells express endogenous $T1\alpha$ mRNA and protein. SV40T type II cells and MLE 15 cells were grown in Dulbecco's modified Eagle's medium and 10% (v/v) heat-inactivated FBS (fetal bovine serum; Life Technologies), 100 units/ml penicillin G and 100 μ g/ml streptomycin sulphate. C6 cells were grown as recommended by A.T.C.C.

Type II cells were purified from adult pathogen-free Sprague–Dawley rats (180–200 g) (Charles River) by standard methods [32]. Cells were divided into two aliquots and either immediately prepared for extraction of DNA or RNA or plated (10^6 cells) on plastic dishes for tissue culture. Plated cells were cultured in minimal essential medium containing 10% (v/v) FBS, non-essential amino acids, 2 mM L-glutamine and penicillin/streptomycin (as above). DNA and RNA were extracted on culture day 6. We have previously reported [27,33,34] the time course of $T1\alpha$ mRNA/protein expression after plating cells on plastic. By culture day 7, the $T1\alpha$ protein level is approx. 25-fold that on day 0.

DNA extraction, modification with bisulphite and sequencing

Genomic DNA was isolated from SV40T type II and C6 cells and from freshly isolated and day-6-cultured type II cells with a Qiagen extraction kit. DNA was isolated from liver, heart, kidney, brain and trachea and fetal brain by standard methods. DNA (0.5 μ g) was modified chemically in accordance with the manufacturer's directions with the CpGenomeSM DNA modification kit (ONCOR) [35] in which bisulphite deaminates unmethylated cytosine residues. The deamination product uracil is amplified as thymidine [36]. Oligonucleotide primers used for amplification of bisulphite-modified DNA, targeting the $T1\alpha$ gene promoter, were as follows: M1, 5'-TTAGTATTAGGAG-GTTTTATG-3'; M2, 5'-AAACAAACACAACAACTTAAC-3'; M3, 5'-ATTAGTTGTATAGGTTATTG-3'; M4, 5'-TAACAACCTTCTCAACACAATC-3'. PCR conditions were as follows: 95 °C for 5 min, 30 cycles at 94 °C (40 s), 55 °C (40 s), 72 °C (60 s), terminating with 72 °C for 10 min. The PCR mixture contained 1 $\times$ PCR buffer (10 $\times$ PCR buffer diluted 1:10; Life Technologies), 2 mM $MgCl_2$, 4 ng/ μ l M1 primer, 4 ng/ μ l M2 primer and 0.2 mM dNTPs. Amplicons were purified with a PCR purification kit (Qiagen). Secondary PCR was performed with the M1 + M2 PCR products by using each primer (M3, M4) at 4 ng/ μ l with 1.5 mM $MgCl_2$ and 0.2 mM dNTPs. The PCR protocol was as above. Secondary products were purified by gel isolation and sequenced at the Boston University core sequencing facility. Sequences from bisulphite-modified DNA from normal organs occasionally show both major and minor peaks at the locus of chemically modified cytosine residues. The minor peaks seem to be analogous to the 'shadow' bands observed on sequencing gels and might represent failure of the bisulphite procedure to modify bases with 100% efficiency. Relative peak heights were measured (see Figure 2); 'off-scale' peak heights are shown as 100%.

Northern blots

RNA was isolated by using TRIZOL reagent (Life Technologies). Northern blots were prepared by the glyoxal/DMSO denaturation method. Total RNA (10 μ g) was subjected to electrophoresis on 1.5% (w/v) agarose gels, blotted, hybridized and washed by standard procedures [19]. ³²P-labelled probes, prepared by the hexamer random primer method, were as follows:

T1 α , 520 bp (rat) or 642 bp (mouse) coding sequence; AQP-5, 366 bp PCR-generated murine coding sequence; caveolin-1, 838 bp coding sequence (human); and SP-B, 550 bp coding sequence (rat). After the first exposure, blots were stripped and reprobed for β -actin [19] to normalize for loading. Film exposure times were selected to ensure that signals were in a linear range. Densitometry (ImageQuant; Molecular Dynamics) of autoradiograms was used for the semiquantification of gene expression.

Methylation of *T1 α* 5' untranslated region *in vitro*

Construction of the *T1 α* promoter-luciferase reporter plasmids –1.3 kb Luc (–1251 to +101) and –170 bp Luc (–170 to +101) has been described previously [19]. The plasmid 0-Luc (pGL3) was purchased from Promega. The –1251 Luc and –170 Luc constructs were digested with *Kpn*I and *Sac*I. *T1 α* promoter fragments were gel-purified and divided into two aliquots. One aliquot was methylated *in vitro* with *Sss*I methylase (New England Biolabs) in accordance with the manufacturer's directions (4 h, 37 °C, 320 mM *S*-adenosylmethionine, 60 units/ml enzyme), which methylates CpG dinucleotides [37] in both strands [38]. A second aliquot was incubated similarly but without the donor *S*-adenosylmethionine (mock-methylated). Methylated and mock-methylated promoters were religated by standard methods into the original vector containing the luciferase reporter. This procedure therefore prevents methylation of the coding region of the luciferase protein, which would be expected to decrease reporter expression.

Cytosine residues (–89 to A and –95 to T) within the Sp1/3 site were mutated by PCR with a promoter fragment from –104 to +101 and a 20-mer 5' primer (5'-CAAACAGAATTGGG-GGAGG-3') and a 3' primer complementary to the wild type. Fragments, purified with QiAquick kit (Qiagen) and digested with *Apo*I and *Sac*I, were ligated into pLuc such that the –170 bp promoter fragment was reconstructed. Successful mutation was confirmed by sequence analysis. Mutated promoters were methylated or mock-methylated as described above.

DNA transfection and transient expression assays

SV40T type II and MLE 15 cells were transfected with the methylated or mock-methylated promoter-Luc constructs (4 μ g/ml) with the DEAE-dextran/chloroquine method [19]. LacZ constructs were used to normalize for transfection efficiency. Cells were harvested at 48 h and extracted by standard methods. Luciferase activity was measured as described previously [19]. Fold increases in expression in three experiments were calculated after adjusting for the baseline 0-Luc value.

Drug studies

The methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-azaC; Sigma) was used to test the effects of demethylation in non-expressing cell lines. Preliminary studies were performed to determine drug concentrations that did not inhibit cell proliferation or viability (results not shown; 2.5–5.0 μ M was toxic for MLE 15 cells but not for C6 cells). MLE 15 cells were treated with 0.1 μ M 5-azaC; C6 cells were treated with 5.0 μ M 5-azaC. After treatment for 24 h, cells were washed in PBS and returned to normal medium. Control plates were treated with PBS/medium for 24 h. Cells were harvested for Northern blot analyses and densitometry. For the purposes of quantifying the fold increase in *T1 α* mRNA whose expression could not be detected in untreated MLE 15 or C6 cells, the lower limit of sensitivity of the densitometric reading was used as the day 0 value.

RESULTS

Sequence analysis

A sequence analysis of the rat –1251 bp *T1 α* 5' flanking sequence revealed that this fragment contains more than 70 palindromic CpG dinucleotides that are putative sites for methylation. Within the –170 bp promoter that controls much of the transcriptional activity [19] there are more than 10 trinucleotide or tetranucleotide sequences that are preferred targets for methylation. These include two consensus elements for the Sp1/3 family of transcription factors at –56 to –42 bp and at –87 to –95 bp.

Northern analysis

We selected rat cells for analysis that either did or did not express endogenous *T1 α* (Figure 1). *T1 α* mRNA was easily detectable in SV40T type II cells but not in C6 cells when compared with adult rat lung. No signal, or only a faint one, was visible in primary type II cells after isolation and purification (Figure 1, lane 4); much longer exposures of the same autoradiogram showed a weak signal (lane 5). In our hands the detection of *T1 α* mRNA in freshly isolated type II cells is variable. As reported previously, type II cells cultured on plastic show approx. 5-fold and 25-fold increases in *T1 α* mRNA and T1 α protein respectively by culture day 8 [27,33]. MLE 15 cells did not express detectable *T1 α* mRNA (see Figure 5A).

DNA methylation in cell lines, primary lung cells and whole organs from adult rats

Bisulphite mapping was used to compare methylation patterns between *T1 α* -expressing SV40T immortalized type II cells and non-expressing C6 cells (Figure 2). No methylated cytosine residues were detected in the –170 bp proximal promoter of the SV40T type II cells (Figure 2A), whereas cytosine residues at positions –95, –89, –75, –62, –54, –48 and –42 were methylated in the C6 cells (Figure 2B). In both cell lines sequence analysis showed peak heights of more than 90% for the appropriate nucleotide. C6 methylation sites were clustered

Figure 1 *T1 α* and β -actin mRNA expression in lung and cell lines

(A) Northern-blot analysis of *T1 α* mRNA expression. Both adult rat lung RNA (lane 1, 10 μ g) and immortalized rat type II cell RNA (SV40T type II cells, lane 2) show *T1 α* transcripts that can be readily detected; rat glial C6 cells (lane 3) are negative. With the same autoradiogram exposure as in lanes 1 and 2, *T1 α* transcripts cannot be detected in freshly isolated rat type II cells (lane 4). Longer exposures of the same blot (lane 5) show a low level of *T1 α* transcripts; this is true of some but not all primary isolates. *T1 α* mRNA is also not detectable in the mouse type II cell line MLE 15, as shown in Figure 5(A). The high level of expression of *T1 α* in type II cells cultured for 6–7 days on plastic has been reported previously (Figure 1A in [27]). (B) Stripped blots were reprobed for β -actin to normalize *T1 α* data for loading. Lane 3 was intentionally overloaded to demonstrate the absence of *T1 α* mRNA from this cell line.

Figure 2 Examples of primary sequence data obtained by bisulphite mapping of the proximal *T1 α* promoter in expressing [SV40T type II (A)] and non-expressing [C6 (B)] cell lines, and in normal adult rat heart (C) and brain (D)

Methylated cytosine residues are marked with arrows. In C6 cells sites at -48 , -54 , -62 , -75 , -89 and -95 bp are methylated; in SV40T cells they are unmethylated. The maps for normal adult heart and brain show methylated sites at only -89 and -95 bp. Results from other organs are summarized in Figure 3 (lower panel).

between bp -42 and -56 and between bp -87 and -95 , previously identified as important domains for transcriptional regulation [19]. We assumed that the methylation pattern of C6 cells represents the maximum number of modifiable sites and examined these sites in other systems. A similar analysis of DNA from normal adult rat heart (Figure 2C) and brain (Figure 2D) showed methylation of cytosine residues at positions -89 and -95 only (arrows).

We compared the methylation patterns of freshly isolated adult rat type II cells ($n = 5$) with those of type II cells cultured for 6 days on plastic dishes ($n = 3$) (Figure 3, upper panel). During this time they switch from the type II to the type I cell phenotype and express type I cell markers including *T1 α* . In freshly isolated cells there is variability in the degree of methylation between different isolates of cells. In three experiments, uncultured type II cells had methylated nucleotides at bp -95 , -89 , -54 , -48 and -42 but not at the -75 and -62 loci that are methylated in cell lines. Although the general methylation patterns were similar in all experiments, the percentage of the peak height indicating methylation in freshly isolated type II cells was variable. This variability was not unexpected and might be related to the lengthy procedure of type II cell isolation (approx. 4 h) rather than to highly reproducible cell purity. It is possible that demethylation of *T1 α* begins during cell isolation but without inhibitors of demethylase enzyme(s) we cannot test this directly.

In cultured type II cells, expressing abundant *T1 α* mRNA and *T1 α* protein, the -95 bp site was totally demethylated in one experiment with a mean methylated peak height of approx. 35% ($n = 3$). The -89 bp site was totally demethylated in all three experiments (Figure 3, upper panel).

Promoter methylation patterns were also characterized for liver, kidney, heart and trachea; none of these organs expressed detectable (Northern analysis) *T1 α* in the adult. We also compared the methylation pattern of non-choroidal areas of brain of

Figure 3 Cytosine methylation in primary type II cells and normal adult rat organs

Upper panel: summary of bisulphite mapping of the *T1 α* promoter in primary type II cells, both freshly isolated ($n = 5$; circles) and cultured on plastic for 6 days ($n = 3$; squares). After 6 days of culture both *T1 α* mRNA and *T1 α* protein levels are high and the promoter is mostly unmethylated. The variability of methylation at -95 and -89 sites in acutely isolated cells is consistent with the Northern analysis (Figure 1) that shows low levels of *T1 α* expression in some type II cell isolates. Lower panel: bisulphite mapping and sequence data for adult rat organs show similar methylation at -95 and -89 bp ($n = 3$ for all organs). The sequence occasionally showed a minor peak at these positions; thus results are reported as percentages of the maximum signal calculated from relative peak heights. Bisulphite maps for day 14 fetal brain DNA show complete demethylation at -89 and -95 bp (results not shown).

adult rats (Figure 3, lower panel) with that of day 14 embryonic rat brain, i.e. comparing non-expressing with expressing tissues. The major methylation targets in these non-expressing organs are cytosine residues at positions -95 and -89 : heart, liver and kidney showed 100% methylation and adult brain and trachea more than 50%. Methylation is more variable at -75 and -62 (Figure 3, lower panel). The most proximal putative methylation sites at -42 , -48 and -54 , methylated in non-expressing cell lines (see above), were unmethylated in these organs. Whereas the -89 and -95 sites were methylated in adult brain (non-expressing), they were unmethylated in the day 14 embryonic brain (expressing).

Effects of promoter methylation *in vitro* on reporter gene expression

The -170 and -1251 *T1 α* promoters were methylated or mock-methylated *in vitro*; successful methylation was confirmed with

Figure 4 Decrease in *T1 α* expression by promoter methylation *in vitro*, which is reversed by mutation of cytosines

(A, B) Methylation of the *T1 α* proximal promoter *in vitro* decreases luciferase reporter protein expression by approx. 50% (complete suppression = 100%) compared with mock-methylated promoter–luciferase constructs in both SV40T type II cells [(A), *T1 α* -expressing] and MLE 15 cells [(B), *T1 α* -non-expressing]. Luciferase activity, normalized to β -galactosidase activity, was measured 48 h after the transduction of either methylated or mock-methylated promoter–luciferase constructs. The zero value is from cells transduced with the empty luciferase vector. Decreases in transcription seem to result from methylated elements in the –170 bp fragment; no additional decrease in expression is seen with the 1.3 kb promoter. (C) Mutation of both cytosine residues (–89 and –95) decreases expression by approx. 25% compared with the unmethylated promoter, which is consistent with the known role of this Sp1 site in transactivation. Methylation of the mutated promoter decreases the inhibition from approx. 55% (wild type, methylated compared with unmethylated) to approx. 80% (mutated, methylated compared with unmethylated).

mCyt-sensitive and mCyt-insensitive restriction enzymes (results not shown). Promoter activity was tested by the transduction of SV40T type II and MLE 15 cells. *lacZ*-normalized luciferase activity after 48 h was compared with that from the promoterless construct 0-luc. Mock-methylated –170 and –1251 bp promoters drove expression approx. 8–10 fold (MLE cells) and

approx. 25–28 fold (SV40T type II cells) over baseline (Figures 4A and 4B). These results are similar to those that we have previously reported for SV40T type II cells [19]. Methylation of the –170 and –1251 bp promoters decreased expression by approx. 50% (47–56%) in SV40T type II cells and approx. 60% (58–68%) in MLE cells compared with controls. Although at first glance these changes seem relatively small compared with strong inductive responses in which gene expression might be many times that of the baseline, the maximum decrease in expression is 100%. Thus an overall 50% decrease in expression is comparable to a complete inhibition of expression in half of the cells, clearly not a trivial response. The approx. 1.3 kb methylated promoter shows the same level of expression as the –170 bp methylated fragment, leading us to conclude that methylation sites, if any, 5' to the –170 bp promoter fragment are unlikely to add further to transcriptional repression.

We have previously shown that the mutation of four out of eight bases in the proximal Sp1 site in the –170 bp promoter decreases expression by 76% compared with the wild type [19]. To test the effects of methylation we mutated the –89 and –95 bases to A or T respectively within this Sp1 consensus element. This mutation decreased expression from the unmethylated –170 bp promoter fragment by more than 20% in both MLE (Figure 4C) and SV40T type II cells (results not shown), confirming our earlier observations. Methylation of the wild-type –170 bp promoter *in vitro* decreased expression by 43% compared with the mock-methylated wild-type fragment. In contrast, methylation of the mutated promoter fragment *in vitro* partly reversed this decrease in expression to 22% compared with the mutated mock-methylated fragment. Thus, as expected, removal of the inhibitory influence of methylated cytosine residues within the Sp1 site resulted in a modest increase in expression. The magnitude of these changes in expression is probably related to the number of bases altered in the Sp1 site; mutating only two would be expected to allow some Sp1 protein binding and transcription but at lower levels.

Treatment of cell lines with 5-azaC induces *T1 α* expression

C6 and MLE 15 cells were treated with the methyltransferase inhibitor 5-azaC as described. These cells have no detectable expression of *T1 α* (Northern analysis or reverse-transcriptase-mediated PCR); AQP-5 and caveolin-1 mRNA species are present in small amounts in MLE 15 cells (Figure 5A). After treatment, MLE cells were harvested on days 2, 4 and 11; C6 cells were harvested on days 4, 6 and 8. Northern analyses of MLE 15 cells showed increasing amounts of type I cell mRNA species with longer culture. On culture day 2 (24 h after removal of the drug) the mRNA species for *T1 α* and caveolin-1 were readily detected and the amount of AQP-5 mRNA was increased approx. 5-fold from the control value (Figure 5B, a–c). Thus the increases in expression of these three type I cell genes were substantial and rapid. C6 cells (Figure 5C) were analysed only for *T1 α* expression, which was detectable on day 4 and markedly up-regulated by day 8.

By day 11, *T1 α* mRNA expression in MLE 15 cells was approx. 130-fold that on day 0. Both AQP-5 and caveolin-1 were also markedly up-regulated by 5-azaC on day 11 (17-fold and approx. 200-fold the day 0 values), whereas SP-B mRNA, a type II cell gene expressed by control MLE 15 cells [31], increased less than 2-fold (Figure 5B, d). Thus in lung epithelial cell lines the expression of a group of type I cell genes could be induced by inhibiting methyltransferase activity; there was little effect on the type II cell genes already expressed by these cells.

Figure 5 Induction of type I cell marker gene expression in cell lines by the demethylating agent 5-aza-C

(A) Representative Northern blot of MLE 15 cells probed for expression of marker genes for type I cells (T1 α , AQP-5 and caveolin-1) and type II cells (SP-B) after treatment for 24 h with 5-azaC as described in the Materials and methods section. mRNA species for T1 α and caveolin-1, not detected in control (C) cells, are induced within 24 h of drug removal (day 2) and increase in expression from 4 to 11 days of culture. Similarly, AQP-5 expression is markedly increased, whereas SP-B expression is increased less than 2-fold. (B) Densitometric analyses of Northern blots of 5-azaC-treated MLE cells ($n = 3$) show time courses of increased expression of marker genes; numbers on day 11 columns are fold increases. The two type I cell genes not expressed in this cell line [those for T1 α (a) and caveolin-1 (c)] are increased more than 100-fold, with smaller increases in AQP-5 transcripts (b). SP-B transcripts (d), highly expressed in this line, are increased less than 2-fold. (C) Similarly, T1 α expression can be induced by 5-azaC in the non-lung C6 cell line.

DISCUSSION

Using a variety of methods and cell and organ systems, we tested the hypothesis that the expression of T1 α is negatively regulated by methylation of specific sites that we have previously shown to be important in transactivation. Although there have been many studies of methylation patterns in cell lines, there are relatively few data on the methylation of specific genes in primary cells and normal organs. In cell lines many genes become unmethylated or hypermethylated, as do non-integrated DNA constructs used for various types of study [39,40]. Thus the methylation status of genes in cell lines might not reflect that of cells in intact animals.

We show here that cytosine residues in a key region in the T1 α proximal promoter are consistently methylated in conditions under which T1 α is not expressed. This was shown in primary type II cells and in non-expressing organs such as liver, kidney and others as well as in cell lines. Conversely, when T1 α is expressed, these proximal sites are unmethylated. Therefore there is a strong negative correlation between the methylation status of the proximal promoter and T1 α expression. This association is true for both developing and adult animals.

The key methylation sites seem to be cytosine residues at -75 , -89 and -95 bp, as shown especially by analyses of adult non-expressing organs. This 20 bp sequence, which meets the criteria for a high-density methylation site [16], encompasses two Sp-family *cis* elements. Methylation of Sp *cis* elements has been demonstrated in several other genes, leading us to wonder whether this is a common mechanism of transcriptional repression. Our present results on intact animals are correlative; complex experiments such as those of Siegfried et al. [41] will be required to prove definitively that methylation at these two sites represses T1 α expression in intact animals. These investigators used transgenic animals to demonstrate [41] that a transgene whose methylation status can be switched *in vivo* is not expressed in normal tissues when methylated.

These results support the conclusion that promoter methylation has a role in the regulation of the developmental patterns of expression of T1 α in the central nervous system. In early rodent embryos (rat, days 10–15), T1 α mRNA and T1 α protein are abundantly expressed throughout the central nervous system; by days 16–17 expression begins to decline and falls to undetectable levels in the adult brain (Northern analysis) except

in the choroid plexus epithelium [34,42]. The promoter methylation pattern again mimics the protein/mRNA expression patterns, showing no methylation in fetal brain (day 14) and high levels of methylation at four sites in the proximal promoter in the adult. Future studies will include a detailed analysis of the developing brain to determine precisely how *Tl α* silencing matches increases in promoter methylation.

We have shown earlier that the -100 to -1 bp promoter fragment confers an approx. 6-fold activation to *Tl α* . Within the next 5' approx. 50 bp (-101 to -150) there are several strong enhancers including a TGT3 (forkhead) domain and a TTF-1 domain. If the -95 bp (and -89 bp) sites are silenced via the MeCP2-histone deacetylase complex, this might in fact interfere with nearby upstream elements, particularly those close to the -100 bp site. Several other 3' methylated sites (-75 , -62 , -54 , -48 and -42) were identified in C6 cells but these sites are not consistently methylated in most normal non-expressing organs and are only partly methylated in primary type II cells. These cytosine residues are therefore not likely to be critical for *Tl α* silencing and might reflect the relatively non-specific hypermethylation of genes in cell lines noted above.

Studies of *Tl α* regulation in primary type II cells, with or without culture, in which *Tl α* transcription is reversibly off or on, also support our conclusion. As expected, cultured type II cells lack promoter methylation, whereas acutely isolated cells have a methylation pattern similar to non-expressing organs and cell lines. However, in the freshly isolated cells the peak heights at the -95 and -89 methylated residues do not reach 100%; Siegfried et al. [41] also reported lower levels of methylation in a methyl-sensitive promoter transgene, although the reason for this is unknown.

We believe that the acutely isolated type II cells might initiate the expression of *Tl α* mRNA during the isolation procedure because both protein and mRNA can be detected in cells cultured for approx. 12 h [27]. This is consistent with Northern-blot analyses that show a marginally detectable level of *Tl α* mRNA after the 4 h isolation in some isolates; it is also consistent with other observations that expression of type II cell genes is extinguished within approx. 12 h of isolation. Because neither *Tl α* protein nor *Tl α* mRNA can be detected in type II cells *in vivo* [33,34,42], we do not believe that normal type II cells in lung express *Tl α* ; rather there is a change in gene expression and phenotype that begins when the cells are detached from the alveolar basement membrane or shortly thereafter.

We also showed that promoter methylation *in vitro* decreases expression from the -170 bp promoter by approx. 50% but does not abolish expression in lung epithelial cell lines. In general it is believed that the higher the methylation density is, and the closer it is to the transcription initiation site, the stronger the gene silencing becomes [16,43]. Even distal sites, if of high methylation density, can be strong repressors. We interpret our results as indicating that methylation has a role, albeit incomplete, in *Tl α* repression. It is also known that strong transcriptional activators can override the repressive effects of a nearby methylated site [44]. MLE 15 and SV40T type II cell lines express some of the important transcription factors, such as TTF-1, that drive *Tl α* transcription [19] and might be able to overcome the inhibitory influence of methylation of the nearby Sp1 site.

The factors that target specific genes for methylation are not known and there is little information relating to control of cell and organ specificity. The identification of four new methyltransferases (Dnmt 3 α , 3 β 1, β 2 and β 3) [8] that are developmentally regulated and are differentially expressed in several organs, including lung, might provide some specificity. Given that Clara and type II cells (two of the more than 40 cell types

in lung) differentially regulate methyltransferase activity in response to a chemical carcinogen [45], there is a possibility that these cells express different methyltransferases that are differentially regulated.

On the basis of earlier studies of several differentiation genes, it has been argued that methylation might not have a central role in development [1] or normal cell differentiation. However, this argument is based largely on the Dnmt1 knock-out mouse that shows normal expression patterns of several differentiation genes. Interpretation of the phenotype of Dnmt-1-null mouse is now less certain because of the identification of two methyltransferase enzymes with some organ and developmental specificity. Combined null mutations in the two enzymes produce severe developmental phenotypes and early embryonic lethality [46]. These enzymes and the newly identified demethylase might mediate dynamic changes [47] in cell-specific gene regulation both in developing and adult animals.

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