Cell type-specific methylation of an intronic CpG island controls expression of the MCJ gene

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Over 50% of human genes are associated with CpG islands and DNA methylation within such CpG islands has been clearly correlated with inhibition of expression. Whereas changes in DNA methylation play a key role in a number of human diseases, in particular cancer, in normal DNA CpG islands are nearly always methylation free, regardless of the expression status of the associated gene. Only limited evidence supports a role for DNA methylation in controlling tissue-specific expression in adult somatic tissue. Loss of expression of the MCJ gene has previously been linked to increased chemotherapeutic drug resistance in ovarian cancer. We report that loss of expression of MCJ in drug-resistant ovarian cancer cell lines depends on methylation of a CpG island within its first exon, but is independent of methylation within the promoter region. Furthermore, cell type-specific expression of the MCJ gene in normal cells also depends on the methylation status of the CpG island within its first exon. The MCJ CpG island is methylated and the gene is not expressed in cells of epithelial origin, but unmethylated and expressed in cells of lymphocyte or fibroblast origin. Chromatin immunoprecipitation assays determined that MCJ CpG island methylation was associated with loss of histone acetylation in ovarian epithelial cells compared with unmethylated fibroblast cells. Reduced acetylation was observed not only within the CpG island, but also within the promoter region, suggesting that CpG island methylation may direct alterations in chromatin structure within the promoter region, leading to gene inactivation.

Introduction

Methylation at the 5' position of cytosines that form part of CpG dinucleotides is the only commonly occurring covalent modification of DNA and ~70% of CpG sites in the human genome are methylated (1). CpG dinucleotides are under-represented throughout the genome, with the exception of short stretches of DNA known as CpG islands. These CpG islands are GC-rich stretches of DNA of up to a few kilobases in length with close to the expected number of CpG dinucleotides and are frequently associated with human genes, often mapping to the promoter/first exon of the gene. In contrast to the bulk of DNA, the CpG sites within CpG islands are almost always methylation free (1).

Alterations in DNA methylation appear to play a key role in a number of diseases, in particular in cancer, where the inactivation of many genes known to be important in tumour development has been found to be associated with increased methylation within promoter-associated CpG islands (2). Indeed, increased methylation of CpG sites within CpG islands has been clearly demonstrated to be associated with transcriptional silencing of linked genes (3). However, although DNA methylation was originally proposed as a mechanism for control of tissue specific expression (4), analysis of the methylation status of CpG islands in human adult somatic tissues determined that CpG islands were almost always methylation free even in tissues where the associated gene was not expressed (5).

Recently, however, Futscher et al. (6) reported the first example of a human gene in which cell type-specific expression is associated with specific differences in methylation of its associated CpG island. The maspin gene is specifically expressed in cells of epithelial origin, but not expressed in other cell types. Futscher et al. (6) demonstrated that the maspin CpG island exhibited a corresponding pattern of DNA methylation in which the CpG island was unmethylated in maspin-expressing epithelial cells, but methylated in other, non-expressing cell types. Furthermore, treatment of non-expressing cells with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine resulted in re-expression of the maspin gene, suggesting that DNA methylation was required for continued suppression of maspin expression.

Here we identify a second human gene, the MCJ gene, in which cell type-specific patterns of methylation are associated with cell type-specific expression, although in this case the tissue-specific methylation patterns are exactly the opposite to that of the maspin gene. Shridhar et al. (7) recently identified the methylation-controlled DNAJ (MCJ) gene as a novel gene exhibiting reduced expression in ovarian cancer cell lines and tumours and demonstrated a role for loss of MCJ expression in vitro in chemoresistance. We now demonstrate that dense methylation of a CpG island, which begins within the first exon of the MCJ gene (Figure 1), is associated with transcriptional silencing in both drug-resistant cancer cells and also in normal cells of epithelial origin. In contrast, only low levels of methylation of CpG sites within the island are seen in cells of lymphocyte and fibroblast origin in which the MCJ gene is expressed. Treatment of non-expressing cell lines in which the MCJ CpG island is methylated with a DNA methyltransferase inhibitor results in re-expression of MCJ, suggesting that DNA methylation is required for suppression of MCJ expression. Chromatin immunoprecipitation (ChIP) assays determined that CpG island methylation was associated with loss of histone acetylation not only within the CpG island, but also within

Abbreviations: ChIP, chromatin immunoprecipitation; OSE, ovarian surface epithelial; IL-4, interleukin-4.

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the promoter region, suggesting that spreading of chromatin changes to regions outside the CpG island may be important for gene inactivation.

Materials and methods

Tissue culture and cells
The primary lines HEK78 and WI-38 were generous gifts from Dr K. Parkinson and Dr N. Keith, respectively. The primary 142BR and immortalized 1BR.3.G fibroblast cell lines were obtained from ECACC (Cambridge, UK). Primary cultures of human OSE (ovarian surface epithelial) cells were generated from women undergoing hysterectomy or oophorectomy for benign conditions as described previously (8). Primary cultures were immortalized using retroviruses modified to express a temperature-sensitive form of SV40 large T antigen and the catalytic subunit of telomerase, hTERT, and immortalized OSE cells were maintained at a temperature permissive for growth (33°C). Fibroblast cell lines were grown as monolayer cultures in minimal essential Eagle’s medium, 1% non-essential amino acids (Sigma, Poole, UK) with 10% (1BR.3.G) or 15% (142BR) fetal calf serum in 95% air/5% CO₂ at 37°C. Genomic DNA was extracted for methylation analysis as previously described (9). The ovarian carcinoma cell line A2780 and its cisplatin-resistant derivative were maintained in RPMI with 10% fetal calf serum in 95% air/5% CO₂ at 37°C. Derivation of the cisplatin-resistant lines has been described before (10). For reversal of methylation the A2780/MCP1 and A2780/MCP3 lines were treated with 5-azacytidine (Sigma) as described before (9).

Tissue samples
Grossly normal tissue adjacent to tumour samples was obtained from the Western Infirmary and Stobhill General Hospitals, Glasgow, UK. Ethical approval for all samples collected had been obtained and samples were collected according to MRC operational and ethical guidelines on ‘Human tissues and biological samples for use in research’. All samples were stored frozen at −70°C. Peripheral blood lymphocytes were obtained from healthy volunteers. Genomic DNA was extracted for methylation analysis as previously described (9).

COBRA analysis
COBRA analysis was performed largely as described before (11). A sample of 1 μg of genomic DNA was modified with sodium bisulphite using the CpGenome modification kit (Intergen, Oxford, UK) as per the manufacturer’s instructions. All samples were resuspended in 40 μl of TE and 1 μl of this was used for subsequent PCR reactions. The samples were ampliﬁed in 25 μl volumes containing 1× manufacturer’s buffer, 1 U FastStart Taq polymerase (Roche, Lewes, UK), 2 (pro primers) or 4 (CpG primers) mM MgCl₂, 10 mM dNTPs, and 75 ng each primer. PCR was performed with one cycle of 95°C for 6 min, 35 cycles of 95°C for 30 s, 63°C for 30 s and 72°C for 30 s, followed by one cycle of 72°C for 5 min. All PCRs were carried out on a PTC-225 DNA engine tetrad (MJ Research, Warrington, MA). Following amplification, the PCR products were digested with a restriction enzyme speciﬁc for the methylated sequence after sodium bisulphite modiﬁcation. For the CpG island primers, digestion of the total PCR products was carried out with 20 U TaqI (Invitrogen, Paisley, UK) in 1× manufacturer’s buffer for 2 h at 65°C. Digested PCR products were separated on 2% agarose gels and visualized by ethidium bromide staining on GelDoc 1000 (Bio-Rad, Hemel Hempstead, UK). The primers used for the PCRs (and positions relative to the transcriptional start site) were: for the CpG island, MCI/CpG, forward (+122 bp) 5′-GTTTGGGGAGGGGATTAGG-3′, reverse (+547 bp) 5′-CTAAACAAATCCACCGGACCGTGACACCTGTA-3′; for the MCI promoter, MCI pro, forward (−316 bp) 5′-AGATAGTTGAGTTATAGGG-3′, reverse (−145 bp) 5′-CTTAAACCGTAATCCCCCCTCCTCC-3′ (for normal cell lines) and MCI pro2, forward (−126 bp) 5′-GTTATATTTGAGGGTTGGAG-3′, reverse (−144 bp) 5′-TTTAAACCTAACCTCCTCCC-3′ (for ovarian cancer cell lines).

Bisulfite sequencing
Sodium bisulphite modification and PCR was carried out as above. PCR products were then separated on 1.5% agarose gels and visualized using ethidium bromide. Bands corresponding to the expected size were cut out of the gel and DNA extracted using GenElute agarose spin columns (Sigma), as per the manufacturer’s protocol. Isolated DNA was resuspended in 10 μl of ddH₂O and 3 μl of this was used for ligation to PGM-t vector system II PCR cloning vector (Promega, Southhampton, UK). Ligation were carried out overnight at 4°C and the ligated products transformed into JM109 cells as per the manufacturer’s protocol. These were then plated out on LB agar plates containing 100 μg/ml ampicillin, 80 μg/ml X-Gal and 0.5 mM IPTG and left overnight at 37°C. White (insert containing) colonies were picked and grown up in 4 ml of LB containing 100 μg/ml ampicillin and plasmid DNA isolated using a QIAprep Spin miniprep kit (Qiagen, Crawley, UK). Sequencing was then carried out on a CEQ 2000XL DNA analysis system (Beckman Coulter, High Wycombe, UK) using the sp6 or T7 sequencing primers. The accuracy of the quantitation of methylation levels was confirmed by analysis of mixtures (75:25, 50:50 and 25:75) of known methylated [in vitro methylated DNA (Intergen)] and known unmethylated DNA [derived from peripheral lymphocytes (see results for lack of effect on MCI methylation in these cells)]. This analysis confirmed that the bisulfite sequencing results were indeed reflective of the methylation status of the input DNA.

RT-PCR
RNA was isolated from cell lines using Trizol reagent (Invitrogen) as per the manufacturer’s protocol. An aliquot of 5 μg RNA was then used for cDNA synthesis using the SuperScript first strand cDNA synthesis kit (Invitrogen). RT-PCR was then performed in 25 μl volumes containing 1× manufacturer’s buffer, 1 U FastStart Taq polymerase, 2 mM MgCl₂, 10 mM dNTPs, 75 ng each primer and 2 μl of cDNA. PCR was performed with one cycle of 95°C for 6 min, 20 (GAPDH) or 25 (MCI) cycles of 95°C for 30 s, 61°C for 30 s and 72°C for 30 s, followed by one cycle of 72°C for 5 min. All PCR reactions were carried out on a PTC-225 DNA engine tetrad. RT-PCR products were separated on 1.5% agarose gels and stained with ethidium bromide. Quantitation was carried out using the GelDoc 1000 system. For RFLP analysis 12.5 μl of RT-PCR product was digested with 7.5 U BsmA1 (New England Biolabs) in a 20 μl volume with 1× manufacturer’s buffer for 2.5 h at 55°C. Products were separated on a 2% agarose gel. The primers used were: for MCI, forward 5′-TGGCGAGAGTTTGGCCTA-3′, reverse 5′-CTAGAGGTCCCTCCAGGTCC-3′; for GAPDH, forward 5′-GTCAGAGCTATTTCTGTTAGT-3′, reverse 5′-GTCTGATGCAGAAGATGAG-3′.
Chromatin immunoprecipitation

ChIP assays were carried using the acetylhistone H3 immunoprecipitation assay kit (Upstate) as described in the manufacturer’s instructions. Briefly, 1 x 10^6 cells were plated out in T75 tissue culture flasks and left overnight at 37°C. The following day the cells were cross-linked by adding 405 μL of 37% formaldehyde to 15 mL of culture medium and incubating for 10 min at 37°C. The cells were then washed with ice-cold phosphate-buffered saline containing protease inhibitors, scraped off the flasks and sonicated in 1.5 mL centrifuge tubes for 4 min at 4000 r.p.m. The pellet was resuspended in 200 μL of SDS lysis buffer (including protease inhibitors) and sonicated to produce fragments of 200–1000 bp. Immunoprecipitation was carried out using 10 μL of the anti-acetylhistone H3 antibody overnight at 4°C. Following washing and elution of the chromatin complexes as per the manufacturer’s instructions, the cross-links were reversed by incubating at 65°C for 4 h and the DNA was recovered by phenol/chloroform extraction and ethanol precipitation. The DNA samples were resuspended in 20 μL of TE and 1 μL of this was used in subsequent PCRs to detect the presence of immunoprecipitated DNA. The samples were amplified in 25 μL volumes containing 1 x manufacturer’s buffer, 1 U FastStart Taq polymerase, 1 (MCJ pro and β-actin ChIP primers) or 3 (MCJ CpG ChIP primers) mM MgCl₂, 10 mM dNTPs and 75 ng each primer. PCR was performed with one cycle of 95°C for 6 min, 35 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 30 s, followed by one cycle of 72°C for 5 min. All PCRs were carried out on a PTC-225 DNA engine tetrad. Following amplification, the PCR products were separated on 2% agarose gels and quantitated using ethidium bromide staining in a GelDoc 1000 system. Quantitative PCRs (qPCR) was carried out using a panel of serially diluted genomic DNA (25 to 0.1 ng). The primers used for the qPCRs (and positions relative to the transcriptional start site) were: MCJ pro ChIP, forward (−253) 5′-GAGGTTTCACTCTGTCGG-3′, reverse (−91) 5′-GAGGCAATTTGCTTACCTCAC-3′; MCJ CpG ChIP, forward (−356) 5′-AATCTGTTGTCCCACTGACG-3′; reverse (−480) 5′-TACTCTGAGCGCCAAC-3′; β-actin ChIP, forward (−268) 5′-TCATCCTCCTGCTCTCCT-3′, reverse (−135) 5′-AAGGCAATTTGCTGGAAGGC3′. Relative quantitation of the signals at the promoter and CpG island were determined by normalizing to the signal obtained at the β-actin gene.

Results

Methylation of the MCJ CpG island, but not the promoter, is associated with loss of MCJ expression and cisplatin resistance in ovarian cancer cell lines

Loss of MCJ expression was reported to increase the resistance of ovarian cancer cells to a number of chemotherapeutic agents, including cisplatin (7). To investigate the potential role of MCJ in influencing sensitivity to chemotherapeutic agents, gene expression was studied using RT–PCR in the epithelial ovarian carcinoma cell line A2780 and 10 cisplatin-resistant derivatives of this cell line. While MCJ expression could be clearly detected in the A2780 cell line, 8 of 10 of the resistant derivatives exhibited loss of MCJ expression (Figure 2B). These results are consistent with MCJ non-expressing cells having a selective advantage following exposure to cisplatin. Previously, loss of MCJ expression has been attributed to DNA methylation (7). In agreement with this, treatment of two of the cisplatin-resistant A2780 derivatives, A2780/MCP1 and A2780/MCP3, with 5-azacytidine, an inhibitor of DNA methyltransferase activity, resulted in re-expression of MCJ in these cell lines (Figure 2B), confirming that the absence of MCJ expression was at least partially dependent on DNA methylation.

DNA methylation-dependent transcriptional inactivation is usually due to hypermethylation of a CpG island within the gene promoter. The MCJ locus exhibits only a limited number of CpG sites in the proximal promoter region, however, a CpG island was identified beginning within the first exon (Figure 1). To more clearly define the role of DNA methylation in loss of MCJ expression in the ovarian carcinoma cell lines, bisulfite sequencing was used to examine the methylation status of the promoter and the CpG island in seven of the lines. This analysis detected no apparent correlation between methylation of the CpG sites in the proximal promoter region and expression of MCJ, as high levels of promoter methylation were detected in both expressing and non-expressing cell lines (examples in Figure 2C and Table I). However, bisulphite sequencing analysis of the CpG island identified a clear difference between expressing and non-expressing cell lines. All alleles sequenced from the non-expressing cell lines exhibited extensive methylation of the CpG island, however, in the expressing cell lines a mixture of both unmethylated and methylated alleles was identified (examples in Figure 2D and Table I). These results indicate that methylation within the CpG island, but not within the promoter region, is critical in determining expression of the MCJ gene.

DNA methylation of the MCJ gene in normal tissue is associated with cell type-specific expression

In normal tissue CpG islands are usually unmethylated regardless of the expression status of the associated gene (5). To determine if this was also the case for the MCJ CpG island, the methylation status of the island was examined in 12 samples of morphologically normal ovarian tissue using the COBRA technique (11). This analysis detected the presence of methylation of MCJ in 11/12 normal samples (examples in Figure 3B). More exact quantitation of the level of DNA methylation was obtained using bisulphite sequencing analysis. An average of 40% of clones derived from the normal ovarian tissue samples were found to be methylated (Table II). The accuracy of the quantitation of methylation levels using the bisulphite sequencing technique was confirmed by analysis of mixtures of DNA of known methylation status (see Materials and methods). Given that most genes in normal cells are unmethylated, the high level of methylation of MCJ in normal ovarian tissue was surprising. The level of methylation was not related to the age of the patient from which the sample was taken (data not shown) and was not due to imprinting, as both MCJ alleles were expressed (Figure 3C). This suggests that methylation may be restricted to a specific cell type or types within the normal tissue samples and that the partial methylation observed may be due to the mixture of cell types in these samples.

To investigate the possibility of cell type-specific methylation, studies were carried out to determine MCJ methylation levels in primary and immortalized cells derived from multiple cell types. Methylation was examined at both the small number of CpG sites in the proximal promoter and at the CpG island. The CpG island exhibited low or absent methylation, by COBRA analysis, in all samples derived from fibroblasts (0/3 cell lines) or lymphocytes (0/6 samples), however, methylation of the CpG island was seen in >95% of alleles derived from three epithelial samples; two derived from ovarian surface epithelium (>90% cytokeratin-positive cells) (OSE-C1 and OSE-C2) and one from skin epithelium (HEK78) (Figure 4B). A more detailed examination of the methylation of the promoter and CpG islands was then carried out using bisulphite sequencing. This analysis confirmed the high levels of methylation of both the promoter and CpG island in the three epithelial cell lines, with much lower levels of methylation, in particular of the CpG island, being seen in the non-epithelial lines (Table III). OSE cells are poorly committed cells that also exhibit some mesenchymal characteristics in vivo and frequently lose epithelial markers and differentiate to...
mesenchymal-like cells in culture (12). A third OSE-derived line (OSE-C3), in which 50% of the cells retain expression of the epithelial marker cytokeratin but 95% of cells express the mesenchymal marker collagen type III, was also examined. The OSE-C3 cell line exhibited a methylation pattern similar to the other non-epithelial cells (Figure 4B and Table III), suggesting that loss of MCJ methylation may occur early during the epithelial to mesenchymal transition.

Fig. 2. MCJ expression correlates with methylation of the CpG island, but not the promoter. (A) Examples of parts of sequencing chromatograms of methylated (upper chromatogram, A2780/MCP6) and unmethylated (lower chromatogram, A2780) clones from bisulphite sequencing of the MCJ CpG island. The positions of differentially methylated CpG sites are marked by R* in the consensus sequence above the chromatograms. (B) Expression of MCJ was determined by RT-PCR in A2780 and its 10 cisplatin-resistant derivatives (A2780/CP70 and A2780/MCP1–A2780/MCP9). 1-5aza and 3-5aza are A2780/MCP1 and A2780/MCP3, respectively, treated with 5-azacytidine. Amplification of GAPDH was used as a positive control. (C) Examples of methylation pattern of the MCJ promoter in expressing (A2780/MCP8) and non-expressing (A2780/MCP6) cell lines, as determined by bisulphite sequencing analysis. Between 12 and 14 clones from each of the two cell lines were sequenced, including eight CpG sites within the proximal promoter (between −150 and +150 bp relative to the transcriptional start site). (D) Examples of methylation pattern of the MCJ CpG island in expressing (A2780) and non-expressing (A2780/MCP6) cell lines, as determined by bisulphite sequencing analysis. Between 15 and 18 clones from each of the two cell lines were sequenced, including 35 CpG sites within the CpG island. U, unmethylated CpG; M, methylated CpG; −, not determined.
Table I. Expression of MCJ correlates with CpG island methylation

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cisplatin sensitivity</th>
<th>MCJ expression</th>
<th>Promoter methylation(a)</th>
<th>CpG island methylation(b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2780</td>
<td>Sensitive</td>
<td>+</td>
<td>46</td>
<td>73</td>
</tr>
<tr>
<td>A2780/MCP1</td>
<td>Resistant</td>
<td>–</td>
<td>83</td>
<td>100</td>
</tr>
<tr>
<td>A2780/MCP3</td>
<td>Resistant</td>
<td>–</td>
<td>81</td>
<td>100</td>
</tr>
<tr>
<td>A2780/MCP6</td>
<td>Resistant</td>
<td>–</td>
<td>81</td>
<td>100</td>
</tr>
<tr>
<td>A2780/MCP8</td>
<td>Resistant</td>
<td>+</td>
<td>88</td>
<td>81</td>
</tr>
<tr>
<td>A2780/CP70</td>
<td>Resistant</td>
<td>+</td>
<td>nd(c)</td>
<td>74</td>
</tr>
<tr>
<td>A2780/MCP1-5-aza(d)</td>
<td>Sensitive</td>
<td>+</td>
<td>nd(c)</td>
<td>44</td>
</tr>
</tbody>
</table>

\(a\)Percentage of CpG sites methylated between –150 and +150 bp, identified by bisulfite sequencing (at least 12 clones sequenced for each sample).
\(b\)Percentage of clones methylated, identified by bisulfite sequencing. A methylated clone is defined as >50% of CpG sites methylated (at least 12 clones sequenced for each sample).
\(c\)Not determined.
\(d\)A2780/MCP1 cells treated with 5-azacytidine.

Table II. Methylation of the MCJ gene in normal ovarian tissue

<table>
<thead>
<tr>
<th>Sample</th>
<th>CpG island methylation(a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal 1</td>
<td>58</td>
</tr>
<tr>
<td>Normal 2</td>
<td>0</td>
</tr>
<tr>
<td>Normal 3</td>
<td>67</td>
</tr>
<tr>
<td>Normal 4</td>
<td>58</td>
</tr>
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<td>Normal 5</td>
<td>23</td>
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<tr>
<td>Normal 6</td>
<td>31</td>
</tr>
<tr>
<td>Normal 7</td>
<td>38</td>
</tr>
<tr>
<td>Normal 8</td>
<td>43</td>
</tr>
</tbody>
</table>

\(a\)Percentage of clones methylated, identified by bisulfite sequencing. A methylated clone is defined as >50% of CpG sites methylated (at least 12 clones sequenced for each sample).

Fig. 3. The MCJ CpG island is methylated in normal ovarian tissue. (A) Examples of parts of sequencing chromatograms of unmethylated (upper chromatogram) and methylated (lower chromatogram) clones from bisulphite sequencing of the MCJ CpG island in normal ovarian tissue samples. The positions of differentially methylated CpG sites are marked Y* in the consensus sequence above the chromatograms. (B) Examples of COBRA analysis of the MCJ CpG island in normal ovarian tissue. The bands representing unmethylated DNA (undigested) and methylated DNA (digested) are indicated. IVM, in vitro methylated DNA. (C) RFLP analysis demonstrates the expression of both MCJ alleles. A polymorphism in the first exon of MCJ creates a BsmAI RFLP. The more common allele (termed allele 1) contains the restriction site and the less common allele (termed allele 2) does not. Digestion of the RT-PCR product with BsmAI results in the presence of only alleles 1 in the homozygous N9 sample, but both alleles 1 and 2 in the heterozygous N10 sample. RT-PCR was carried out with both gene-specific primers (GSP) and oligo(dT) primers (dT).
RT-PCR was then used to study the relationship between methylation of MCJ and expression. The highly methylated epithelial cell lines exhibited complete loss (OSE-C1) or barely detectable levels (OSE-C2 and HEK78) of MCJ expression. In contrast, in the fibroblast and lymphocyte cells, in which methylation levels were much lower, much higher levels of MCJ expression were detected (Figure 4C). Thus high levels of methylation of the MCJ CpG island are restricted to epithelial cells and correlate with inhibition of MCJ expression, while in lymphoid and mesenchymal cells CpG island methylation is low or absent and MCJ expression levels are high. Therefore, cell type-specific expression of MCJ in normal cells is dependent on DNA methylation of the gene, in particular within the CpG island.

To determine if methylation of the CpG island was associated with alterations in the local chromatin structure, chromatin immunoprecipitation assays were used to assess histone

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**Table III.** Methylation of the MCJ gene in different cell types

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell type</th>
<th>Promoter methylationa</th>
<th>CpG island methylationb</th>
<th>MCJ expressionc</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEK78</td>
<td>Epithelial</td>
<td>95</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>OSE-C1</td>
<td>Epithelial</td>
<td>93</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>OSE-C2</td>
<td>Epithelial</td>
<td>90</td>
<td>100</td>
<td>2</td>
</tr>
<tr>
<td>OSE-C3</td>
<td>Epithelial/ mesenchymald</td>
<td>76</td>
<td>29</td>
<td>49</td>
</tr>
<tr>
<td>PBL</td>
<td>Lymphocyte</td>
<td>68</td>
<td>8</td>
<td>419</td>
</tr>
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<td>WI-38</td>
<td>Fibroblast</td>
<td>67</td>
<td>21</td>
<td>303</td>
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<tr>
<td>1BR.3.G</td>
<td>Fibroblast</td>
<td>84</td>
<td>42</td>
<td>59</td>
</tr>
<tr>
<td>142BR</td>
<td>Fibroblast</td>
<td>60</td>
<td>8</td>
<td>51</td>
</tr>
</tbody>
</table>

*aPercentage of CpG sites methylated between −315 and +145 bp, identified by bisulfite sequencing (at least 12 clones sequenced for each sample).*

*bPercentage of clones methylated, identified by bisulfite sequencing. A methylated clone is defined as >50% of CpG sites methylated (at least 12 clones sequenced for each sample).*

*cExpression level of MCJ relative to the HEK78 cell line.*

*dThis cell line has, at least partially, undergone epithelial to mesenchymal transition (see text for details).*
acetylation both within the CpG island and also within the promoter region. As shown in Figure 5B, the high levels of methylation of the MCJ CpG island seen in the A2780/MCP1 cell line were associated with a clear reduction in the level of histone acetylation within the CpG island compared with the 1BR.3.G fibroblast cell line, which has low levels of CpG island methylation. However, alterations in the chromatin structure were not restricted to the region of the CpG island. Analysis of the region just upstream of the transcriptional start site demonstrated that the A2780/MCP1 cell line again exhibited reduced histone acetylation compared with the 1BR.3.G cell line, normalized to the signal obtained at the β-actin locus, is indicated below. No Ab, no antibody control. (C) Results of the ChIP analysis for the MCJ promoter (first panel), CpG island (second panel) and the β-actin locus (third panel), following treatment of cells with 5-azacytidine. The fold increase in the signal from the 5-azacytidine-treated A2780/MCP1 cells (+5-aza) versus untreated cells (A2780/MCP1), normalized to the signal obtained at the β-actin locus, is indicated below. No Ab, no antibody control.

Discussion

Until recently it was believed that in normal adult somatic tissue CpG islands remained free from DNA methylation even in cells where the corresponding gene was not expressed. However, the recent report by Futscher and colleagues (6) identified the maspin gene as the first human gene in which DNA methylation played a role in cell type-specific expression, with DNA methylation and lack of expression being found in non-epithelial cells, but no DNA methylation and expression of maspin in epithelial cells. The present report identifies MCJ as a second gene in which cell type-specific expression is controlled at least in part by DNA methylation. In this case the opposite pattern is seen to that observed for the maspin gene, with DNA methylation of the CpG island and lack of MCJ expression being found in epithelial cells, but low or absent CpG island methylation and expression of MCJ in non-epithelial cells. It could be suggested that the methylation differences detected in the cell lines could be an artefact of growth of the cells in tissue culture, however, as analysis of normal ovarian samples indicates that significant levels of methylation of MCJ are present in primary tissue and analysis

Fig. 5. ChIP analysis identifies reduced histone acetylation associated with CpG island methylation. (A) Diagram showing methylation of the CpG sites within the 5'-end of the MCJ locus in the A2780/MCP1 and 1BR.3.G cell lines. , 75-100% methylated; , 50-75% methylated; , 25-50% methylated; , 0-25% methylated. The position of primers used for the ChIP analysis of the promoter and CpG island are indicated below. (B) Results of the ChIP analysis for the MCJ promoter (first panel), CpG island (second panel) and the β-actin locus (third panel), using anti-acetylated H3 antibody. The fold reduction in the signal from the A2780/MCP1 cell line compared with the 1BR.3.G cell line, normalized to the signal obtained at the β-actin locus, is indicated below. No Ab, no antibody control. (C) Results of the ChIP analysis for the MCJ promoter (first panel), CpG island (second panel) and the β-actin locus (third panel), following treatment of cells with 5-azacytidine. The fold increase in the signal from the 5-azacytidine-treated A2780/MCP1 cells (+5-aza) versus untreated cells (A2780/MCP1), normalized to the signal obtained at the β-actin locus, is indicated below. No Ab, no antibody control.
of the cell lines identified essentially identical patterns of methylation in primary (HEK78, WI38 and 142BR) and immortalized (OSE-C1, OSE-C2 and 1BR.3G) cell lines, indicating that neither immortalization nor prolonged growth in culture significantly affect MCJ methylation levels, epithelial-specific methylation of MCJ appears to be the only reasonable explanation for the results presented.

In addition to its role in control of expression of genes with CpG islands, DNA methylation also appears to play a role in normal cells in the control of expression of genes which exhibit only rare CpG sites, for example interleukin-4 (IL-4) (13). However, in contrast to maspin and MCJ, although the presence of methylation is required for appropriate control of gene expression, demethylation of the gene does not appear to be required for re-expression (14). Instead, it appears that methylation of the limited number of CpG sites allows binding of the methyl-binding domain protein MBD2, which competes with positively acting transcription factors for binding to the IL-4 promoter (15). Activation of IL-4 in T cells is achieved with positively acting transcription factors for binding to the methyl-binding domain protein MBD2, which competes from its binding sites without requiring any change in algorithms (16) (http://www.usccancer.com/cpgislands). This would also explain the apparently contradictory results obtained for some other genes in which methylation of CpG islands within the gene did not affect expression [e.g. in exon 2 of the p16 gene (20)]. In such cases the CpG islands lie at greater distances from the promoter region and thus the promoter would likely lie outside the region of modified chromatin induced by methylation of the CpG island. Thus although CpG islands are frequently found within exonic sequences, a comprehensive search for CpG islands on chromosomes 21 and 22 found similar levels of exonic and promoter-associated CpG islands (16), the majority of these will likely be too distal from the promoter region to affect gene expression. Nevertheless, the MCJ gene may not be unique in this mechanism of methylation-dependent repression. A number of other genes that can be inactivated by DNA methylation have CpG islands largely within the transcribed region [for example 14-3-3σ (21) and the endothelin receptor B Δ3 transcript (22)] and most CpG islands implicated in affecting gene expression overlap both the promoter and exonic sequences. Although attention has generally focused on promoter methylation, it may be that methylation of transcribed sequences is as important as methylation of the promoter in many of these genes.

As yet no functional role has been assigned to the MCJ protein and indeed MCJ has no apparent homologues in other non-human mammals, although the presence of a DNAJ domain suggests that it may well interact with the hsp70 protein (7). The cell type-specific methylation and expression of MCJ identified in this report indicates that its function is specifically required in non-epithelial cells and that MCJ expression may be important in differentiation in cells of non-epithelial lineages. Alternatively, MCJ expression may be incompatible with epithelial cell differentiation. The analysis of MCJ methylation in the OSE cell lines suggests that reversal of MCJ methylation may be an early event in the epithelial to mesenchymal transition. Immunofluorescence of the OSE-C3 cell line identified loss of the epithelial-specific marker cytokeratin in >50% of the cells and a marked increase in expression of a mesenchymal marker, collagen type III (>98% cells positive), compared with the primary culture from which it was derived, and the OSE-C1 and OSE-C2 cell lines, suggesting that the OSE-C3 cell line has, to some extent, undergone an epithelial to mesenchymal transition in culture. Analysis of MCJ in this cell line identified loss of epithelial cell-specific methylation and re-expression of MCJ. Thus reversal of MCJ CpG island methylation and re-expression of MCJ may play a role during the epithelial to mesenchymal transition of OSE cells.

In normal ovarian tissue samples a degree of variation was seen in the levels of MCJ methylation and indeed no methylation was detected in one of the samples. This is almost certainly due to variation in the percentage of OSE cells, which are present in only a thin layer on the surface of the ovary (12), in the normal tissue samples. However, variation in the extent of methylation of MCJ in OSE cells in vivo cannot be excluded. In addition, as these samples were taken from tissue adjacent to tumour samples, there could be a low level of infiltrating tumour cells. However, the levels of methylation detected (~50%) are much too high to be readily explained by such contamination and indeed in one normal sample 50% methylation was detected despite the absence of any detectable methylation in the adjacent tumour. In addition, we have analysed the methylation of a number of other loci in a subset (seven) of these and other normal samples taken from ovarian cancer patients and only identified very infrequent and low levels of methylation of genes methylated in the associated
tumour (23). Therefore the methylation detected in the normal ovarian samples is almost certainly derived from normal epithelial cells.

The analysis of MCJ methylation in ovarian cancer cell lines reported here and in the previous study of Shridhar et al. (7) suggests that methylation of MCJ may be an important determinant of chemosensitivity. However, it should be noted that many genes may become methylated in such drug-resistant cell lines and ovarian tumours (24) and that MCJ may be one of several methylated genes involved in drug resistance. Nevertheless, the observed methylation of MCJ may represent a potential opportunity for therapeutic intervention. A number of inhibitors of DNA methylation are currently undergoing clinical trials, including investigating the possible use of one such inhibitor to re-activate genes, such as MCJ, involved in determining chemosensitivity (25). We are currently analysing a large cohort of ovarian tumours, using the bisulphite sequencing technique, to determine the levels of methylation of the MCJ CpG island in ovarian tumours and identify any links between MCJ methylation and patient responses to chemotherapy.

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