The enzyme 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2) is selectively expressed in aldosterone target tissues, where it confers aldosterone selectivity for the mineralocorticoid receptor by inactivating 11β-hydroxyglucocorticoids. Variable activity of 11βHSD2 is relevant for blood pressure control and hypertension. The present investigation aimed to elucidate whether an epigenetic mechanism, DNA methylation, accounts for the rigorous control of expression of the gene encoding 11βHSD2, HSD11B2. CpG islands covering the promoter and exon 1 of HSD11B2 were found to be densely methylated in tissues and cell lines with low expression but not those with high expression of HSD11B2. Demethylation induced by 5-aza-2′-deoxycytidine and procarbamide enhanced the transcription and activity of the 11βHSD2 enzyme in human cells in vitro and in rats in vivo. Methylation of HSD11B2 promoter–luciferase constructs decreased transcriptional activity. Methylation of recognition sequences of transcription factors, including those for Sp1/Sp3, Arnt, and nuclear factor 1 (NF1) diminished their DNA-binding activity. Herein NF1 was identified as a strong HSD11B2 stimulatory factor. The effect of NF1 was dependent on the position of CpGs and the combination of CpGs methylated. A methylated-CpG–binding protein complex 1 transcriptional repression interacted directly with the methylated HSD11B2 promoter. These results indicate a role for DNA methylation in HSD11B2 gene repression and suggest an epigenetic mechanism affecting this gene causally linked with hypertension.

Introduction

The intracellular access of glucocorticoids to their receptors is modulated by the 11β-hydroxysteroid dehydrogenase (11βHSD) enzymes, which interconvert biologically active 11β-hydroxyglucocorticoids and inactive 11-ketosteroids (1–3). The enzyme 11βHSD2 catalyzes the dehydrogenation of 11β-hydroxyglucocorticoids, has a nanomolar Kₘ for glucocorticoids, utilizes NAD⁺ as a cofactor, and is localized in the endoplasmic reticulum membrane with a cytoplasmic orientation of its catalytic domain (4, 5). The enzyme exhibits cell-specific expression in mineralocorticoid target tissues, such as epithelial cells from colon or renal cortical collecting tubules, where its main function is to protect the nonselective mineralocorticoid receptor (MR) from activation by 11β-hydroxyglucocorticoids (1, 4). Reduced activity of 11βHSD2 leads to overactivation of the MR by cortisol with renal sodium retention, hypokalemia, and a salt-sensitive increase in blood pressure (6, 7). Compromised 11βHSD2 activity can be caused by two mechanisms: first, by loss-of-function mutations of the gene encoding 11βHSD2 (HSD11B2), and second, by xeno- or endobiotics or shear stress (4). Slightly reduced activity of the 11βHSD2 enzyme is associated with a salt-sensitive increase in blood pressure and polymorphic markers in the gene in normal volunteers or in patients with essential hypertension (8, 9). Furthermore, associations between microsatellites or variants of the HSD11B2 gene and the occurrence of end-stage renal failure have been observed (10, 11). All these findings suggest that 11βHSD2 is relevant for blood pressure control. However, the frequency of mutated exons in the HSD11B2 gene is extremely low (11). Thus, other mechanisms accounting for the inter-individual variable 11βHSD2 enzyme activities have to be considered.

The mechanism of the cell-specific constitutive expression of HSD11B2 in mineralocorticoid target tissues is poorly understood. Others and we have reported that the transcription factors Sp1 and/or Sp3 (Sp1/Sp3) interact with the HSD11B2 gene promoter (12, 13). Sp1/Sp3 are ubiquitously expressed and therefore cannot sufficiently account for the distinct cell type–specific expression of HSD11B2. The HSD11B2 promoter comprises a highly G + C–rich (or GC-rich) core, contains more than 80% GC, lacks a TATA-like element, and has two typical CpG islands. This raises the possibility that CpG dinucleotide methylation may play a role in the cell type–specific and possibly in the epigenetically determined inter-individual variable expression of HSD11B2. Here we present evidence that CpG methylation regulates HSD11B2 expression in vitro and in vivo.

Results

The HSD11B2 promoter comprises CpG islands. Analysis of a 13,400-bp DNA fragment ranging from nucleotide −3,731 to nucleotide +9,669 in the human HSD11B2 gene showed two CpG islands located in the promoter and exon 1 (nucleotides −633 to −97 and −77 to +460) and two islands in exon 5 and the downstream region (nucleotides +5,659 to +5,721 and +7,357 to +7,515) (Figure 1). Analysis of rat HSD11B2 revealed one CpG Island in the promoter and exon 1 (nucleotides −358 to +763) (Figure 7C).

The HSD11B2 gene is upregulated by 5-aza-2′-deoxycytidine and procarbamide. To examine the role of DNA methylation in HSD11B2 expression, we grew cell lines expressing various levels of the HSD11B2 with and without 5-aza-2′-deoxycytidine (5-aza-CdR),

Nonstandard abbreviations used: × (50x, 100x), excess competitor; Ab., antibody against; 5-aza-CdR, 5-aza-2′-deoxycytidine; Cons., consensus sequence; EMSA, electrophoretic mobility-shift assay; GR, glucocorticoid receptor; HDAC, histone deacetylase; 11βHSD, 11β-hydroxysteroid dehydrogenase; HSD11B2, gene encoding 11βHSD2; MBD, methylated-CpG−binding domain protein; Me-, methylated; MeCP, methylated-CpG−binding protein complex; MMTV, mouse mammary tumor virus; MMTV-Luc, MMTV-luciferase; MR, mineralocorticoid receptor; NF1, nuclear factor 1; P, probe; THA, 11-dehydrocorticosterone; THB, 5α-THB, corticosterone; TK, thymidine kinase; TSA, trichostatin A; U-, unmethylated.

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: J Clin Invest. 2004;114:1146–1157 (2004). doi:10.1172/JCI200421647.
a methyltransferase inhibitor. Dose-dependent activation of the gene was observed (Figure 2A), suggesting that demethylation enhances the expression of \textit{HSD11B2}. Promoters with methylated CpG islands are hypoacetylated and repressed (14). To test whether histone hyperacetylation enhances \textit{HSD11B2} expression, we treated cells with the histone deacetylase (HDAC) inhibitor trichostatin A (TSA). TSA alone did not induce \textit{HSD11B2} gene expression in JEG-3, MCF-7, and SW620 cells (Figure 2A), but the addition of TSA for 12–24 hours after 48 hours of treatment with 5-aza-CdR increased the expression in MCF-7 and SW620 cells, an effect not observed in JEG-3 cells (Figure 2A). When carcinoma cell lines are established, certain genes are methylated de novo. Therefore, we examined the effect of 5-aza-CdR on \textit{HSD11B2} expression in primary human kidney cells and again observed a dose-dependent activation of the gene (Figure 2A). Like 5-aza-CdR, procainamide, a less effective DNA methyltransferase inhibitor (15), increased the activity and mRNA of 11βHSD2 in a dose-dependent way (Figure 2B).

\textit{Reduced glucocorticoid receptor transactivation by cortisol after 5-aza-CdR treatment.} The intracellular access of glucocorticoids to the receptor is modulated by 11βHSD2. In humans it converts cortisol into biologically inactive cortisone. To demonstrate that demethylation of the \textit{HSD11B2} gene causes a reduced enzyme activity in intact cells, we used a transactivation assay (3). For that purpose, the mouse mammary tumor virus (MMTV) promoter, which contains essential glucocorticoid response elements, was linked to a luciferase gene (MMTV-Luc) and was cotransfected with a glucocorticoid receptor (GR) expression vector and a thymidine kinase–Renilla luciferase (TK–Renilla luciferase) internal control plasmid in JEG-3 cells. Transfected cells express luciferase only when an intracellular 11β-hydroxyglucocorticoid such as cortisol, but not when an 11-ketosteroid such as cortisone, is present (3). Increased 11βHSD2 activity enhances the conversion of cortisol to cortisone, thus causing a diminished luciferase expression. Diminished transactivation of MMTV-Luc was consistently observed after 5-aza-CdR treatment (Figure 3D), indicating an enhanced 11βHSD2 activity. That the reduced transactivation upon 5-aza-CdR treatment was caused by decreased cortisol concentrations due to increased 11βHSD2 activity rather than by modulation of the GR itself was demonstrated by the fact that dexamethasone-mediated transactivation by GR was not altered by 5-aza-CdR treatment (Figure 3D). Both dexamethasone and 11-keto-dexamethasone, the product of 11βHSD2, are potent GR agonists (16).

\textit{Hypermethylation of the \textit{HSD11B2} promoter correlates with an absence of \textit{HSD11B2} expression.} Hypermethylation of normally unmethylated CpG islands correlates with transcriptional repression (17, 18). To define the methylation status of \textit{HSD11B2} CpG islands, we performed bisulfite sequencing (19). Examples of sequencing are shown in Figure 3B. The cytosines in the CpG dinucleotides remained unchanged at several positions after bisulfite treatment and therefore were methylated. In contrast, all non-CpG dinucleotide cytosines appeared after PCR as thymidines, indicating complete bisulfite modification. After 5-aza-CdR treatment, the thymidine signals (unmethylated CpGs) increased compared with the cytosine signals (methylated CpGs), indicating reduced DNA methylation (Figure 3B). Figure 3C shows the methylation pattern of the \textit{HSD11B2} CpG islands in normal human tissues and in cell lines before and after 5-aza-CdR treatment. The methylation levels were low in SW620 cells, placenta, and distal renal tubules when part 1 (nucleotides –704 to –380) and part 2 (nucleotides –403 to –76) were considered (Figure 3C). In contrast, the same regions were methylated in proximal tubules, liver, and skeletal muscle as well as in JEG-3 and MCF-7 cells. Methylation of CpGs was reduced by 5-aza-CdR in JEG-3 and MCF-7 cells (Figure 3C, parts 1 and 2), an effect that correlated with higher expression of the \textit{HSD11B2} gene (Figure 2A). In part 3 (nucleotides –102 to +215), normal tissues but not cell lines were slightly methylated, whereas in part 4 (nucleotides +5,510 to +5,784 and part 6, nucleotides +7,295 to +7,597) were fully methylated in all samples before and after 5-aza-CdR treatment. Liver, muscle, and proximal kidney tubules, with strong CpG methylation, are well known to express almost no \textit{HSD11B2}, whereas placenta and distal kidney tubules, with a lower degree of CpG methylation, express \textit{HSD11B2} (20). Thus, these data reveals a strong negative correlation between the methylation status and gene expression of the \textit{HSD11B2} promoter in tissues, especially in parts 1 and 2, an observation in line with the lower degree of methylation and higher 11βHSD2 activity in SW620 than in JEG-3 or MCF-7 cells (Figure 2).
Methylated CpG sites in the HSD11B2 promoter are targets for protein binding. Sites with high scores for potential binding for transcription factor complexes including nuclear factor 1 (NF1), AP2, SP1, Ah-Arnt, Arnt1, AP4, GC-box, and Ik2 in methylated CpG sites were identified. To prove that methylation-dependent protein-DNA interactions occur, we performed electrophoretic mobility-shift assays (EMSA) with methylated (Me-) and unmethylated (U-) probes (Ps) containing binding sites for Arnt and Ah-Arnt (nucleotides -518 to -489, including six CpGs), Sp1/Sp3 (nucleotides -287 to -268, including three CpGs), and NF1 (nucleotides -419 to -397, including three CpGs) (Table 1).

EMSA with MCF-7 cell nuclear protein extracts revealed two specific complexes with the unmethylated probe (U-PArnt) and one with the methylated probe (Me-PArnt) (Figure 4A). Unmethylated excess competitor (U-PArnt 100×) displaced both complexes, but methylated competitor (Me-PArnt 100×) displaced only the lower one, indicating methylation sensitivity of the upper complex. The lower complex comprises Ah-Arnt, because antibody against Ah-Arnt (Ab.Ah-Arnt) but not against Arnt1 (Ab.Arnt1) inhibited formation of the lower complex. EMSA with MCF-7 nuclear protein extracts using an Sp1/Sp3 probe (nucleotides -287 to -268) revealed three abundant protein-DNA complexes when the probe was unmethylated (U-PSp1), while the complexes were reduced when the methylated counterpart (Me-PSp1) was used (Figure 4B). Unmethylated probe in excess (U-PSp1 100×) competed more efficiently with U-PSp1 than did methylated probe (Me-PSp1 100×), an observation confirmed by another set of methylated (Me-PSp1-2 100×) and unmethylated (U-PSp1-2 100×) competitors and an unmethylated probe containing the Sp1/Sp3 recognition binding site (U-PSp1 100×) (Figure 4B). Supershift studies showed antibodies against Sp1, Sp3, or both together (Figure 4B, Ab.Sp1, Ab.Sp3, and Ab.Sp1/Sp3), but not the unrelated antibody (Ab.Ap4), shifted the complexes. Thus, methylation decreases Sp1/Sp3 binding.

NF1 isoforms interact with methylated-CpG binding proteins at NF1 binding sites. EMSA performed on SW620 cell nuclear protein extracts after overexpression of different murine HA-tagged NF1 isoforms with an NF1 recognition site (nucleotides -419 to -397) revealed the formation of a main complex with all four NF1 isoforms, whether fully methylated (Me-NF1) or unmethylated (U-NF1) NF1 probes were considered (Figure 5, A and B). Above and below the main complex, several additional bands restricted...
Figure 3
The effect of 5-aza-CdR on the DNA methylation pattern of HSD11B2 and glucocorticoid-mediated transactivation of MMTV-Luc. (A) Schematic representation of HSD11B2 gene. P1–P6 indicate the stretches of DNA amplified for methylation analyses and refer to parts 1–6 in C. N1-F, N1-R, N2-F, and N2-R are nested primers. (B) Example of bisulfite sequencing (nucleotides –456 to –391). The sequences before (top) and after bisulfite treatment either without (middle) or with (bottom) 5-aza-CdR treatment are presented. After bisulfite treatment, C-to-T conversion did not occur at several CG sites, indicating methylation of those CpG dinucleotides. Treatment with 5-aza-CdR increased unmethylated forms of alleles, as shown by increased T versus C signals (compare middle and bottom). (C) Methylation pattern of the HSD11B2 promoter and exonic and downstream CpG islands in various cells and tissues. The CpG number indicates the number of CpG dinucleotides along the promoter and CpG position denotes the position of this CpG along the transcription start site. The color of the circle reflects the degree of methylation (key). (D) Effect of 5-aza-CdR on glucocorticoid-mediated MMTV-Luc transactivation in JEG-3 cells. The dose-response curve of cortisol was blunted by 5-aza-CdR. Transfections were performed in triplicate; all results were confirmed by at least two different independent experiments.
to the methylated NF1 probe (Me-NF1) appeared, suggesting presence of methylated-CpG binding proteins (Figure 5, A and B). Me-NF1 was completely displaced by excess of unlabeled Me-NF1 (Me-NF1 100x) (Figure 5B), while unmethylated competitor (U-NF1 100x) caused only a partial displacement, an effect confirmed by the use of an excess of unmethylated probe containing an NF1 binding site (U-NF1-Cons. 100x) (Figure 5B). The main complex and methylation-specific complexes were shifted by antibody against HA (Figure 5E). Antibody against methylated-CpG–binding domain protein 1 (MBD1) but not against MBD2 and Arnt1 (Figure 5E). Antibody against HA again showed a supershifted band. These data indicate that the MBD1 interacts with NF1 at the NF1 binding site.

We confirmed this using assay with antibody against HA (Figure 5E). Antibody against methylated-CpG–binding domain protein 1 (MBD1) but not against MBD2 and Arnt1 (Figure 5E). Antibody against HA again showed a supershifted band. These data indicate that the MBD1 interacts with NF1 at the NF1 binding site.

To study the type of methylated-CpG binding protein involved (22), we demonstrated by supershift assays that methylation-specific complexes in the main complex shifted with antibodies against methylated-CpG–binding domain protein 1 (MBD1) but not against MBD2 and Arnt1 (Figure 5E). Antibody against HA again showed a supershifted band. These data indicate that the MBD1 interacts with NF1 at the NF1 binding site.

In vitro methylation represses the HSD11B2 promoter activity. In order to determine whether the HSD11B2 promoter activity is regulated by CpG methylation and/or NF1, we performed reporter gene assays. Promoter-luciferase constructs containing various lengths of upstream regulatory sequences between nucleotides –1,778 and –1,778 (Figure 6, A and B) were fully methylated by Sso1 methylase.

### Table 1

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence, 5′–3′</th>
<th>Nucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td>U-NF1</td>
<td>CAGGCTTGGCCGCGCGTGCTGTT</td>
<td>–419 to –397</td>
</tr>
<tr>
<td>Me-NF1(1CG)</td>
<td>CAGGCTTGGCCGCGCGTGCTGTT</td>
<td>–419 to –397</td>
</tr>
<tr>
<td>Me-NF1(2CG)</td>
<td>CAGGCTTGGCCGCGCGTGCTGTT</td>
<td>–419 to –397</td>
</tr>
<tr>
<td>Me-NF1</td>
<td>CAGGCTTGGCCGCGCGTGCTGTT</td>
<td>–419 to –397</td>
</tr>
<tr>
<td>U-NF1(Tg)</td>
<td>CAGGCTTGGCCGCGCGTGCTGTT</td>
<td>–419 to –397</td>
</tr>
<tr>
<td>Me-NF1(Tg)</td>
<td>CAGGCTTGGCCGCGCGTGCTGTT</td>
<td>–419 to –397</td>
</tr>
<tr>
<td>NF1-Con.</td>
<td>TCCAAAGCGACCGCGCCACAGCGGC</td>
<td>–287 to –268</td>
</tr>
<tr>
<td>U-Psp1</td>
<td>GTTGGTGGGCGCGCGGGA</td>
<td>–287 to –268</td>
</tr>
<tr>
<td>Me-Psp1</td>
<td>GTTGGTGGGCGCGCGGGA</td>
<td>–287 to –268</td>
</tr>
<tr>
<td>U-Psp1-2</td>
<td>ACAGGCGGCGGCGCCAGCGCG</td>
<td>–491 to –467</td>
</tr>
<tr>
<td>Me-Psp1-2</td>
<td>ACCAGGCGGCGGCGCCAGCGCG</td>
<td>–491 to –467</td>
</tr>
<tr>
<td>U-Sp1</td>
<td>GATCGAAGTACGACCGCGCGCGC</td>
<td>–518 to –489</td>
</tr>
<tr>
<td>U-PRam</td>
<td>CGAGACGCTTGGAGTTGCGGCGCGTGACG</td>
<td>–518 to –489</td>
</tr>
<tr>
<td>Me-PRam</td>
<td>CGGAGACGCTTGGAGTTGCGGCGCGTGACG</td>
<td>–518 to –489</td>
</tr>
<tr>
<td>NR-F</td>
<td>CATTGTAGCTGCAATTGCCTGCTGCG</td>
<td>–718 to –688</td>
</tr>
<tr>
<td>NR-R</td>
<td>CCGTGGAGCTATTCTCTCTACCTCCC</td>
<td>+599 to +629</td>
</tr>
<tr>
<td>N1-F</td>
<td>TTGTGGAGCAAGATGTGTGCTG</td>
<td>–715 to –690</td>
</tr>
<tr>
<td>N1-R</td>
<td>CCTCCTTACCTTTCTCCTCCTCCC</td>
<td>–97 to –76</td>
</tr>
<tr>
<td>N2-F</td>
<td>GGGGGAAGAGTAAGAAGAAC</td>
<td>–97 to –76</td>
</tr>
<tr>
<td>N2-R</td>
<td>CTCATTTTCCTCCACTCCCTGCC</td>
<td>+599 to +623</td>
</tr>
<tr>
<td>P1-F</td>
<td>GGAATTGGGTGGTGTGGAATTTATGTATGGTTATG</td>
<td>–704 to –672</td>
</tr>
<tr>
<td>P1-R</td>
<td>ATACCTTCTTACACTCTACCC</td>
<td>–403 to –380</td>
</tr>
<tr>
<td>P2-F</td>
<td>GTTGTGGYTAGTTAAGAGTAT</td>
<td>–403 to –380</td>
</tr>
<tr>
<td>P2-R</td>
<td>ACTTCTTCTTCTCTCTCTCACCC</td>
<td>–103 to –76</td>
</tr>
<tr>
<td>P3-F</td>
<td>GTTGGGAGGAAGATGGAAAGGATT</td>
<td>–102 to –75</td>
</tr>
<tr>
<td>P3-R</td>
<td>CTTTCAaacctataaccactaacaaca</td>
<td>+188 to +215</td>
</tr>
<tr>
<td>P4-F</td>
<td>GTTGTGGATGTGTGGTTTATAAT</td>
<td>+187 to +213</td>
</tr>
<tr>
<td>P4-R</td>
<td>CAATGGAATACATCTAATAATACC</td>
<td>+448 to +476</td>
</tr>
<tr>
<td>P5-F</td>
<td>AATTGTGTGTGTGTTAATTTTGATAG</td>
<td>+5,510 to +5,538</td>
</tr>
<tr>
<td>P5-R</td>
<td>CAAACATATACAAATAAAAAACTCACAA</td>
<td>+5,755 to +5,784</td>
</tr>
<tr>
<td>P6-F</td>
<td>GTATATGGGATGTGTTTTTGATGTT</td>
<td>+7,295 to +7,322</td>
</tr>
<tr>
<td>P6-R</td>
<td>ACCACATCTACTTCATTCTCCACACCA</td>
<td>+7,573 to +7,597</td>
</tr>
</tbody>
</table>

CmG, cytosine methylation.

with Me-NF1, Me-NF1(1CG) increased but Me-NF1(2CG) slightly decreased affinity for the complexes. Competition experiments revealed that Me-NF1(1CG) and Me-NF1(2CG) were highly displaced from the main but less so from the methylation-specific complexes by an excess of U-NF1 100x, while Me-NF1(1CG) 100x, Me-NF1(2CG) 100x, and Me-NF1 100x completely displaced the Me-NF1(1CG) and Me-NF1(2CG) probes (Figure 5C). Unmethylated and methylated cold probes competed better with the Me-NF1(2CG) probe than with the Me-NF1(1CG) probe, confirming that Me-NF1(1CG) has a higher affinity for the NF1 main complex.

All NF1 proteins bind as a dimer to DNA in the major groove and recognize a dyad symmetric consensus sequence (Cons.) TTTGGC(N)5GCCAA which similarity with individual “half-sites” (TTGGC or GCCAA) (21). The core binding site of NF1 (TTGGC) contains a cytosine at the 3′ end, with the potential to be the cytosine of another CpG. In the HSD11B2 gene promoter we identified both configurations: the C followed by a G or the C followed by another nucleotide. To investigate the relevance of these two possible configurations with respect to the methylation status by EMSA, we used two mutated NF1 probes, U-NF1(Tg) and Me-NF1(Tg), and SW620 nuclear extracts overexpressing NF1-X2 (Table 1). The U-NF1(Tg) and U-NF1 probes produced an identical pattern of shifted bands (Figure 5D). NF1-specific binding was confirmed by competition assays using cold U-NF1 100x and U-NF1(Tg) 100x probes and by supershift assays with an antibody against HA (Figure 5D). Interestingly, when methylation occurred at CpGs outside of the core NF1 binding site of the mutated probe Me-NF1(Tg), the NF1 main complex and the methylation-specific complexes decreased when compared with the complexes obtained when unmethylated [U-NF1 and U-NF1(Tg)] and methylated (Me-NF1) probes were used (Figure 5D). The specific binding of the complexes was confirmed using an excess of U-NF1 100x, U-NF1(Tg) 100x, Me-NF1(Tg) 100x, or Me-NF1 100x (Figure 5D). These data demonstrate the pivotal role of the core neighboring nucleotide for DNA-NF1 interactions. This conclusion was confirmed by the use of end-labeled Me-NF1 probe (Figure 5E).

To study the type of methylated-CpG binding protein involved (22), we demonstrated by supershift assays that methylation-specific complexes and parts of the main complex shifted with antibodies against methylated-CpG–binding domain protein 1 (MBD1) but not against MBD2 and Arnt1 (Figure 5E). Antibody against HA again showed a supershifted band. These data indicate that the MBD1 interacts with NF1 at the NF1 binding site.

In vitro methylation represses the HSD11B2 promoter activity. In order to determine whether the HSD11B2 promoter activity is regulated by CpG methylation and/or NF1, we performed reporter gene assays. Promoter-luciferase constructs containing various lengths of upstream regulatory sequences between nucleotides –1,778 and –1,778 (Figure 6, A and B) were fully methylated by Sso1 methylase.
and were subsequently transfected with and without an NF1-A1.1 expression vector into SW620 cells. The construct containing nucleotides –210 to +117 produced strong luciferase activity (Figure 6, A and B), whereas the construct containing nucleotides –400 to +117 showed consistently less activity, suggesting presence of a repressor/silencer between nucleotides –400 and –210 (12). This repression was overcome by the construct containing nucleotides –720 to +117, indicating the presence of an activator/enhancer between nucleotides –720 and –400. Methylation of the constructs completely suppressed their activity (Figure 6A). The major activity of the HSD11B2 promoter appears to be within sequences between nucleotides –720 and +117 (Figure 6, A and B). To assess the effect of methylation on the promoter alone, we separately methylated the promoter fragment (nucleotides –720 to +117), religated this into the unmethylated plasmid, and transfected cells with this construct. The methylation decreased the relative luciferase activity from 50 to less than 3, a decrease comparable to the values in those without procainamide treatment (Figure 7B). Similarly, in animals treated with procainamide, the urinary 11β-HSD2 activity was higher in kidneys from rats treated with procainamide than in those without procainamide treatment (Figure 7B). Similarly, in 10 rats treated with 5-aza-cytidine (10 mg/kg/day i.p.), the urinary THB + 5α-THB) / THA ratio declined in all animals (mean ± SD: 2 ± 0.4 versus 1.2 ± 0.2, day 0 versus day 3). No further analyses beyond day 3 could be performed because the animals appeared sick after day 4 on 5-aza-cytidine. A third demethylating agent, 5-aza-CdR, given i.p. (1 mg/kg/day) for 3–7 days also caused a steady decline in the (THB + 5α-THB) / THA ratio of the same magnitude as that observed for procainamide, whereas no changes were observed in control rats given vehicle only (results not shown). For methylation-sensitive restriction enzyme analyses, genomic DNA from different tissues of control and 5-aza-CdR–treated rats was extracted and subjected to digestion with methylation-sensitive restriction enzymes. The resulting fragments were separated by electrophoresis and visualized by ethidium bromide staining.

**Figure 4**

Binding of nuclear proteins on methylated or unmethylated putative Amt- or Sp1/Sp3-binding sites in the HSD11B2 gene. (A and B) The positions of specific complexes (Specific, Sp1/Sp3, and Sp3) or nonspecific bands (N.S.) are indicated along the left margins. Supershifts (S.S.) were performed with specific antibodies (Ab.Amt, Ab.Ac-Amt, Ab.Sp1, or Ab.Sp3), combinations (Ab.Sp1/Sp3), or anti-Ap4 as a nonspecific control (Ab.Ap4). For competition studies, a 100-fold (100×) molar excess of cold methylated or unmethylated probe was used.
were digested with EcoRI and either HpaII, a methylation-sensitive enzyme, or MspI, a methylation-insensitive enzyme, and was probed in Southern blots with an 805-bp fragment derived from the rat HSD11B2 promoter (nucleotides –1,097 to –292). DNA from kidney digested with EcoRI alone produced the anticipated 3,350-bp fragment (Figure 7D, lane 1). In both control rats and rats treated for 7 days with 5-aza-CdR, digestion of genomic DNA with the methylation-insensitive enzyme MspI together with EcoRI yielded the predicted 1,053-bp band in all three tissues analyzed (Figure 7D). When instead of MspI the methylation-sensitive HpaII was used, a different pattern of bands was observed (about 1,791 bp, 1,539 bp, and 1,053 bp). In rats not given 5-aza-CdR, digestion of DNA from kidney with EcoRI and HpaII revealed a more intense band at 1,053 bp than did digestion of DNA from lung and liver (Figure 7D). This observation is in line with results derived from bisulfite sequencing (Figure 3C) and indicates that tissues not expressing HSD11B2 (lung and liver) exhibit a higher level of DNA methylation on the HSD11B2 promoter than does the HSD11B2-expressing kidney. Treatment with 5-aza-CdR and digestion with EcoRI and HpaII resulted in an increase in the intensity of the

Figure 5
EMSA with differentially methylated or unmethylated NF1 probes. (A and B) EMSA with fully methylated (Me-NF1) or unmethylated (U-NF1) NF1 probes of the human HSD11B2 promoter was performed on SW620 cell nuclear protein extracts after overexpression of HA-tagged murine NF1 isoforms NF1-A1.1, NF1-B2, NF1-C2, or NF1-X2 (A) and NF1-C2 or NF1-X2 (B). The position of methylation-specific complexes (Meth. Sp. compl.) and NF1-specific complexes (NF1 compl.) or nonspecific bands (N.S.) are indicated along the left margins. Supershifts were performed with HA-specific antibodies (Ab.HA). For competition studies, a 100-fold (×100) molar excess of a cold methylated (Me-), unmethylated (U-) or a consensus unmethylated (NF1-Cons. 100×) NF1 probe was used. The sequences and methylation sites of the probes are given in Table 1. (C–E) EMSA with site-specific methylated probes. EMSA was performed on SW620 cell nuclear protein extracts after overexpression of the HA-tagged murine NF1-X2 isoform. Different DNA probes [Me-NF1(1CG), Me-NF1(2CG), Me-NF1, and U-NF1], including mutated probes [U-NF1(Tg) or Me-NF1(Tg)] and their corresponding cold competitors (×100), were used (see Table 1). Supershifts were performed with specific (Ab.HA, Ab.MBD1) or unrelated (Ab.MBD2 and Ab.Arnt) antibodies. Whereas C demonstrates that the position of CpG methylation and D, that the composition of core neighboring nucleotides affect the binding of NF1, E suggests that MBD1 interacts with NF1 at the NF1 binding site. Sp. compl.; specific complexes.

1152 The Journal of Clinical Investigation http://www.jci.org Volume 114 Number 8 October 2004
1,053-bp band compared with the intensity of the band from DNA of the same organ from untreated rats (Figure 7D, kidney, lung, and liver) and a slight decrease in the corresponding 1,791-bp band (Figure 7D, lung and liver). These changes induced by 5-aza-CdR are compatible with a decline in \(HSD11B2\) promoter DNA methylation and confirm the effect in cell lines assessed by bisulfite sequencing (Figure 3C). The decreased level of promoter methylation causing a higher expression of the \(HSD11B2\) gene was further evidenced by Northern blotting (Figure 7E).

**Discussion**

The \(HSD11B2\) gene promoter resembles the promoters of housekeeping genes, characterized by a GC-rich region, multiple GC boxes, and a lack of TATA or CCAAT sequences with CpG Islands along the gene (Figure 1). Thus, the cell type–specific expression of \(HSD11B2\) is rather surprising, because housekeeping genes are usually ubiquitously expressed. The region between nucleotides -213 and +156, which contains several Sp1/Sp3 binding sites, is critical for basal promoter activity of 1\(\beta\)HSD2 (12, 13). Sp1/Sp3 are ubiquitously expressed. Therefore, the mechanism accounting for the cell type–specific expression of the \(HSD11B2\) gene is not completely understood. The presence of CpG islands suggested that the \(HSD11B2\) gene might be regulated at least in part through CpG methylation. In general, hypermethylation of normally unmethylated CpG islands correlates with transcriptional repression. Here we have provided evidence that \(HSD11B2\) expression and activity are inversely correlated with the presence of methylation at the promoter. In support of the idea of methylation suppression, DNA methyltransferase inhibitors enhanced transcription and activity of 1\(\beta\)HSD2 in different cell types. Moreover, these inhibitors increased mRNA abundance in various tissues and decreased the urinary glucocorticoid metabolite ratios in rats, indicating higher 1\(\beta\)HSD2 activity. Furthermore, the relevance of DNA methylation for the tissue-specific expression and regulation of the \(HSD11B2\) gene was demonstrated by methylation-sensitive restriction enzyme analyses followed by Southern blotting using genomic DNA from different tissues of control and 5-aza-CdR–treated rats.

The present investigation supports the notion that DNA methylation is dominant to histone acetylation, since treatment with an HDAC inhibitor increased the expression of \(HSD11B2\) only after removal of the cytosine methylation in most of the cells analyzed (Figure 2) (14). In JEG-3 cells, TSA did not increase \(HSD11B2\) expression, suggesting that chromatin modification in the \(HSD11B2\) promoter may vary between cell types. The mechanisms discussed previously that lead to the differential effect of
research article

TSA include a cooperative inhibition of histone deacetylation and DNA methylation linked through the DNA methyl-binding proteins and/or an effect on other transcription factors (25, 26). An inverse correlation between promoter methylation and HSD11B2 expression is apparent when JEG-3, MCF-7, SW620, placenta, skeletal muscles, liver, lung, and renal proximal and distal tubules are considered as a group (20). DNA methylation in the upstream region was decreased by 5-aza-CdR, an effect associated with an enhanced expression of HSD11B2. In contrast, the exonic and downstream CpG islands were fully methylated in all normal tissues and cell lines before and after treatment with 5-aza-CdR (Figure 3C). The active expression of HSD11B2 despite the presence of fully methylated exonic and downstream CpG islands is in agreement with observations about other genes (17).

One mechanism by which promoter methylation affects expression of the corresponding gene is to modulate the binding of transcription factors, as shown, for instance, for Myc/Myn or AP2 (27, 28). Because the HSD11B2 promoter contains several Sp1/Sp3 binding sites that are methylated at core and neighboring CpGs, we investigated the effect of methylation on Sp1/Sp3 binding. In agreement with a study about the p21(10g) gene (29), the fully methylated Sp1/Sp3 probe with methylated-CpG dinucleotides at the core and outside of the consensus Sp1/Sp3 element reduced Sp1/Sp3 binding (Figure 4B).

Similarly, methylation of the CpG dinucleotides at Ah-Arnt/Arnt binding sites in the erythropoietin gene (30), the fully methylated Ah-Arnt/Arnt probe (Figure 4B). Similarly, methylation of the CpG islands at Ah-Arnt/Arnt binding sites in the HSD11B2 promoter appears to be relevant. In line with observations about the erythropoietin gene (30), the fully methylated Ah-Arnt/Arnt probe formed only one of two specific complexes (Figure 4A).

Because we found here that NF1 upregulates HSD11B2 (Figure 6B) and several methylated CpGs are present within or immediately around the NF1 binding sites, we explored the relevance of CpG methylation to NF1 binding. Previously the effect of methylation on NF1 binding was investigated by Ben-Hattar et al. (31), who showed that methylation-specific cytosine residues within or immediately around the CCAAT-box binding transcription factor (CTF) binding site of herpes simplex virus TK promoter had no effect on the affinity of CTF (28, 31), but the efficiency of transcription was reduced at least 50-fold compared with the efficiency when the unmethylated promoter was used. It is conceivable that methylated cytosines in a different configuration in the consensus NF1 binding site might modulate NF1 binding. All NF1 proteins bind as a dimer to DNA in the major groove and recognize a dyad symmetric consensus sequence [TTGGC(N)5GCAA] on duplex DNA. NF1 binds specifically to individual half-sites (TTGGC or GCAA) (21). Previous investigations focusing on the effect of the NF1 half-palindrome and mutations in the spacer region between the two palindromes revealed that using only an NF1 half-palindrome decreased the affinity of the NF1 and mutations of the first two nucleotides in the spacer modulated the NF1 binding (32, 33).

The core binding site of NF1 (TTGGC) contains a cytosine at the 3' end, which has the potential to be a part of a CpG dinucleotide. Our investigation demonstrates for the first time, to our knowledge, that probes with fully methylated or “positionally methylated” CpGs either at the core and/or adjacent to the core increase the main NF1 complex and recruit additional methylation-specific complexes compared with unmethylated probe (Figure 5), whereas methylation of adjacent CpGs considerably decreases binding of the NF1 main complex and methylation-specific complexes when the core CpG is mutated concomitantly (Figure 5D). These observations indicate that both the position of CpGs and the combination of CpGs methylated determine NF1 binding.

NF1 and MBD1 coexist in the main and methylation-specific NF1 complexes, because either NF1 or MBD1 antibody shifted

Figure 7

Effect of procainamide and 5-aza-CdR on HSD11B2 in rats. (A and B) After treatment with procainamide (PA), the urinary (THB + 5c-THB) / THA ratios declined in all animals (A), indicating increased activity of 11βHSD2. This observation is in line with the elevated mRNA levels in kidney tissue of rats given procainamide (B). The y axis indicates the difference between the cycle threshold values (dCT) of HSD11B2 mRNA and ribosomal RNA as internal control. A low value indicates high content of HSD11B2 mRNA. (C) Schematic representation of rat HSD11B2 CpG islands spanning from nucleotide −358 to nucleotide +763 with respect to the transcription initiation site (arrow at +1). A fragment spanning nucleotides −1,097 to −292 was used for Southern blot analysis. Horizontal bars with numbers (below diagram) indicate the expected size of the hybridized fragments. (D) Methylation-sensitive restriction enzyme analyses. Genomic DNA from different tissues of control rats (−) and 5-aza-CdR-treated rats (+) was double-digested with EcoRI and either HpaII or MspI and was fractionated by agarose gel electrophoresis followed by Southern blotting. For interpretation, see the Results section. (E) Northern blot analyses without and with 5-aza-CdR treatment. An XhoI fragment of HSD11B2 was used as a probe. Expression of HSD11B2 increased after administration of 5-aza-CdR in the three tissues analyzed.
the complexes (Figure 5E). Therefore, we speculate that MBD1 interacts with NF1 to stabilize its binding to the methylated DNA sequence and mediates transcriptional repression. MBD1 (formerly PCML) is the least characterized of the methylated-CpG binding proteins. MBD1 comprises an N-terminal MBD that binds to methylated DNA and a C-terminal transcriptional repression domain associated with HDAC (34). Earlier studies demonstrated that MBD1 partners with the p150 subunit of chromatin assembly factor 1 to form a multiprotein complex that also contains HP1a, suggesting a role for MBD1 in methylation-mediated transcriptional repression and inheritance of epigenetically determined chromatin states (34). A role for NF1 in chromatin remodeling, in addition to its directly enhancing transcriptional effect, has been reported by Hebbel et al. (35). NF1 interacts with a variety of coactivator or cosuppressor proteins in a cell-type- and/or promoter-specific manner (21). Our observations indicate for the first time, to our knowledge, that NF1 interacts with MBD1.

Direct binding of a specific transcriptional repressor complex is the second major mechanism of transcriptional repression of genes exhibiting methylated CpGs (22, 27). A conserved family of proteins that bind to methylated CpGs with little apparent specificity for flanking nucleotide sequences has been identified (22). MeCP1 was the first complex identified that discriminates between methylated and unmethylated DNA and represses transcription through preferential binding to, remodeling of, and deacetylation of methylated nucleosomes (23, 36). Here we have presented evidence that a similar complex binds to the methylated HSD11B2 promoter. Blocking MBD2 with anti-MBD2 shifted the MeCP1-like complex (Figures 6, C–E), supporting a previous report that MBD2 is a component of the MeCP1 and/or related complexes (24). A definite role for transcriptional repressors such as MeCP1 and MBD1 should be established by studies with the corresponding knockout animals in the future.

In vitro methylation of promoter-luciferase constructs considerably reduced the promoter activity after transfection. Reporter assays provided evidence for enhancer elements between nucleotides –720 and –400. The higher methylated CpGs in part 1 (nucleotides –704 to –380) and its effect on HSD11B2 expression is consistent with this notion. Procarcinamide increased 11βHSD2 activity and mRNA levels in cell lines and in vivo. Given the known effect of procarcinamide (15), it is likely that this effect is attributable to decreased DNA methylation. The increased mRNA abundance and activity of HSD11B2 observed within 5 days of procarcinamide dosing in vivo might be unexpected, as the best investigated inhibitor of DNA methyltransferase, 5-aza-CdR, acts mainly in the S phase of the cell cycle and the proliferation rate of the renal tubular cells is generally considered to be low. However, the mechanism of the demethylating effect of the procarcinamide is not well defined and is possibly not confined to the S phase. Furthermore, quantitative data of the proliferation rate of cortical collecting ducts without and with procarcinamide are not available. The enhancing effect of procarcinamide on the expression of HSD11B2 in vivo might also be the consequence of activation of the gene in other dividing cells. Of interest, evidence is growing that expression of HSD11B2 exhibits a “pro-proliferative” effect on its own (37).

Our investigation has demonstrated evidence of an epigenetic mechanism, providing a tentative explanation for the highly cell-specific expression of 11βHSD2. Potentially more importantly, changes in methylation patterns might explain the inter-individual differences in HSD11B2 expression in mineralocorticoid target tissues and by that mechanism modulate blood pressure. The HSD11B2 gene was induced by three structurally different DNA methyltransferase inhibitors. The magnitude of these changes appears to be clinically important, a contention supported by the observation that moderate changes in 11βHSD2 activity affected blood pressure significantly in normal volunteers (38). DNA methylation of many genes changes with age, disease states, and environmental signals, including diet (18). Thus, we hypothesize that epigenetic modifications of genes with pivotal relevance for blood pressure control, as shown here for HSD11B2, are linked to the development of hypertension. Future studies in humans and/or knockout mice with distinct deficiencies in compounds relevant to CpG methylation must be performed to define the ultimate relevance of this epigenetic mechanism to blood pressure.

Methods

Supplies. Supplies were obtained from the following companies: chemicals and radiochemicals, from Sigma, Roche Diagnostics, Fruka, Merck, or Amersham Pharmacia Biotech; oligonucleotides, from Microsynth AG; Taq polymerase, from Qiagen; enzymes and vectors, from Roche Diagnostics, Gibco BRL, New England Biolabs, or Promega; and TLC plates, from Macherey-Nagel.

Human cell line and tissues. SW620, a colon carcinoma cell line, and MCF-7, a breast adenocarcinoma cell line, were grown in DMEM (13), and JEG-3, a placenta carcinoma cell line, in MEM (39). Human tissues were provided by the Department of Surgery (University Hospital of Berne, Switzerland). Informed consent was obtained from the patients. Renal proximal and distal tubular cells were isolated as described (40).

Animal studies. The committee on animal research (University Hospital of Berne, Switzerland) approved the protocol. Wistar rats weighing 190–210 g were kept in metabolic cages under controlled conditions for 5 days for adaptation. Thereafter, animals were fed powdered normal chow diet or with or without the addition of a watery solution of procarcinamide (250 mg/day/rat) or were fed powdered normal chow diet and injected daily with 5-aza-CdR (1 mg/kg in 0.4 ml of PBS) or PBS alone (0.4 ml) i.p. for 7 days. Urine was collected over 24 hours from 2 days before until 5–7 days after treatment. At the end animals were sacrificed and organs were removed and kept at –80°C.

Real-time PCR and Northern blotting. RNA extraction and TaqMan assays were performed (13) using a forward primer of positions 802–821, a reverse primer of positions 850–869, and a probe of positions 823–847 for human HSD11B2 mRNA, and a forward primer of positions 1,269–1,288, a reverse primer of positions 1,336–1,313, and a probe of positions 1,290–1,311 for rat HSD11B2 mRNA. Total cellular RNA (20 μg) was separated by electrophoresis through a 1.2% formaldehyde agarose gel for Northern blotting and was transferred to a nylon membrane. A 529-bp Xhol fragment of 11βHSD2 cDNA was used for hybridization, with GAPDH cDNA as control.

Assay of 11βHSD2 activity. The conversion of cortisol to cortisone was assessed (39). The assay was repeated up to four times using different protein concentrations.

Nuclear transactivation assay. To demonstrate that reduced CpG methylation enhances the 11βHSD2 enzyme activity in whole cells, thereby reducing the intracellular availability of cortisol to the receptor, we performed nuclear transactivation assays (3). JEG-3 cells were plated onto 24-well plates and 12–18 hours thereafter were transfected using Fugene-6. Each well contained MMTV-Luc (0.8 μg), TK–Renilla luciferase internal control (0.11 μg), and rat GR vector (0.11 μg). At 16 hours after transfection, cells were treated with 5-aza-CdR (1.5 μM) or PBS for 48 hours, were washed with PBS, and were incubated for 24 hours in char-
To treat cells with 5-aza-CdR, TSA, and procainamide, the cells were split at low density (15). Various concentrations of the drugs were added for 72 hours, and the medium was changed every 24 hours. TSA was added the last day for an additional 12–24 hours. On day 4, mRNA and enzyme activity were determined.

**In vivo methylation of reporter plasmids.** SstI methylase was used for the methylation of HSD11B2 promoter–luciferase constructs in pGL3-Basic. The constructs consisted of various lengths of the promoter and 117 bp of exon 1 (12). The reporter plasmid p–720/+117 was obtained by PCR cloning using the NR-F and NR-R primers (Table 1). All fragments had at their 3’ end an Nco I site corresponding to the translation initiation codon. The efficiency of methylation was determined with the methylation-sensitive restriction enzyme HpaII. Region-specific methylation was carried out on the promoter fragment of p–720/+117 after excision and isolation of the fragment (30, 41). In each case, half of the DNA was methylated with SssI methylase and the other half was incubated with methylase in the absence of 5-adenosylmethionine as a mock methylation. Methylated and mock-methylated fragments were reaggregated into the vector from which they had been excised.

**Transfections and reporter gene assays.** These techniques have been described previously (13). The normalized values of β-galactosidase activity in the Dual-Light system (Tropix) in triplicate samples varied by less than 5%. Plasmid DNA was prepared using the Qiagen columns (Qiagen) following the manufacturer’s instructions.

**Bisulfite sequencing.** Bisulfite genomic sequencing allows discrimination of 5-methyl-cytosine versus cytosine (19). Genomic DNA was isolated as described (19). Briefly, 2.5 mg of DNA cut with EcoRI was denatured for 10 minutes at 37 °C with 4.5 ml of 3 M NaOH. Then, 750 ml of sodium bisulfite (40.5%) and 42 ml of hydroxyquinone (10 mM) were added. The reaction was allowed to proceed at 55 °C for 5 hours with the following program: 95 °C for 1 minute for the first cycle, followed by 94°C for 30 seconds and 55°C for 59 minutes and 30 seconds (repeated five times). After the addition of 160 ml of 100% ethanol, DNA was purified with the DNeasy tissue kit (Qiagen). Desulfonation was performed for 20 minutes after samples were washed on a column with 50 ml of 10 mM Tris-HCl (pH 8.0). Modified DNA was amplified by nested PCR with the HotStarTagTM PCR kit from QIAGEN using two sets of forward and reverse primers, which were designed for an area from the sequence of strand-specific primers containing the modified cytosine bases. The primers used for the methylation analysis were described previously (13). The normalized values of β-galactosidase activity in the Dual-Light system (Tropix) in triplicate samples varied by less than 5%. Plasmid DNA was prepared using the Qiagen columns (Qiagen) following the manufacturer’s instructions.

**In vitro methylation of reporter plasmids.** SstI methylase was used for the methylation of HSD11B2 promoter–luciferase constructs in pGL3-Basic. The constructs consisted of various lengths of the promoter and 117 bp of exon 1 (12). The reporter plasmid p–720/+117 was obtained by PCR cloning using the NR-F and NR-R primers (Table 1). All fragments had at their 3’ end an Nco I site corresponding to the translation initiation codon. The efficiency of methylation was determined with the methylation-sensitive restriction enzyme HpaII. Region-specific methylation was carried out on the promoter fragment of p–720/+117 after excision and isolation of the fragment (30, 41). In each case, half of the DNA was methylated with SssI methylase and the other half was incubated with methylase in the absence of 5-adenosylmethionine as a mock methylation. Methylated and mock-methylated fragments were reaggregated into the vector from which they had been excised.

**Transfections and reporter gene assays.** These techniques have been described previously (13). The normalized values of β-galactosidase activity in the Dual-Light system (Tropix) in triplicate samples varied by less than 5%. Plasmid DNA was prepared using the Qiagen columns (Qiagen) following the manufacturer’s instructions.

**Bisulfite sequencing.** Bisulfite genomic sequencing allows discrimination of 5-methyl-cytosine versus cytosine (19). Genomic DNA was isolated as described (19). Briefly, 2.5 mg of DNA cut with EcoRI was denatured for 10 minutes at 37 °C with 4.5 ml of 3 M NaOH. Then, 750 ml of sodium bisulfite (40.5%) and 42 ml of hydroxyquinone (10 mM) were added. The reaction was allowed to proceed at 55 °C for 5 hours with the following program: 95 °C for 1 minute for the first cycle, followed by 94°C for 30 seconds and 55°C for 59 minutes and 30 seconds (repeated five times).

After the addition of 160 ml of 100% ethanol, DNA was purified with the DNeasy tissue kit columns. Desulfonation was performed for 20 minutes after samples were washed on a column with 500 ml of 0.3 M NaOH/80% ethanol. After being washed, the modified DNA was eluted from the column with 50 ml of 10 mM Tris-HCl (pH 8.0). Modified DNA was amplified by nested PCR with the HotStarTagTM PCR kit from QIAGEN using two sets of forward and reverse primers, which were designed for an area without or with a single CpG only (N1-F, N1-R, N2-F, and N2-R, Table 1). The sequences of strand-specific primers containing the modified cytosine bases are summarized in Table 1. The PCR fragments amplified were gel-purified and were directly sequenced or were cloned using the pCR4-TopoTA Cloning kit (Invitrogen Corp.). Eight clones were sequenced to gel-purified and were directly sequenced or were cloned using the pCR4-TopoTA Cloning kit (Invitrogen Corp.). Eight clones were sequenced to