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DNA methylation and Sp1 binding determine the tissue-specific transcriptional activity of the mouse *Abcc6* promoter

Vanessa Douet, **Matthew B. Heller**, and **Olivier Le Saux**
Department of Cell and Molecular Biology, John A. Burns School of Medicine, University of Hawaii, Honolulu, Hawaii, 96813

Summary

The gene encoding the ABCC6 protein, an ABC transporter of the multidrug resistance-associated protein (MRP), is mainly expressed in liver and kidney. Mutations in ABCC6 are responsible for the development of the pseudoxanthoma elasticum (PXE) phenotype. PXE is a recessive disease characterized by the calcification of elastic fibers resulting in dermal, vascular and ocular clinical manifestations. The physiological function of ABCC6 and the rodent orthologs Abcc6 is unknown and their precise relationship to elastic fibers is only a matter of speculation. Despite several studies focused on the transcriptional regulation of *ABCC6/Abcc6*, the molecular signals conferring the tissue-specificity to the *ABCC6/Abcc6* expression are not well defined. In this report, we determined the level of the mouse *Abcc6* promoter methylation in tissues with low level of expression (tail extremity and skin), intermediate (kidney) and high level of expression (liver). We observed that high and moderate levels of methylation correlated with low levels of *Abcc6* expression. Moreover, we determined that CpG methylation of the *Abcc6* proximal promoter region was interfering with the binding of the Sp1 transcription factor thereby inhibiting Sp1-dependent transactivation. Thus, our data provides the first direct evidence that an epigenetic mechanism regulates the binding of the transcription factor Sp1 to the proximal promoter and participates in the tissue-specific expression control of the mouse *Abcc6* gene.

Introduction

ABCC6 encodes an ATP-binding cassette (ABC) transporter of the sub-family C previously referred to as multidrug-associated resistance proteins 6 or “MRP6” [1]. *ABCC6* expression is primarily found in liver and kidneys but can also be detected in other cell types albeit at lower levels [2–4]. The ABCC6 protein is a transmembrane protein located in the basolateral side of polarized cells and exports metabolite(s) of unknown nature [2,3]. However, vesicular studies have demonstrated that leukotriene-C4 and N-ethylmaleimide-S-glutathione can be actively transported by the human ABCC6 while the BQ123 cyclic peptide is only efficiently transported by the rat Abcc6 [5]. The overall function of ABCC6 is unclear but relates in some ways to elastic fibers maintenance or integrity. Indeed, mutations in the *ABCC6* gene were associated with the development of pseudoxanthoma elasticum (PXE, OMIM #264800, 177850) [6–9]. PXE is a heritable disorder characterized by mineralization of elastic fibers and other connective tissue alterations [10,11]. The genetics of PXE are now well defined, however, the pathomechanism that links the deficiency of ABCC6 activity to calcification of elastic

Address for correspondence: Dr. Olivier Le Saux, University of Hawaii, John A. Burns School of Medicine, Department of Cell and Molecular Biology. 651 Ilalo Street, BSB 222, Honolulu, HI 96813, Tel. 808 692-1504. Fax 808 692-1970. e-mail: lesaux@hawaii.edu.

fibers remains to be elucidated. After the identification of the causative gene (*ABCC6*), it became evident that PXE was not a connective tissue disease *stricto sensu* but rather a metabolic disorder [12,13] and several recent studies provided support to this notion [14–16]. Because the tissue distribution of ABCC6 was a major argument in suggesting PXE as a metabolic disease, there has been much interest in determining the transcriptional regulation signals governing the tissue-specificity of *ABCC6* expression. The regulation of gene expression of both human and mouse *ABCC6/Abcc6* was found to depend in part on the binding of the Sp1 transcription factor to the proximal promoter *in vivo* and *in vitro* [17,18]. In addition, the *ABCC6* proximal promoter was also described as transcriptionally dependent on DNA-methylation in cell lines [19].

In this report, we describe evidences of an association between the DNA methylation status of the mouse *Abcc6* proximal promoter and Sp1 binding in tissues with high, intermediate and low level of expression. We notably found that the methylation of the mouse *Abcc6* proximal promoter inversely correlates with Sp1 binding and transcriptional activation and that DNA methylation repressed the mouse *Abcc6* transcriptional activity in liver and kidney cell lines. This work provides the first direct evidence that concerted effects of genetic and epigenetic factors plays an essential role in the tissue-specific expression of the mouse *Abcc6* gene.

Materials and methods

Cell culture and transient transfection

TIB-73 cells from the American Type Culture Collection (ATCC, Manassas, VA) were maintained in Dulbecco's modified Eagle medium (DMEM), complemented with 10% (v/v) fetal bovine serum. Transient transfections were performed using the GeneJammer transfection reagent (Stratagene, La Jolla, CA) as described by the manufacturer. Cells were co-transfected with the specific *Abcc6* promoter::*firefly luciferase* reporter construct and the *renilla* luciferase plasmid (pRL-SV40, Promega, Madison, WI) as a control of transfection efficiency.

In vivo methylation promoter studies

Tissues collected from 10 month-old mice were rapidly frozen and stored at −80°C before experiments. Genomic DNA was extracted from tissues using the Qiagen DNeasy kit (Qiagen, Valencia, CA). Methylated DNA was isolated using the Promoter Methylation PCR kit according to the manufacturer's instructions (Panomics, Redwood city, CA). The methylated genomic DNA was analyzed by PCR using primers specific to the mouse *Abcc6* promoter: 5′-CAC CAG CTC CAC CTC TGT AT –3 and 5′- GTT TGT CTT ACA GCT TCC CG –3′. A positive PCR amplification indicates that the promoter is methylated and as the level of amplification is directly proportional to the concentration of (methylated) DNA isolated, the quantification of the PCR products reflected the level of DNA methylation.

Quantification of methylation

The PCR products were amplified, electrophoresed on a 2% agarose gel and stained with SYBRGreen I (Molecular Probe, Carlsbad, CA). After staining, the intensity of each band was measured using the Kodak Gel Logic 200 and associated software Molecular Imager v4.0. As standard controls, the PCR fragment was amplified using the input from total genomic DNA (prior using the Promoter Methylation PCR kit). The ratio of methylation was calculated by comparing the intensity of the methylated fragment with that present in the total genomic DNA extract (input).

Chromatin Immunoprecipitation Assays (ChIP) and quantitative PCR

ChIPs were performed from skin, kidney and liver isolated from 10 month-old mice, as previously described [17]. Immunoprecipitated DNA was purified and analyzed for specific enrichment by quantitative PCR analysis using SYBRGreen™ qPCR SuperMix (Invitrogen, Carslbad, CA) and primers specific to the mouse *Abcc6* proximal promoter (see above). As the elution volume of the immunoprecipitated chromatin samples may vary, we used the neomycine-resistance gene present on the pCMV plasmid, which was added in each sample prior to performing the ChIP assays, to account for deviation in the measurement of DNA levels. The sequence of the oligonucleotides specific to the neomycine-resistance gene is as follow: mNeo (5′-GAACAAGATGGATTGCACGCAGG-3′) and aNeo (5′-CGCTGACAGCCGGAACACG-3′). The quantification of co-immunoprecipitated promoter fragments was based on Chakrabarti et *al*. [20]. Samples were quantified in triplicate from three independent immunoprecipitations.

Electromobility shift assay (EMSA)

20ng of purified Sp1 protein was incubated for 20 minutes at room temperature with a DNA fragment corresponding to the −152/+162bp region of the mouse *Abcc6* promoter that was either methylated or not. The DNA methylation was performed as described below. DNA/protein complexes were resolved on a 4% polyacrylamide gel in 0.5X TBE at 120V and detected by SYBR Green/SYPRO Ruby staining (Molecular Probes, Carlsbad, CA).

In vitro methylation of the proximal promoter sequence

A region encompassing the −152/+162bp domain of the *Abcc6* gene has been excised from its plasmid vector (pGL3-Basic, Promega, Madison, WI) using *Kpn*I and *Eco*RI restriction enzymes. The excised DNA fragment was gel-purified and subsequently methylated with the *Ss*I DNA methyltransferase (New England Biolabs, Beverly, MA) according to the manufacturer's instructions. Full methylation was confirmed by resistance to *Hpa*II digestion (New England Biolabs, Beverly, MA). The methylated promoter fragment was re-inserted into the pGL3-Basic vector. The resulting construct was transfected into TIB-73 and RAG cell lines and the luciferase activity was measured as described below. The same unmethylated DNA fragment was used as positive control.

Luciferase activity assays

Cells were washed in phosphate-buffered saline (PBS), scraped from the flasks and resuspended in a Passive Lysis Buffer (PLB from the Promega Dual-Luciferase Reporter Assay System) for luciferase reporter assays. Both *firefly* and *renilla* luciferase activities were measured using Turner Designs Luminometer Model TD-20/20 Genetic Reporter System. The transfection efficiency was normalized to *renilla* luciferase activity.

Statistical analysis

Values were used in paired two-tailed Student’s t-tests to determine statistical significance of difference between 2 groups. Differences were considered significant when $p<0.05$.

Results

Methylation of the *mouse Abcc6* proximal promoter

To investigate the level of *in vivo* methylation of the proximal promoter, genomic DNA samples were isolated from two groups of tissues. Tail extremity and skin were chosen for their very low level of *Abcc6* expression whereas liver and kidney were selected because these tissues present the highest levels of expression. One should note that in spite of being second to the

liver in *Abcc6* expression levels, the kidney expression is relatively low and somewhat comparable to the expression levels found in tail extremity. Indeed, when compared to liver, kidney, tail extremity and skin expression levels amounted to 5.2%, 0.9% and 0.1% respectively. However, we described in this report the kidney expression of *Abcc6* as intermediate between the liver expression and that of the tail extremity and skin.

Methylated genomic DNA was isolated using a methylation promoter PCR kit (Panomics). We found that 88% and 57% of the DNA isolates corresponding to the −152bp/+162bp promoter region was methylated in skin and tail extremity respectively (Figure 1A). In contrast, the proximal region was remarkably hypomethylated in DNA isolates from liver and kidney with values of 4% and 18% respectively (Figure 1A). These results showed an inverse correlation between methylation of the proximal promoter and the level of expression. It is noteworthy that a relatively low and moderate levels of methylation such as those found in kidney (18%) and even in tail extremity tissues (57%) equated to a sharp drop in expression level with 95 to 99% decrease, which clearly underscored the importance of the −152bp to +162bp region for the expression of *Abcc6* (Figure 1B). Interestingly, the analysis of DNA regions upstream of the proximal promoter did not reveal any significant methylation in all DNA sample eluates (data not shown) indicating that the distal region of the promoter is not subject to significant methylation.

Effect of methylation on *Abcc6* transcription

To analyze the effect of DNA methylation on the expression of *Abcc6* gene *in vitro*, a reporter plasmid (pGL3) bearing either a CpG methylated proximal promoter sequence or an unmethylated copy was used in transient transfection with hepatocytes (TIB-73) and kidney (RAG) cell lines. DNA fragments corresponding to the −152/+162bp region were excised from the vector, methylated *in vitro* with the *SssI* enzyme and inserted into the reporter plasmid. One microgram of the resulting constructs was directly transfected into TIB-73 cells without further bacterial amplification to preserve the methylation profile. We compared the relative luciferase activity of the constructs with the methylated and unmethylated fragments (Figure 2). As previously shown [17], the reporter construct carrying the −152/+162bp fragment (without methylation) induced a high transcriptional activity both in TIB-73 and RAG cells as compared to an empty vector with a 8.8-fold and 7.8-fold induction level, respectively. In contrast, the presence of a methylated fragment in the reporter construct resulted in a sharp decrease of luciferase activity in TIB-73 (99.6%) and in RAG (85.2%) (Figure 2). Similar results were obtained when higher amounts of plasmid constructs (4 μg) were used for transfection (data not shown). These results clearly indicated that the DNA methylation of the *Abcc6* proximal promoter region affected the *Abcc6* gene transcription efficiency.

Sp1 binding

The Sp1 transcription factor was previously shown to be essential for both human and mouse *ABCC6/Abcc6* gene expression [17,18]. To assess whether the methylation level of the proximal promoter influenced Sp1 binding, the relative association of Sp1 to the *Abcc6* proximal promoter was examined by chromatin immunoprecipitation assays (ChIP) in samples derived from liver, kidney and skin. Quantitative PCR (qPCR) was performed as previously described [17] to determine the relative level of association. Our results indicated that the relative Sp1 association with the proximal promoter in liver is more than twice that found in kidney genomic DNA while the level of association in skin was minimal with values similar to the negative control (Figure 3).

Effect of methylation on Sp1 binding

Since the *Abcc6* proximal promoter region that binds Sp1 exhibits a low level of methylation in high expressing tissues, we investigated whether Sp1 binding might directly depend on DNA methylation. To test this possibility, electromobility mobility shift assay was carried out using DNA fragments corresponding to the *Abcc6* proximal promoter (−152/+162bp fragment). The DNA fragments were amplified by PCR, subjected to *in vitro* methylation (See Methods) and incubated in the presence of purified Sp1 protein. Non-methylated fragments were used as controls. The control fragments were able to bind Sp1 while the methylated DNA could not as documented on Figure 4. This suggested that CpG dinucleotide methylation decreased the ability of Sp1 to bind to this promoter region.

Discussion

After a comprehensive series of reports on the genetics of pseudoxanthoma elasticum focusing notably on identifying mutations in the *ABCC6* gene [21–25] and the generation of mouse models [14,26], there is now a growing interest in the transcriptional regulation of *ABCC6* expression. Indeed, *ABCC6* and its rodent orthologs *Abcc6* are primarily expressed in liver and kidneys whereas the PXE phenotype affects dermal, vascular and ocular tissues. The apparent discrepancy between the sites of *ABCC6* expression and affected tissues as well as recent data has prompted some investigators to suggest that the etiology of PXE might originate in liver and/or kidneys [13,15,27]. To date, a few studies focusing on the *ABCC6/Abcc6* promoters have been initiated [17–19]. In this report, we present evidences that the tissue-specificity of the mouse *Abcc6* gene expression strongly depends on synergy between CpG methyaltion and Sp1 binding on the proximal promoter region (−152/+162bp).

We previously reported that the proximal region of the mouse *Abcc6* gene corresponds to a TATA-less promoter containing Sp1 binding sites. These sites may participate in the tissue-specific gene expression along with a liver-specific enhancer region located in the distal promoter region [17]. The proximal promoter region is GC-rich and binds Sp1 which can trans-activate both human and mouse *ABCC6/Abcc6* gene *in vitro* [17,18]. CpG methylation levels of the human proximal promoter of *ABCC6* was recently found inversely correlated to the transcriptional activity in cell lines [19]. Although, no CpG Island *per se* could be predicted in the mouse proximal promoter sequence, this GC-rich region requires the binding of Sp1 for its basal activity [17]. As DNA methylation can occur on CpG islands as well as on non-CpG sequences at the binding sites of Sp-type transcription factor [28–32], we hypothesized that the transcription of *Abcc6* could depend on the methylation status of the proximal promoter in a tissue-specific manner. Indeed, we found such correlation in mouse tissues between high, moderate and low levels of expression (liver, kidney, tail extremity and skin) and the degree of methylation (Figure 1). In addition, we observed that the *Abcc6* proximal promoter transcriptional activity in hepatocytes and kidney cells is significantly repressed following *in vitro* methylation (Figure 2). These results clearly suggested that this epigenetic mechanism is involved in the control of tissue-specific expression of the *Abcc6* gene.

Furthermore, as several studies have previously reported that CpG and non-CpG methylation do inhibit the binding of Sp1 proteins [33–35], methylation of the proximal promoter of *ABCC6/Abcc6* that contains Sp1 binding sites [17,18] could directly prevent Sp1 from binding and promoting gene transcription. Our results indeed showed that the Sp1 transcription factor binds to the proximal *Abcc6* promoter only in tissues where the promoter is hypomethylated, that is liver and to a lesser extent in kidney (Figure 3). Moreover, we clearly demonstrated that *de novo* methylation of the proximal promoter blocked the binding of Sp1 to the proximal promoter (Figure 4) strongly suggesting that both Sp1 and methylation synergistically regulate the basal transcriptional activity of the *Abcc6* gene thereby controlling tissue-specificity.

In summary, we report the first evidences that the proximal promoter region of the *Abcc6* gene is a critical domain that determines the tissue-specific transcription by way of DNA methylation and binding of Sp1 transcription factor. Together with previously published data, one could now describe the transcriptional regulation of *ABCC6*/*Abcc6* as dependent of several distinct genetic and epigenetic mechanisms involving an ubiquitous regulatory protein (Sp1) and DNA methylation determining tissue-specificity in addition to liver and erythroid-specific enhancers (HNF-4a and NF-E2) [17] and at a different level, pro-inflammatory cytokines [18].

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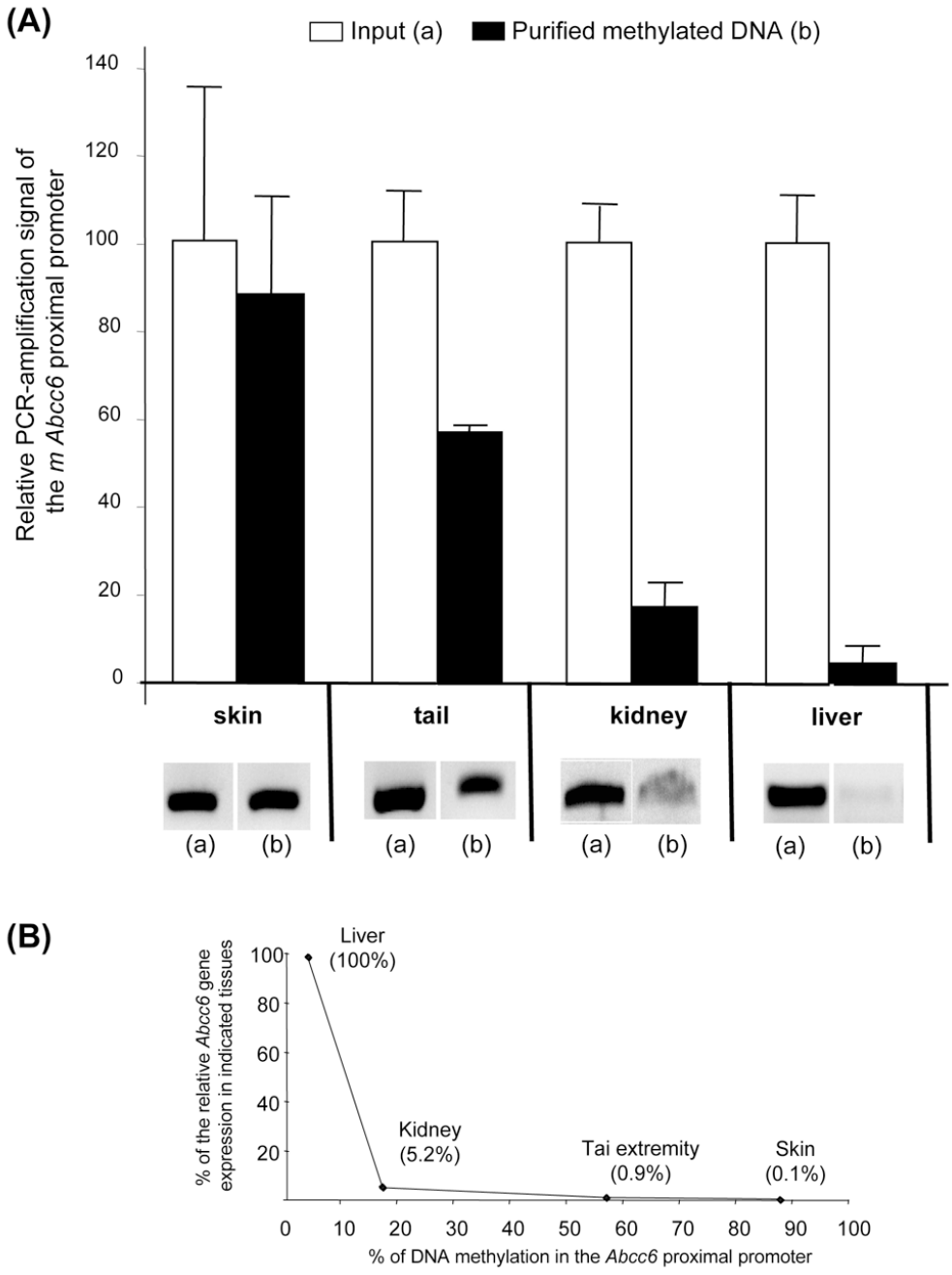

Figure 1. *In vivo* methylation of the proximal *Abcc6* promoter

(A) Genomic DNA from tail extremity, skin, kidney and liver was analyzed with the Promoter Methylation PCR kit (Panomics). Methylated DNA fragments isolated from genomic DNA were submitted to PCR-amplification with primers specific to the proximal promoter region (−152/+162bp). The presence of a PCR product reflects the methylation status in the genomic DNA sample. After staining, the intensity of each band was measured using the Kodak Gel Logic 200 imager and associated Molecular Imager software 4.0. The ratio of methylation was calculated by comparing the intensity of the methylated fragment to that present in the total genomic DNA extract (input). The experiment was performed in triplicate. (B) Correlation between the level of *Abcc6* gene expression (% of liver expression) and DNA methylation.

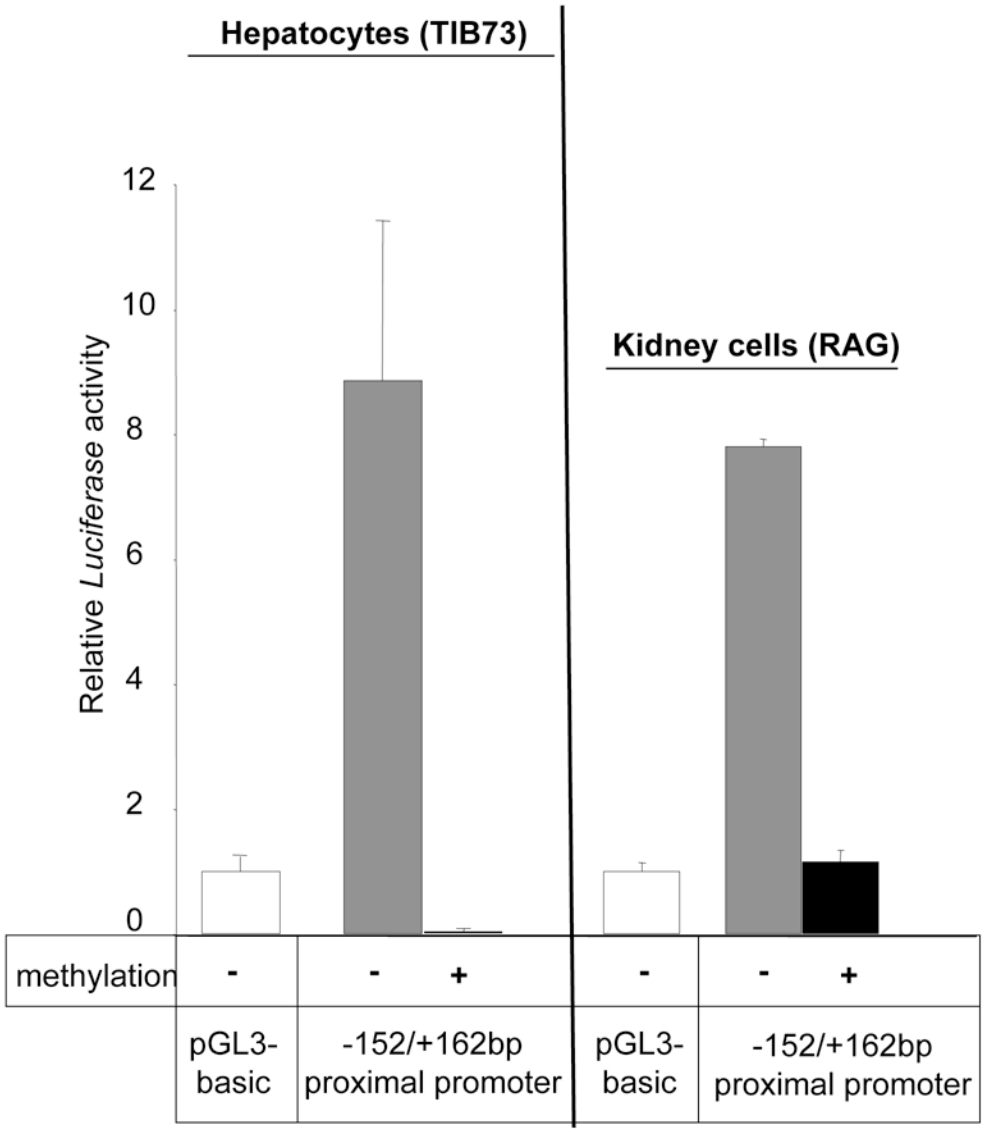

Figure 2. Effect of DNA methylation on the *Abcc6* proximal promoter activity

A DNA fragment corresponding to the–152/+162bp fragment was methylated *in vitro* and inserted into a reporter vector (pGL3). This plasmid construct was transfected into hepatocytes (TIB-73) and renal adenocarcinoma (RAG). These cell lines were transfected with 1μg of plasmid (as indicated) and 0.1 μg of pRL-SV40 plasmid for control purposes, and cultured for 24h. As a positive control, an identical unmethylated DNA fragment was used. *Luciferase* activity was normalized to the *renilla luciferase* activity. Each value represents the mean ± standard deviation of at least three independent transfection experiments, each performed in triplicate. The *luciferase* activities are represented as a ratio of the promoter-less plasmid (pGL3-basic) activity, which was given a value of 1.

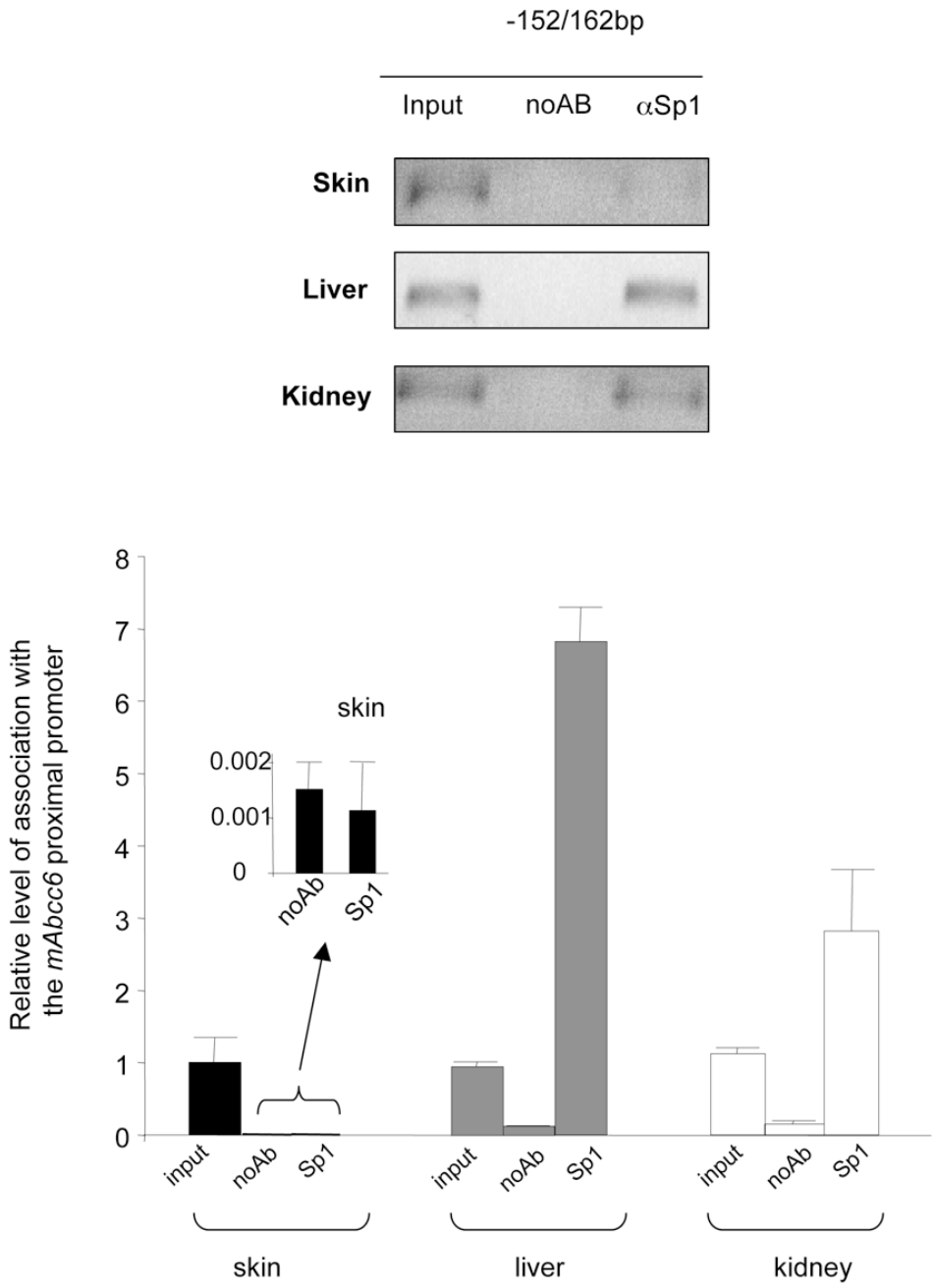

Figure 3. Relative association of the *Abcc6* promoter with the Sp1 transcription factor *in vivo*

Fragments of chromatin from skin, liver and kidney were immunoprecipitated with anti-Sp1 antibodies and quantified by qPCR using primers specific to the proximal region of the *Abcc6* promoter (−152/+162bp). The data was normalized to the total input of DNA used prior to immunoprecipitation and to the control assay (noAB). The differences in association are shown as relative to the total DNA input (input). Data represents the mean of 3 independent immunoprecipitations quantified in duplicate assays. Standard errors are indicated.

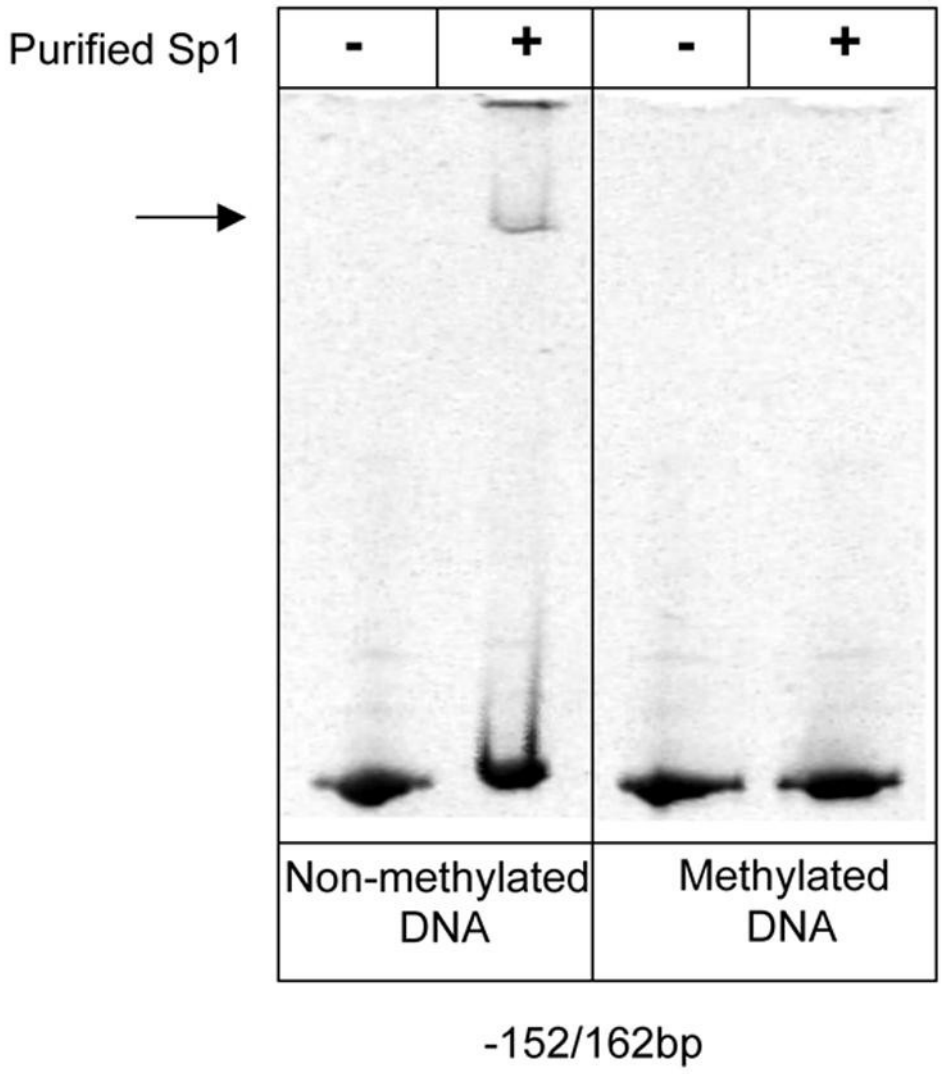

Figure 4. Sp1 binding to methylated *Abcc6*proximal promoter

Electromobility shift assays (EMSA) performed with methylated and unmethylated DNA fragments corresponding to the *Abcc6* proximal promoter (−152/+162bp) and purified Sp1 proteins. The arrow indicate the Sp1-induced shift.