Published in final edited form as:
Biochim Biophys Acta. 2006 ; 1759(8-9): 426–436.

HNF4α and NF-E2 are key transcriptional regulators of the murine *Abcc6* gene expression

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SUMMARY

Mutations in an ABC transporter gene called *ABCC6* are responsible for pseudoxanthoma elasticum (PXE), a rare heritable disease characterized by elastic fiber calcification in skin, ocular and vascular tissues. The presumed function of this ABC transporter is to export metabolites from polarized cells. However, the endogenous substrate(s) are unknown and the exact relationship with elastic fibers is unclear. As *ABCC6* is expressed at high level only in liver and kidneys, tissues seemingly unrelated to the PXE phenotype, we explored the transcriptional regulation of the murine *Abcc6* gene to define transcriptional regulation conferring tissue specificity and to gather clues on its possible biological function. We cloned 2.9 kb of the *mAbcc6* 5′-flanking region and several deletion constructs linked to a luciferase reporter gene. We delineated a proximal promoter and a liver-specific enhancer region. We also demonstrated that the proximal region is a TATA-less promoter requiring an intact CCAAT-box and Sp1 binding for its basal activity. By using reporter assays and chromatin immunoprecipitations, we showed that HNF4α and surprinsingly, NF-E2, enhanced the *mAbcc6* promoter activity. The involvement of both HNF4α and NF-E2 in the *mAbcc6* gene regulation suggests that Abcc6 might be involved in a detoxification processes related to hemoglobin or heme.

Keywords

Abcc6; ABC transporter; pseudoxanthoma elasticum; transcriptional regulation; reporter gene analysis

INTRODUCTION

The *ABCC6* gene encodes an ATP-binding cassette (ABC) transporter that belongs to the sub-family C also known as the multidrug-associated resistance protein family (MRP) [1,2]. Based on homology with the well-characterized ABCC1 protein (~45% homology) and its localization on the basolateral membrane of hepatocytes, ABCC6 is thought to be a metabolite pump exporting compounds from these cells [3]. Ilias et al. [4] demonstrated in vesicular studies that glutathione S-conjugates, including leukotriene-C4 and N-ethylmaleimide-S-glutathione, are actively transported by the human ABCC6 whereas the endothelin-1 receptor antagonist BQ123 is not efficiently transported in contrast to Abcc6 in rat [5]. In spite of these

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various studies, the endogenous substrates and the overall function(s) of ABCC6 are presently unknown.

The first indicator of ABCC6 function was unveiled when mutations in the *ABCC6* gene were associated with the development of pseudoxanthoma elasticum (PXE, OMIM #264800, 177850) [6–9]. PXE is a rare heritable disorder characterized by progressive dermal, ocular and cardiovascular abnormalities that arise through connective tissue alterations and mineralization of elastic fibers [9–12], which suggested that the lack of ABCC6 function relates to either elastic fibers maintenance or deposition. To date, more than 100 mutations have been characterized in PXE patients [13–18]. Most nucleotide variants cluster in specific regions of ABCC6, notably the ATP-binding domains, suggesting that the PXE result lack of transport activity [19]. This suggestion was later verified experimentally [4].

Because *ABCC6* is primarily expressed in liver and kidneys and hepatic and renal functions of PXE patients are seemingly normal, it was suggested that PXE might be a metabolic disease rather than a connective tissue disorder [20,21]. Several studies have indeed reported alterations in plasma lipoproteins or vitamin D metabolism from PXE patients [22] and the recent generation of the PXE mouse model with altered HDL and creatinine levels support these findings [23]. Also, Maccari et al. recently reported abnormal excretion of glycoaminoglycans in the urine of PXE patients, which may indicate abnormal kidney functions [24]. Furthermore, we recently reported that metabolites present in serum of PXE patients interfered with the assembly of elastic fibers produced by skin fibroblasts and aortic smooth muscle cells [25]. All these results do support the notion that the pathology of PXE may indeed derives from the lack of ABCC6 function in either liver or kidney or both.

Tissue-specific function may be considered as a consequence of tissue- or cell-specific gene expression [26]. The regulation of the *ABCC6/Abcc6* gene expression appears to be different from that of the other ABC transporter genes [5,26,27], which implies a distinct physiological function of ABCC6/Abcc6. Indeed, the proximal promoter of the human *ABCC6* gene contains an activator sequence transcriptionally dependent on DNA-methylation which may play a role in the tissue specificity [28]. In addition, Jiang et al. [29] showed in vitro that the human *ABCC6* proximal promoter can be modulated by pro-inflammatory cytokines. However, inflammation processes are not a major characteristic of PXE and the regulation signals responsible for the tissue-specific control of ABCC6 expression remain to be defined.

In the present study, we cloned 2.9kb segment of mouse *Abcc6* gene promoter. After a predictive analysis, we determined that the 5′-flanking region of the *mAbcc6* gene contains a proximal TATA-less promoter requiring an intact CCAAT-box and Sp1 (stimulating-protein 1) binding for its basal expression. We also found a liver-specific cis-acting enhancer region located between −1.6kb and 2.9kb. Further analysis showed for the first time that the HNF4α and NF-E2 transcription factors trans-activate the *mAbcc6* gene expression *in vitro*, while HNF1α does not.

MATERIALS AND METHODS

DNA sequence analysis

The murine *mAbcc6* gene is located on chromosome 7 in a divergent arrangement with a gene called *pM5*. The intervening region between *mAbcc6* and *pM5* is 3.5kb in length and the sequence of this region was determined by combination of database mining at the NCBI (www.ncbi.nlm.nih.gov/) and gaps and non-sequenced segments were characterized by direct sequence of PCR products. The primers used to generate these PCR products are described in Table 1. Putative promoter/enhancer elements in the 5′-flanking promoter region of the

mAbcc6 were identified using MOTIF, MATINSPECTOR and TFSEARCH (motif.genome.ad.jp, www.genomatix.de, transfac.gbf.de).

Identification of transcription initiation sites

The 5′ untranslated sequence of the *mAbcc6* gene was obtained by 5′ RACE using the SMART RACE cDNA Amplification kit (Clontech). The 5′ RACE-ready first-strand cDNA was synthesized using 1μg of total RNA from mouse liver. The 5′ flanking sequence of *mAbcc6* was amplified using a universal primer (UMP, Clontech) and a *mAbcc6*-specific primer: 5′-GCCTGCTGCACAGAGTTGATT-3′. The resulting PCR products were gel-purified, cloned into the pGEM-T easy vector (Promega) and sequenced.

The *mAbcc6* gene promoter constructs

Six *mAbcc6* gene fragments were generated by PCR amplification using Pfu DNA polymerase (Stratagene). The primers sequences are described in Table 1. The PCR fragments were cloned into the pGL3-basic plasmid (Promega). The *mAbcc6* gene promoter activity was analyzed using vectors bearing the six constructs inserted 5′ to the firefly luciferase reporter gene. The largest construct spanned −2926bp to +162bp of DNA adjacent to the *mAbcc6* gene putative translation start site. For site-directed mutagenesis, the CCAAT-binding site located at −94bp from the most 5′ ATG (A is +1) was mutated to GCTAT using the Transformer site-directed mutagenesis kit (BD Biosciences). The −2926/+162bp promoter construct was used for this experiment. The primers: used to generate this mutated construct were designated BamKpn and CCAAT* (see Table 1). Each *mAbcc6* gene promoter construct was verified by sequencing.

Cloning of the *Hnf1*α, *Hnf4*α, *p45-Nf-E2* and *Sp1* cDNAs

Total RNA isolated (1μg) from mouse liver was reversely transcribed with the Superscript III reverse transcriptase kit primed with Oligo(dT) (Invitrogen). cDNAs were then PCR-amplified with the specific primers (see Table 2), using the PCR Platinum Supermix (Invitrogen). Each PCR product was cloned into an expression vector, pTarget (Promega), using TA-cloning. The expression of the cloned fragments was driven by the cytomegalovirus enhancer/promoter elements (CMV). After cloning, the sequence of all cDNAs was verified by direct sequencing.

Cell culture and transient transfection

RAG, Hepa1-6, NIH/3T3 and TIB-73 cells from the American Type Culture Collection (ATCC) 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) as described by the manufacturer. Cells were harvested for luciferase activity assays after a 24 h incubation period. The transfection efficiency was determined by co-transfecting cells with a vector expressing the *Renilla luciferase* gene under the control of the SV40 enhancer (pRL-SV40) and calculating the ratio *Firefly* / *Renilla* luciferase activity. To compare the transcriptional activity of the *Abcc6* promoter between cell lines, the data was normalized to the total proteins extracted. For co-transfection experiments, cells were grown for 48 hours with complete medium supplemented with G-418 antibiotic then harvested and tested for luciferase activity. For luciferase activity assays, a Turner Designs Luminometer Model TD-20/20 Genetic Reporter System was used.

Electrophoretic mobility shift assay (EMSA) and Chromatin Immunoprecipitation (ChIP)

Nuclear extracts were prepared from TIB-73 liver cells using a nuclear extraction kit (Panomics) according to the manufacturer's instructions. 3μg of nuclear extract was used. For gel mobility super-shift assays, 2μg of antibody specific to Sp1 (PEP2, sc-59, Santa Cruz Biotechnology), were pre-incubated with the nuclear extract mixture for 20 minutes before

adding the nucleotide probe corresponding to the −152/+162bp region of the mAbcc6 promoter. DNA/protein complexes were resolved on a 4% polyacrylamide gel in 0.5×TBE at 120V and detected by SYBR Green/SYPRO Ruby staining (Molecular Probes). ChIP were performed according to standard protocol but with some modifications [30]. For negative control, an aliquot of crosslinked chromatin was immunoprecipitated with goat serum instead of a specific antibody (referred to as “noAb” fraction). Immunoprecipitated DNA was purified with a PCR purification kit (Qiagen) and analyzed for specific enrichment by quantitative PCR analysis.

Quantitative PCR (qPCR)

Total RNA was isolated from TIB-73 cells using the Qiagen RNeasy purification kit and reverse-transcribed with the Superscript III reverse transcriptase kit primed with Oligo(dT) (Invitrogen). PCR reactions were carried out using Taqman universal PCR mastermix and TaqMan probes specific to *mAbcc6* and *GAPDH* for reference gene normalization (Applied Biosystems). Because the transfection efficiency also determine the level of target mRNAs, we used the level of expression of the neomycine-resistance gene encoded by the expression vector bearing the transcription factor of interest to account for deviation in the measurement of mRNA levels with the following oligonuleotides mNeo: 5′-gaacaagatggattgcacgcagg-3′ and aNeo: 5′-cgctgacagccggaacacg-3′. These primers yielded a unique 117bp PCR product. Samples were quantified by real time qPCR using SYBRGreen I chemistry. The quantification of the co-immunoprecipitated promoter fragment was based on Chakrabarti et al. [31]. The relative proportions of co-immunoprecipitated *mAbcc6* promoter were determined based on the threshold cycle value (TC) for each PCR reaction. qPCR data analysis followed the methodology described in a recent report [32]. For each TF analyzed, samples were quantified in triplicate from three independent immunoprecipitations. A melting curve analysis was performed for each sample after PCR amplification to ensure that a single product was obtained. The primers “forward” and “152/+162” (Table 1) were used to amplify the proximal promoter of *mAbcc6* gene, giving a 314bp product. For these experiments, we used a 7300 Real Time PCR system (Applied Biosystems). Each reaction was performed in triplicate with default conditions: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min.

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 $p<0.05$ and are indicated by an asterisk on the figures.

RESULTS

Identification of the transcription initiation sites of the murine *Abcc6* gene in liver

To identify the initiation site(s) of the murine *Abcc6* gene, we used a ligation-mediated 5′-RACE method that requires the presence of a capped 5′-end on the original RNA template. For this purpose, we performed 5′-RACE on total RNA extracted from 10 month- mouse liver. Sequencing of multiple clones showed that three different transcription start sites were identified at 12, 16 and 21 nucleotides upstream from the most 5′ putative translation initiation codon (designated +1, Figure 1). Remarkably, the murine cDNA sequence contains two in-frame ATGs (designated +1, +18, Figure 1), which could be used as translation initiation codons as shown in the rat Abcc6 gene [5]. Therefore, we included both ATGs in the PCR fragment used for the promoter studies.

Predictive nucleotide sequence analysis of the 5′ region of the murine *Abcc6* gene

Clustering analysis of the ortholog genes can reveal conserved DNA sequences, which reflect putative promoter regions and/or common regulatory elements. The human, rat and mouse genes are located on different chromosomes. The human and rodent *ABCC6/Abcc6* genes are located on different chromosomes. The human *ABCC6* gene is at 16p13.1, the rat gene (*rAbcc6)* is positioned at 1q22 and the mouse gene, *mAbcc6* at 7B3. The region immediately 5′ to the *ABCC6/Abcc6* genes is divergent between the rodent and human sequences and probably represents a syntenic breakpoint. In mouse and rat, the sequence extending 5′ of the *Abcc6* gene is bordered by a gene in a divergent arrangement called *pM5* also referred to as *Nodal Modulator 1* (NOMO1) at the relatively close distance of 3.5kb in contrast to human. After this preliminary analysis, we performed an alignment of the human, rat and mouse *ABCC6/Abcc6* 5′-flanking regions. The murine and rat *Abcc6* genes shared an extensive sequence identity (63%) in the 5′-flanking region but lower sequence conservation was found between the human and the rodent sequences. In the distal region, a well-conserved sequence (60% identity) contained a predicted promoter element with a canonical TATA-box on the reverse strand. In the human sequence, that region was previously shown to contain a CpG island [28] and our analysis also predicted the presence of a CpG island in the mouse but not in the rat sequence. That distal region is likely to contain the promoter elements specific to the*Nodal Modulator 1* gene (Alias *pM5*) positioned in an orientation opposite to the *Abcc6* gene. Another CpG island region was identified in the proximal region of the human *ABCC6* gene [28]. Although no CpG island could be predicted in the rodent sequences, this proximal region is GC-rich (60.5%) and shares 41% identity overall with 78% identity between mouse and rat. The intervening region showed a lower degree of identity (~33%). Despite this reduced conservation, that region presented short DNA segments highly conserved between all three species, suggesting that these segments contain potential common regulatory elements involved in the *ABCC6/Abcc6* gene regulation (data not shown).

For subsequent analysis, we focused on the murine sequence. We considered 2926bp upstream to the most 5′ ATG and an additional 162bp downstream, as the potential promoter region of the murine *Abcc6* gene (*mAbcc6*). We analyzed this sequence for possible transcription factor binding sites with the TRANSFACT, MOTIF and MATINSPECTOR transcription factor databases. This *in silico* analysis showed no canonical TATA-box element in the proximal region (Region C, Figure 2). However, we identified several GC-boxes corresponding to putative Sp1 binding sites, and a CCAAT-box at −94bp from the most 5′ ATG (Figure 2). These elements are often found in core the proximal regions of TATA-less promoters [33]. Additional putative *cis*-acting liver-specific elements were identified as members of the HNF family and the leucine zipper C/EBP family (CCAAT/enhancer binding protein) [34,35]. Other potential transcription factor binding sites for GATA-1 and NF-E2 (nuclear-factor erythroid 2) were found. These transcription factors are known to regulate the expression of genes involved in the erythroid development and maturation. Finally, several putative environmental response cis-elements, such as NFκB (nuclear factor κB), MTF-1 (metal transcription factor), SRF (serum response factor) and USF (upstream stimulating factor) were also identified.

Transcriptional activity of the 5′ region of the *mAbcc6* gene

To define the murine *Abcc6* gene promoter and delineate its tissue-specific enhancer/inhibitor element(s), six fragments with sizes ranging from −152/+162bp to −2926/+162bp were derived by PCR from the genomic mAbcc6 5′ flanking region (Table 1). The sequence of these PCR fragments were all verified by sequencing and inserted into a vector with a *luciferase* reporter gene (pGL3). Based on *mAbcc6* tissue expression profile, three mouse cell lines were chosen for analysis. The Hepa1-6, a hepatocellular carcinoma cell line, represented the liver, the RAG cell line derived from a renal adenocarcinoma, represented the kidneys. The embryonic

fibroblast cell line NIH/3T3 was used as a negative control because the endogenous expression of *ABCC6/Abcc6* in skin fibroblasts is very low [36]. All fragments achieved transcriptional activation of the *luciferase* reporter gene in hepatoma, kidney cells and in fibroblasts (Figure 3). Despite similar transfection efficiency in all cell lines (data not shown), the NIH/3T3 cells exhibited the lowest level of promoter activation with little variability between the 6 promoter fragments examined (Figure 3). In contrast, the transfected Hepa 1–6 and RAG cells showed a greater variability of *luciferase* expression. Interestingly, the smaller −152/+162bp fragment displayed the maximum promoter activity in RAG cells but the highest activity in Hepa1-6 cells was obtained with the −2926/+162bp fragment. The reporter activity profile of vectors carrying fragment of increasing length from −152/+162bp to −928/+162bp, decreased gradually in Hepa1–6 and RAG cells (Figure 3). The level of *luciferase* activity returned progressively to high level with the −1640/+162bp and −2926/+162bp fragments in Hepa1–6 cells but remained at a lower level in RAG cells. These results demonstrated that the elements present in the proximal regulatory region, defined as the region between −152bp and +162bp of the *mAbcc6* promoter are sufficient to recruit the basal transcriptional machinery but additional upstream segments conferred appeared necessary for a tissue-specific expression. Indeed, we found that regulatory element(s) present between −1640bp and −2926bp gave a high liver-specific activity of transcription but only provided a moderate transcriptional activity in kidney cells, which correlated to the known *mAbcc6* expression levels in these tissues [17]. Moreover, the region between −350bp and −1640bp presented an inhibitory effect in both liver and kidney cells. Therefore, the proximal *mAbcc6* gene promoter is comprised within the first −152bp region and the tissue-specific expression of the *mAbcc6* gene requires a combination of *cis*-acting enhancer element(s) located between −1640bp and −2926bp upstream from the start of translation and inhibitory domain(s) between −350bp and −1640bp. In addition, the reporter activity of the −2926/+162bp promoter construct was shown to be higher in TIB-73 cells (non-tumorogenic cells) than in Hepa1–6 cells (data not shown). Therefore, subsequent experiments were carried out with the TIB-73 cell line.

The *mAbcc6* proximal promoter region is a TATA-less promoter

The −152/+162bp fragment of the *mAbcc6* promoter appeared important for the basal expression. The computer analysis of this sequence revealed a potential CCAAT-box located at −94bp from the translation start and GC- boxes potentially associated with Sp1 binding sites without a canonical TATA-element. The presence of these boxes suggested that the *mAbcc6* proximal promoter region exhibits features of TATA-less promoters [37,38]. Using the −2926/+162bp fragment, we performed site-directed mutagenesis on the putative CCAAT-box to confirm that the *mAbcc6* promoter activity was dependent on this *cis*-element. Substitution of the two nucleotides in the CCAAT-box (GCTAT) resulted in a drastic loss (99.7%) of promoter activity in TIB-73 cells (Figure 4A).

To examine the role for the Sp1 transcription factor in the *mAbcc6* gene expression, TIB-73 cells were co-transfected with pGL3 carrying the −152/+162bp promoter fragment and an expression vector bearing the Sp1 cDNA. The co-transfection led to a 4-fold induction of the −152/+162bp promoter activity as compared to its activity when cells were co-transfected with an empty expression vector (Figure 4B). As expected, a low promoter activity was observed when fibroblasts were transfected with the −152/+162bp construct. However, when fibroblasts were co-transfected with the Sp1 expression vector, a 4-fold induction of the −152/+162bp promoter activity was observed, indicating that Sp1 promoted the *mAbcc6* gene expression. Therefore, to test whether Sp1 was physically bound to the *mAbcc6* proximal promoter, we carried out an electromobility shift assay (EMSA) using the −152/+162bp fragment of the *mAbcc6* promoter and nuclear extract from TIB-73 cells. We observed a band shift on the gel (Figure 4C, lane 2) and we verified that Sp1 was responsible for this shift using an Sp1-specific

antibodies pre-incubated with the TIB-73 nuclear extract (Figure 4C, lane 3). The super-shift assay showed that Sp1 was indeed bound to the *mAbcc6* proximal region *in vitro*. The ability of Sp1 to bind in vivo the −152/+162bp region was further tested by chromatin immunoprecipitation (ChIP). Cross-linked protein-chromatin complexes from TIB-73 cells were precipitated using either the anti-Sp1 antibody or goat serum as negative control. The −152/+162bp *mAbcc6* promoter region was amplified from the immunoprecipitated sample with specific primers (Table 1), whereas no significant amplification was observed with the negative control (Figure 4D).

Altogether, these results demonstrated that the proximal region of *mAbcc6* is a TATA-less promoter that can be trans-activated by the binding of Sp1.

Trans-activation of the *mAbcc6* gene expression by HNF4α and p45-NFE2

As the CCAAT-box and Sp1 participate in the basal expression of the *mAbcc6* gene, one can reasonably infer the presence of additional transcription factors, which would modulate the gene expression in a tissue-specific manner. For this purpose, several transcription factors were co-expressed with the *luciferase* reporter constructs −2926/+162bp and −152/+162bp in TIB-73 cells and in NIH/3T3 cells. The previous *in silico* analysis revealed the presence of four HNF-type binding sites potentially involved in the *mAbcc6* transcriptional regulation. We tested the trans-activation of the two potential HNF-type transcription factors, HNF4α and HNF1α in TIB-73 cells. The co-transfection of *HNF4*α cDNA expression vector and the −2926/+162bp::*luciferase* construct led to a 4-fold increase of *luciferase* activity (Figure 6A). A similar induction was obtained when TIB-73 cells were co-transfected with the reporter vector bearing the shorter −152/+162bp fragment and the *HNF4*α cDNA expression vector. This result suggested the presence of HNF4α-specific binding site(s) within the −152/+162bp proximal promoter. Interestingly, no significant trans-activation by HNF4α was detected in fibroblasts (Figure 5B), suggesting that HNF4α requires additional element(s) absent in fibroblasts. In addition, the co-expression of *HNF1*α cDNA did not induce any significant trans-activation of the −2926/+162bp and −152/+162bp promoters, indicating that HNF1α does not play a major role in the *mAbcc6* gene regulation.

Surprisingly, we identified four putative binding sites for the NF-E2 erythroid transcription factor in the *mAbcc6* gene promoter sequence, each within AP-1 motifs similar to those found in the globin gene regulatory sequences. Two of these NF-E2 putative binding sites were located in the distal *mAbcc6* promoter region and two additional motifs were localized downstream of the initiator codon (Figure 2). To test whether this transcription factor was involved in the *mAbcc6* gene regulation, TIB-73 cells were transfected with the large and the short *mAbcc6* promoter constructs along with a vector expressing the *p45-NF-E2* cDNA. This cDNA encodes the 45kDa subunit of the NF-E2 transcription factor. The *p45-NF-E2* cDNA expression led to a 3-fold induction of the *luciferase* activity with the shorter −152/+162bp fragment and a 8-fold induction was observed with the larger −2926/+162bp fragment (Figure 5A). In the NIH/3T3 fibroblasts, similar induction levels were obtained with each promoter construct (Figure 5B). The difference of induction obtained with the larger −2926/+162bp fragment and the shorter −152/+162bp fragment both in hepatocytes and fibroblasts (Figure 5A and B) is suggestive of additive and/or cooperative effects between the four NF-E2 binding sites on the mAbcc6 gene expression.

Finally, we examined the *in vivo* association of the HNF4α, p-45-NF-E2 and HNF1α transcription factors to the *mAbcc6* promoter in TIB-73 cells, using the Chromatin Immunoprecipitation assay (ChIP). qPCR was used to determine the relative level of association between the *mAbcc6* promoter and the transcription factors as described in a previous report [31], with primers specific to the −152/+162bp fragment. The ChIP assays

showed that with the antibodies specific to HNF4α and p45-NF-E2, the level of association with the *mAbcc6* proximal promoter is 4 to 5-fold greater than with the one obtained with the negative control (Figure 7). As expected, HNF1α showed no significant association with the *mAbcc6* proximal promoter (Figure 7).

In conclusion, these results demonstrated that the HNF4α and p45-NF-E2 transcription factors are able to bind *in vivo* the *mAbcc6* gene promoter and trans-activate its expression.

DISCUSSION

PXE is a complex disorder characterized by multi-organ involvement with progressive and uneven severity. Since the gene responsible for PXE has been identified, the exact functional relationship between an ABC transporter and elastic fiber defects has remained elusive. The ABCC6 transport activity was recently demonstrated and a few substrate molecules identified [4], however the nature of the endogenous substrate(s) is still unknown. The pathomechanism of PXE is also unexplained, as the major sites of the *ABCC6/Abcc6* gene expression seem to be unrelated to the affected tissues. The *ABCC6* gene is expressed primarily in the liver and to a lower extent in the kidneys in human as well as in rodents, whereas markedly lower levels of expression have been observed in many other tissues [2] [36] [17]. This restricted profile of expression indicates that the human *ABCC6* and the rodent *Abcc6* genes are under the control of strong tissue-specific transcriptional signals. In this study, we report that two transcription factors, HNF4α and NF-E2, are key regulator of the murine *Abcc6* gene transcriptional regulation.

Based on the premise that conserved regulatory sites may provide some clues as to what the function of *ABCC6/Abcc6* might be, we first compared the 5′-flanking region sequence of the human *ABCC6* gene and its rodents ortholog. An *in silico* analysis of the *ABCC6/Abcc6* genes of the human, rat and mouse predicted that the potential promoter regions were similar with a high degree of conservation within the first 300bp upstream of the translation start. Following these predictive analyses, we identified 3 transcription initiation starts for the murine *Abcc6* expressed in liver using a “cap” dependent 5′ RACE method. Each transcription initiation site was located within the first 30 nucleotides upstream from the predicted start of translation, which was similar to those described for the rat *Abcc6* [5]. Using transient transfection assays, we defined a proximal promoter for the murine *Abcc6* promoter within 152bp upstream from the translation start. In addition, we found that this proximal region conferred high liver- and kidney-specific levels of expression but not in fibroblasts, which is consistent with the *in vivo ABCC6/Abcc6* expression profile [39]. In the human sequence, a similar proximal region of the *ABCC6* promoter containing a GC-rich domain was defined as conferring tissue specificity and a potential target for transcription factors [28]. Our results showed that the binding of the Sp1 transcription factor trans-activated the *mAbcc6* proximal promoter even in fibroblasts, which present a very low level of endogenous expression. These results suggested that Sp1 might in part determine the tissue specificity of the *mAbcc6* gene expression. Because tissue specificity can be determined by methylation at Sp1 sites, the binding of Sp1 transcription factor was suggested to protect the genomic DNA from being methylated [40] [41] [42] [43]. Aranyi et al. [28] described the presence of a CpG island in the human proximal promoter and suggested that methylation at this site could influence the tissue specific gene expression of *ABCC6*. Although no CpG island per se could be predicted in the proximal region in both rat and mouse sequences, the GC-rich region of the *mAbcc6* proximal promoter required the binding of Sp1 for its basal activity. Therefore, one could suggest that Sp1 binding sites in tissues with low level of *mAbcc6* gene expression could be methylated, thus preventing Sp1 from promoting the gene expression.

Another remarkable feature of this promoter sequence was the lack of any identifiable canonical TATA-element. Instead, multiple putative Sp1 binding sites were found in close proximity to the consensus sequence CCAAT at −94bp from the translation start, defining this promoter as a TATA-less promoter [33] [37,38]. We showed that this CCAAT-box was indeed critical to the *mAbcc6* gene expression. Interestingly, the human *ABCC1*, *ABCC2* and the rat *Abcc3* genes, which are closely related to *ABCC6/Abcc6*, are also under the control of TATA-less promoters with Sp1 involvement [44–49]. However, there are significant differences in the tissue and temporal expression profiles of those genes indicating that the *ABCC6/Abcc6* gene expression depends on other transcription factors and/or regulatory mechanisms [50, 51]. Distal elements were proposed to confer the liver-specific expression of the human *ABCC6* gene *in vivo* but not *in vitro* [29]. We found a similar *cis*-acting enhancer region between −1.64kb and −2.92kb in vitro by using the TIB73 cells, indicating that these cells as an *in vitro* model fully reproduced the in vivo mechanisms governing the *mAbcc6* gene regulation. The *in silico* analysis of this distal region predicted the presence of multiple other putative regulatory elements such as C/EBP and HNF3 transcription factors, which are known to be highly abundant in normal liver and involved in liver-specific expression [34]. Further analysis will determine whether those transcription factors are involved in the tissue specific expression of the *mAbcc6* gene.

The present study is the first showing that *mAbcc6* is a target gene of the transcription factor HNF4α, but not of HNF1α. Our results are consistent with the fact that the HNF4α gene provides transcriptional signals essentially in liver and kidney and the HNF1α gene gives transcriptional signals primarily in kidney, spleen and to a lower extent in liver [34]. In addition, we found that the ectopical expression of *HNF4α* cDNA in fibroblasts did not trans-activate the *mAbcc6* gene expression, suggesting that the HNF4α transcription factor cooperates with another element in hepatocytes. This result showed that HNF4α plays a role in the regulation of the *mAbcc6* gene in liver. As HNF4α is broadly involved in the hepatic maintenance by responding to metabolic status indicators [52–54], one could propose that ABCC6/Abcc6 might participate in a maintenance function and/or in a detoxification process.

We also found that the subunit p45-NF-E2 is also a major contributor to the *mAbcc6* gene regulation in hepatocytes and can trans-activate the *mAbcc6* promoter in fibroblasts. These results were unexpected because NF-E2 is an essential regulatory element controlling the pathways of heme and globin synthesis [55] [56]. NF-E2 is a heterodimer composed of two subunits of 45kDa and 18kDa protein of the Maf family, respectively. The heterodimeric association between Maf and the p45-NF-E2 subunit directly controls the DNA-binding properties of p45-NF-E2 and thus determines the positive transcriptional activity of NF-E2 [57] [58] [59] [60]. Our results clearly showed that NF-E2 is important for the transcriptional regulation of *mAbcc6* in liver. This is particularly intriguing as there is a high incidence of PXE manifestations in Mediterranean patients with beta-thalassemia, sickle cell and sickle thalassemia. These diseases all derive from genetic lesions leading to an abnormal or reduced production of beta-globin whereas the PXE manifestations associated with the beta-thalassemias don’t seem to derive from ABCC6 mutations [61]. Therefore, one could speculate that the *ABCC6* expression may be influenced by altered NF-E2 binding in beta-thalassemia patients and a functional correlation could then be suggested between a detoxification function of ABCC6/Abcc6 and the hemoglobin/heme metabolism in liver.

In conclusion, we showed that the proximal region of the murine *Abcc6* gene corresponds to a TATA-less promoter and contains Sp1 binding sites, which may participate in its tissue-specific gene expression along with a liver-specific enhancer region located in the 5′ distal promoter region. We found that HNF4α and NF-E2 transcription factors are key regulators of

the *mAbcc6* gene expression in liver, which possibly implicate the mouse Abcc6 function in detoxification processes and/or hemoglobin-related metabolism.

Acknowledgements

This study was supported by the National Institutes of Health grant RR16453 and the McKee funds of the Hawaii Community Foundation.

References

1. Kruh GD, Belinsky MG. The MRP family of drug efflux pumps. Oncogene 2003;22:7537–7552. [PubMed: 14576857]
2. Kool M, van der Linden M, de Haas M, Baas F, Borst P. Expression of human MRP6, a homologue of the multidrug resistance protein gene MRP1, in tissues and cancer cells. Cancer Res 1999;59:175–182. [PubMed: 9892204]
3. Scheffer GL, Hu X, Pijnenborg AC, Wijnholds J, Bergen AA, Scheper RJ. MRP6 (ABCC6) detection in normal human tissues and tumors. Lab Invest 2002;82:515–518. [PubMed: 11950908]
4. Ilias A, Urban Z, Seidl TL, Le Saux O, Sinko E, Boyd CD, Sarkadi B, Varadi A. Loss of ATP-dependent transport activity in pseudoxanthoma elasticum-associated mutants of human ABCC6 (MRP6). J Biol Chem 2002;277:16860–16867. [PubMed: 11880368]
5. Madon J, Hagenbuch B, Landmann L, Meier PJ, Stieger B. Transport function and hepatocellular localization of mrp6 in rat liver. Mol Pharmacol 2000;57:634–641. [PubMed: 10692506]
6. Bergen AA, Plomp AS, Schuurman EJ, Terry S, Breuning M, Dauwerse H, Swart J, Kool M, van Soest S, Baas F, ten Brink JB, de Jong PT. Mutations in ABCC6 cause pseudoxanthoma elasticum. Nat Genet 2000;25:228–231. [PubMed: 10835643]
7. Le Saux O, Urban Z, Tschuch C, Csiszar K, Bacchelli B, Quaglino D, Pasquali-Ronchetti I, Pope FM, Richards A, Terry S, Bercovitch L, de Paepe A, Boyd CD. Mutations in a gene encoding an ABC transporter cause pseudoxanthoma elasticum. Nat Genet 2000;25:223–227. [PubMed: 10835642]
8. Ringpfeil F, Nakano A, Uitto J, Pulkkinen L. Compound heterozygosity for a recurrent 16.5-kb Alu-mediated deletion mutation and single-base-pair substitutions in the ABCC6 gene results in pseudoxanthoma elasticum. Am J Hum Genet 2001;68:642–652. [PubMed: 11179012]
9. Struk B, Neldner KH, Rao VS, St Jean P, Lindpaintner K. Mapping of both autosomal recessive and dominant variants of pseudoxanthoma elasticum to chromosome 16p13.1. Hum Mol Genet 1997;6:1823–1828. [PubMed: 9302259]
10. Lebwohl M, Phelps RG, Yannuzzi L, Chang S, Schwartz I, Fuchs W. Diagnosis of pseudoxanthoma elasticum by scar biopsy in patients without characteristic skin lesions. N Engl J Med 1987;317:347–350. [PubMed: 3600730]
11. Neldner KH. Pseudoxanthoma elasticum. Int J Dermatol 1988;27:98–100. [PubMed: 3360560]
12. Tiozzo Costa R, Baccarani Contri M, Cingi MR, Pasquali Ronchetti I, Salvini R, Rindi S, De Luca G. Pseudoxanthoma elasticum (PXE): ultrastructural and biochemical study on proteoglycan and proteoglycan-associated material produced by skin fibroblasts in vitro. Coll Relat Res 1988;8:49–64. [PubMed: 3345648]
13. Katona E, Aslanidis C, Remenyik E, Csikos M, Karpati S, Paragh G, Schmitz G. Identification of a novel deletion in the ABCC6 gene leading to Pseudoxanthoma elasticum. J Dermatol Sci 2005;40:115–121. [PubMed: 16183260]
14. Chassaing N, Martin L, Mazereeuw J, Barrie L, Nizard S, Bonafe JL, Calvas P, Hovnanian A. Novel ABCC6 mutations in pseudoxanthoma elasticum. J Invest Dermatol 2004;122:608–613. [PubMed: 15086542]
15. Gheduzzi D, Guidetti R, Anzivino C, Tarugi P, Di Leo E, Quaglino D, Ronchetti IP. ABCC6 mutations in Italian families affected by pseudoxanthoma elasticum (PXE). Hum Mutat 2004;24:438–439. [PubMed: 15459974]
16. Hendig D, Schulz V, Eichgrun J, Szliska C, Gotting C, Kleesiek K. New ABCC6 gene mutations in German pseudoxanthoma elasticum patients. J Mol Med 2005;83:140–147. [PubMed: 15723264]

17. Beck K, Hayashi K, Nishiguchi B, Le Saux O, Hayashi M, Boyd CD. The distribution of Abcc6 in normal mouse tissues suggests multiple functions for this ABC transporter. J Histochem Cytochem 2003;51:887–902. [PubMed: 12810839]

18. Noji Y, Inazu A, Higashikata T, Nohara A, Kawashiri MA, Yu W, Todo Y, Nozue T, Uno Y, Hifumi S, Mabuchi H. Identification of two novel missense mutations (p.R1221C and p.R1357W) in the ABCC6 (MRP6) gene in a Japanese patient with pseudoxanthoma elasticum (PXE). Intern Med 2004;43:1171–1176. [PubMed: 15645653]

19. Le Saux O, Beck K, Sachsinger C, Silvestri C, Treiber C, Goring HH, Johnson EW, De Paepe A, Pope FM, Pasquali-Ronchetti I, Bercovitch L, Marais AS, Viljoen DL, Terry SF, Boyd CD. A spectrum of ABCC6 mutations is responsible for pseudoxanthoma elasticum. Am J Hum Genet 2001;69:749–764. [PubMed: 11536079]

20. Uitto J. Pseudoxanthoma elasticum-a connective tissue disease or a metabolic disorder at the genome/environment interface? J Invest Dermatol 2004;122:ix–x. [PubMed: 15086584]

21. Uitto J. The gene family of ABC transporters--novel mutations, new phenotypes. Trends Mol Med 2005;11:341–343. [PubMed: 15996518]

22. Wang J, Near S, Young K, Connelly PW, Hegele RA. ABCC6 gene polymorphism associated with variation in plasma lipoproteins. J Hum Genet 2001;46:699–705. [PubMed: 11776382]

23. Gorgels TG, Hu X, Scheffer GL, van der Wal AC, Toonstra J, de Jong PT, van Kuppevelt TH, Levelt CN, de Wolf A, Loves WJ, Scheper RJ, Peek R, Bergen AA. Disruption of Abcc6 in the mouse: novel insight in the pathogenesis of pseudoxanthoma elasticum. Hum Mol Genet 2005;14:1763–1773. [PubMed: 15888484]

24. Maccari F, Gheduzzi D, Volpi N. Anomalous structure of urinary glycosaminoglycans in patients with pseudoxanthoma elasticum. Clin Chem 2003;49:380–388. [PubMed: 12600949]

25. Le Saux O, Bunda S, Vanwart CM, Douet V, Got L, Martin L, Hinek A. Serum Factors from Pseudoxanthoma Elasticum Patients Alter Elastic Fiber Formation In Vitro. J Invest Dermatol. 2006E-pub ahead of print.

26. Zhang W, Morris QD, Chang R, Shai O, Bakowski MA, Mitsakakis N, Mohammad N, Robinson MD, Zirngibl R, Somogyi E, Laurin N, Eftekharpour E, Sat E, Grigull J, Pan Q, Peng WT, Krogan N, Greenblatt J, Fehlings M, van der Kooy D, Aubin J, Bruneau BG, Rossant J, Blencowe BJ, Frey BJ, Hughes TR. The functional landscape of mouse gene expression. J Biol 2004;3:21. [PubMed: 15588312]

27. Hirohashi T, Suzuki H, Ito K, Ogawa K, Kume K, Shimizu T, Sugiyama Y. Hepatic expression of multidrug resistance-associated protein-like proteins maintained in eisai hyperbilirubinemic rats. Mol Pharmacol 1998;53:1068–1075. [PubMed: 9614210]

28. Aranyi T, Ratajewski M, Bardoczy V, Pulaski L, Bors A, Tordai A, Varadi A. Identification of a DNA methylation-dependent activator sequence in the pseudoxanthoma elasticum gene, ABCC6. J Biol Chem 2005;280:18643–18650. [PubMed: 15760889]

29. Jiang Q, Matsuzaki Y, Li K, Uitto J. Transcriptional regulation and characterization of the promoter region of the human ABCC6 gene. J Invest Dermatol 2006;126:325–335. [PubMed: 16374464]

30. Kuo MH, Allis CD. In vivo cross-linking and immunoprecipitation for studying dynamic Protein:DNA associations in a chromatin environment. Methods 1999;19:425–433. [PubMed: 10579938]

31. Chakrabarti SK, James JC, Mirmira RG. Quantitative assessment of gene targeting in vitro and in vivo by the pancreatic transcription factor, Pdx1. Importance of chromatin structure in directing promoter binding. J Biol Chem 2002;277:13286–13293. [PubMed: 11825903]

32. Christenson LK, Stouffer RL, Strauss JF 3rd. Quantitative analysis of the hormone-induced hyperacetylation of histone H3 associated with the steroidogenic acute regulatory protein gene promoter. J Biol Chem 2001;276:27392–27399. [PubMed: 11346648]

33. McKnight S, Tjian R. Transcriptional selectivity of viral genes in mammalian cells. Cell 1986;46:795–805. [PubMed: 3530495]

34. Xanthopoulos KG, Prezioso VR, Chen WS, Sladek FM, Cortese R, Darnell JE Jr. The different tissue transcription patterns of genes for HNF-1, C/EBP, HNF-3, and HNF-4, protein factors that govern liver-specific transcription. Proc Natl Acad Sci U S A 1991;88:3807–3811. [PubMed: 2023930]

35. Courtois G, Morgan JG, Campbell LA, Fourel G, Crabtree GR. Interaction of a liver-specific nuclear factor with the fibrinogen and alpha 1-antitrypsin promoters. Science 1987;238:688–692. [PubMed: 3499668]

36. Matsuzaki Y, Nakano A, Jiang QJ, Pulkkinen L, Uitto J. Tissue-Specific Expression of the ABCC6 Gene. J Invest Dermatol 2005;125:900–905. [PubMed: 16297187]

37. Mantovani R. A survey of 178 NF-Y binding CCAAT boxes. Nucleic Acids Res 1998;26:1135–1143. [PubMed: 9469818]

38. Mantovani R. The molecular biology of the CCAAT-binding factor NF-Y. Gene 1999;239:15–27. [PubMed: 10571030]

39. Klement JF, Matsuzaki Y, Jiang QJ, Terlizzi J, Choi HY, Fujimoto N, Li K, Pulkkinen L, Birk DE, Sundberg JP, Uitto J. Targeted ablation of the abcc6 gene results in ectopic mineralization of connective tissues. Mol Cell Biol 2005;25:8299–8310. [PubMed: 16135817]

40. Lee LT, Tan-Un KC, Pang RT, Lam DT, Chow BK. Regulation of the human secretin gene is controlled by the combined effects of CpG methylation, Sp1/Sp3 ratio, and the E-box element. Mol Endocrinol 2004;18:1740–1755. [PubMed: 15118068]

41. Siegfried Z, Eden S, Mendelsohn M, Feng X, Tsuberi BZ, Cedar H. DNA methylation represses transcription in vivo. Nat Genet 1999;22:203–206. [PubMed: 10369268]

42. Philipsen S, Suske G. A tale of three fingers: the family of mammalian Sp/XKLF transcription factors. Nucleic Acids Res 1999;27:2991–3000. [PubMed: 10454592]

43. Kuramasu A, Saito H, Suzuki S, Watanabe T, Ohtsu H. Mast cell-/basophil-specific transcriptional regulation of human L-histidine decarboxylase gene by CpG methylation in the promoter region. J Biol Chem 1998;273:31607–31614. [PubMed: 9813077]

44. Kauffmann HM, Vorderstemann B, Schrenk D. Basal expression of the rat, but not of the human, multidrug resistance protein 2 (MRP2) gene is mediated by CBF/NF-Y and Sp1 promoter-binding sites. Toxicology 2001;167:25–35. [PubMed: 11557127]

45. Stockel B, Konig J, Nies AT, Cui Y, Brom M, Keppler D. Characterization of the 5′-flanking region of the human multidrug resistance protein 2 (MRP2) gene and its regulation in comparison withthe multidrug resistance protein 3 (MRP3) gene. Eur J Biochem 2000;267:1347–1358. [PubMed: 10691972]

46. Takada T, Suzuki H, Sugiyama Y. Characterization of 5′-flanking region of human MRP3. Biochem Biophys Res Commun 2000;270:728–732. [PubMed: 10772892]

47. Tanaka T, Uchiumi T, Hinoshita E, Inokuchi A, Toh S, Wada M, Takano H, Kohno K, Kuwano M. The human multidrug resistance protein 2 gene: functional characterization of the 5′-flanking region and expression in hepatic cells. Hepatology 1999;30:1507–1512. [PubMed: 10573531]

48. Tzeng SJ, Huang JD. Transcriptional regulation of the rat Mrp3 promoter in intestine cells. Biochem Biophys Res Commun 2002;291:270–277. [PubMed: 11846400]

49. Zhu Q, Center MS. Cloning and sequence analysis of the promoter region of the MRP gene of HL60 cells isolated for resistance to adriamycin. Cancer Res 1994;54:4488–4492. [PubMed: 8044800]

50. Haimeur A, Conseil G, Deeley RG, Cole SP. The MRP-related and BCRP/ABCG2 multidrug resistance proteins: biology, substrate specificity and regulation. Curr Drug Metab 2004;5:21–53. [PubMed: 14965249]

51. Maher JM, Slitt AL, Cherrington NJ, Cheng X, Klaassen CD. Tissue distribution and hepatic and renal ontogeny of the multidrug resistance-associated protein (Mrp) family in mice. Drug Metab Dispos 2005;33:947–955. [PubMed: 15802388]

52. Duncan SA, Nagy A, Chan W. Murine gastrulation requires HNF-4 regulated gene expression in the visceral endoderm: tetraploid rescue of Hnf-4(−/−) embryos. Development 1997;124:279–287. [PubMed: 9053305]

53. Guo H, Wei J, Inoue Y, Gonzalez FJ, Kuo PC. Serine/threonine phosphorylation regulates HNF-4alpha-dependent redox-mediated iNOS expression in hepatocytes. Am J Physiol Cell Physiol 2003;284:C1090–1099. [PubMed: 12466152]

54. Hayhurst GP, Lee YH, Lambert G, Ward JM, Gonzalez FJ. Hepatocyte nuclear factor 4alpha (nuclear receptor 2A1) is essential for maintenance of hepatic gene expression and lipid homeostasis. Mol Cell Biol 2001;21:1393–1403. [PubMed: 11158324]

55. Andrews NC. The NF-E2 transcription factor. Int J Biochem Cell Biol 1998;30:429–432. [PubMed: 9675875]

56. Talbot D, Grosveld F. The 5′HS2 of the globin locus control region enhances transcription through the interaction of a multimeric complex binding at two functionally distinct NF-E2 binding sites. Embo J 1991;10:1391–1398. [PubMed: 1902783]

57. Fujiwara KT, Kataoka K, Nishizawa M. Two new members of the maf oncogene family, mafK and mafF, encode nuclear b-Zip proteins lacking putative trans-activator domain. Oncogene 1993;8:2371–2380. [PubMed: 8361754]

58. Igarashi K, Kataoka K, Itoh K, Hayashi N, Nishizawa M, Yamamoto M. Regulation of transcription by dimerization of erythroid factor NF-E2 p45 with small Maf proteins. Nature 1994;367:568–572. [PubMed: 8107826]

59. Kataoka K, Igarashi K, Itoh K, Fujiwara KT, Noda M, Yamamoto M, Nishizawa M. Small Maf proteins heterodimerize with Fos and may act as competitive repressors of the NF-E2 transcription factor. Mol Cell Biol 1995;15:2180–2190. [PubMed: 7891713]

60. Hamlin N, Beck K, Bacchelli B, Cianciulli P, Pasquali-Ronchetti I, Le Saux O. Acquired Pseudoxanthoma elasticum-like syndrome in beta-thalassaemia patients. Br J Haematol 2003;122:852–854. [PubMed: 12930400]

61. Aessopos A, Farmakis D, Loukopoulos D. Elastic tissue abnormalities resembling pseudoxanthoma elasticum in beta thalassemia and the sickling syndromes. Blood 2002;99:30–35. [PubMed: 11756149]

Fig 1. Transcription initiation sites of the murine *Abcc6* gene

Asterisks indicate the position of the transcription initiation sites that were identified by 5′-RACE. The putative initiator codons (ATG) are indicated.

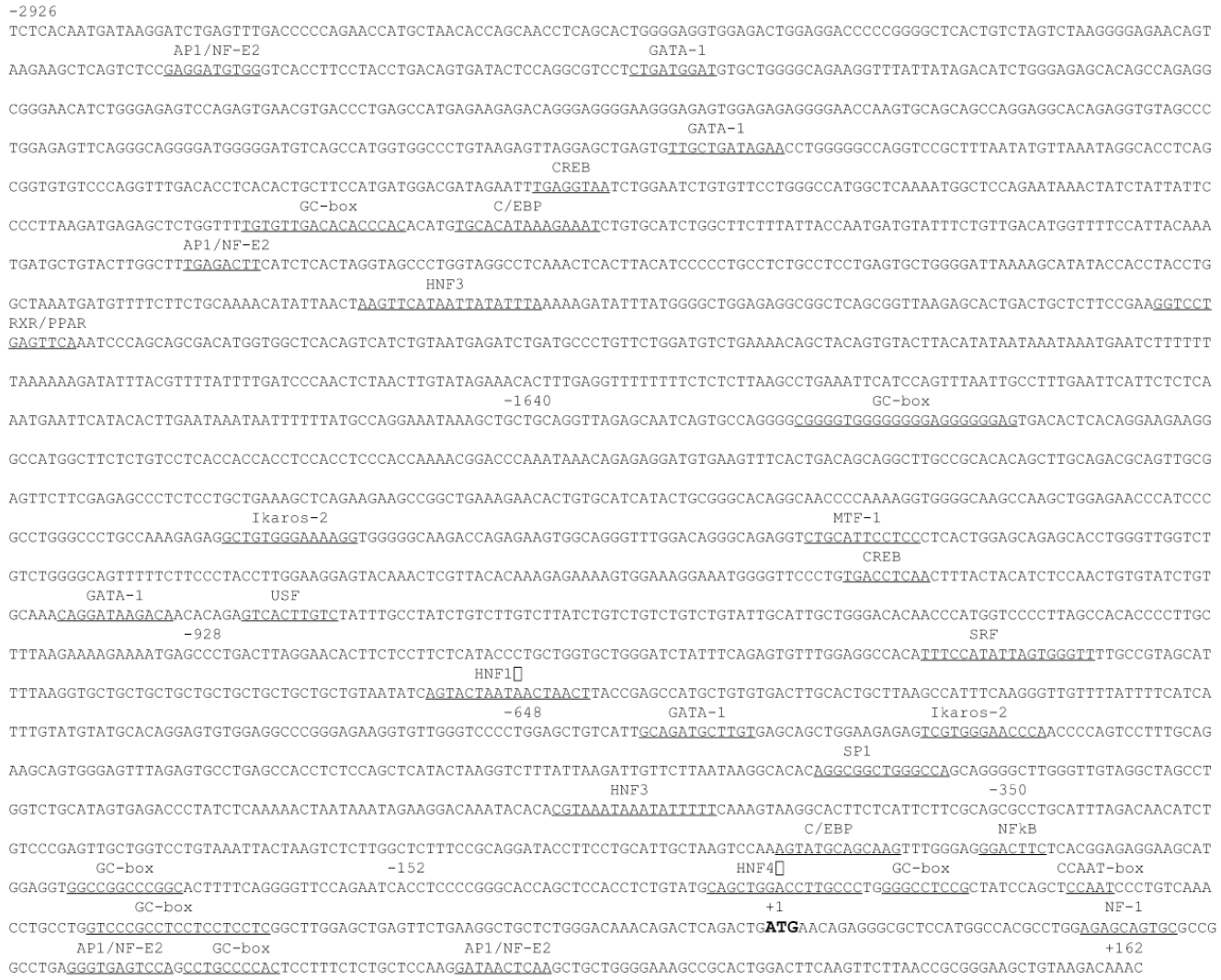

Fig 2. The 5′ flanking sequence of the murine *Abcc6* gene

This sequence encompasses a genomic region from −2928bp to +162bp starting from the adenosine nucleotide of the most 5′ ATG indicated by “+1”. The predictive analysis of putative promoter/enhancer elements in the 5′-flanking promoter region of the murine *Abcc6* was performed using MOTIF, MATINSPECTOR, TFSEARCH. The putative *cis*-elements are underlined.

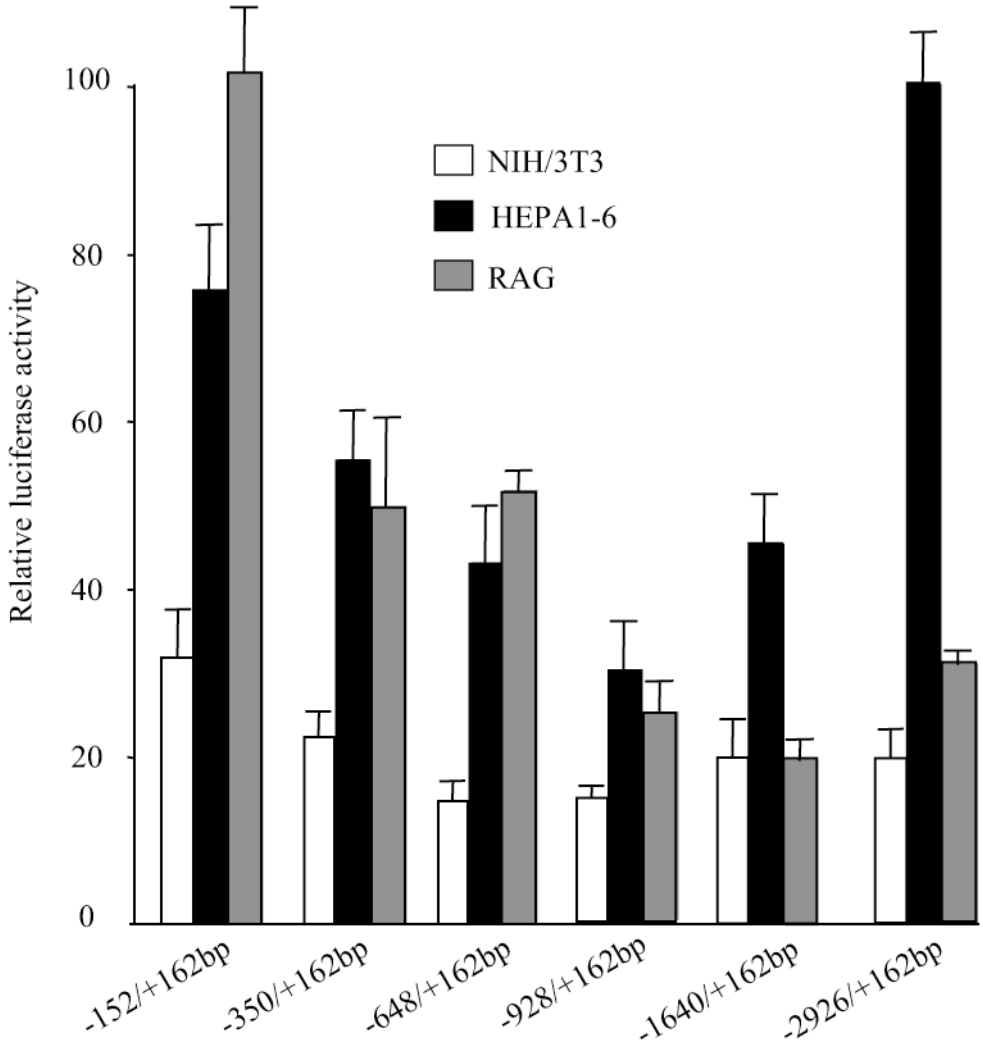

Fig 3. Transcriptional activity of the *mAbcc6* gene promoter

Six reporter constructs were derived from the pGL3 vector and contained fragment of various length of the murine *Abcc6* promoter (−2629, −1640, −928, −648, −350 and −152 to +162bp from the transcription start site). These constructs were transfected into fibroblast (NIH/3T3), hepatoma (Hepa1-6) and kidney cells (RAG). These cell lines were transfected with 1μg of plasmid and 0.1 μg of pRL-SV40 plasmid for control purposes and cultured for 24h. *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 percentage of the −2926/+162bp promoter construct activity obtained in hepatoma (Hepa1-6).

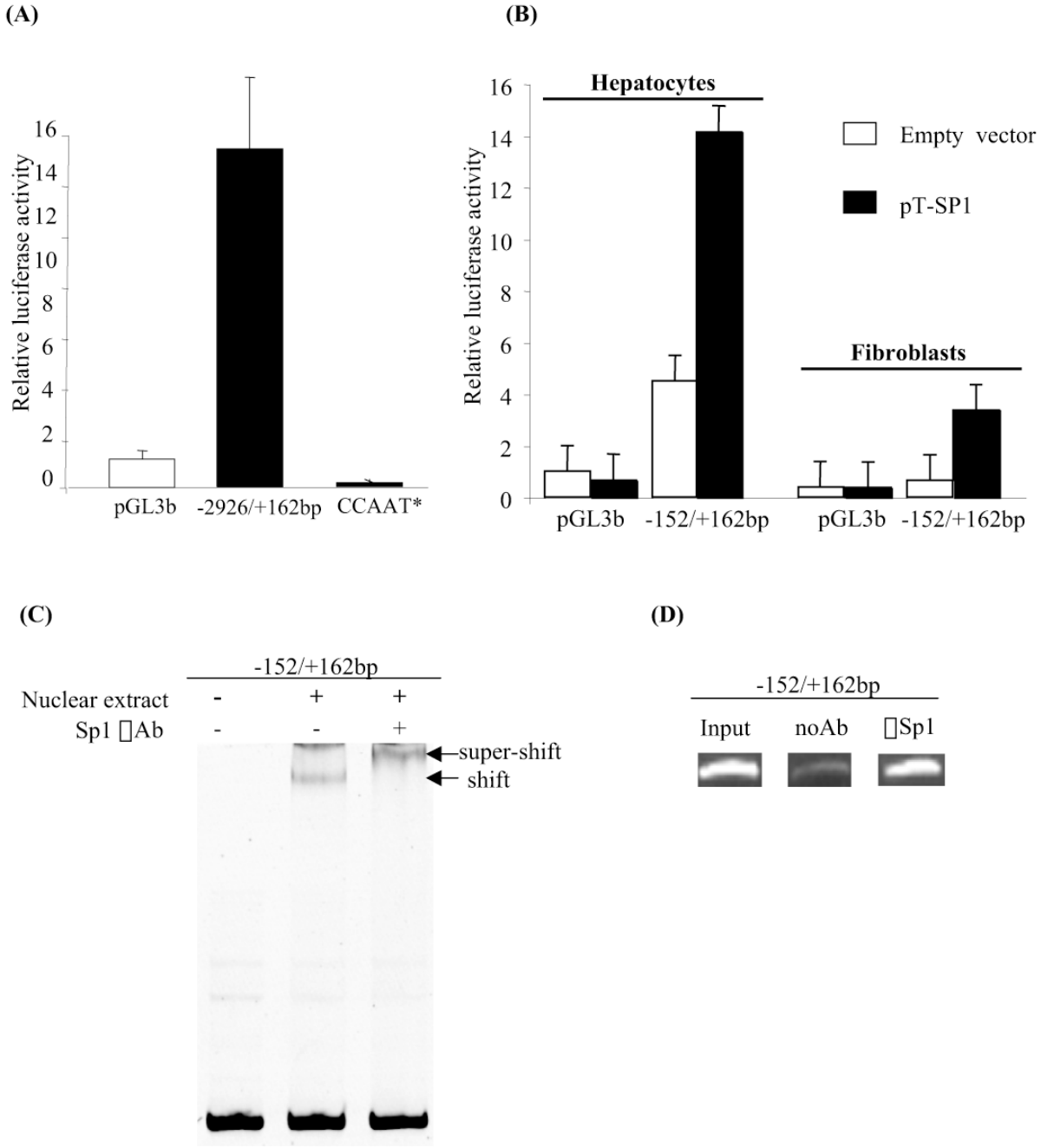

Fig 4. The *mAbcc6* proximal promoter region is a TATA-less gene promoter

(A) *Luciferase* reporter plasmids bearing the −2926/+162bp fragment of the *mAbcc6* promoter and the same fragment with a mutated CCAAT box (indicated as CCAAT*) were transfected into hepatocytes (TIB-73). The *luciferase* activity that was normalized to the transfection efficiency is shown as relative to the promoter-less vector (pGL3 basic). Results were obtained from at least three independent transfection experiments performed in triplicate. **(B)** Hepatocytes (TIB-73) and fibroblasts (NIH/3T3) were co-transfected with a *luciferase* reporter plasmids (pGL3-basic) containing the −152/+162bp fragment of the *mAbcc6* promoter and an expression vector bearing the Sp1 transcription factor cDNA. An empty expression vector was used as a negative control. The level of *luciferase* activity induction is shown as relative to the

pGL3 basic and was normalized to the transfection efficiency. Results were derived from at least three independent transfection experiments performed in triplicate. **(C)** Electrophoretic mobility shift assays (EMSA) were performed with the *mAbcc6* proximal promoter (−152/+162bp) fragment and nuclear extract from hepatocytes (TIB-73). For the supershift assay, 2μg of the Sp1 antibody was pre-incubated with nuclear extracts before adding the −152/162 *mAbcc6* fragment. *(D)* Chromatin Immunoprecipitation assays. Soluble chromatin from TIB-73 cells was immunoprecipitated with the Sp1 antibody (αSp1) or incubated with normal goat serum (noAb) for control purposes. The total extracted DNA (Input) prior to immunoprecipitation and the immunoprecipitated samples (αSp1, noAB) were PCR-amplified using primers specific to the −162/+152bp fragment.

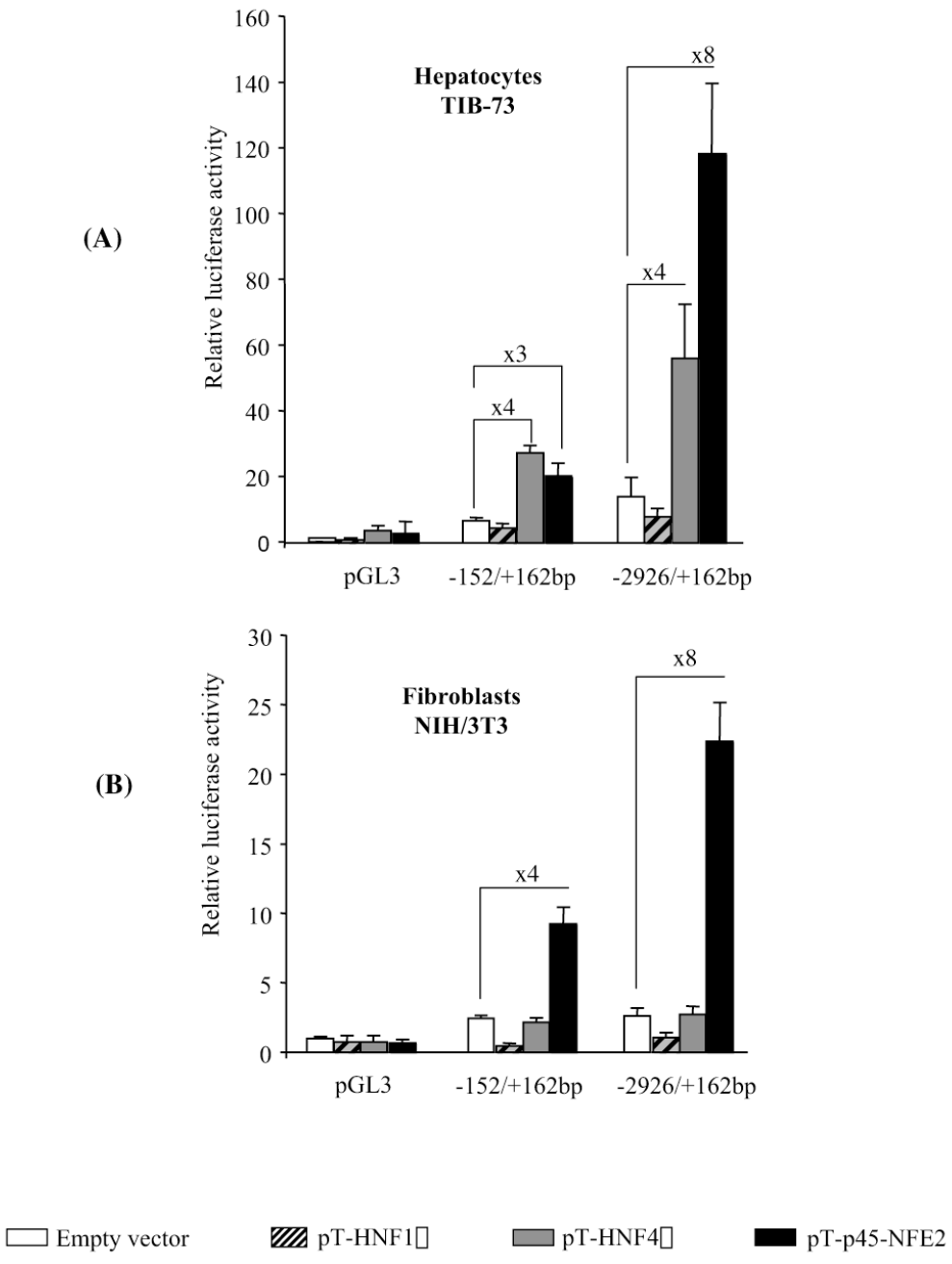

Fig 5. Trans-activation of the *mAbcc6* gene promoter

(A) Hepatocytes (TIB-73) and **(B)** fibroblasts (NIH/3T3) were co-transfected with the *luciferase* reporter vectors bearing two *mAbcc6* promoter fragments (−152/+162bp or −2926/+162bp) and vectors expressing the transcription factors *HNF1α*, *HNF4α* or *p45-NFE2*. For negative control, an empty vector was used. The *luciferase* activities are represented as a relative level of induction (in parentheses) of the −152/+162bp and −2926/+162bp promoter activities normalized to the value obtained from cells co-transfected with each of the promoter constructs and the negative control vector (pTarget) for transcription factor expression. The assays were performed at least three times and in triplicate. Standard errors are indicated.

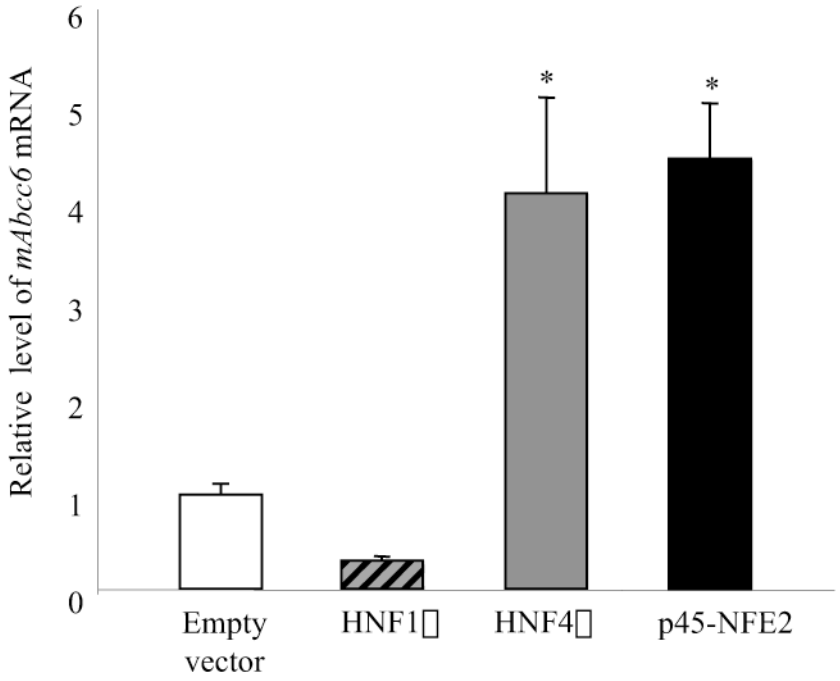

Fig 6. Trans-activation of the endogenous *Abcc6* gene in TIB-73 cells

Hepatocytes were transfected with a vector expressing the *HNF1α*, *HNF4α* or *p45-NFE2* cDNAs. As a negative control, the cells were transfected with an empty vector. The levels of the endogenous *mAbcc6* gene were determined by quantitative PCR with TaqMan probes specific to the murine *mAbcc6* cDNA. The results were normalized to the transfection efficiency by measuring the level of expression of the neomycin-resistance gene present on the vector expressing the transcription factors. The assays were performed three times at least in triplicate. Standard errors are indicated.

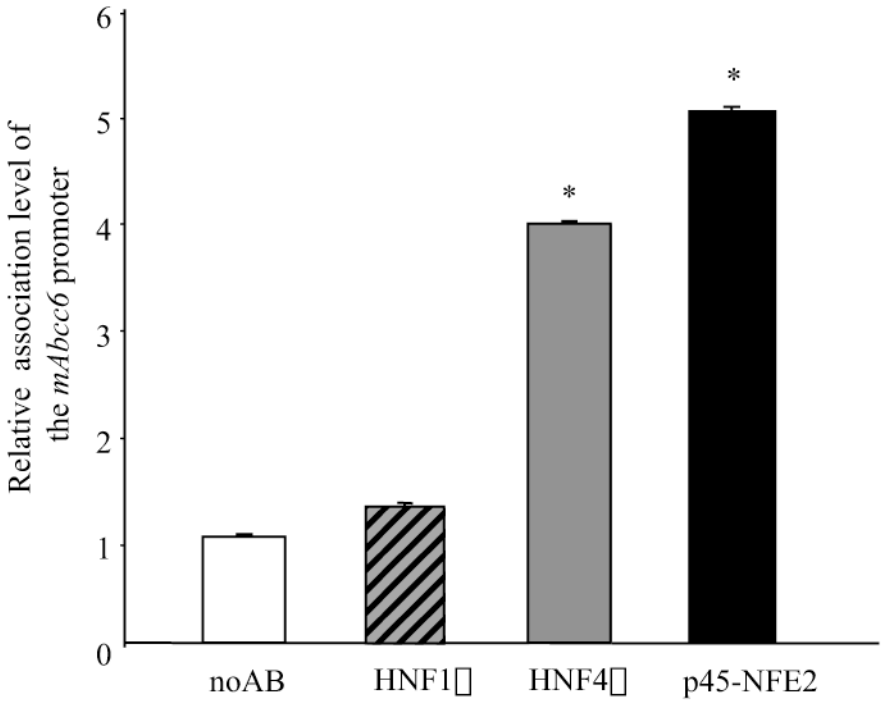

Fig 7. Relative *in vivo* association of the *mAbcc6* promoter with the HNF1α, HNF4 α and p45-NFE2 transcription factors

Fragments of chromatin from TIB-73 cells were immunoprecipitated with anti-HNF1α, -HNF4α and -p45-NFE2 antibodies and quantified by real-time PCR using primers specific to the proximal region of the *mAbcc6* promoter (−152/+162). The data was normalized to the total input of DNA used prior to immunoprecipitation and the control assay (noAB). The differences in relative association are shown as relative to the negative control (noAB). Data represents the mean of 5 independent immunoprecipitations quantified in duplicate assays. Standard errors are indicated.

Table 1

Oligonucleotides used to PCR amplify fragments of the *mAbcc6* promoter

A unique reverse primer was used in combination with 6 other primers that were named according to their position relative to the *mAbcc6* initiator codon

Designation	Sequence
+162bp (Reverse)	5′- GTT TGT CTT ACA GCT TCC CG –3′
−152/+162 (forward)	5′- CAC CAG CTC CAC CTC TGT AT –3′
−350/+162 (forward)	5′- GCG CCT GCA TTT AGA CAA CA –3′
−648/+162 (forward)	5′- CTG GAG CTG TCA TTG CAG AT –3′
−928/+162 (forward)	5′- GCC CTG ACT TAG GAA CAC TT –3′
−1640/+162 (forward)	5′- GCT GCA GGT TAG AGC AAT CA –3′
−2926/+162 (forward)	5′- CAC AAT GAT AAG GAT CTG AG –3′
BamKpn_pGL	5′- TCG ATA AGG TAC CGT CGA CCG –3′
CCAAT*	5′- ATC CAG CTG CTA TCC CTG TC –3′

Table 2

Oligonucleotides used to PCR amplify the cDNA of the transcription factors p45-NF-E2, Sp1, HNF4α and HNF1α.

Designation	Sequence
NFE2_m	5′- ATG CCC CCG TGT CCT CCT CAG C –3′
NFE2_a	5′- TCA ATC TGT AGC CTC CAT TTT GGT TCC –3′
SP1_a	5′- TTA GAA ACC ATT GCC ACT GAT ATT AAT GG –3′
SP1_m	5′- ATG AGC GAC CAA GAT CAC TCC –3′
HNF4_a	5′- CTA GAT GGC TTC TTG CTT GGT GAT CG –3′
HNF4_m	5′- ATG GAT ATG GCC GAC TAC AGC GC –3′
HNF1_a	5′- TTA CTG GGA AGA GGA GGC C –3′
HNF1_m	5′- ATG GTT TCT AAG CTG AGC CAG –3′