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ABCC6 expression is regulated by CCAAT/Enhancer binding protein activating a primate-specific sequence located in the first intron of the gene

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Abstract

Pseudoxanthoma elasticum (PXE), a rare recessive genetic disease causing skin, eye and cardiovascular lesions is characterized by the calcification of elastic fibers. The disorder is due to loss-of-function mutations of the *ABCC6* gene but the pathophysiology of the disease is still not understood. Here we investigated the transcriptional regulation of the gene, using DNase I hypersensitivity assay followed by luciferase reporter gene assay. We identified three DNase I hypersensitive sites (HS) specific to cell lines expressing *ABCC6*. These HS are located in the proximal promoter and in the first intron of the gene. We further characterized the role of the HS by luciferase assay and demonstrated the transcriptional activity of the intronic HS. We identified the CCAAT/Enhancer binding protein β (C/EBP β) as a factor binding the second intronic HS by chromatin immunoprecipitation and corroborated this finding by luciferase assays. We also showed that C/EBP β interacts with the proximal promoter of the gene. We propose that C/EBP β forms a complex with other regulatory proteins including the previously identified regulatory factor HNF4 α . This complex would account for the tissue-specific expression of the gene and might serve as a metabolic sensor. Our results point toward a better understanding of the physiological role of *ABCC6*.

INTRODUCTION

Pseudoxanthoma elasticum (PXE, OMIM 264800) is a rare Mendelian disorder characterized by calcification and fragmentation of the elastic fibers (Varadi *et al.* 2011; Li *et al.*, 2009a; Uitto *et al.*, 2010). Patients in their first decades of life develop yellowish papules at the neck and flexor areas of the body. Plaques of papular lesions and redundant skin may appear during the slowly progressing disease in the axillae, the inguinal regions and the periumbilical area. The skin loses its elasticity and can become wrinkled. Ultrastructural analyses of the dermis show the fragmentation of the elastic fibers and the

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accumulation of calcium deposits, which can be visualized by von Kossa staining. Fragmentation of the elastic fibers of the Bruch's membrane in the eye may lead to neovascularization and potentially severe loss of vision. In parallel, cardiovascular complications and coronary artery disease may complete the syndrome.

Recessive mutations in the *ABCC6* ATP-binding cassette transporter gene lead to the development of the disease (Bergen *et al.*, 2000; Le Saux *et al.*, 2000; Ringpfeil *et al.*, 2000). The mutation detection rate is over 90% (Vanakker *et al.*, 2008) with more than 300 mutations reported and compiled in the *ABCC6* mutation database. Stop codon mutation p.R1141X represents 25% of all observed mutations. It should be noted that PXE is also associated with a promoter polymorphism of the gene and other rare cases of promoter mutations exist as well (Hendig *et al.*, 2008; Schulz *et al.*, 2006). The biochemically well characterized *ABCC6* gene product is a plasma membrane protein transporting a currently unknown molecule to the blood stream (Ilias *et al.*, 2002). Surprisingly the gene has a very restricted tissue-specific expression pattern since it is expressed predominantly in the liver and it is absent from the tissues affected by the disease (Matsuzaki *et al.*, 2005). Skin transplantation experiments between wild-type and knock-out mice and development of parabiotic animals confirmed the role of this expression pattern by showing that the disease should be considered metabolic (Jiang *et al.*, 2009; Jiang *et al.*; Uitto *et al.*, 2010). Recent data on acquired PXE after liver transplantation in human patients (Bercovitch *et al.*, 2011) also corroborate the idea that PXE is a metabolic disorder caused either by the absence of a molecule from the serum secreted by the hepatocytes or the presence of a molecule, which is normally not present (Le Saux *et al.*, 2006).

Several other pathologies are also associated to the PXE phenotype. While PXE is an autosomal recessive disorder and carriers of one loss-of-function mutant *ABCC6* allele have no obvious clinical phenotype, they probably have an increased risk to develop coronary artery disease (Koblos *et al.*, 2010; Trip *et al.*, 2002). Furthermore, when they are carriers of other mutations as well, e.g. in the *GGCX* gene, PXE-like phenotype can develop (Li *et al.*, 2009b). A significant portion (approximately 5%) of beta-thalassemic patients develops PXE-like phenotype (Aessopos *et al.*, 2008; Hamlin *et al.*, 2003) most probably due to the down-regulation of *ABCC6* without any mutation in the gene (Martin *et al.*, 2011).

Although several studies have been conducted to understand the transcriptional regulation of the gene (Aranyi *et al.*, 2005; de Boussac *et al.*, 2010; Jiang *et al.*, 2006) and (Varadi *et al.*, 2011 and references therein), to date only the essential role of hepatocyte nuclear factor 4 α (HNF4 α) is known in the tissue-specific expression of the gene from ours and others work (Bolotin *et al.*, 2010; de Boussac *et al.*, 2010; Douet *et al.*, 2006). We have also shown the regulatory role of the extracellular signal related kinase 1 and 2 (ERK1/2), which exerts its negative action on the expression of the gene via the inhibition of HNF4 α (de Boussac *et al.*, 2010). The role of DNA methylation and several other minor factors has been uncovered however, the mechanisms of relatively high expression levels of the gene in hepatocytes remained poorly understood (Varadi *et al.*, 2011 and references therein). In light of future potential allele-specific therapy of PXE (Le Saux *et al.*, 2011), improvement of PXE-related phenotypes and in order to decrease the *ABCC6* haploinsufficiency associated cardiovascular risks, we aimed to identify crucial missing players in the transcriptional regulation of the human *ABCC6* gene. We therefore searched for further regulatory sequences and identified a primate-specific activator element in the first intron of *ABCC6* with strong activity in the human hepatoma HepG2 and the differentiated colon carcinoma Caco-2 cells. We also demonstrated that the activator interacts with the proximal promoter and binds C/EBP β and probably other presently unidentified factors.

RESULTS

Expression of the *ABCC6* gene in model cell lines

In order to investigate the transcriptional regulation of the *ABCC6* gene, we first chose model cell lines. We selected two cell lines characterized by high and two by low expression level of the gene. From our previous studies we already knew that HepG2 cells have a high expression level (Aranyi *et al.*, 2005); HEK293 and HeLa have very low expression level or do not express the gene (Aranyi *et al.*, 2005; de Boussac *et al.*, 2010). Previous data showed that *Abcc6* has a relatively high expression level in rodent small intestine (Maher *et al.*, 2006; Maher *et al.*, 2005). Indeed, we previously observed an intermediate expression level in Caco-2 cells (Aranyi *et al.*, 2005). These cells are known to spontaneously differentiate to intestinal epithelial-like cells in post-confluent culture (Grasset *et al.*, 1984). We now performed spontaneous differentiation experiments with Caco-2 cells. As a control we confirmed the increased expression of some differentiation markers (Gelebart *et al.*, 2002; Ribiczey *et al.*, 2007). The expression level changes of several ABC transporter genes were also analyzed. While *ABCC1* levels progressively decreased, *ABCC2*, *ABCC3* and *ABCG5* expression level increased in parallel with that of *ABCC6* (not shown). Indeed, after the cells reached 50% confluency, the expression level of *ABCC6* consistently increased until post-confluent day 6 and became similar to that observed in HepG2 cells. These data confirmed that Caco-2 cells can be used as a model system in our study for high expression level of *ABCC6*.

DNase I assay revealed an active genomic region

To identify regulatory sequences of the *ABCC6* gene we performed DNase I hypersensitivity assay (DHA) (Lu and Richardson, 2004). The partial digestion of the genomic DNA in its nuclear context by DNase I reveals transcriptionally active regions due to their increased sensitivity to the enzyme. The great advantage of this technique is that it maps physiologically relevant regulatory sequences in a hypothesis free manner *i.e.* without any prior analysis of the region. We investigated the first intron of the *ABCC6* gene and a 10kb upstream region between *ABCC6* and *NOMO3*, the neighboring gene (Figure 1A). We mapped the hypersensitive sites (HS) of this region by Southern blot. We designed the *ABCC6* specific probe to recognize the 3' end of the first intron of the gene. This location allows the screening for potential HS in the *ABCC6* regulatory sequences with high accuracy (Figure 1A).

After performing DHA in HepG2 cells, three HS were revealed in the studied region (Figure 1B). We identified the 11kb fragment as a product of enzymatic digestion at a HS in the first intron of *NOMO3* gene. The two other fragments of 2.2kb and 1.8kb revealed HS close to the transcription start site (TSS) of the *ABCC6* gene. We decided to further study only the two HS close to the TSS of the *ABCC6* gene since no data indicate that the HS in the *NOMO3* gene plays any role in the regulation of *ABCC6*.

The two closer HS were thus further investigated by the analysis of a 4.7kb DNA fragment containing the first intron and the proximal promoter of *ABCC6*. DHA analyzed by this higher resolution Southern blot showed that the two HS previously identified around the TSS of *ABCC6* were in fact three different HS, represented in the Southern blot now by bands at 2.2kb, 1.7kb and 1.3kb. We named these HS sites from 5' to 3', HS1 to HS3 (Figure 1C).

Both the DNase I digestion and our Southern blot conditions have a resolution of approximately 150bp thus we located HS1 in the proximal promoter of the *ABCC6* gene between -200bp and -50bp, while both HS2 and HS3 are situated in the first intron of

ABCC6 between +310 and +460bp and between +600 and +750bp, respectively (Figure 1C).

Hypersensitive sites are cell-type specific

As *ABCC6* has tissue-specific expression, we next asked whether the HS observed in HepG2 are also cell-type specific. To answer this question we performed DHA on Caco-2, HEK and HeLa cells (Figure 1D and E). Caco-2 cells were approximately 95% confluent. We observed HS1 in the 4.7 kb genomic DNA fragment studied as earlier in the HepG2 cells. However, we obtained only very weak signals for HS2 and HS3, therefore we could not confirm undoubtedly the presence of these sites in Caco-2 cells.

In contrast, when we performed DHA in HEK and HeLa cells, we did not observe any HS in the same region. Indeed, HS1, 2 and 3 observed previously were absent from HEK and HeLa cells when we analyzed the larger 15.7 kb fragment. The entire region seemed to have a much more compact chromatin structure, as the HS in the first intron of *NOMO3* appeared only upon using higher DNase I concentrations in the assay. The detection of this HS served also as a positive control for the experiment (Figure 1E) demonstrating the cell-type specificity of HS1-3.

Functional characterization of the HS in the proximal promoter and the first intron

In the following experiments we investigated the functionality of the different HS and more specifically we focused on the two intronic HS by luciferase assay. We assumed from the localization of HS1 in the proximal promoter that it marks the binding of HNF4 α (de Boussac *et al.*, 2010) (and references therein). We mapped by luciferase assay the different HS with constructs starting at -332 (relative to the translation start site) and giving maximal promoter activation (Aranyi *et al.*, 2005). The constructs were progressively truncated at the 3' end from +960 (Figure 2A).

In HeLa and HEK cells we saw a low basal activity of the -332/+72 and -332/+513 constructs, which corresponds to the activation caused by the -145/+72 region observed in every cell type tested so far, as reported in our previous studies (Aranyi *et al.*, 2005; de Boussac *et al.*, 2010). The addition of the +514/+629 fragment to the -332/+513 construct led to the doubling of the reporter gene activity, which was not further increased by the addition of further intronic fragments (Figure 2A).

Surprisingly in HepG2 and Caco-2 cells, the activity of -332/+960 was approximately 6-8 times higher than the activity of the proximal promoter -332/+72. This activation remained at the same level when the construct was truncated at +688 but dropped to the level of the activity of the proximal promoter when the construct was truncated at +629 (Figure 2A). When we tried to further narrow down the response element, an intermediate activity was observed for the construct -332/+652 relative to the constructs -332/+629 and -332/+688 in both HepG2 and Caco-2 cells (Figure 2B). In our following experiments we have also shown that the 60bp intronic sequence has no enhancer activity in plasmids specifically designed for testing this (not shown). Altogether, our data suggest the presence of at least one strong cell-type specific activator element in the first intron of the *ABCC6* gene between +629 and +688bp, which overlaps with HS3.

Identification of primate specific regulatory elements in the *ABCC6* gene

Seeing the major role in the regulation of *ABCC6* of the sequence overlapping with HS3, we next wanted to identify the regulatory proteins binding the HS3 region. Therefore we performed *in silico* analysis of the 60bp intronic segment. First, we investigated the evolutionary conservation of the region (Supplementary figure 1A). The +629/+652

fragment of the human gene was completely absent from the majority of investigated species except for primates and small conservation in the rabbit and the African elephant. The +653/+688 region remained completely absent from mouse, rat and chicken, while it was present in addition to primates in the African elephant and approximately 50% conserved in guinea pig, cow and rabbit. The *ABCC6* proximal promoter was also investigated by the same bioinformatic approach, which demonstrated the presence of another primate specific sequence, while efficiently identified the HNF4 α binding site in almost all analyzed species (Supplementary Figure 1B).

C/EBP β activates *ABCC6* expression via HS3

Next, we investigated the human sequence for the presence of transcription factor binding sites in the 60bp long intronic region. The search engines indicated only few sites. Interestingly, some of the factors binding the putative sites are enriched in the liver (C/EBP β , HNF3).

We tested the potential role of the transcription factors identified by co-expressing them with our -332/+688 construct and HNF4 α in HeLa cells. We co-expressed HNF4 α since we hypothesized that its presence might be necessary for the activity of the factor(s) binding the putative intronic response element(s). The only transcription factor, which induced the expression of our construct after the co-expression with HNF4 α was C/EBP β (Figure 2C).

Next, we wished to show the binding of C/EBP β to the *ABCC6* intronic activator in the chromatin environment of HepG2 cells, where the gene is endogenously expressed. Therefore, we performed chromatin immunoprecipitation assay with an anti-C/EBP β antibody. We observed a more than 3-fold enrichment of the target region (+503/+700) in the immunoprecipitated fraction, demonstrating thereby the binding of C/EBP β to the first intron of the human *ABCC6* gene in the chromatin context in HepG2 cells (Figure 2D). Altogether, these data prove a major role for C/EBP β in the transcriptional regulation of the human *ABCC6* gene.

Interplay between the proximal promoter and the first intron

In our following experiment we mapped the region responsible for the binding of C/EBP β , as the resolution of the ChIP is not sufficient to locate exactly the response element. Different luciferase constructs were expressed alone or in the presence of C/EBP β or HNF4 α or both in HeLa cells. We found in these experiments at least two C/EBP β binding sites between +513/+629 and +630/+652 (Figure 3A).

In these experiments we have also observed that the overexpression of C/EBP β alone can reach a similar pattern of induction of the constructs than in the presence of HNF4 α , which is therefore not an obligatory partner of C/EBP β in this experimental context (Figure 3A). Nevertheless, these experiments also suggested an interplay between the proximal promoter and the intronic region as concluded by the comparison of luciferase activities of constructs -145/+688 and -332/+688. We further explored the possible interactions between the proximal promoter of *ABCC6* and the intronic hypersensitive sites in HepG2 and Caco-2 cells (Figure 3B) by testing new constructs. The new -234/+688 and -209/+688 constructs were compared with the previously described -332/+688 construct. The -332/+688 and the -234/+688 constructs showed similarly high luciferase activities (Figures 3B). According to our observations in HeLa cells, and in contrast to the longer constructs, the -209/+688 construct showed a low luciferase activity, confirming that the intronic activator sequence is interacting with the -234/-209 fragment and not with the -165/-145 sequence, which binds HNF4 α (de Boussac *et al.*, 2010). Interestingly, the -234/-209 fragment corresponds to the primate specific sequence identified in the proximal promoter (Supplementary Figure 1B).

In our final set of experiments we performed luciferase assays in HepG2, Caco-2 and HeLa cells with constructs harboring mutations at the predicted C/EBP β binding sites to determine the major site of action of the protein (Figure 3C and D). While the mutagenesis of site 1 (+539/+547) had only small effect, the mutagenesis of site 2 (+585/+594) increased the activity of the construct in HeLa cells when co-expressed with C/EBP β (Figure 3C). Therefore the role of the second site could not be evaluated. Interestingly, the mutagenesis of the third, most active site located between +632 and +640 abolished completely the activation of the site 3 by C/EBP β in HeLa cells ($p < 0.01$). Indeed, the activity of the construct mut3(-332/+688) in the presence of C/EBP β was not different from that of the construct truncated at +629 (see Figure 3A). Double mutation of sites 1 and 3 had similar effect as the single mutation of site 3, while the triple mutation of the sites had smaller effect due to the adverse behavior of the mutation at site 2. Deletion of the HS2 region had no significant effect. Similar behavior of the mutant constructs was observed in HepG2 and Caco-2 cells (Figure 3D). However, it should be noted that constructs with mutations at site 3 expressed in HepG2 and Caco-2 still maintained a higher residual activity of the region than expected. This demonstrates that the +630/+652 sequence binds specifically C/EBP β and suggests that additional endogenous activator factor(s) bind(s) the +652/+688 fragment (Figure 3D and not shown).

DISCUSSION

ABCC6 is characterized by a strict tissue-specific expression overlapping with the expression pattern of the HNF4 α transcription factor (Matsuzaki *et al.*, 2005; Sladek FM, 1990). However, HNF4 α and the other previously identified factors seemed to have only a small transcriptional activator potential in the regulation of *ABCC6*, suggesting that other elements are also required since the expression level of the gene is relatively high in hepatocytes. In the present study we have demonstrated that the human *ABCC6* gene is under the control of a strong cell-type specific activator located in the first intron of the gene. We also showed that this sequence specifically binds C/EBP β and at least one further activator protein. Finally, we have shown that to develop its full activity, C/EBP β requires the interaction with the proximal promoter of the gene. Our results are summarized in Figure 3E.

We investigated model cell lines where *ABCC6* is expressed (HepG2 and Caco-2) or is not expressed (HeLa and HEK) to identify further response elements and factors playing an important role in the regulation of the gene. We used DNase I hypersensitivity assay followed by luciferase reporter gene assay. It should be noted that the genome contains two human-specific *ABCC6* pseudogenes, as a result of evolutionarily recent segmental duplications (Pulkkinen *et al.*, 2001; Symmons *et al.*, 2008). Due to the 99% sequence identity of the three loci it was impossible to perform an *ABCC6* specific DHA, *i.e.* the observed HS may reflect any of the three genomic loci. Nevertheless, we considered that since both pseudogenes are expressed (Aranyi *et al.*, 2005; Pulkkinen *et al.*, 2001) and are located within large, almost identical duplicated genomic regions the same HS pattern might characterize all three loci. However, in the following luciferase reporter gene experiments, which were performed to study the functional role of the HS the plasmid constructs contained the *ABCC6* specific sequence.

The approach proved to be efficient and cell-type specific activator elements (HS2 and 3) were found in the first intron of the gene similarly to *CFTR*, another member of the *ABCC* gene family (Smith *et al.*, 1996). The physiologic role of HS3 was in the focus of this study. Interestingly this strong activator sequence turned out to be primate-specific as observed when aligned with other *ABCC6* sequences from different species (Supplementary Figure 1A).

Our bioinformatic analysis predicted the binding of different transcription factors to the 60bp long intronic region of the human gene characterized by increased transcriptional activity. In our experiments only C/EBP β was found to specifically bind and transactivate the sequence. We showed the specific binding of the protein by several methods. ChIP assays demonstrated the binding of C/EBP β to at least one functional site between +200bp and +1kb considering the resolution of our technique (Figure 2D). We have extensively mapped the binding of C/EBP β in the first intron of *ABCC6* by luciferase reporter gene assays as well (Figures 2C, and 3): i/ at least two binding sites exist between +513 and +688 conferring a 5-6-fold activation to the reporter constructs, as shown by co-expression of C/EBP β and HNF4 α with wild-type constructs; ii/ as shown by the study of mutant constructs, the major site is located at +632/+640, which overlaps with the 5' end of a 60bp region of HS3 (Figure 3C and D).

We have also demonstrated that the intronic activator region overlapping with HS3 interacts with HS1 located in the proximal promoter (Figures 3B). We have found that C/EBP β (HS3 region) to elicit its full activity has to interact with the proteins binding the -332/-145 (HS1) region. This latter sequence binds HNF4 α and also harbors the -234/-209 sequence, the site for the second important cell-type specific activator of the proximal promoter needing HNF4 α for its binding according to our previous data (Ratajewski *et al.*, 2009).

In the present study we have shown that the activation of the intronic sequence by C/EBP β needs the presence of the -234/-209 sequence (Figures 3A and B). We hypothesize that in HeLa cells when C/EBP β was overexpressed a protein complex formed including C/EBP β and the protein binding the -234/-209 sequence even in the absence of HNF4 α (Figure 3A, comparison of constructs -332/+688 and -145/+688 coexpressed with C/EBP β). We consider therefore that in these cells the primary interaction is between C/EBP β and the factor binding the -234/-209 sequence. This result also suggests that this hypothetical binding protein (HBP1), which is present in the cells, is unable to fully activate the construct in the absence of C/EBP β (Figure 3E). Although we considered previously that the activator that binds the -234/-209 sequence is a tissue-specific factor, this observation also suggests that HBP1 might be more widely expressed than previously thought (Ratajewski *et al.*, 2009). Since our previous data indicated the dependence of HBP1 binding on HNF4 α binding, we hypothesize that in HepG2 and Caco-2 cells, which reflect much better the physiological regulation of *ABCC6* than HeLa cells, a complex composed of HNF4 α , C/EBP β and HBP1 is playing the major regulatory role in the expression of the gene. Detailed luciferase analyses also showed that the -332/+652 construct had an intermediate activity compared to the -332/+688 and -332/+629 luciferase reporter gene constructs in both HepG2 and Caco-2 cells (Figure 2B). Our data clearly indicate that C/EBP β is the main activator of the +630/+652 region, while another hypothetical binding protein (HBP2) also binds the +652/+688 region. Most probably HBP2 is also included in the complex since in the absence of -332/-145 region the entire +630/+688 region loses its transcriptional activator potential (Figure 3B and E).

Interestingly and in agreement with our findings on the intronic segment, the -234/-209 sequence containing the binding site for HBP1 is not conserved evolutionarily according to our *in silico* analysis (Supplementary Figure 1), suggesting that the highlighted regulatory network is entirely primate specific. This strongly suggests that the rodent *Abcc6* genes are not appropriate models of the transcriptional regulation of the human gene.

C/EBP β is a member of the CCAAT/enhancer binding protein family (Akira *et al.*, 1990). These transcription factors have a basic leucine zipper domain and bind DNA as homodimers or heterodimerize with another member of the family and recognize very similar or identical sequences (Okazaki *et al.*, 2002; Ramji and Foka, 2002). In hepatocytes two C/EBPs, α and

β are highly expressed. Our data therefore do not exclude the potential role of C/EBP α or other C/EBPs in the transcriptional regulation of *ABCC6* in addition to that of C/EBP β . Interestingly, both C/EBP α and β typically activate the transcription of genes playing a role in metabolism by binding their enhancer (Ramji and Foka, 2002). They often act in concert with HNF4 α (Gautier-Stein *et al.*, 2005; Schmidt *et al.*), with which they are major sensors of the metabolic state of hepatocytes.

Currently major objectives of the PXE research field are to understand the pathomechanism of the disease and to develop potential treatments of the various PXE symptoms and PXE associated phenotypes. Our results reported here and in our previous report (de Boussac *et al.*, 2010) both point toward a potential unique role of *ABCC6* among the other ABC transporters in the metabolic processes of the cell. This might indicate that the severity of PXE or various PXE-related phenotypes is directly linked to the metabolic status of the patients, potentially explaining the great variation of the clinical phenotype even within the same family (Varadi *et al.*, 2011). This idea might serve as a basis of paradigm shift after the recently disproved hypothesis on the role of *ABCC6* in vitamin K metabolism (Borst *et al.*, 2008; Fulop *et al.*, 2011) and help to decipher the pathomechanism of the disease.

Finally, our results contribute to the development of treatments of PXE and PXE related conditions as well. Indeed the better understanding of the transcriptional regulation of *ABCC6* might contribute to the success of the recently proposed allele-specific therapy of PXE caused by mis-sense mutations, when the protein is mislocalized but is still active (Le Saux *et al.*, 2011). In such a case inducing the expression of the gene and helping by chemical chaperones the proper targeting of the protein might cure the disease. In conclusion, our results may open new perspectives for targeting research and development for the treatment of PXE, PXE-like and *ABCC6* mutation carrier-state associated phenotypes by focusing on the induction of *ABCC6* expression as a new therapeutic target.

Materials and Methods

Cell culture

Cell lines were obtained from ATCC and cultured according to the manufacturer's instructions. More specifically HepG2 human hepatoma cell line was cultured in advanced MEM supplemented with 10% FBS, 2mM glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin. HeLa and HEK293 cells were cultured in RPMI supplemented with 10% FBS, 2mM glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin. Caco-2 cells were cultured in advanced MEM supplemented with 20% FBS, glutamine and antibiotics like the other cell lines until reaching confluency (D0) and 14 more days as described previously (D14) (Ribiczey *et al.*, 2007). Post-confluent Caco-2 cell cultures were re-fed by renewal of medium every second day.

DNase I hypersensitivity assay

6×10^6 Cells were collected and resuspended in 100 μ l of buffer Ψ (11 mM K-phosphate pH 7.4; 108 mM KCl; 22 mM NaCl; 1 mM MgCl₂; 1 mM DTT; 1 mM ATP) at 4°C, and were then treated with DNase I for 3 minutes on ice. The DNase I digestion was stopped by the addition of 2.5ml of lysis buffer (50 mM Tris-HCl pH 8; 20 mM EDTA; 1% SDS) followed by phenol extraction of the genomic DNA.

Southern blot

20 μ g of DNase I-treated DNA was digested overnight with BsmI or NspI as indicated in the text. A probe located between +1435 and +1735bp (relative to the translation start site). Primers to amplify the probe were designed and tested for specificity (as all primers used in

this study) by BiSearch (Aranyi *et al.*, 2006). The probe was radiolabeled with the DecaLabel™ DNA Labeling Kit (Fermentas) and used for the Southern blot assay. The assay was performed according to standard protocols. Hybridization was carried out in QuickHyb (Stratagene) at 65°C for 3h. Membranes were exposed to hyperfilm (Amersham) for 24-36h at -80°C.

Promoter constructs transfections and Luciferase assay

Luciferase reporter gene assays were performed as described previously (de Boussac *et al.*, 2010). C/EBPβ and other transcription factor expression plasmids were purchased from Origene Technologies Inc. (Rockville, USA). New reporter plasmid vectors containing the ABCC6-PXE promoter sequences were constructed by PCR cloning. Human genomic DNA was used as template for amplification of the construct -332/+960, then this construct was used as template for the amplification of the others. The restriction sites (KpnI site for forward primers and HindIII site for reverse primers) were added to the 5'-end of primers, and promoter sequences were amplified using high fidelity thermostable DNA polymerase (TaqPfu). PCR products were cloned into the pGL3-Basic vector (Promega). The constructs were sequenced to avoid using constructs, which do not match the *ABCC6-PXE* gene sequence.

Site-directed mutagenesis

Mutations of the potential C/EBPβ binding sites in the *ABCC6* promoter were generated by a similar PCR-based method as in our previous study (Ratajewski *et al.*, 2008). C/EBPβ consensus binding site is TKNNGNAAK. Three putative sites are present in the 1st intron of the gene. The sites between +539/+547 (TAATACAAC mutated to TAATTCCCC – mutations in bold) and +585/+593 (GAAGGAAAC mutated to GAAGTACCC) are degenerate, while the third site between +632/+640 (TTGTGAAAC mutated to TTGTTACCC) is consensus. The introduced mutation of C/EBPβ sites was based on a previous report of Hayashida *et al.* (Hayashida *et al.*, 2009; Ratajewski *et al.*, 2008).

The sequences of the mutated inserts were verified by automated sequencing. In double and triple mutant constructs the same mutations were included. To create a HS2 deletion construct we used the same method.

ChIP assay

Chromatin immunoprecipitation and quantification of the transcription factor-bound genomic DNA fragment were performed as described previously (de Boussac *et al.*, 2010). The following antibodies were used: Normal Mouse IgG (part of the EZ-ChIP kit, Millipore) and anti-C/EBPβ (E299, Abcam). Results were normalized to total extracted DNA amount in samples taken prior to immunoprecipitation by the deltaCt method.

Bioinformatics

In silico analysis of the genomic sequence were performed using the following web servers: P-Match <http://gene-regulation.com/cgi-bin/pub/programs/pmatch/bin/p-match.cgi>; Matinspector <http://www.genomatix.de/cgi-bin/sessions/login.pl?s=ed1de7682d64bd03ebafefad4b12b450>; TF-search <http://www.cbrc.jp/research/db/TFSEARCH.html>.

ABCC6 promoter regions from 13 different species were identified and downloaded using the UCSC genome browser (Kent *et al.*, 2002) (<http://genome.ucsc.edu/>). Genomic sequences used are as follows: *Homo sapiens* (GRCh37/hg19 assembly), *Pan troglodytes* (CGSC 2.1/panTro2 assembly), *Macaca mulatta* (MGSC Merged 1.0/rheMac2 assembly),

Callithrix jacchus (WUGSC 3.2/calJac3 assembly), *Mus musculus* (NCBI37/mm9 assembly), *Rattus norvegicus* (Baylor 3.4/rn4 assembly), *Cavia porcellus* (Broad/cavPor3 assembly), *Oryctolagus cuniculus* (Broad/oryCun2 assembly), *Ailuropoda melanoleuca* (BGI-Shenzhen 1.0/ailMel1 assembly), *Loxodonta africana* (Broad/loxAfr3 assembly), *Pongo pygmaeus abelii* (WUGSC 2.0.2/ponAbe2 assembly), *Bos Taurus* (Baylor 4.0/bosTau4 assembly) and *Gallus gallus* (WUGSC 2.1/galGal3 assembly). Multiple sequence alignment was performed using DIALIGN program with default parameters (Morgenstern, 2004).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

PXE	pseudoxanthoma elasticum
C/EBPβ	CCAAT/Enhancer binding protein β
HNF4α	hepatocyte nuclear factor 4 α
DHA	DNase I hypersensitivity assay
HS	hypersensitive site
TSS	transcription start site
HBP1, 2	hypothetical binding protein 1, 2

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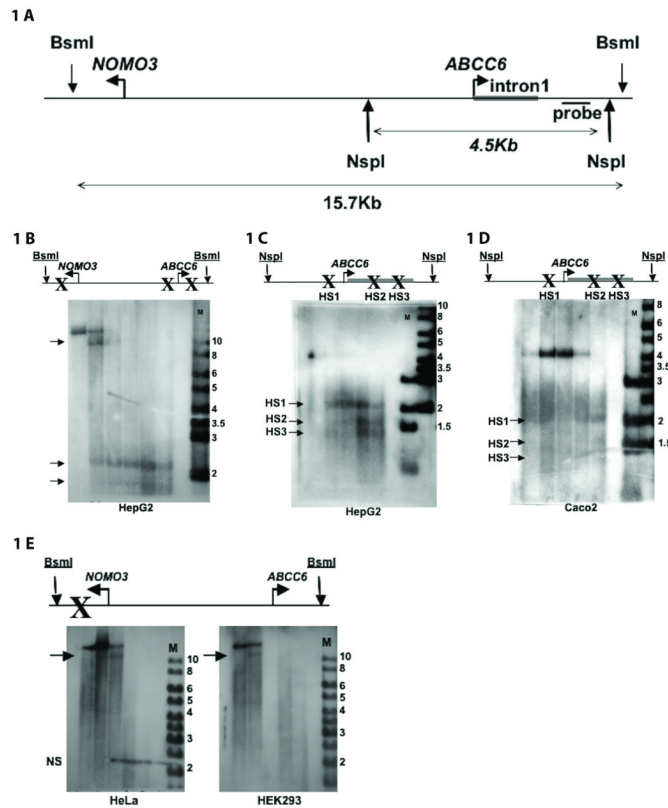


Figure 1. DNase I hypersensitivity assay identifies cell-type specific hypersensitive sites
 A) Map of the *NOMO3* – *ABCC6* intergenic region. B) Detection of HS in HepG2 cells. C) Higher resolution analysis. D) Detection of HS in Caco-2 cells (95% confluency). E) Detection of HS in HeLa and HEK293 cells. Please note that the non-specific (“NS”) band on panel E) does not respect the borders of the loaded lanes. Arrows indicate HS, (M) is a 1kb marker (numbers indicate fragment sizes in kb). HS are indicated by “X” on the schematic genomic map. DNase I partial digestion was carried for 3 minutes with 0, (9), 11, 13, 15, 17 μ g (HepG2), 0, 5, 10, 15, 20 μ g (Caco-2) and 0, 15, 20, 25, 30 μ g (HeLa and HEK293) enzyme.

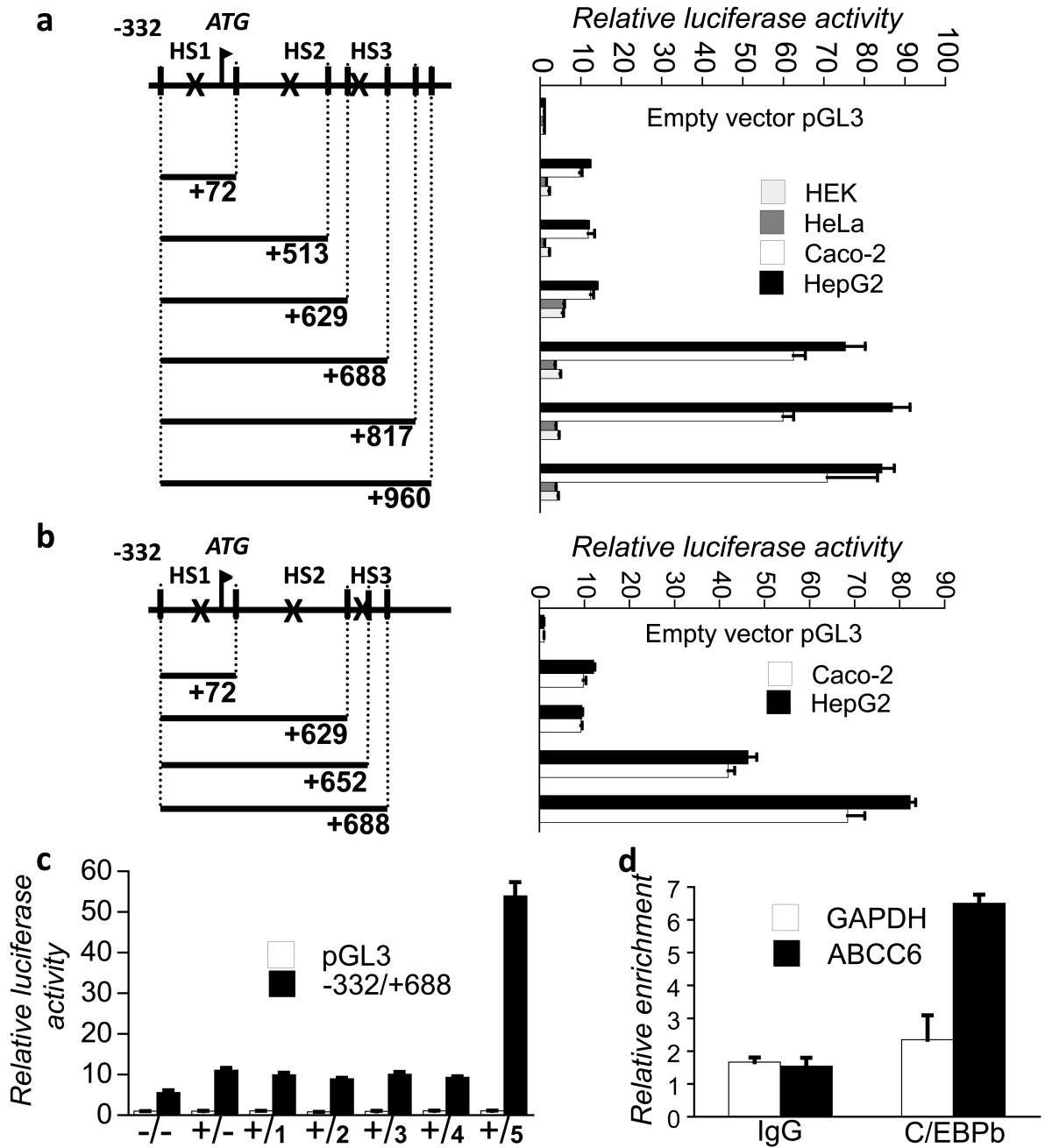


Figure 2. HS3 overlaps with a primate-specific intronic activator sequence, which binds C/EBPβ
 Results of luciferase assays were standardized and expressed as fold induction of the empty pGL3-basic vector, mean±SEM (n = 5). A) Transcriptional activity of the first intron of *ABCC6*. The genomic map indicates the HS by “X”. B) Further mapping of the +629/+688 region. C) Putative transcription factors binding the 60bp sequence were tested in HeLa cells by luciferase assay in co-expression experiment with the -332/+688 construct and a vector containing HNF4α. Factors tested were as follows: 1: USF2; 2: RELA; 3: HNF3beta; 4: ELK1; 5: C/EBPbeta. HNF4alpha overexpression is indicated by + and empty vector is indicated by -. D) ChIP of C/EBPβ in HepG2 cells. *GAPDH* and IgG were used as controls.

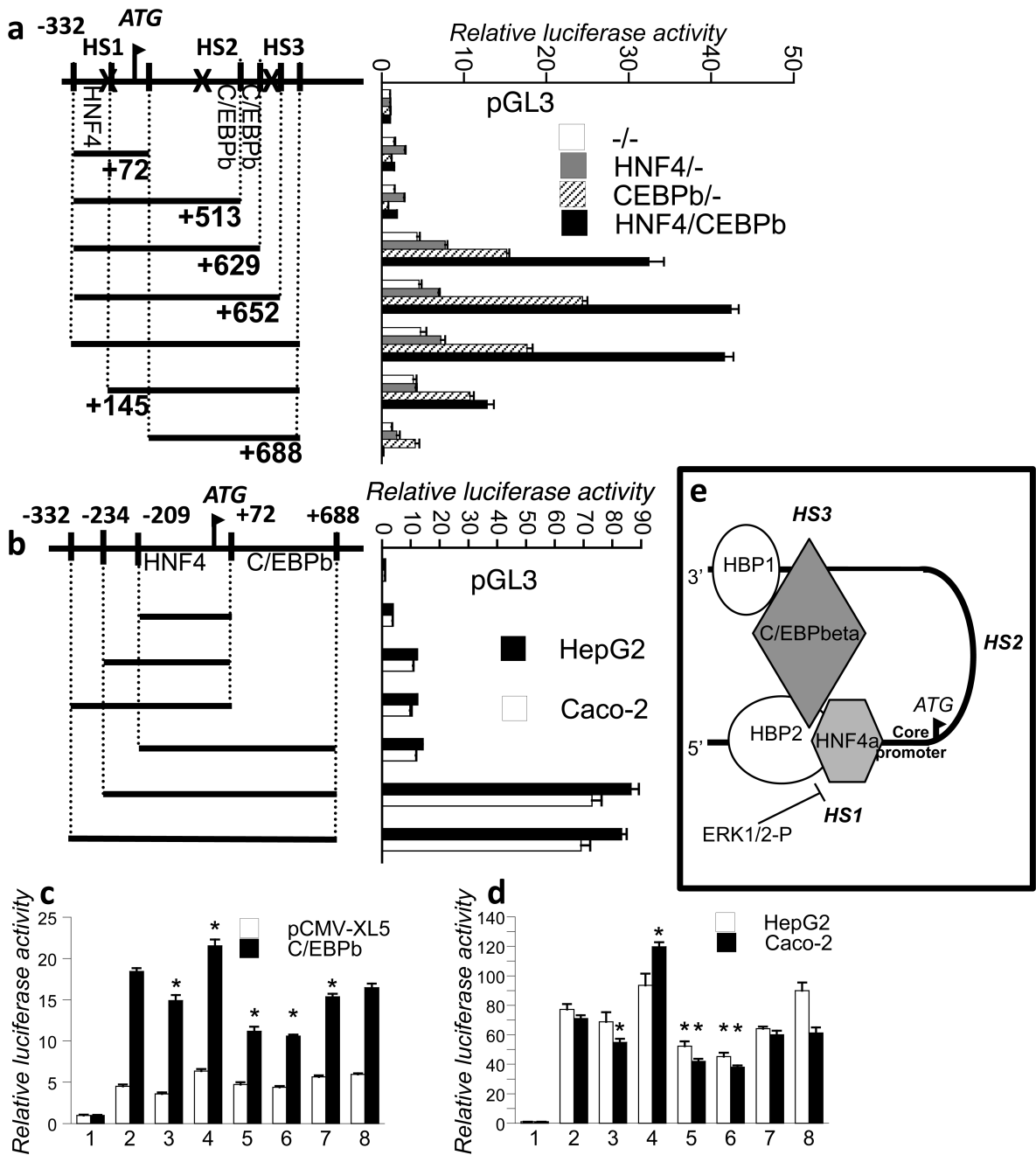


Figure 3. Characterization of C/EBPβ binding to the intronic activator region

Results of luciferase assays were standardized as in figure 2. A) Mapping of C/EBPβ binding site. Luciferase constructs were co-expressed with HNF4α or C/EBPβ or both or none in HeLa cells. B) Interaction of the C/EBPβ binding region and the promoter in HepG2 and Caco-2 cells. ABCC6 luciferase constructs were tested in HeLa cells overexpressing C/EBPβ (C) and in HepG2 and Caco-2 cells (D). The following constructs were tested: 1: pGL3; 2: ABCC6(-332/+688); 3: ABCC6mut1(-332/+688); 4: ABCC6mut2(-332/+688); 5: ABCC6mut3(-332/+688); 6: ABCC6mut1,3(-332/+688); 7: ABCC6mut1,2,3(-332/+688); 8: ABCC6delHS2(-332/+72 +629/+688). Constructs are

described in Materials and Methods. Asterisks indicate significant effect relative to wt. E)
The scheme summarizes a working model for the transcriptional regulation of ABCC6.

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