An Alternative Splice Variant in Abcc6, the Gene Causing Dystrophic Calcification, Leads to Protein Deficiency in C3H/He Mice*

Zouhair Aherrahrou1, Lars C. Doehring1, Eva-Maria Ehlers1, Henrike Liptau1, Reinhard Depping5, Patrick Linsel-Nitschke1, Piotr M. Kaczmarek1, Jeanette Erdmann1, and Heribert Schunkert5

From the 4Department of Medicine II and the 5Institute of Physiology, University of Luebeck, 23538 Luebeck, Germany

Received for publication, October 5, 2007, and in revised form, January 11, 2008
Published, JBC Papers in Press, January 16, 2008, DOI 10.1074/jbc.M708290200

Dystrophic cardiac calcification (DCC) is an autosomal recessive trait characterized by calcium phosphate deposits in myocardial tissue. The Abcc6 gene locus was recently found to mediate DCC; however, at the molecular level the causative variants remain to be determined. Examining the sequences of Abcc6 cDNA in DCC-resistant C57BL/6 and DCC-susceptible C3H/He mice, we identified a missense mutation (Cys to Thr at codon 619, rs32756904) at the 3′-border of exon 14 that creates an additional donor splice site (GT). Accordingly, an alternative transcript variant was detected, lacking the last 5 bp of exon 14 (-AGG(C/T)GCTgtga- in DCC-susceptible C3H/He mice that carry the Thr allele. The 5-bp deletion was found to result in premature termination at codon 684, in turn leading to protein deficiency in DCC-susceptible mouse tissue as well as in cells transfected with Abcc6 cDNA lacking the last 5 bp of exon 14. All mouse strains that were found to carry the Thr allele, including C3H/He, DBA/2J, and 129S1/SvJ, were also found to be positive for DCC. In summary, we identified a splice variant leading to a 5-bp deletion in the Abcc6 transcript that gives rise to protein deficiency both in vivo and in vitro. The fact that all mouse strains that carry the deletion also develop dystrophic calcifications further suggests that the underlying splice variant affects the biological function of MRP6 protein and is a cause of DCC in mice.

Dystrophic cardiac calcifications (DCC)2 are calcium phosphate deposits outside osseous tissue that occur independently from calcium and phosphate homeostasis. In western countries such calcification can affect the arterial system in patients with atherosclerosis, diabetes mellitus, and chronic renal failure. Interestingly, similar deposits are also observed in pseudoxanthoma elasticum (PXE), a heritable disorder of the connective tissue that prominently affects the arteries of skin, eye, and heart.

Both DCC and PXE were reported to develop spontaneously in aging mice and human, respectively. DCC, however, may also be induced in mice by various stimuli such as infectious agents (1, 2), hormonal status (3–5), diet (5–9), and freeze-thaw injury (10, 11).

Based on gene mapping data, recent studies identified Abcc6 as causative gene for both DCC and PXE, in mice and in human, respectively. The genetic basis of DCC was initially facilitated by the identification of a major QTL locus named Dyscale1 on mouse chromosome 7 that contributes to DCC (12). We ultrafine-mapped this locus to an 80-kb region on proximal mouse chromosome 7 that contains only two known genes, epithelial membrane protein-3 (EMP-3) and the ATP binding cassette C 6 (Abcc6), and one gene of unknown function (BC013491) using an in silico mapping strategy (13). In parallel, the locus for PXE was identified on human chromosome 16p13.1 to a 500-kb region that also includes Abcc6 (14, 15). Subsequently, using knock-out and transgenic mouse models, the Abcc6 gene was demonstrated to cause both DCC and PXE (16–18).

Abcc6 belongs to the large ABC family containing nearly 48 genes. ABC proteins bind and hydrolyze ATP to meet the energy requirement for transportation of various molecules across the plasma membrane. Similar processes may take place at intracellular membranes of the endoplasmic reticulum, peroxisome, and mitochondria (19). ABC proteins are involved in the transport or removal of toxic metabolites using substrates such as glutathione (20). The Abcc6 gene encodes a 165-kDa protein named MRP6. It is predominantly expressed in the liver and to a lesser extent in the kidney. Interestingly, these tissues are not predominantly affected by either DCC or PXE (21, 22). Here we present an approach for identification and functional characterization of a primary genetic variant causing DCC in mice.

EXPERIMENTAL PROCEDURES

Animal Housing and Histological Analysis—Animal studies were performed in accordance with the German animal studies committee of Schleswig-Holstein. Female mice from C57BL/6j (C57) and C3H/Hej (C3H) inbred strains were purchased from Charles River Laboratories.

Five mice in each group were sacrificed at age 6–8 months under anesthesia by cervical dislocation. Tissues were prepared...
Abcc6 Splice Variant Leads to Protein Deficiency in Mice

| Gene symbol | Primers | Forward | Reverse | Product (bp) |
|-------------|---------|---------|---------|--------------|
| Abcc6       | 1       | CCAACCTGGAGAGGGAAT | CCAACACTGTGGCCCTTTT | 814 |
|             | 2       | GCCTCTGTGACGCTACAGGG | GCTCTTGACGCTGAGCTGT | 812 |
|             | 3       | AGAAGAGGCGCTCCCATCACG | CCTCTGAGGAGCAGTACAGC | 687 |
|             | 4       | GAAATCTCCTCTCTACTGCTG | TCTCTGACTCATCTGCTG | 751 |
|             | 5       | ATGGAGGACCTCTCTGATGGA | GACGAGATGGAGAAGCACA | 670 |
|             | 6       | GCTGAGGAGCTGACGCTCT | GACGAGCTGAGATGGAGT | 690 |
|             | 7       | AAGTCCTCCCCGCTGCTG | TCTGAGGCTCATTGATCC | 672 |
|             | 8       | GGGTCCTGTTAAGATAGAT | TGAGAGCTGACTGATCCT | 447 |
|             | 9       | GTGAGGAGCAGATTAGCAGG | GAGTAGAAATACGAGGAG | 700 |
|             | 10      | GAAATACACGGAAGGAG | CGAGAGCTGAGAAGCACA | 653 |
| del-5bp-ex14 |         | AGAAGAGGCGCTCCCATCACG | CCTCTGAGGAGCAGTACAGC | 156 |

as previously described (13). Slides were stained using alizarin red S and calcine stains for analysis of calcium phosphate deposits (13, 23).

Reverse Transcriptase PCR and mRNA Quantification—Total RNA was extracted from liver and reverse-transcribed into complementary DNA (cDNA) as previously described (13). Changes in mRNA levels were determined using the ΔΔCt method as previously reported (11, 13).

Abcc6 cDNA Sequencing—Primer pairs (Abcc6 1–10) covering the 5'-UTR, the coding region, and the 3'-UTR of the Abcc6 gene (Ensembl Transcript ID ENSMUST00000002850) were designed on-line (Table 1). Direct sequencing was performed as described previously (13) on PCR fragments amplified from C57 and C3H mice. Sequencing of PCR products was performed on both strands by a commercial sequencing service (Seqlab, Goettingen, Germany).

PCR Amplification and Electrophoresis—An Eppendorf MasterMix™ (2.5×) containing dNTP, buffer, and polymerase was used to amplify Abcc6 cDNA fragments following the instruction of the manufacturer. Amplicons were analyzed after electrophoresis on a 1% QBA-Agarose™ gel (Qbiogene) and Sybr Green red S and calcein stains for analysis of calcium phosphate deposits (13, 23).

Changes in mRNA levels were determined using the in vitro system for [35S]methionine (TNT Coupled Reticulocyte Lysate System; Promega) according to the manufacturer’s protocol. [35S]Methionine was obtained from Hartmann Analytic (Brasnchewig, Germany). After incubation, 1 μl of the reaction batch was dissolved in Laemmli buffer and proteins were separated by SDS-PAGE (10%). To detect the [35S]-labeled proteins, the dried gels were autoradiographed (16 h). The gels were analyzed using the software PCAS 2.09g (Raytest Isotopenmessgeräte GmbH).

Cell Culture and Cell Transfection—Human embryonal kidney cells (HEK-293) were kindly provided by Dr. Stefanie Stoelting (University of Luebeck), and the cells were grown in complete Dulbecco’s modified Eagle’s medium (1×) with glucose 4.5 g/liter and L-glutamine-pyruvate, containing 15% fetal calf serum and penicillin/streptomycin (1×) at 37 °C and 5% CO2. One day prior to transfection, cells were plated at a density of 1 × 10^5 into Lab-Tek® 4-well glass chamber slides (Nalge Nunc Int.). The cells were transfected with DNA from pSG5, pSG5-Abcc6, and pSG5-Abcc6-5bpdel plasmids using nanojet transfection reagent and following the instruction of the manufacturer (PAA Laboratorie GmbH). Two days after transfection, cells were fixed in 70% ethanol and analyzed immunohistologically. Similarly, cells were plated into 24-well plates and transfected with the three constructs for Western blot analysis.

Immunohistological Analysis—Immunostaining was performed with a polyclonal anti-mouse MRp6-S20 antibody at 5 μg/ml (Santa Cruz Biotechnology, Inc.) as previously described (11). Briefly, bound MRp6 primary antibodies were detected using biotinylated secondary antibodies that were visualized using a streptavidin-horseradish peroxidase complex and diaminobenzidine as supplied with the Cell and Tissue Staining kit (R&D systems). Slides were then counterstained with hematoxylin. Controls were performed without primary antibodies.

Western Blot Analysis—Protein extracts were prepared from the liver of mice following standard protocols. Liver tissue samples frozen in liquid nitrogen were homogenized using a mortar. Lysis buffer (1× cell lysis buffer (Cell Signaling), 1× Roche cocktail (Roche Diagnostics GmbH), and 1 mM Phenylmethane-
**Abcc6 Splice Variant Leads to Protein Deficiency in Mice**

suflonyl fluoride (Sigma) containing urea (8 M) was added, and samples were centrifuged. After protein quantification, 30 µg were sampled and separated on a 6.5% SDS-PAGE and afterward electrotransferred to an Immobilon-P transfer membrane (Millipore, Bedford, MA). After blocking with 5% lowfat milk, membranes were incubated with the primary antibodies MRP6-S20 (Santa Cruz Biotechnology) and α-actin from (Abcam plc). The epitope of the polyclonal MRP6 has a length of ~15–25 aa and maps within aa 1–50 at the N terminus of MRP6 of mouse origin (Swiss Prot protein accession number Q9R1S7). For signal detection, the ECL-plus Western blotting detection system (RPN, 2132; GE Healthcare) was used. Chemiluminescence detection was performed with a Molecular Imager ChemiDoc XRS system (Bio-Rad).

**TABLE 2**

Genomic sequence analysis of Abcc6 and its respective SNP genotypes repartition on DCC-resistant C57 and DCC-susceptible C3H mouse strains

| Mbp     | SNPs      | Flanking sequence                  | Amino acid substitution | Position in cDNA |
|---------|-----------|------------------------------------|-------------------------|------------------|
| 45897184 | NT_039424.6_6916921 | GTGTATGAGCC (G/A) CAGCTCCTTG | p.A28V                  | ENSMUST0000002850 |
| 45897156 | NT_039424.6_6916895 | CGGCTTCTCAC (A/G) AGCCACAGGC | p.V705M                 | c.283            |
| 45888663 | NT_039424.6_6908400 | CACAGAGGCA (C/T) GGGGAGTCCG | p.S138A                 | c.412            |
| 45888260 | NT_039424.6_6907997 | GGGGATGCGG (T/G) TACCGGGGTT | p.J151V                 | c.451            |
| 45888221 | NT_039424.6_6907958 | CCTGCGAAGA (T/G) GCGCAAGAGC |                         |                  |
| 45882232 | rs3275366 | CTGCGACGAG (C/T) GAGGAGTGGC |                         |                  |
| 45880089 | NT_039424.6_6900426 | CACCGCAGCC (G/A) ACCGGAGACT |                         |                  |
| 45880062 | rs32756118 | CACCGGCTG (G/A) TGTCCACGCG | p.R619S                 |                  |
| 45880584 | rs32756117 | AGGCAGGCAA (C/T) ACCAGCGTG |                         |                  |
| 45873318 | rs32429747 | ACCACACAG (C/T) GCTCAAGGAA |                         |                  |
| 45870021 | rs32756094 | AGGAGGCTG (G/A) GCCGAGAGAC |                         |                  |
| 45866857 | rs32753990 | GAGGCAGCAA (A/C) GCCGGAGAAC |                         |                  |
| 45866816 | rs32753988 | CCAGGTCTCC (A/C) CCAGAGGCT |                         |                  |
| 45866788 | rs32753987 | AGAGCGAGG (A/C) TCTGAGACCT |                         |                  |
| 45865814 | NT_039424.6_6848551 | TTCTGTAACG (A/G) GCCGAGAGCA |                         |                  |
| 45867806 | rs32757904 | GGGAGCTCTG (T/G) TGGTACACGCG |                         |                  |
| 45857780 | rs32757080 | GTATAAGGG (G/A) TCTTACACCG |                         |                  |
| 45854592 | New       | TGAGAGAGGC (A/T) GCACATCCTC |                         |                  |
| 45854451 | NT_039424.6_6874188 | CTGCCAGGCC (G/A) AAGTGTTCTCG |                         |                  |
| 45847761 | rs32753295 | TCCAGCCTGC (G/C) CTGAGTCCTG |                         |                  |
| 45845359 | rs32753722 | CACGGCTTCC (A/G) GGGGGCTGTC |                         |                  |
| 45845291 | rs32753714 | CCTTCCCTGCC (A/G) AGTCCTACCA |                         |                  |
| 45844875 | rs32757080 | TTGAGACTCT (T/G) AGTTCCAGGA |                         |                  |
| 45844865 | rs32752435 | CGGGGTTTGG (T/A) AAGATCCTT |                         |                  |
| 45844518 | New       | CCCTGATCAC (T/C) CACCAGGACG |                         |                  |
| 45844358 | NT_039424.6_6864095 | TAAGTTGAAT (C/T) CTTAATCCAA |                         |                  |
| 45844325 | rs32751538 | CAAGCAGAAA (G/C) AAGATTTCCG |                         |                  |

**FIGURE 1. MRP6 expression.** Western blot analysis of MRP6, the encoding protein of Abcc6. A, dramatically reduced MRP6 expression is demonstrated in the liver of C3H mice as well as congenic (Cg1) mice in comparison to C57 mice. 30 µg of total protein was loaded in each lane. α-Actin was used as loading control. B, quantification of the level of MRP6 expression from Western blots (n = 5) in each mouse strain, C57, C3H, and Cg1, after normalization to α-actin. The band intensity and size was determined using the software Quantity One® (Bio-Rad). The expression of MRP6 in the wild type C57 mice is set as 100%. Error bars indicate S.E.

**Statistical Analysis**—Data analyses were performed by Student’s t test, and results are expressed as the means ± S.E., with p < 0.05 as significant.

**RESULTS**

**MRP6 Protein Expression in Mice**—Previous reports have shown that the Abcc6 gene product MRP6 is highly expressed in the liver. To find evidence of an association or correlation between the level of MRP6 protein expression and the observed DCC phenotype in mice, we analyzed MRP6 expression in the liver of DCC-susceptible C3H and DCC-resistant C57 mice as well as congenic mice (Cg1). Western blot analysis revealed a dramatic decrease in MRP6 expression in the liver of both DCC-susceptible C3H and Cg1 mice compared with liver tissue from DCC-resistant C57 mice (Fig. 1, A and B). With the MRP6 protein expression level in the liver of C57 mice defined as 100%, the expression levels were determined to be 34 ± 12 and 33 ± 17% in C3H and Cg1 mice, respectively.

**Sequencing of Abcc6 cDNA**—To search for novel genetic regulatory elements that might affect the Abcc6 gene expression and thus of the corresponding encoded protein MRP6, we sequenced the 5’-UTR, the coding region, and the 3’-UTR in the cDNA of Abcc6 from DCC-resistant C57 and DCC-susceptible C3H mouse. Comparing the genomic sequences between the two inbred strains of mice, we...
identified a total of 26 SNPs (Table 2). Of these, 25 were known and one SNP was not published previously. Among these SNPs nine resulted in amino acid substitutions (p.A28V, p.V95M, p.S138A, p.I151V, p.R619S, p.V706A, p.K767R, p.T927I, p.A1368T). In addition, we identified two novel small deletions within this region. The 3′-UTR of DCC-susceptible C3H mice was found to contain a 10-bp deletion (GAGCA(TCACAC-GAGAC/del)TCTGA), which is located in a region important for mRNA stability. C3H mice were also found to be heterozygous for another novel 5-bp deletion that was identified at the 3′-border of exon 14.

Alternative Splice Variant in DCC-susceptible C3H Mice—To confirm the above mentioned heterozygosity for the 5-bp deletion in exon 14, we designed new primer pairs flanking this region (Table 1, del-5bp-ex14 F/R), producing an ~150-bp PCR product, suitable for separating on a high resolution 4% MetaPhor gel. Fig. 2 shows three bands in C3H mice (Fig. 2A, lane C3H, arrows): the 5-bp deletion (Mut), the wild type (Wt), and an extra band (Hd). In contrast, the C57 mice contain only one band (Fig. 2A, lane C57). To further analyze the bands in C3H mice, we subcloned the PCR product using the

FIGURE 2. Identification of an alternatively spliced Abcc6 transcript in DCC-susceptible C3H mice. Arrows designate bands of the Abcc6 PCR product flanking the splice site. A, lanes C57 and C3H are PCR products from DCC-resistant C57 and DCC-susceptible C3H mice, respectively; lanes C-1 and C-2 are PCR products from two clones after subcloning of the reverse transcription PCR product from C3H mice. Hd, heteroduplex band; Wt, wild type (C57, 156 bp); Mut, mutant (5-bp deletion from C3H mice, 151 bp) B, PCR products from each clone: C-1 (lane C-1) and C-2 (lane C-2) as well as the mixture of C-1 and C-2 (lane C-1/C-2) were separated by electrophoresis. M, the 100-bp scale marker is used to show the position of the predicted PCR products (the 100-bp band has moved out of the gel and the remaining 200-bp band is shown in lane M).

FIGURE 3. A single base pair mutation creates an additional donor splice site in C3H mice. A, presentation of the exon-intron boundary in the Abcc6 pre-mRNA transcript between ex14 and ex15 in C3H and C57 mice. Exon and intron regions are represented by capital and lowercase letters, respectively. Original splice donor (gu) and splice acceptor (ag) are underscored in the intron between ex14 and ex15. SNPs (C/U) are shown in red in the pre-mRNA sequence between C57 and C3H. The new splice donor site (GU) is underscored in ex14 of C3H mice. B, DNA sequence electropherograms of Abcc6 gDNA (genomic DNA) and cDNA (complementary DNA) in C57 and C3H mice. Arrows depict the Cys to Thr base pair change between C57 and C3H mice. The 5-bp deletion in the cDNA sequence in C3H mice (triangle) is shown after transcription in comparison to the wild type C57 mice (underscored).
Abcc6 Splice Variant Leads to Protein Deficiency in Mice

TOPO TA Cloning® kit and screened insert clones for differences in insert size. We found inserts with two different sizes (Fig. 2A, lanes C-1 and C-2), the wild type (C-1) and the 5-bp deletion (C-2). We then sequenced the two inserts after PCR amplification using the M13 universal primers (C-1 and C-2).

We did not find clones with an insert that corresponds in size to the 5-bp deletion. Fig. 2B shows the extra band (Hd) present in this mixture.

pR619S Mutation Creates an Alternative Splice Variant—To find the genomic mutation creating the alternative splice variant, we further analyzed the genomic sequence variation that had been found between C57 and C3H mice in exon 14 (ex14) and identified a single base pair mutation (C/T) within the 5-bp deletion in ex14 (−AGG(C/T)GCTgtga−). In C3H mice, this mutation leads to an amino acid substitution at position 619 (p.R619S). Interestingly, in pre-mRNA of C3H mice, this mutation creates an additional splice donor site (GU) at codon 619, thus effectively resulting in deletion of the last 5 bp (GUGCU) of exon 14 (Fig. 3). This deletion creates a premature stop codon at codon 685, in turn giving rise to a truncated protein of only 684 aa in C3H mice instead of 1449 aa in C57 mice (Fig. 4).

In Vitro Overexpression of the Truncated Protein from a Construct Carrying the 5-bp Deletion—To examine whether the Abcc6-del5bp mRNA construct encodes an expressed protein, we tested the transcription and translation of pSG5, pSG5-Abcc6, and pSG5-Abcc6-del5bp plasmids in a TNT Coupled Reticulocyte Lysate System. Fig. 5A shows two bands with two different molecular masses, respectively: The predicted wild type protein band (MRP6) of nearly 165 kDa and an additional band (~50 kDa) corresponding to the truncated protein (MRP6-trun). Similar protein bands could be also demonstrated by Western blot analysis using an anti-MRP6 polyclonal antibody that recognizes epitope in the N-terminal region after transcription of HEK-293 cells with both pSG5-Abcc6 and pSG5-Abcc6-del5bp (Fig. 5B).

Moreover, we analyzed the expression level of both pSG5-Abcc6 and pSG5-Abcc6-del5bp constructs in HEK-293 cells at RNA and protein level using relative real-time reverse transcription PCR and immunohistological analysis, respectively. In comparison to negative controls (empty pSG5 vector), induction in cells transfected with pSG5-Abcc6-del5bp and pSG5-Abcc6 was 73 ± 12 (p < 0.001) and 475 ± 85 (p < 0.001), respectively (Table 3). Thus, using relative real-time reverse transcription PCR, the gene expression of Abcc6 was found to reach only 15% in cells transfected with pSG5-Abcc6-del5bp and pSG5-Abcc6 was 73 ± 12 (p < 0.001) and 475 ± 85 (p < 0.001), respectively (Table 3). Thus, using relative real-time reverse transcription PCR, the gene expression of Abcc6 was found to reach only 15% in cells transfected with pSG5-Abcc6-del5bp and pSG5-Abcc6 was 73 ± 12 (p < 0.001) and 475 ± 85 (p < 0.001), respectively (Table 3). Thus, using relative real-time reverse transcription PCR, the gene expression of Abcc6 was found to reach only 15% in cells transfected with pSG5-Abcc6-del5bp and pSG5-Abcc6 was 73 ± 12 (p < 0.001) and 475 ± 85 (p < 0.001), respectively (Table 3). Thus, using relative real-time reverse transcription PCR, the gene expression of Abcc6 was found to reach only 15% in cells transfected with pSG5-Abcc6-del5bp and pSG5-Abcc6 was 73 ± 12 (p < 0.001) and 475 ± 85 (p < 0.001), respectively (Table 3). Thus, using relative real-time reverse transcription PCR, the gene expression of Abcc6 was found to reach only 15% in cells transfected with pSG5-Abcc6-del5bp and pSG5-Abcc6 was 73 ± 12 (p < 0.001) and 475 ± 85 (p < 0.001), respectively (Table 3). Thus, using relative real-time reverse transcription PCR, the gene expression of Abcc6 was found to reach only 15% in cells transfected with pSG5-Abcc6-del5bp and pSG5-Abcc6 was 73 ± 12 (p < 0.001) and 475 ± 85 (p < 0.001), respectively (Table 3). Thus, using relative real-time reverse transcription PCR, the gene expression of Abcc6 was found to reach only 15% in cells transfected with pSG5-Abcc6-del5bp and pSG5-Abcc6 was 73 ± 12 (p < 0.001) and 475 ± 85 (p < 0.001), respectively (Table 3). Thus, using relative real-time reverse transcription PCR, the gene expression of Abcc6 was found to reach only 15% in cells transfected with pSG5-Abcc6-del5bp and pSG5-Abcc6 was 73 ± 12 (p < 0.001) and 475 ± 85 (p < 0.001), respectively (Table 3). Thus, using relative real-time reverse transcription PCR, the gene expression of Abcc6 was found to reach only 15% in cells transfected with pSG5-Abcc6-del5bp and pSG5-Abcc6 was 73 ± 12 (p < 0.001) and 475 ± 85 (p < 0.001), respectively (Table 3). Thus, using relative real-time reverse transcription PCR, the gene expression of Abcc6 was found to reach only 15% in cells transfected with pSG5-Abcc6-del5bp and pSG5-Abcc6 was 73 ± 12 (p < 0.001) and 475 ± 85 (p < 0.001), respectively (Table 3). Thus, using relative real-time reverse transcription PCR, the gene expression of Abcc6 was found to reach only 15% in cells transfected with pSG5-Abcc6-del5bp and pSG5-Abcc6 was 73 ± 12 (p < 0.001) and 475 ± 85 (p < 0.001), respectively (Table 3). Thus, using relative real-time reverse transcription PCR, the gene expression of Abcc6 was found to reach only 15% in cells transfected with pSG5-Abcc6-del5bp and pSG5-Abcc6 was 73 ± 12 (p < 0.001) and 475 ± 85 (p < 0.001), respectively (Table 3). Thus, using relative real-time reverse transcription PCR, the gene expression of Abcc6 was found to reach only 15% in cells transfected with pSG5-Abcc6-del5bp and pSG5-Abcc6 was 73 ± 12 (p < 0.001) and 475 ± 85 (p < 0.001), respectively (Table 3). Thus, using relative real-time reverse transcription PCR, the gene expression of Abcc6 was found to reach only 15% in cells transfected with pSG5-Abcc6-del5bp and pSG5-Abcc6 was 73 ± 12 (p < 0.001) and 475 ± 85 (p < 0.001), respectively (Table 3).
11 laboratory inbred strains as well as the B6.C3H\(^{Dyscalt}\) congenic mice (Cg1), i.e. mice that carry the \(\text{Abcc6}\) allele from C3H mice on a C57 genetic background. As shown in Table 3 we tested for genotype-phenotype association (Table 4) (13) in the following strains, C57BL/6J (C57), C57BL/10J (B10), C3H/HeJ (C3H), DBA/2J (DBA), 129S1/Sv (129S1), Balb/c (Balb/c), CBA/J (CBA), A/J (A), FVB/N (FVB), MRL/Mp (MRL), and NZB/BINJ (NZB), as well as in Cg1 mice.

C3H, DBA, and 129S1 mice as well as B6.C3H\(^{Dyscalt}\) congenic mice were found to carry the Thr allele at the 3’-border of exon 14 (at codon 619). All these mice were found to display DCC after freeze-thaw injury (13). In contrast, mice that carry the Cys allele were found either negative (C57, FVB, MRL, A, and CBA) or positive (NZB and Balb/c) for DCC. We suggest that other mutations in \(\text{Abcc6}\) might explain the susceptibility of NZB and Balb/c mice to develop DCC.

**Predisposition of DCC-susceptible C3H Mice to PXE**—We previously reported the predisposition of C3H mice to cardiovascular calcification (DCC). Particularly, these mice demonstrated calcium phosphate deposits some days following freeze-thaw injury of myocardial tissue as well as of the abdominal aorta (11, 13, 23). In the \(\text{Abcc6}\) knock-out mice, MRP6 disruption led to a rather similar phenotype called PXE that, however, occurs rather late during the aging process. Specifically, these \(\text{Abcc6}\) knock-out mice displayed calcium phosphate mineral deposits in small- and medium-sized vessels predominantly in kidney tissue at 6 months of age and progressed to affect more organs, including skin, eye, and adipose tissue as well as aorta, vena cava, and Bruchs membrane, at 17–22 months of age (16, 17). The C57 wild type mice studied here displayed no such calcification (Fig. 7D). No calcification was observed in age-matched C57 mice (\(n = 3\)). No clear differences were seen either in the dermal elastic fibers or in retina between the tested C3H and C57 mice.

**DISCUSSION**

The ATP binding cassette C 6 (\(\text{Abcc6}\)) gene has been identified to cause both DCC and PXE (16–18, 24); however, the mutations responsible for DCC remained elusive. In this study, we present a variant occurring in several mouse strains that relates to phenotypic characteristics of both DCC and PXE. Specifically, examining the genomic DNA of DCC-susceptible C3H and DCC-resistant C57 mice, a missense mutation p.R619S (rs32756904) in the \(\text{Abcc6}\) gene has been identified that gives rise to an alternative splice variant in the corresponding mRNA, which in turn leads to protein expression deficiency. The functional implications of this deletion were then tested in some detail.

The \(\text{Abcc6}\) gene encodes MRP6, a 1449-aa protein of 165 kDa that consists of three transmembranous domains and two intracellular nucleotide binding folds (25). It is involved in the ATP-driven transport of various molecules across the cell membrane. MRP6 is mainly expressed in the liver and to a lesser extent in kidney. In contrast, very low levels have been found in tissues affected by dystrophic calcification, such as heart and muscle (18, 21, 22). Thus, the preventive mechanism of \(\text{Abcc6}\) on cardiac calcification seems to be mediated by systemic rather than tissue-specific factors. Accordingly, alterations of hepatic expression of MRP6 may explain the development of DCC and PXE.
**Abcc6 Splice Variant Leads to Protein Deficiency in Mice**

**TABLE 4**

SNP genotypes spanning the Cys to Thr mutation at codon 619 (rs32756904) in Abcc6 genomic DNA and DCC phenotype information in 11 laboratory inbred strains as well as congenic mice (Cg1)

| Strains | DCC* | SNPs | Position | B10 | CBA | FVB | MRL | A | C57 | Cg1 | C3H | DBA | 129 | NZB | Balb |
|---------|------|------|----------|-----|-----|-----|-----|---|-----|-----|-----|-----|-----|-----|-----|-----|
|         |      | rs32756904 | 45870623 | CC  | CC  | CC  | CC  | CC | CC  | TT* | TT* | TT* | TT* | CC  | CC  |

* Design DCC phenotype as previously published (13).

* SNPs that diverge from the DCC-resistant C57 strains.

Using Western blot analysis, we found a dramatic decrease of the MRP6 protein in hepatocytes of both C3H and congenic mice compared with wild type C57 mice. To determine the genetic variant that might lead to protein deficiency, we examined the 5'-UTR, the coding region, and 3'-UTR of Abcc6 in both C3H and C57 mice. Comparing the cDNA sequence of Abcc6 in these mice, we found two relevant deletions that might affect mRNA stability, a 10-bp deletion at the 3'-UTR region and a 5-bp deletion at exon 14. We therefore hypothesized that one of these deletions might contribute to the observed deficiency in MRP6 expression. Meng et al. (18) previously found no effect of the 10-bp deletion on the mRNA stability, therefore excluding this deletion as causal mutation for DCC.

In this study, we focused on the 5-bp deletion at exon 14 that was newly detected. This deletion was related to an alternative splice variant in C3H mice that is caused by a newly uncovered donor splice site (GU) in the Abcc6 pre-mRNA transcript. The variant leads to a deletion that skips the last 5 bp (GUGCUL) of exon 14. The translation of the corresponding mRNA results in a premature stop codon at position 685 that in turn gives rise to a dysfunctional protein lacking nearly half of the expected sequence (aa 685–1449). The introduction of such premature stop codons had been shown by Stamm et al. (26) to enhance mRNA degradation by nonsense-mediated decay and thus results in protein deficiency. To examine the expression of the truncated protein we used an MRP6 N terminus antibody and analyzed liver tissues from DCC-susceptible C3H and Cg1 mice using Western blot analysis. We first tested whether this antibody recognizes the truncated protein overexpressed by pSG5-Abcc6-del5bp construct in HEK-293 cells. Transfected HEK-293 cells display expression of the truncated protein. By contrast, we could not detect the truncated protein in liver tissue of DCC-susceptible mice. In fact, this finding suggested early nonsense-mediated decay degradation processes resulting in loss of MRP6 immunoreactivity. Furthermore, to examine the expression level of the Abcc6 variants in transfected HEK-293 cells, we employed relative real-time reverse transcription PCR and immunohistological analysis. We observed a decrease in Abcc6 expression both at RNA and protein levels in cells expressing the truncated protein as compared with cells expressing the wild type Abcc6 cDNA.

Moreover, to find an association between the identified rs32756904 missense mutation at codon 619 and DCC phenotype in mice, we genotyped 11 laboratory mouse strains for this mutation and found three mouse strains that carry the Thr mutation and found three mouse strains that carry the Thr allele at the 3'-UTR region of exon 14, C3H, DBA, and 129S1. All of these mice were found to display dystrophic cardiac calcification (13).

Finally, we sought to investigate whether the novel alternative splice variant in Abcc6 gene might also be associated with the development of PXE in mice. Interestingly, a more detailed phenotypic characterization of DCC-susceptible mice carrying the 5-bp deletion displayed features of PXE-like phenotype as well, thus suggesting that this variant may also affect this disease phenotype. Further functional analyses are needed to clarify the downstream mechanisms related to this splice variant on the initiation and development of calcification in mice.

**CONCLUSION**

We identified a missense mutation in the Abcc6 gene, the gene causing both DCC and PXE. Our series of experiments...
shows that this mutation gives rise to an alternative splice site leading to a 5-bp deletion in the Abcc6 transcript that in turn results in protein deficiency. We suggest that the protective effect of MRP6 seems to be mediated by systematic factors and that alterations of hepatic expression of MRP6 may explain the development of DCC and PXE.

Acknowledgments—We thank A. Thiemig, J. Stegmann, and S. Wrobel for technical assistance. We also thank Prof. Dr. M. Bader, Dr. N. Alenina, and Dr. F. Kaiser for fruitful discussions during this work. We are indebted to Dr. R. Noel for advice in matters of animal maintenance and welfare.

REFERENCES

1. Gang, D. L., Barett, L. V., Wilson, E. J., Rubin, R. H., and Medearis, D. N. (1986) Am. J. Pathol. 124, 207–215
2. Price, P., Eddy, K. S., Papadimitriou, J. M., Faulkner, D. L., and Shellam, G. R. (1991) Am. J. Pathol. 138, 59–67
3. Lestroh, A., and Li, C. H. (1955) Nature 176, 504
4. Sparks, L. L., Rosenau, W., Macalpin, R. N., Daane, T. A., and Li, C. H. (1955) Nature 176, 503–504
5. Eaton, G. J., Custer, R. P., Johnson, F. N., and Stadenow, K. T. (1978) Am. J. Pathol. 90, 173–186
6. Highman, B., and Daft, F. S. (1965) Arch. Pathol. 52, 221–229
7. Van Vleet, J. F., and Ferrans, V. F. (1987) Am. J. Vet. Res. 48, 255–261
8. Everitt, J. I., Ross, P. W., Neptum, D. A., and Mangum, J. B. (1988) Lab. Anim. Sci. 38, 426–429
9. Brunnett, S. R., Shi, S., and Chang, B. (1999) Genomics 59, 105–107
10. Ivanic, B. T., Utz, H. F., Kaczmarek, P. M., Aherrahrou, Z., Axtnr, S. B., Klepsch, C., Lusis, A. J., and Katus, H. A. (2001) Physiol. Genomics 6, 137–144
11. Aherrahrou, Z., Axtnr, S. B., Kaczmarek, P. M., Jurat, A., Korff, S., Doehring, L. C., Weichenhan, D., Katus, H. A., and Ivanic, B. T. (2004) Am. J. Pathol. 164, 1379–1387
12. Ivanic, B. T., Qiao, J. H., Machleder, D., Liao, F., Drake, T. A., and Lusis, A. J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5483–5488
13. Aherrahrou, Z., Doehring, L. C., Kaczmarek, P. M., Liptau, H., Ehlers, E. M., Pomarino, A., Wrobel, S., Grotz, A., Mayer, B., Erdmann, J., and Schunkert, H. (2007) Physiol. Genomics 28, 203–212
14. Van Soest, S., Swart, I., Tijmes, N., Sandkuijl, L. A., Rommers, J., and Bergen, A. A. (1997) Genom. Res. 7, 830–834
15. Cai, L., Struk, B., Adams, M. D., Ji, W., Haaf, T., Kang, H. L., Dho, S. H., Xu, X., Ringsfeil, F., Nancarrow, J., Zach, S., Schaen, L., Stumpp, M., Niu, T., Chung, J., Lunke, K., Verreccchia, B., Goldsmith, L. A., Viljoen, D., Figuera, L. E., Fuchs, W., Lebohwol, M., Uitto, J., Richards, R., Hohl, D., and Rame-sar, R. (2000) J. Mol. Med. 78, 36–46
16. Klement, I. F., Matsuzaaki, Y., Jiang, Q. J., Terlizzi, J., Choi, H. Y., Fujimoto, N., Li, K., Pulkkinen, L., Birk, D. E., Sundberg, J. P., and Uitto, J. (2005) Mol. Cell Biol. 25, 8299–8310
17. Gorgels, T. G., Hu, X., Scheffer, G. I., van der Wal, A. C., Toonstra, J., de Jong, P. T., van Kuppevelt, T. H., Levelt, C. N., de Wolf, A., Loves, W. J., Scheper, R. J., Peek, R., and Bergen, A. A. (2005) Hum. Mol. Genet. 14, 1763–1773
18. Meng, H., Vera, I., Che, N., Wang, X., Wang, S. S., Ingram-Drake, L., Schadt, E. E., Drake, T. A., and Lusis, A. J. (2007) Proc. Natl. Acad. Sci. U. S. A. 104, 4530–4535
19. Dean, M., Rzhetsky, A., and Allikmets, R. (2001) Genome. Res. 11, 1156–1166
20. Ilias, A., Urban, Z., Seidl, T. L., Le Saux, O., Sinko, E., Boyd, C. D., Sarkadi, B., and Varadi, A. (2002) J. Biol. Chem. 277, 16860–16867
21. Beck, K., Hayashi, K., Nishiguchi, B., Le Saux, O., Hayashi, M., and Boyd, C. D. (2003) J. Histoch. Cytochem. 51, 887–902
22. Matsuzaki, Y., Nakano, A., Jiang, Q. J., Pulkkinen, L., and Uitto, J. (2005) J. Investig. Dermatol. 125, 900–905
23. Doehring, L. C., Kaczmarek, P. M., Ehlers, E., Mayer, B., Erdmann, J., Schunkert, H., and Aherrahrou, Z. (2006) Ann. Anat. 188, 235–242
24. Bergen, A. A., Plomp, A. S., Schuurman, E. J., Terry, S., Breuning, M., Dauwerse, H., Swart, J., Kool, M., van Soest, S., Baas, F., ten Brink, J. B., and de Jong, P. T. (2000) Nat. Genet. 25, 228–231
25. Hendig, D., Schulz, V., Eichgrun, J., Szliska, C., Gotting, C., and Kleesiek, K. (2005) J. Mol. Med. 83, 140–147
26. Stamm, S., Ben-Ari, S., Rafalska, I., Tang, Y., Zhang, Z., Toiber, D., Thanaraj, T. A., and Soreq, H. (2005) Gene 344, 1–20