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**DOI**

[10.1002/hep.510250635](https://doi.org/10.1002/hep.510250635)

**Publication date**

1997

**Published in**

Hepatology

[Link to publication](#)

**Citation for published version (APA):**

Paulusma, C. C., Kool, M., Bosma, P. J., Scheffer, G. L., ter Borg, F., Scheper, R. J., Tytgat, G. N. J., Borst, P., Baas, F., & Oude Elferink, R. P. J. (1997). A mutation in the human canalicular multispecific organic anion transporter gene causes the Dubin-Johnson syndrome. *Hepatology*, 25, 1539-1542. <https://doi.org/10.1002/hep.510250635>

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# A Mutation in the Human Canalicular Multispecific Organic Anion Transporter Gene Causes the Dubin-Johnson Syndrome

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**The human Dubin-Johnson syndrome (DJS) is a rare autosomal recessive liver disorder characterized by chronic conjugated hyperbilirubinemia. Patients have impaired hepatobiliary transport of non-bile salt organic anions. A highly similar phenotype has been described for a mutant Wistar rat strain, the transport-deficient (TR<sup>-</sup>) rat, which is defective in the canalicular multispecific organic anion transporter (cmoat). This protein mediates adenosine triphosphate-dependent transport of a broad range of endogenous and xenobiotic compounds across the (apical) canalicular membrane of the hepatocyte. The complementary DNA (cDNA) encoding rat cmoat has recently been cloned, and the mutation underlying the defect in TR<sup>-</sup> rats has been identified. In the present study, we have isolated the human homologue of rat cmoat, human cMOAT, and analyzed the corresponding cDNA from fibroblasts of a DJS patient for mutations. Our results show that a mutation in this gene is the cause of DJS. (HEPATOLOGY 1997;25:1539-1542.)**

The typical phenotypic and diagnostic features of Dubin-Johnson syndrome (DJS)<sup>1,2</sup> (Omim 237500) are highly similar to those in the transport-deficient (TR<sup>-</sup>) rat.<sup>3</sup> First, there is chronic conjugated hyperbilirubinemia. Second, the hepatic clearance of intravenously injected bromosulfophthalein is delayed, and the glutathione conjugate, which is normally excreted into bile via the canalicular multispecific organic anion transporter (cMOAT),<sup>4</sup> regurgitates into the plasma.<sup>5</sup> Third, there is an increased urinary excretion of coproporphyrin I, a metabolic byproduct of heme synthesis, which also is a substrate for cMOAT.<sup>6</sup> Finally, liver biopsies from patients with the DJS display a characteristic lysosomal accumulation of black pigment with otherwise normal histology<sup>1-3</sup>; this pigment also is observed when TR<sup>-</sup> rats are fed a diet supplemented with aromatic amino acids.<sup>7</sup> Studies in the TR<sup>-</sup> rat have greatly contributed to the biochemical characterization of the transport system involved in this defect. These mutant animals lack the hepatobiliary excretion of many organic anions including bilirubin-glucuronide,<sup>8,9</sup> cysteinyl-leukotrienes,<sup>10,11</sup> and some divalent bile salt conjugates<sup>12,13</sup> (among various other glutathione and glucuronide conjugates) (re-

viewed by Oude Elferink et al.<sup>4</sup>). The transport of these compounds is impaired in DJS as well.<sup>3,14</sup> The characterization of the human multidrug resistance-associated protein (MRP1)<sup>15</sup> as an organic anion pump with a highly similar substrate specificity as the putative cMOAT<sup>16,17</sup> has recently led to the cloning of the rat *cmoat* complementary DNA (cDNA), a liver-specific homologue of MRP1.<sup>18,19</sup> The identification of a single-nucleotide deletion in this gene in TR<sup>-</sup> rats has unambiguously demonstrated its role in canalicular organic anion transport.<sup>18</sup> Indeed, transfection studies revealed an increased organic anion efflux from cells expressing cMOAT (Paulusma CC, unpublished data, July 1996). In view of the highly similar phenotypes of TR<sup>-</sup> rats and DJS patients, we postulated that a mutation in the human *cMOAT* gene underlies the transport defect in the DJS.

## MATERIALS AND METHODS

**Cloning of Human cMOAT cDNA.** A human liver 5' stretch-plus cDNA library (Clontech, Palo Alto, CA) was screened, using a 5-kb fragment of rat *cmoat* as a probe, as described previously.<sup>18</sup> Three overlapping cDNAs were isolated. The sequence of the 5' end, encoding the first 11 amino acids of cMOAT, was determined from a human cDNA clone (no. 124379, Soares fetal liver spleen library 1NFLS) obtained from the I.M.A.G.E. consortium. *cMOAT* was sequenced using the ABI377 automatic sequencer (accession number GenBank U49248).

**Patient, Tissues, and Fibroblast Culture.** One caucasian, female patient was studied. Liver from this patient was obtained by a needle biopsy. Normal control liver was obtained from surgical pathology specimens. Biopsies were fixed for histology in 4% formaldehyde and embedded in paraffin. Skin fibroblasts from the patient and normal control were obtained by skin biopsy and cultured in Ham F-10 (Life Technologies, Gaithersburg, MD), supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 50 units/mL penicillin, and 50 µg/mL streptomycin, at 37°C.

**Immunohistochemistry.** Formaldehyde-fixed, paraffin-embedded liver sections were deparaffinized in xylene and rehydrated. Endogenous peroxidase activity was blocked with 0.3% (vol/vol) H<sub>2</sub>O<sub>2</sub> in methanol for 30 minutes. Before staining, the sections were pretreated with 0.01 mol/L citric acid (pH 6.0) for 3 × 5 minutes at 100°C. The sections were blocked with normal rabbit serum for 10 minutes and incubated with monoclonal antibody M<sub>2</sub>III-6 for 1 hour. This antibody was produced against a bacterial fusion protein containing the 202-amino acid COOH-terminal end of rat *cmoat*, and characterized as described previously.<sup>18</sup> Immunoreactivity was visualized with biotinylated rabbit anti-mouse Fab<sub>2</sub> (Dako Copenhagen, Denmark), followed by streptavidin-conjugated horseradish peroxidase (Dako) in phosphate-buffered saline/1% bovine serum albumin, and subsequent staining with 3,3'-diaminobenzidine tetrahydrochloride and 0.02% (vol/vol) H<sub>2</sub>O<sub>2</sub> in phosphate-buffered saline. P-glycoproteins were detected with monoclonal antibody JSB-1.<sup>20</sup> All sections were (counter)stained with hematoxylin and mounted.

**RNA Extraction and cDNA Synthesis.** Total RNA was extracted from fibroblasts according to the acid-phenol single-step method.<sup>21</sup> cDNA synthesis was performed with 6 µg of total RNA and random hexamer primers with Moloney murine leukemia virus reverse transcriptase (Life Technologies), at 37°C for 1 hour, followed by 10 minutes at 65°C to inactivate the Moloney murine leukemia virus reverse transcriptase.

Abbreviations: DJS, Dubin-Johnson syndrome; TR<sup>-</sup>, transport deficient; cMOAT, canalicular multispecific organic anion transporter; MRP1, multidrug resistance-associated protein; cDNA, complementary DNA; PCR, polymerase chain reaction.

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Received February 10, 1997; accepted March 18, 1997.

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0270-9139/97/2506-0037\$3.00/0

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1 MLEKFCNSTP WNSFLDSE ADLPLCFEOT VLWVPLGFL WLLAPWLLH VYKSRKRS TTKLYLAKOV FVGLLLAA IELALVLTED SGQATVPVAVR
101 NTPSLYLTG WLLVLLIQYS RQWCVOKNSU FLSLFWLISI LCGTQFQTL IRTLLOGDGS NLAYSCLFEFI SYGFOILLI FSAESENES SNNPSSIASF
201 LSSITYSWVD SIIKGYKRP L LTEDVWEVD EEMKTKLVS KFETHMKREL QKARRALORR QEKSSQNSG ARLPLGNKWQ SOSQDALVLE DVEKXKXSG
301 TKQVQKSWL MKALFKTFYM VLKSLFKLKL VNDIFTFVSP QLLKLLISFA SDDGTYLWIG YLCAILLFETA ALIQSFCLOC YFOLCFKLGV KVRTAIMASV
401 YKALTLNLR ARKEYTVEET VNLMSVDAOK LMDVTFNMH LWSVGLIVL SIFFLURELG PSVLAGGVVM VLVIPINAIL STKSTIQVK NNMKNQDKLK
501 IMEILSGIK ILKYFAPEPS FROOVNLRK KELKMLAFS QLOCVIVFV QLPVILVSVV TFSYVYVDS NNILDAQAF TSITLENLR FPLSLMHPMI
601 SSMQASVST ERLEYKGGD DLDTSATRD CNFDKAMQFS EASFVMEHS EATVROVMDL IMAGQLVAVI QVGVGKSSL ISAMLGEMEN VGHYIYKGT
701 TATVPOQSI QNGTIKDMIL FGFEKERYK QOVLACALL PDLEMLPQGD LAETGEGKIN LSGGQKQIS LARATYQMLD LYLLDDPLSA VDAHVQKHIF
801 MKVLGNLNL KGRTRLLVTH SMHFLPQVDE IVVLGNQIV EKGYSALLA KKGFAKMLK TFLRHTGPEE EATHVDSGEE EDDYGLISS VEIEPEDAAS
901 ITNRRENSFR RTLSSRSSN GRHLKSLNNS LKTRNWNLSK EDEELVKQK LKKEFIETG KWKFSIYLEY LQAIGLESIF FIIILAFMNS VAFIGLNLWL
1001 SAWTSQSKIF NSDYFASOR DMRVGVYVGL GLAQGIFVFI AHFVSAFGV HASNHLKQL LNWILRAPMR FFDITPTGRI VNRFAQDST VDDTLPOSLR
1101 SHITCFGLII STLVNIMCAT PVFTIIVLPL GIIVYVQMF VYSTSRQLRR LDSVTRSPIY SHFSETVGL PVIRAFEHQO RFLKHNEERI DTKQCVFSW
1201 ITSNLVAIR LELVGNLTVF FSALMNVLYR DTLSQDYGF VLSNALITO TLNULVMTS EIEHTVAVE RITEYKVEN EAPWVDKRP PPDWPSGKI
1301 QFNHYVQRY PELDLVLRGI TCDIGMEKI QVWRTGAK SSLTNLRFRI LEAAGQRII DGDVJASGL HDRLKTLII PDPILFSDS LRMLDPPFN
1401 YSDEEMKAL ELANLKSFVA SLOGLSHEV TEAGGNLSIG ORQLLCLGRA LLRSKLVIL DEATAAVDLE TDLNLTQTTI NEFAHCTVIT IARHLTMD
1501 SDKVMVLQNG KTIIEVGSPEE LLQIPGPFYF MAKEAGIEVH NSTK*
    
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FIG. 1. Deduced amino acid sequence of human cMOAT (GenBank accession number U49248). Predicted transmembrane regions are underlined. Walker A, B, and signature sequence are double-underlined. \*\*\*Predicted N-glycosylation sites, which correspond with MRP1 and cmoat. Δ, Location (amino acid 1066) at which a stop codon is introduced by a C-to-T transition in DJS cMOAT.

**Polymerase Chain Reaction.** The human cMOAT cDNA was amplified from both patient and control fibroblast cDNA using five sets of cMOAT-specific primers: 5'-tagaagagtcttcgtccagacgag-3'(forward I) and 5'-gcaatttcagcagctgaggactcac-3'(reverse I), 5'-aaatctggtgatg-aaggctctg-3'(forward II) and 5'-tccaggttcacatctcggactctggc-3'(reverse II), 5'-acatctgccattcagatgactgc-3'(forward III) and 5'-caactctcatgt-cctctgagatgc-3'(reverse III), 5'-tgaagtctccatctacctggagtacc-3' (forward IV) and 5'-gatgatggtcagcttctctcggagg-3'(reverse IV), and 5'-gtcatcctcacaactgcctctcagaatcttag-3'(forward V) and 5'-ctgcta-gaatttctgctgttcacattc-3'(reverse V). Polymerase chain reactions (PCR) were performed in a Perkin Elmer GeneAmp PCR system 2400 (Perkin Elmer, Norwalk, CT), in 1× Taq polymerase buffer (Life Technologies), 1.5 mmol/L of MgCl<sub>2</sub>, 0.5 mmol/L of dNTPs, 400 nmol/L of each primer, and 0.5 units of Taq polymerase. The PCR products were obtained after application of the "touch down" PCR protocol<sup>22</sup>; the reactions were denatured at 96°C for 5 minutes, and subjected to five times of two cycles with annealing temperatures of 72, 70, 68, 66, and 65°C, respectively, and subsequently with 30 cycles with an annealing temperature of 64°C. Each cycle started with 20 seconds at 94°C, 30 seconds at the indicated annealing temperature, and 90 seconds at 72°C. The PCR reaction was terminated after an extension step at 72°C for 10 minutes.

**Subcloning and Sequencing of cMOAT PCR Products.** PCR fragments obtained from fibroblasts were excised from agarose gel, purified, ligated into the TA-cloning plasmid pCR II (Invitrogen, Leek,

The Netherlands), and transformed into INVαF' competent cells (Invitrogen). White colonies were picked, grown overnight, and plasmid DNA was isolated using the alkaline lysis method.<sup>23</sup> Nucleotide sequences of 5-8, pooled clones were determined by the dideoxynucleotide chain method.<sup>24</sup>

RESULTS

The human cMOAT cDNA was obtained after screening a human liver cDNA library using a 5-kb fragment of rat cmoat as a probe.<sup>18</sup> Three overlapping cDNAs were isolated, which lacked the 5' 30 coding nucleotides, when compared with the rat cmoat sequence. The missing 5' end was present in a cDNA clone (#124379), which we obtained from the I.M.A.G.E. consortium. The full-length cDNA encoding cMOAT contains a single open reading frame of 1,545 amino acids (Fig. 1), with a predicted molecular weight of 174 kd, which shares 77.7% and 88.7% sequence identity and similarity, respectively, with the rat cmoat protein. The cMOAT cDNA described in this study is identical to the cMRP/cMOAT cDNA recently reported by others.<sup>19,25</sup> The tissue distribution of cMOAT in humans and rats is highly similar, with high expression in liver, and low expression in kidney and duodenum (Kool M, et al., submitted).

We have studied a patient (age 54) who was diagnosed for DJS at the age of 20. She frequently complained of pains in the upper abdomen. General liver function was normal except for chronic elevated conjugated (38 to 70 μmol/L) and unconjugated (12 to 25 μmol/L) serum bilirubin levels. It was not possible to visualize the gallbladder after administration of oral contrast reagent, a characteristic feature of DJS. The patient showed a delayed plasma clearance of intravenously injected bromosulphophthalein, followed by a secondary rise in plasma bromosulphophthalein levels. At the age of 32, the patient underwent cholecystectomy. A characteristic black liver was observed, and microscopic analysis of a liver section revealed mild fibrosis and the pigment accumulation indicative of DJS (Fig. 2B).

Paraffin-embedded liver sections of DJS and control liver were examined for the presence and localization of the cMOAT protein, using monoclonal antibody M<sub>2</sub>III-6. In human control liver (Fig. 2A), the antibody stained the canalicular membrane of the hepatocyte. In DJS liver (Fig. 2B), no canalicular staining was observed, indicating that this patient lacked the cMOAT protein. The same results were obtained in liver slices of Wistar and TR rats (not shown). As

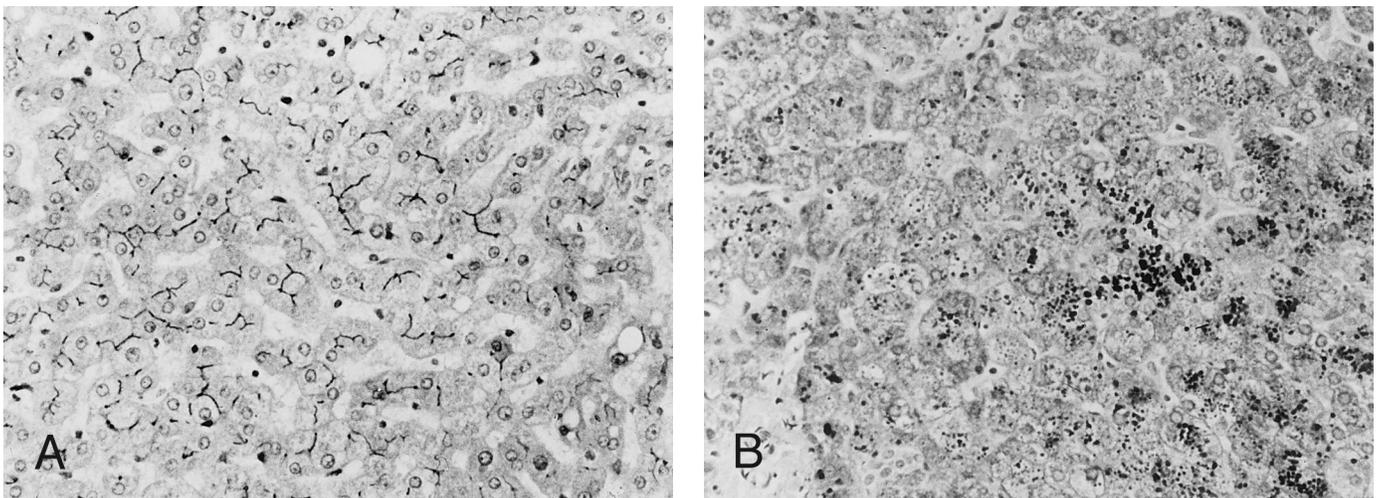


FIG. 2. Immunohistochemical detection of the cMOAT protein in human liver using monoclonal antibody M<sub>2</sub>III-6. (A) Section of a normal liver which shows the exclusive canalicular localization of the protein. (B) Liver section of the DJS patient in which no canalicular staining is observed. In addition, this figure displays the syndrome-characteristic vesicular brown pigment accumulation in hepatocytes. (Original magnification ×20.)

a control, a positive canalicular staining was observed in both DJS and control liver with JSB-1,<sup>20</sup> an antibody against P-glycoprotein (not shown).

To investigate the nature of the genetic defect, total RNA was isolated from cultured fibroblasts obtained from a skin biopsy of both the patient and a normal control. cDNA was prepared and the total *cMOAT* cDNA was amplified by the "touch down" PCR protocol.<sup>22</sup> Sequence analysis of multiple independent clones revealed a mutation in the patient at codon 1066 (CGA to TGA; arginine to stop-codon) (Fig. 3), which leads to premature termination of *cMOAT* protein synthesis, the normal protein being 1,545 amino acids long (see also Fig. 1). The mutation results in the loss of a *TaqI* restriction site, and we have confirmed the absence of this site in the patient by *TaqI* digestion of a *cMOAT* PCR product encompassing the site of the mutation (Fig. 4). Digestion of genomic DNA from patient and control with *TaqI*, and subsequent Southern blot analysis, showed a different hybridization pattern in patient and control, indicating that the patient is homozygous for the mutation in codon 1066 (not shown).

### DISCUSSION

This article describes the identification of the genetic defect that underlies the phenotype observed in patients with DJS, and that corresponds to the genetic defect identified in the animal model for DJS, the TR<sup>-</sup> rat.<sup>18</sup> Kartenbeck et al.<sup>26</sup> previously described the absence of the canalicular immunostaining in a liver section of a DJS patient, using an antibody directed to MRP1. This antibody gave both lateral and canalicular staining in control liver, but only lateral staining in DJS liver. Because MRP1 is only present in the basolateral membrane of epithelial cells,<sup>27</sup> we conclude that the antibody crossreacts with the apically localized, and homologous, *cMOAT* protein, a conclusion supported by more recent work of this group.<sup>19</sup> The antibody used in our work does not cross-react with MRP1 and only stains the canalculus. Indeed, staining with this antibody was completely negative in the DJS patient.

The mutated *cMOAT* gene in the patient under study results in a truncated protein. Our antibody is raised against the C-terminal 202 amino acids (of rat *cmoat*), which lie be-

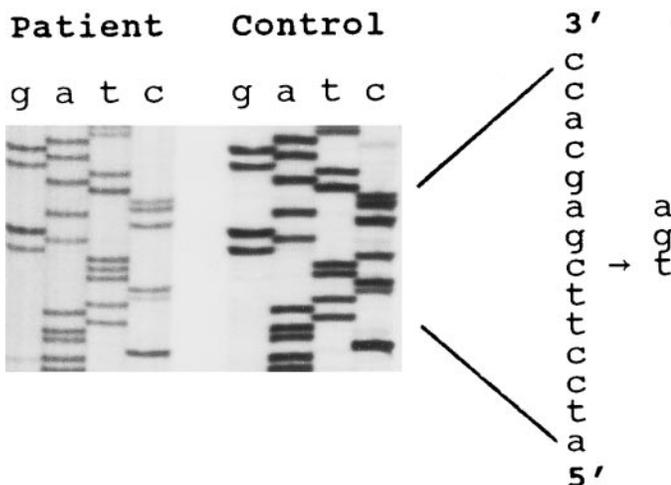


FIG. 3. Part of the *cMOAT* cDNA sequence encompassing the mutation that results in the absence of the functional protein in the patient. The normal sequence is depicted on the right. (Arrow) Site of the mutation at codon 1066. This codon normally encodes an arginine residue (CGA), but is changed into a stop-codon (TGA) in the patient. The mutation of C to T eliminates the recognition site for the restriction enzyme *TaqI* (5'-TCGA-3').

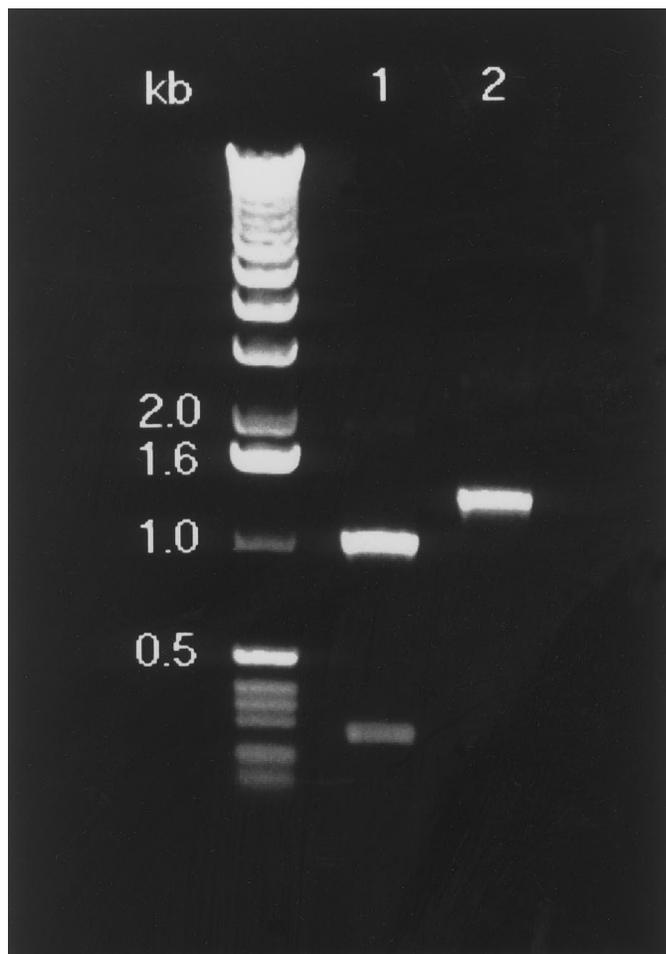


FIG. 4. *TaqI* digest of a part of the *cMOAT* cDNA that was obtained with primer combination forward IV/reverse IV. Lane 1, control and lane 2, the patient cDNA digest. Molecular size markers are indicated on the left in base pairs.

hind the mutation in this patient. We do not know, therefore, whether the truncated protein is present in the canalculus, whether it is mistargeted, or broken down. Because the mutation leads to the absence of four membrane-spanning domains and the complete second adenosine triphosphate-binding cassette, it can be assumed that the protein is not functional. In relation to this, it was recently shown that expression of the NH<sub>2</sub>- or COOH-proximal halves of the MRP1 protein separately (each containing one adenosine triphosphate-binding cassette) did not result in adenosine triphosphate-dependent leukotriene C<sub>4</sub> transport, while coexpression of both half-molecules restored this transport,<sup>28</sup> a phenomenon that also might apply to related adenosine triphosphate-binding cassette transporter proteins, including *cMOAT*.

An association has been observed between DJS and Factor-VII deficiency in a patient group from Jewish communities in Iran and Iraq.<sup>29</sup> In these communities, a high incidence of several genetic disorders, including DJS, is caused by a high degree of consanguinity.<sup>30</sup> The genes encoding human Factor-VII and *cMOAT* have been localized to chromosomes 13q34<sup>31</sup> and 10q23-q24,<sup>25,32</sup> respectively. Thus, a primary linkage between DJS and clotting Factor-VII deficiency can be ruled out. Our demonstration of a low but detectable expression of the *cMOAT* gene in fibroblasts allows a simple identification of this inherited disorder, without the need for liver biopsy.

**Acknowledgment:** The authors thank Petra Mooyer, Evelien Hogenhout, and Patricia Veltman for culturing the human fibroblasts; Marcel de Haas and Nico Ponne for technical assistance; and the PALGA foundation for information on DJS patients.

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