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Advances in Brief

A Human Canalicular Multispecific Organic Anion Transporter (cMOAT) Gene Is Overexpressed in Cisplatin-resistant Human Cancer Cell Lines with Decreased Drug Accumulation[1]

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Abstract

By targeting the ATP binding conserved domain in three ATP binding cassette superfamily proteins (P-glycoprotein, multidrug resistance protein, and cystic fibrosis transmembrane regulator), we isolated the cDNA of a new ATP binding cassette superfamily that was specifically enhanced in a cisplatin-resistant human head and neck cancer KB cell line. A human clone homologous to rat canalicular multispecific organic anion transporter (cMOAT) was found and designated human cMOAT. Fluorescence *in situ* hybridization demonstrated the chromosomal locus of the gene on chromosome 10q24. The human cMOAT cDNA hybridized a 6.5-kb mRNA that was expressed 4- to 6-fold higher by three cisplatin-resistant cell lines derived from various human tumors exhibiting decreased drug accumulation. Human cMOAT may function as a cellular cisplatin transporter.

Introduction

Acquirement of drug resistance by tumor cells is frequently observed in cancer patients receiving chemotherapeutic treatment. Two ABC[3] proteins, the M_r 170,000 Pgp and the M_r 190,000 MRP, are known to confer multidrug resistant phenotypes to cancer cells (1–3). Although the primary structures of Pgp and MRP share only 15% amino acid homology (4), both Pgp and MRP are involved in drug resistance to a similar profile of chemotherapeutic agents that include anthracyclines, vinka alkaloids, and epipodophyllotoxins (1–3). By contrast, cancer cells that overexpress Pgp or MRP do not show cross-resistance to platinum-containing compounds, alkylating agents, and antimetabolites (1–3). MRP-transfectant cells show resistance to anthracyclines, vinka alkaloids, epipodophyllotoxins, and heavy metal anions but are sensitive to platinum-containing compounds (5, 6). However, MRP can transport the cysteinyl leukotriene C4 and other glutathione conjugates (7–9), suggesting that MRP may be a glutathione-conjugate transporter.

Of the platinum-containing compounds, cisplatin is a potent anticancer agent that has been widely used to treat various malignant tumors, including testicular, head and neck, esophageal, lung, ovarian, and bladder cancers. However, clinical application of long duration is often limited because of the development of cisplatin-resistant tumors. To further improve the chemotherapeutic efficacy of this potent anticancer agent, we must understand the mechanisms that control sensitivity to cisplatin (10, 11). Cisplatin accumulation is decreased in many specific cisplatin-resistant cells (12–14), and the ATP-dependent active outward efflux of cisplatin is enhanced in both cisplatin-resistant human epidermoid cancer KB and human prostatic cancer PC-3 cell lines (12, 14).

Because the overexpression of two ABC-superfamily genes, *MDR1/mdr1* (15) and *MRP* (4, 5), is closely associated with Pgp- and non-Pgp-mediated multidrug resistance, we initially isolated other genes belonging to the ABC superfamily to explain the decreasing cellular accumulation of cisplatin by tumor cells. Specifically, we targeted the ATP binding domain conserved in *MDR1*, *MRP*, and *CFTR* genes in the present study and compared the difference in mRNA from both cisplatin-sensitive and -resistant cells. In this study, we isolated full-length human cMOAT cDNA that is highly homologous to rat cMOAT (16), a homologue of the human *MRP* gene. We will also discuss a possible correlation between human cMOAT and cellular cisplatin accumulation.

Materials and Methods

Cell Lines. Cisplatin-resistant cell lines were derived from various drug-sensitive parental counterparts: T24/DDP10 from human bladder cancer T24 cells (13); P/CDP5 from human prostatic cancer PC-3 cells (12); and KB/KCP4 from human head and neck cancer KB cells (14, 17). Etoposide/teniposide-resistant KB/VP4 cells were derived from KB cells (18). These cell lines were cultured at 37°C under a humidified atmosphere of 5% CO_2 in Eagle's MEM (Nissui Seiyaku, Tokyo, Japan) containing 10% fetal bovine serum (Sera-lab, Sussex, United Kingdom), Bactopeptone (1 mg/ml; DIFCO Laboratories, Detroit, MI), glutamine (0.292 mg/ml), kanamycin (100 mg/ml), and penicillin (100 units/ml).

cDNA Cloning. Total RNA was isolated from KB/KCP4 cells and 1–2 μg were then reverse-transcribed using molony muline leukemia virus reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD). The resulting cDNA was used as a template for PCR using various primer combinations. In both nested PCR and heminested PCR, cDNA was reacted with the outer primer pairs (C1; Fig. 1*A*), and an aliquot of the reaction product was then reamplified with the inner primers (C2, C3, and C4). The primer sequences used for C-series PCR are shown in Fig. 1*B*, and the expected sizes of PCR products are shown in Fig. 1*A*. PCR cycle temperatures and times were 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s for a total of 35 cycles for all reactions. The PCR products were subcloned in pMOSBlue vector (Amersham, Buckinghamshire, United Kingdom) and sequenced. The overlapping cDNA clones were obtained by screening a λgt11 HeLa cell cDNA library (Clontech, Palo Alto, CA), a pCMVSPORT human kidney cell cDNA library (Life Technologies, Inc.), and a λzapII human liver cDNA library with the cDNA probe. All overlapping cDNA clones were subcloned into the pUC18

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[3] The abbreviations used are: ABC, ATP binding cassette; Pgp, P-glycoprotein; MRP, multidrug resistant protein; CFTR, cystic fibrosis transmembrane regulator; cMOAT, canalicular multispecific organic anion transporter; cisplatin, *cis*-diamminedichloroplatinum(II); FISH, fluorescence *in situ* hybridization.

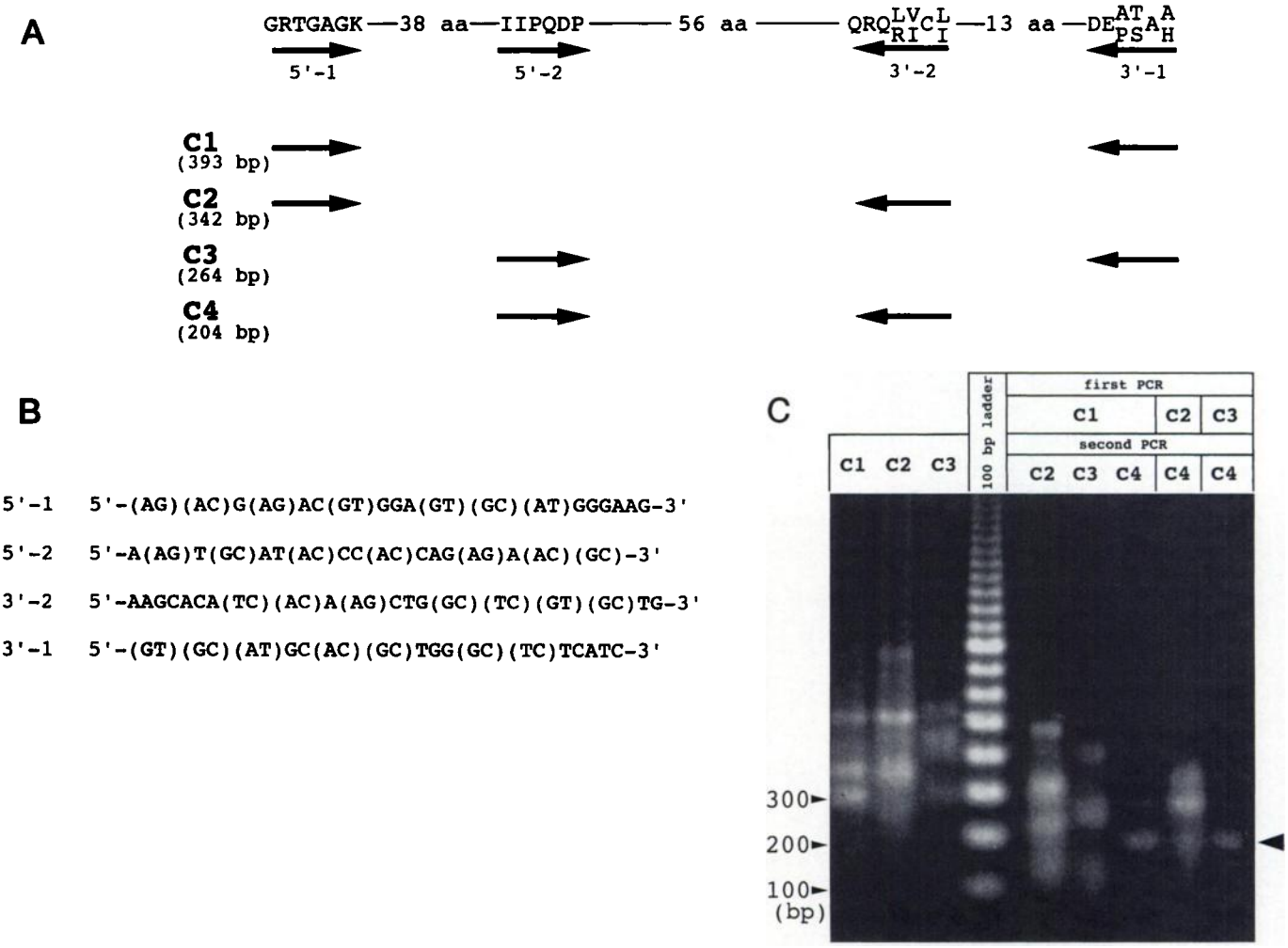

Fig. 1. *A*, schematic representation of primer positions and the nested PCR strategy. *Top*, the conserved amino acid sequence of the ATP binding region. *C1-C4*, primer pairs for PCR and the expected product sizes. *B*, the sequence of primers used for nested PCR. *C*, gel electrophoresis of PCR and nested PCR reaction products. *Arrow*, 204-bp C4 primer pair products.

plasmid. Chain elongation and termination were performed with a DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems, Tokyo, Japan), and nucleotide sequencing was performed with a DNA sequencing system (model 373S; Applied Biosystems). Data were analyzed using GeneWorks software (IntelliGenetics, Mountain View, CA).

Genome DNA Cloning. The EMBL3 human placenta genome DNA library (19) was screened by the previously described method for cDNA library screening. The clones were used for FISH analysis.

Northern Blots. A human multiple tissue Northern blot was commercially obtained (Clontech), and total RNA was extracted from exponentially growing cells by the acid guanidinium thiocyanate-phenol-chloroform method (20). RNA (20 μg) was subjected to electrophoresis in a 1% agarose gel containing 2.2 M formaldehyde and then transferred to a nylon membrane (Hybond N^+; Amersham). The filters were hybridized with a ^{32}P-labeled cDNA probe in a solution containing 50% deionized formamide, 10 × Denhardt's buffer, 5 × SSC [1 × SSC = 0.15 M NaCl and 0.015 M sodium citrate (pH 7.0)], and 0.1% SDS for 24 h at 42°C and then washed twice in 42°C 2 × SSC containing 0.1% SDS. The amount of human cMOAT encoding mRNA was quantified with a Fujix BAS 2000 image analyzer (Fuji, Tokyo, Japan).

FISH Analysis. Probe labeling and *in situ* hybridization were performed as described previously (21). Briefly, chromosome spreads were obtained from phytohemaggulutinin-stimulated blood lymphocytes of a healthy donor after thymidine synchronization and bromodeoxyuridine incorporation. Genomic DNA fragments were labeled with biotin-16-dUTP (Böehringer Mannheim, Mannheim, Germany) by nick translation. *In situ* hybridization was performed in the presence of the COT-1 DNA (Life Technologies, Inc.) competitor. Hybridized probes were detected with FITC-conjugated avidin (Böehringer Mannheim). Chromosomes were counterstained with 0.2 mg/ml propidium iodide for R-banding. Fluorescence signals were analyzed using a Zeiss Axioskop epifluorescence microscope equipped with a cooled Charge Coupled Device camera (model PXL 1400; Photometrics, Tucson, AZ). Image acquisition was performed on a Macintosh Quadra 840AV computer using the IPLab™ (Signal Analytics, Vienna, VA) software. The images were then pseudocolored and merged using Adobe Photoshop™ 2.5J (Adobe Systems Japan, Tokyo, Japan). FITC and propidium iodide images were colored white and gray, respectively. The merged FITC and propidium iodide images were directly printed using a Fuji Pictrography 3000 output device.

Results and Discussion

Human MDR1, MRP, and CFTR have been identified as members of the ABC transporter superfamily (4, 22). The similarity of these three genes resides predominantly in two ATP binding domains. Based on their homology, we designed highly degenerate C-series primers (Fig. 1*A*). We also designed PCR primer mixtures ranging in complexity from 64 to 128. We used the C series of primer sequences (Fig. 1*B*) because we could not produce PCR products of expected size when another series of primer pairs were used. The results obtained for C-series primer pairs show that nested PCR amplification of a unique band from primary amplification products contains several bands (Fig. 1*C*). The PCR products were subcloned, and 43 transformants were subsequently sequenced. Among these clones, only 10 had 5′ and 3′ primer sequences at opposite ends. The sequences of

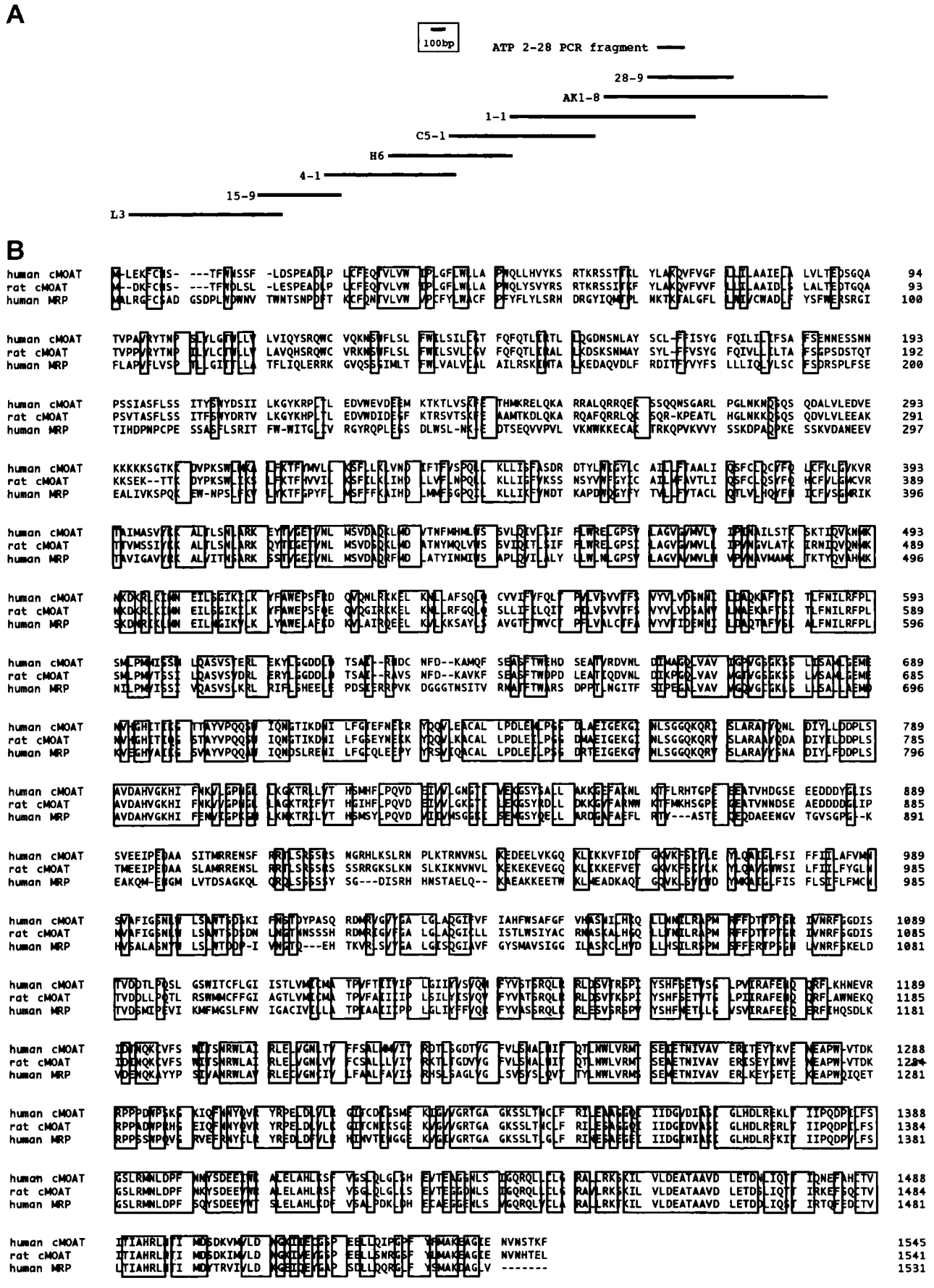

Fig. 2. *A*, schematic representation of overlapping cDNA clones. These cDNA clones encompass the coding region for human cMOAT. *B*, comparison of amino acid sequences of human cMOAT, human MRP (HUMMRPX), and rat cMOAT. *Boxes*, amino acid identity. Amino acid differences are shown in their corresponding positions. *Dashes*, gaps that are introduced to maximize identity.

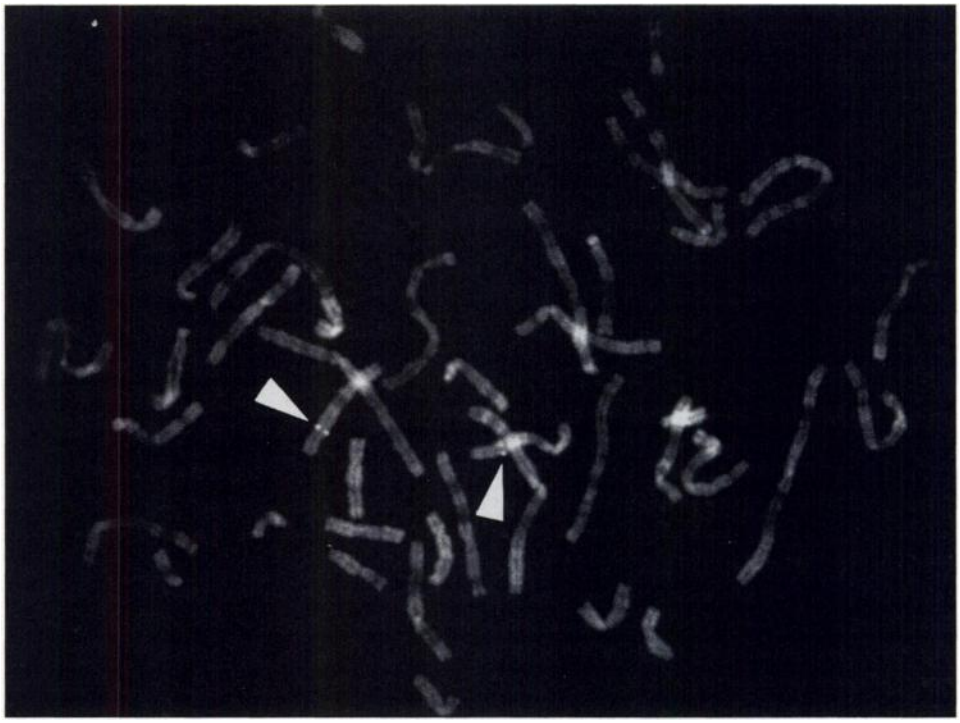

Fig. 3. Mapping of the gene region encoding human cMOAT by FISH. Four genomic clones were isolated using a cDNA probe and subjected to FISH analysis. *Arrowheads*, fluorescence signals on the R-banded metaphase chromosomes. Based on observations of more than 10 metaphase chromosomes, a band is identified at 10q24.

five clones were identical to those of MRP, and four clones had unrecognized sequences with no homology to sequences in the Genbank database. One clone, pATP2-28, showed homology with MRP (4) and, more significantly, with rat cMOAT (16). Therefore, we tentatively designated this product human cMOAT. Screening of the human cervical cancer HeLa cell cDNA library, the human kidney cell cDNA library, and the human liver cDNA library with a 207-bp ATP2-28 and walking probe allowed the isolation of overlapping cDNA clones (Fig. 2*A*). Sequencing each of these clones revealed an open reading frame coding for 1545 amino acids that showed 46% similarity to that of human MRP (Fig. 2*B*). The EMBL3 human placenta genomic DNA library was also screened, and several positive clones were identified and then used for FISH analysis, revealing positive signals at 10q24 (Fig. 3). Because human *MDR1, CFTR,* and *MRP* genes are localized on 7q21.1, 7q31, and 16p13.12-13, respectively (4, 23, 24), the human *cMOAT* gene seems to be different from these other genes.

Multiple-tissue Northern blot analyses with the C5-1 cDNA probe revealed that human cMOAT mRNA was expressed in liver but undetectable in other tissues (Fig. 4*A*). The tissue profile of human cMOAT expression was identical to that of rat cMOAT expression. TR^- rats, an animal model of the Dubin-Johnson syndrome, are defective in cMOAT, which mediates the hepatobiliary excretion of numerous organic anions including glutathione *S*-conjugates. We have previously reported that cellular cisplatin accumulation was markedly decreased in the cisplatin-resistant human head and neck cancer KB cell line KB/KCP4 (14), the cisplatin-resistant human prostatic cancer PC-3 cell line P/CDP5 (12), and the cisplatin-resistant human bladder cancer T24 cell line T24/DDP10 (13). The transport of leukotriene C4 in membrane vesicles prepared from cisplatin-resistant KB/KCP4 cells was facilitated by an ATP-dependent pump that seemed similar to the GS-X pump (17). Therefore, we examined whether expression of the human *cMOAT* gene was enhanced in these drug-resistant cell lines exhibiting decreased cisplatin accumulation. Human cMOAT mRNA was overexpressed 4.0- to 6.0-fold in all three cisplatin-resistant cell lines as compared to their parental drug-sensitive counterparts (Fig. 4*B*). In contrast, human *cMOAT* gene expression was not enhanced in KB/VP4 cells that overexpress the *MRP* gene (Fig. 4, *C* and *D*). The KB/VP4 cell line, derived from KB cells resistant to the epipodophyllotoxin anticancer agent etoposide, had a multidrug resistance phenotype and exhibited decreased drug accumulation (18). The size of the human cMOAT mRNA was estimated to be about 6.5 kb (Fig. 4*B*), similar to that of MRP mRNA (Ref. 4; see Fig. 4*D*) but larger than the 4.5-kb human MDR1 mRNA.

We isolated human cMOAT cDNA by targeting the ATP binding domain conserved in three human ABC superfamily genes [*MDR1* (P-gp), *MRP*, and *CFTR*] and found human cMOAT was a homologue of human MRP (4) and the recently described rat *cMOAT* gene (Ref. 16; see Fig. 2). Mayer *et al.* (25) have reported that the absence of MRP or its isoform from the canalicular membrane is the basis for the hereditary defect of the hepatobiliary excretion of anionic conjugates in the TR^- rat model of human Dubin-Johnson syndrome. Paulusma *et al.* (16) have reported selective expression of rat cMOAT by liver hepatocytes as well as reduced mRNA levels in the TR^- rat. Expression of human cMOAT was observed in liver but was not detectable in other tissues, suggesting that human cMOAT may mediate the hepatobiliary excretion of numerous organic anions and that human cMOAT mutation may result in the Dubin-Johnson syndrome. We are now exploring this possibility in human patients.

Expression of human cMOAT was enhanced in cisplatin-resistant human cancer cell lines with decreased cellular cisplatin accumulation but was not enhanced in lines with normal levels of drug accumulation. We have previously reported that two cisplatin-resistant lines, KB/KCP4 and P/CDP5, that do not overexpress Pgp or MRP show enhanced active ATP-dependent efflux of cisplatin (12, 14). We also demonstrated by a cell-cell hybridization test that both the drug resistance and the accumulation defect found in KB/KCP4 cells are dominant traits (14), suggesting the existence of an active efflux system for cisplatin in KB/KCP4 and P/CDP5 cells. Ishikawa *et al.* (26) have suggested that an ATP-dependent pump may function to transport glutathione-conjugates (GS-X pump). Ishikawa and Ali-Osman (27) have additionally reported

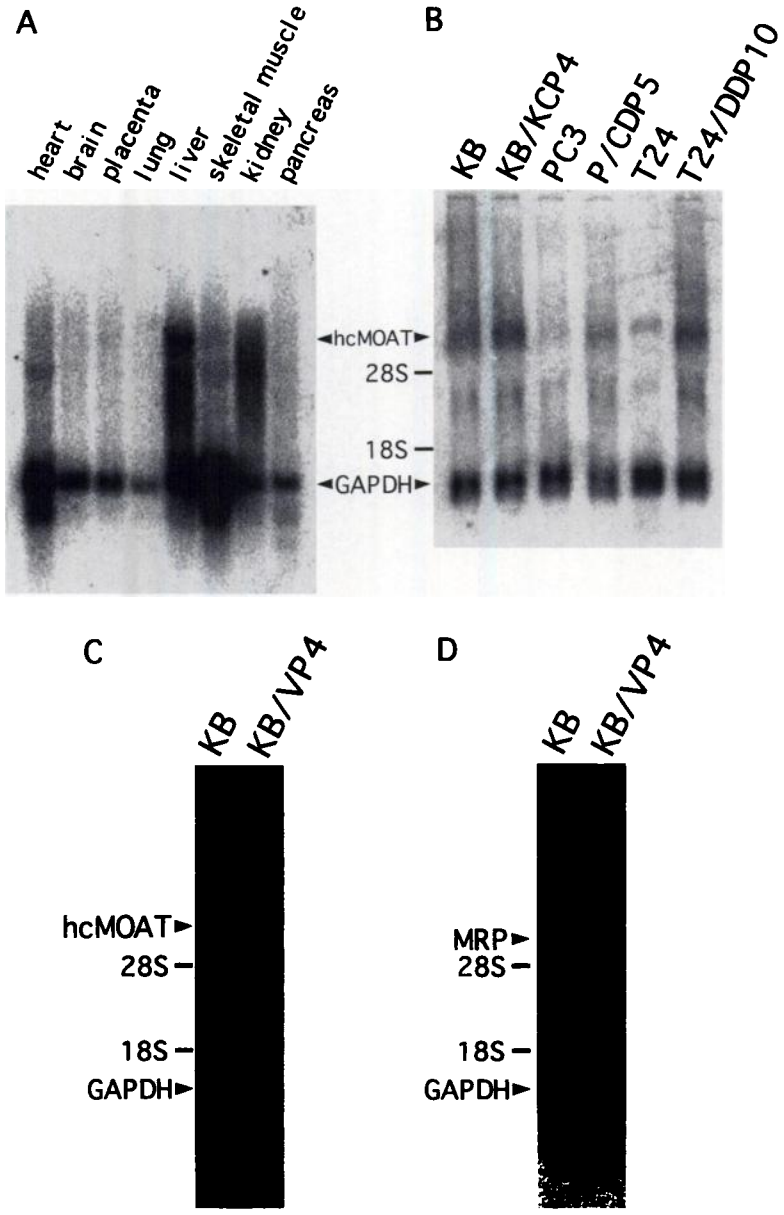

Fig. 4. Northern blot analysis. *A*, human multiple tissue blots were probed with human cMOAT C5-1 cDNA fragment (see Fig. 2*A*). *B*, various parental and cisplatin-resistant cell lines were analyzed using the same probe. Northern blots of KB and KB/VP4 cell lines were probed with human cMOAT (*C*) and MRP (*D*).

that the GS-X pump may effuse glutathione-conjugated cisplatin from L1210 murine leukemia cells. Cisplatin molecules were found to interact very quickly with two glutathione molecules in growing mammalian cells (28). It remains unclear whether human cMOAT is closely coupled with the activity of the glutathione-conjugated cisplatin pump.

On the other hand, MRP functions as an ATP-dependent export pump not only for glutathione conjugates but also for glucuronidated and sulfated endogenous as well as exogenous compounds, including various anticancer agents (29). However, all three cisplatin-resistant lines derived from KB, PC-3, and T24 cells do not overexpress MRP (12–14), and cancer cell lines that overexpress MRP, including KB/VP4 cells, are not cross-resistant to cisplatin (5, 6, 18). It is thus unlikely that MRP is involved in intrinsic or acquired cisplatin resistance due to decreased drug accumulation. Further studies using human cMOAT cDNA transfection will confirm that human cMOAT is directly involved in cisplatin resistance and can serve as a multispecific organic anion transporter. Transfection of the complete human cMOAT cDNA is in progress in our laboratory.

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