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Structure and Expression of the Messenger RNA Encoding the Murine Multidrug Resistance Protein, an ATP-binding Cassette Transporter

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SUMMARY

In vitro, overexpression of the human multidrug-resistance protein (MRP) causes a form of multidrug resistance similar to that conferred by P-glycoprotein, although the two proteins are only very distantly related. Studies with MRP-enriched membrane vesicles have demonstrated that the protein can bind and transport cysteinyl leukotrienes, as well as some other glutathione conjugates, with high affinity. In contrast, there is no direct evidence of the ability of MRP to bind or transport unmodified forms of the drugs to which it confers resistance. To facilitate studies of the physiological function(s) of MRP and its ability to cause multidrug resistance *in vivo*, we cloned and characterized the mRNA specifying its murine homolog. The murine MRP mRNA encodes a protein of 1528 amino acids that is 88% identical to human MRP. Although detectable by Northern blotting at variable levels in a wide range of tissues, *in situ* hybridization experiments revealed that MRP mRNA expression in some tissues is cell-type specific. High levels of the mRNA were detected in epithelia lining bronchi and bronchioles, as well as stage-specific expression in the seminiferous epithelium of the testes. Comparison of the predicted hydropathy profiles of human and murine MRP suggests a highly conserved membrane topology, the most distinctive feature of which is an extremely hydrophobic NH_2-terminal region containing five or six potential transmembrane sequences. This structural feature is shared with the sulfonylurea receptor and the yeast cadmium factor 1 but is not present in members of the superfamily, such as the cystic fibrosis transmembrane conductance regulator and P-glycoproteins. Finally, we used overlapping cDNAs to construct an episomally replicating murine MRP expression vector that was stably transfected into HeLa cells. MRP-transfected cell populations expressed markedly elevated levels of a 180-190-kDa protein that cross-reacted with a polyclonal antiserum raised against a peptide that is completely conserved in murine and human MRPs. The MRP transfectants also displayed increased resistance to vincristine (5-6-fold) and doxorubicin (<2-fold).

Inherent or acquired resistance to multiple chemotherapeutic agents is a major obstacle to the successful treatment of a number of common malignancies. The most extensively characterized mechanism capable of causing multidrug resistance involves overexpression of the ATP-dependent transmembrane transporter P-gp, which in humans is encoded by the *MDR1* gene (1, 2). However, overexpression of P-gp is infrequent in a number of cancers that are often refractory to chemotherapy, suggesting that alternative causes of multidrug resistance exist (2, 3). This supposition was confirmed recently by the cloning of the mRNA encoding human MRP and the subsequent demonstration that its overexpression also conferred multidrug resistance (4-6). Since its initial characterization in the small-cell lung cancer cell line H69AR, MRP has been found to be overexpressed in both selected and inherently multidrug-resistant human tumor cell lines of numerous origins, including small-cell and large-cell lung carcinomas, leukemias, fibrosarcomas, neuroblastomas, gliomas, and carcinomas of the breast, thyroid, bladder, prostate, and cervix (4, 7). In addition, increased expression of a cross-hybridizing mRNA species was detected with a human MRP cDNA probe in a multidrug-resistant mouse leukemia cell line (8). Drug-resistant cell lines that overexpress both MRP and P-gp have also been described (9-11). In these cases, overexpression of MRP preceded that of P-gp

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ABBREVIATIONS: P-gp, P-glycoprotein; MRP, multidrug-resistance protein; ABC, ATP-binding cassette; CFTR, cystic fibrosis transmembrane conductance regulator; SUR, sulfonylurea receptor; PCR, polymerase chain reaction; RT, reverse transcriptase; NBF, nucleotide binding fold; SDS, sodium dodecyl sulfate; SSC, standard saline citrate.

during selection in relatively low drug concentrations. This observation suggests that MRP may be involved in the early stages of drug resistance and that its overexpression may result from exposure to clinically relevant concentrations of drugs (9, 10). Several recent reports have implicated MRP in clinical drug resistance in neuroblastoma and various forms of leukemia (7, 12, 13).

The predicted amino acid sequence of MRP indicates that it is a member of the ABC superfamily of transmembrane transport proteins (4). Members of this superfamily transport a diversity of molecules, including ions, amino acids, sugars, long chain fatty acids, and peptides (14). Many of the predicted characteristics of MRP have been confirmed through biochemical studies in drug-selected and -transfected cell lines (15-17). Evidence of the ability of MRP to function as a transporter has been demonstrated most convincingly with isolated membrane vesicles from drug-selected and MRP-transfected cells (18, 19). MRP was shown to be an ATP-dependent, high affinity transporter of cysteinyl leukotrienes as well as some other glutathione conjugates (18-20). Additional physiological roles suggested for MRP include those of a GS-X pump and an organic anion transporter, although there is little evidence to support these suggestions. Furthermore, it has not been possible to demonstrate that MRP can either bind or transport unmodified forms of the drugs to which it confers resistance (16, 21). Thus, the mechanism by which the protein confers resistance to such a broad spectrum of xenobiotics remains to be established.

As an initial step toward the development of an animal model for the investigation of the physiological functions of MRP and its ability to confer multidrug resistance *in vivo*, we cloned and characterized the mRNA for a highly homologous murine protein. We also determined the tissue distribution of the mRNA and its spatial pattern of expression in murine lung and testis. Extensive sequence comparison of murine and human members of the ABC superfamily indicates that the mRNA encodes a true MRP homolog. These analyses demonstrate that MRP belongs to a subgroup of the superfamily that includes the rat SUR and the yeast cadmium resistance protein YCF1 (22, 23). Alignment of the hydropathy plots of human and murine MRP, rat SUR, and yeast YCF1 revealed a conserved organization of transmembrane regions that differs considerably from those of structures proposed for other members of the superfamily, such as P-gp and CFTR. Comparison between murine and human proteins also suggests that the number of transmembrane domains may have been underestimated in the original model we proposed for human MRP (4). Transfection of HeLa cells with an episomally replicating expression vector containing a cDNA that included the entire predicted coding region of the murine MRP mRNA resulted in production of a protein of the expected size. The vector-encoded protein was recognized by an antiserum raised against a conserved peptide in the NH_2-proximal ATP binding domains of murine and human MRPs. The MRP-transfected cells were also shown to be 5-6-fold resistant to vincristine relative to cells transfected with the parental vector.

Materials and Methods

Cloning and sequence analyses of murine MRP. A mouse skeletal muscle 5′-stretch plus cDNA library (Clontech Laboratories) was screened as described previously (24) with a DNA fragment isolated from a 129SV-CP mouse genomic library (kindly provided by R. Zirngibl, Queen's University, Kingston, Canada) by screening with a human, 5′ proximal MRP cDNA extending from nucleotides −196–1427 of the mRNA (all nucleotide numbering is given relative to the start of translation). The murine genomic fragment contained a putative exon corresponding to exon 2 of the human *MRP* gene (25). The muscle cDNA library was also screened under high stringency conditions with a 3′ proximal cDNA fragment, mrp10.1, corresponding to nucleotides 3881-4815 of human MRP mRNA (4). A total of ~5 × 10^5 plaques were screened with both probes, and four positive cDNA clones were selected and plaque purified (clones 14B, 16, 37, and 41). The cDNA inserts of clones 14B, 16, and 41 were subcloned into a pBluescript vector (Stratagene), and both strands were sequenced, either manually according to the dideoxy chain termination method (26) and with the use of Sequenase version 2.0 (U.S. Biochemicals) or at the Core Facility for Protein and DNA Chemistry (Queen's University, Kingston, Canada) with the use of an Applied Biosystems Automated DNA Sequencer.

PCR. PCR was carried out with *Pyrococcus furiosus* polymerase (Stratagene), under conditions specified by the manufacturer, to amplify specific regions of the isolated cDNAs for additional sequencing. RT-PCR of poly(A)$^+$ RNA from the L138C3-109 murine mastocytoma cell line (a gift from Dr. D. Keppler, German Cancer Center, Heidelberg, Germany) was used to amplify specific regions of the murine mRNA. The DNA amplification products were then subjected to dideoxy sequencing.

Expression vector construction and transfection. A DNA fragment containing the complete coding region of murine MRP mRNA, as well as 6 and 305 nucleotides of the 5′- and 3′-untranslated regions, respectively, was assembled in the vector pBluescript (Stratagene). The fragment was synthesized from two overlapping cDNA clones and two RT-PCR products. The integrity of the resulting fragment was confirmed through complete sequencing of the portions produced by PCR and all cloning junctions. The fragment was then transferred to the modified pCEBV7 vector we described previously, where its expression is driven by the cytomegalovirus promoter (15, 27). This vector contains the *Escherichia coli hph* gene, which permits selection of transfected cells with hygromycin B (Boehringer). HeLa cells were transfected with the expression vector according to a standard calcium phosphate coprecipitation procedure (24). After 48 hr, the medium was replaced with fresh medium supplemented with 50 μg/ml hygromycin B and maintained 7 days. The cells were then maintained in medium supplemented with 100 μg/ml hygromycin B. After a period of 3 weeks in hygromycin B-supplemented media, the cells were harvested, and the mRNA and protein levels of the vector-encoded murine MRP were determined with the use of Northern and immunoblot analyses as described below. Transfected cells were also tested for resistance to vincristine (Sigma Chemical Co.) and doxorubicin (Sigma Chemical Co.) with the use of a tetrazolium salt-based microtiter plate [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, as described previously (15). Resistance was determined in two independent experiments, and within each experiment, assays were carried out in quadruplicate.

RNA isolation and Northern blot analysis. Total RNA was obtained through extraction with TRIzoL reagent (GIBCO-BRL) from various tissues of mature CD1 mice and murine mastocytoma L138C3-109 cells and of HeLa cells transfected with the parental pCEBV7 expression vector (C6), the vector containing the murine MRP mRNA coding sequence (mB), or the pRc/CMV vector containing the human MRP mRNA coding sequence (T5) (5, 15). Poly(A)$^+$ RNA was selected from total RNA with the use of either PoLyATtract (Promega) or Micro-FastTrack (Invitrogen) isolation kits. RNA was analyzed with electrophoresis on formaldehyde/agarose denaturing gels and transferred to Zetaprobe membrane (Bio-Rad) under standard conditions (NEN Products) (28). Blots were prehybridized at 37° for 4-6 hr in 50% formamide, 5× standard saline/phosphate/EDTA buffer (1× = 150 mM NaCl, 10 mM Na_2HPO_4, pH 7.0, 1 mM EDTA),

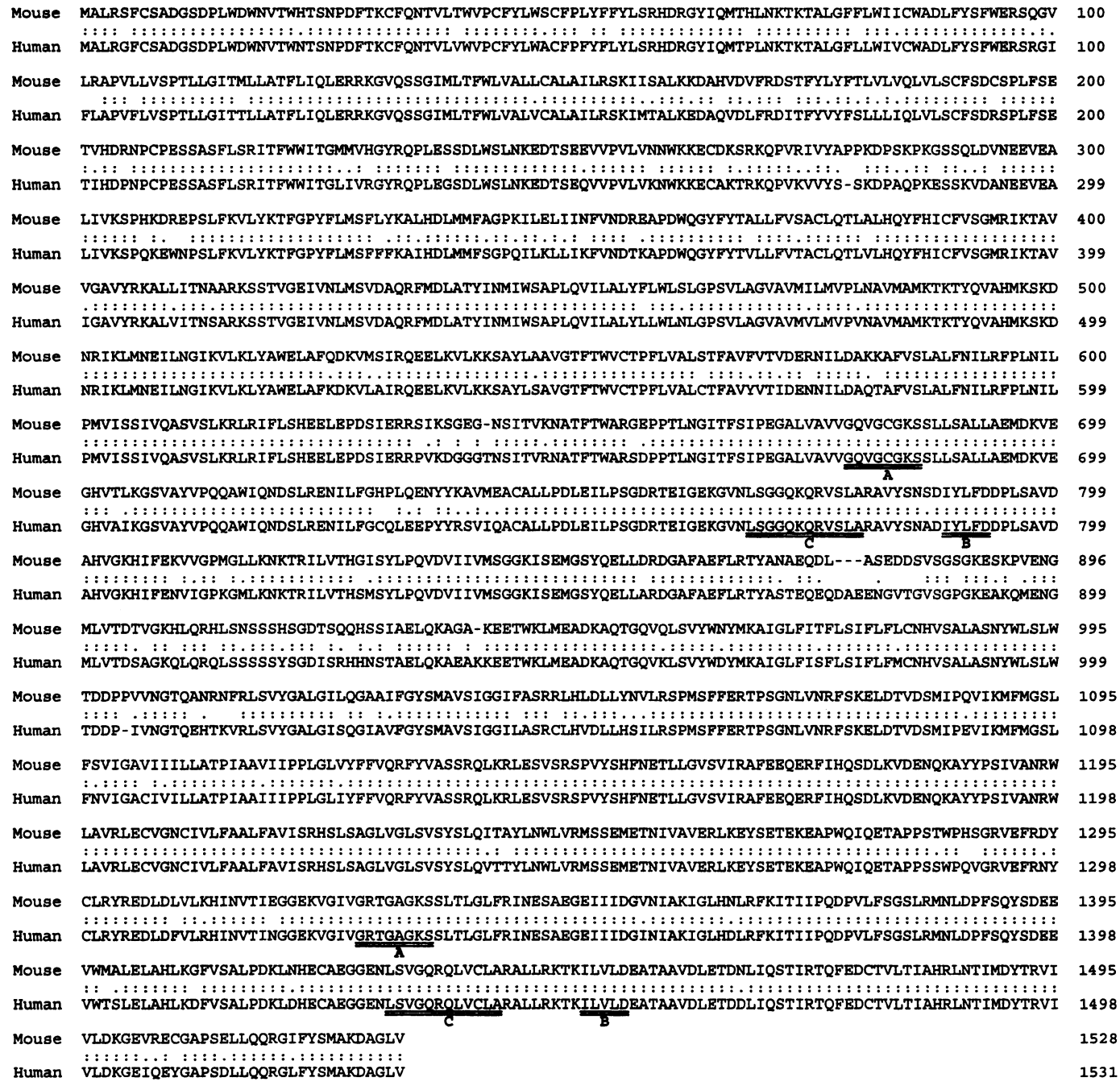

Fig. 1. Alignment of the predicted amino acid sequence of murine and human MRPs. *:*, Conserved residues. *.*, Conservative substitutions. *Double underlining*, Walker A and B motifs and the active transport family signature (*C*), which are conserved characteristics of the NBFs of ABC transporters. The sequence shown for the human protein differs at two residues from the published sequence as the result of two nucleotide changes detected during sequencing of additional cDNA clones from the H69AR cell line. Both changes result in a match with the mouse sequence. The affected residues are Ser^{685} (originally leucine) and Ala^{1282} (originally arginine).

4× Denhardt's, 0.5% SDS, and 100 μg/ml sheared and denatured herring testes DNA. They were hybridized under the same conditions for 12-16 hr with [α-^{32}P]dATP-labeled cDNA fragments and subsequently washed four times for 15 min in 0.1× SSC (1× SSC = 150 mM NaCl, 15 mM sodium citrate) and 0.1% SDS at 52°. Blots were probed with putative murine MRP cDNA fragments and, where stated, were hybridized without stripping with a cDNA corresponding to β-actin.

Protein blot analysis. The relative levels of expression of murine and human MRPs in transfected and control cells were determined through immunoblot analysis of membrane protein fractions. Membrane proteins were prepared, separated through SDS-polyacrylamide gel electrophoresis, and transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore), as described previously (5). Affinity-purified anti-MRP polyclonal antibody, MRP-1, was used to detect both human and murine proteins. This polyclonal antibody was raised against a synthetic peptide corresponding to amino acids 765-779 of human MRP that is entirely conserved in murine MRP. Antibody binding was visualized with the use of horseradish peroxidase-conjugated goat anti-rabbit IgG and enhanced chemiluminescence detection (Amersham).

***In situ* hybridization.** Single-stranded antisense RNA probes were produced through run-off *in vitro* transcription in the presence of digoxigenin/UTP (Boehringer-Mannheim). The template for the

MRP probe was a fragment of clone 14B corresponding to nucleotides 119-1610 of the murine mRNA, which was subcloned in pBluescript II SK$^+$ (Stratagene). As a control, tissue sections were hybridized with an antisense probe that was complementary to the coding region of rabbit β-globin (29). Cryosections (6-8 μm) were mounted onto poly-L-lysine-coated glass microscope slides, fixed for 1 hr in 4% paraformaldehyde, treated with proteinase K (1 μg/μl for 10-20 min), and post-fixed in 4% paraformaldehyde for 20 min before being hybridized overnight in 50% formamide, 5× SSC, 0.5 mg/ml tRNA, 0.005% heparin, 0.1% Tween-20, and 250 μg/ml denatured herring testes DNA. On the next day, the slides were washed in 2× SSC twice for 30 min each at room temperature and once for 30 min at 65°, followed by a 30-min high stringency wash at 65° in 0.1× SSC/0.1% SDS. Remaining unhybridized probe was removed through a 30-min digestion with 20 μg/ml RNase A (Pharmacia) at 37°, followed by 15 min in 2× SSC/0.1% SDS at 65° and 30 min in 1× SSC/0.1% SDS at 65°. Hybridized probe was detected with an alkaline phosphatase-conjugated sheep anti-digoxigenin antibody (Boehringer-Mannheim) and the chromogenic substrates nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate.

Results and Discussion

Cloning of mouse MRP cDNA. Preliminary Northern blotting analyses of several murine tissues were carried out to find a suitable source from which to clone the murine MRP homolog. Analyses with a human MRP cDNA probe and high stringency washing conditions were used to detect a single cross-hybridizing mRNA species of 6.0-6.2 kb in all tissues. The size of the mRNA is consistent with that overexpressed in a multidrug-resistant murine leukemia cell line, which was also detected with a human MRP cDNA probe (8). Because the cross-hybridizing species was present at relatively high levels in skeletal muscle, a mouse skeletal muscle cDNA library was screened using both 5′ and 3′ proximal MRP DNA probes. The 5′ proximal probe was isolated during preliminary screening of a 129SV-CP mouse genomic library with a human MRP cDNA containing nucleotides −196–1427. The murine genomic fragment contained a sequence that was 90% identical to the second exon of the human *MRP* gene, which encodes nucleotides 48-226 of the coding region of the mRNA (25). The 3′ proximal probe was a human cDNA fragment encompassing nucleotides 3881-4815 of MRP mRNA. Sequencing of four positive cDNA clones revealed a potential open reading frame of 1528 amino acids, which was 84% identical at the nucleotide level with the coding region of human MRP mRNA. The open reading frame was interrupted in one clone by a stretch of 65 nucleotides bracketed by potential intron acceptor and donor sites. The region spanning the possible intron was amplified with RT-PCR using RNA from the mouse mastocytoma cell line, which expresses relatively high levels of a 6.0-6.2-kb mRNA that under high stringency conditions cross-hybridizes with human MRP cDNA (data not shown). The sequence of the RT-PCR product matched that of the cDNA clones except that the 65-nucleotide sequence present in clone 14B was absent. Because the library was constructed from total poly(A)$^+$ RNA, we concluded that the sequence that was not present in the majority of the mRNA most likely represents an unspliced intron. The 5′ proximal clone, clone 37, contained a methionine codon at a position corresponding to the initiator methionine of human MRP plus five nucleotides of 5′-untranslated sequence. In addition to the open reading frame, the compiled sequence of 5884 nucleotides contained a 3′-untranslated region of 1295 nucleotides.

Comparison of amino acid sequences. The deduced amino acid sequences of the murine protein and human MRP are 88% identical (Fig. 1). Walker A and B motifs, characteristic of members of the ABC superfamily, are completely conserved in both the NH_2- and COOH-proximal NBFs of the two proteins, as is the atypical spacing of these motifs in the NH_2-proximal domain (4, 30). The highest variability between the predicted human and murine amino acid sequences occurs in the so-called linker region following the NH_2-proximal NBF (defined as amino acids 793-965 in mouse MRP and amino acids 793-969 in human MRP), where identity decreases to 78%. This region is also relatively variable between other homologous members of the ABC superfamily (14). Despite its relative lack of conservation, in the case of both CFTR and P-gp there is substantial evidence that phosphorylation of serine residues in the linker region influences activity of the protein (31-33). Human MRP is also phosphorylated, primarily on serine residues (16, 17). The locations of the phosphoserine residues are not known, but preliminary data suggest that protein kinase C may contribute to their phosphorylation (17). The human and mouse proteins contain 8 and 10 potential serine protein kinase C sites, respectively, of which only 5 are conserved and none are located in the linker region. Other conserved sites of potential serine phosphorylation include a single site for cyclic nucleotide-dependent protein kinase that is present in both proteins and 14 conserved serine casein kinase II sites.

The most striking similarity observed between murine and human MRPs was the complete conservation of 114 amino acids extending from residues 1123-1236 and 1126-1239 of the murine and human proteins, respectively. No such extended region of identity exists between any known murine and human homologs of the other ABC transporters, suggesting that the region may be critical for the function of MRP.

Analysis of the tissue distribution of the murine mRNA. Northern analyses with the cDNA insert from clone 16 as a probe detected an mRNA of 6.0-6.2 kb in all tissues examined (Fig. 2), with high levels present in testes, lung, kidney, heart, and skeletal muscle and very low levels present in liver and intestine. This tissue profile of expres-

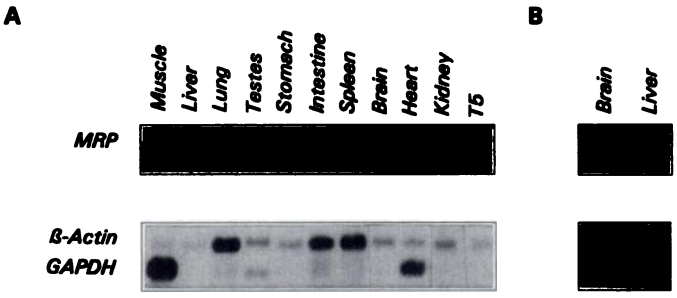

Fig. 2. Northern blot analyses of poly(A)$^+$ RNA isolated from normal mouse tissues. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase. A, Poly(A)$^+$ RNA from the indicated tissues (5 μg) and from the MRP-transfected HeLa cell line (*T5*) (0.5 μg) were analyzed with the use of formaldehyde/agarose gel electrophoresis and blotted, as described in Materials and Methods. The blot was hybridized with a ^{32}P-labeled DNA fragment from murine clone 16, which detected a single-size class of mRNA (6.0-6.2 kb). All tracks shown are from the same gel. B, Poly(A)$^+$ RNA from brain (5 μg) and liver (20 μg) was resolved with the use of gel electrophoresis, blotted, and probed as described for A.

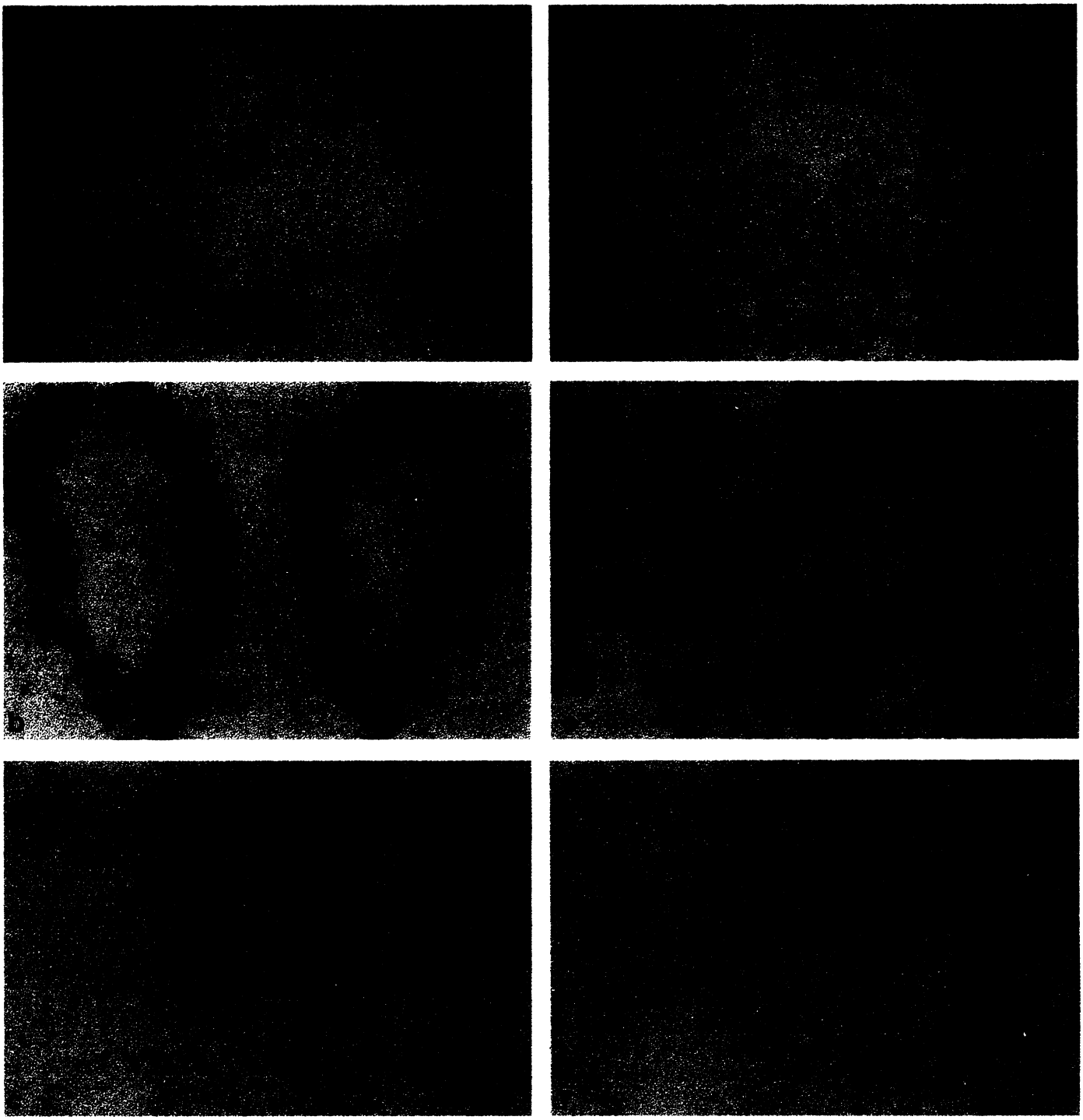

Fig. 3. *In situ* hybridization analysis with single-stranded, digoxigenin-labeled MRP RNA probes. Low-magnification views (64×) of sections from adult mouse testis (a) and lung (d) hybridized with the antisense murine mRNA probe derived from clone 14B. b and e, High-magnification views (320×) of areas shown in a and d, respectively. Identical results were obtained when hybridization was carried out with another RNA derived from clone 41 that corresponds to a region of the transcript distinct from the clone 14B probe (data not shown). The specificity of the staining pattern was also assessed through hybridization with a rabbit β-globin probe. c and f, High-magnification views (320×) of testis and lung sections, respectively, hybridized with the rabbit β-globin antisense probe. Both sections show only a low level of background staining, without any discernible pattern.

sion is qualitatively similar to that reported for human MRP mRNA (4, 34). MRP has a number of functional characteristics that are very similar to the ATP-dependent leukotriene export carrier characterized in rat hepatocanalicular membranes (35). Consequently, it was of interest to determine whether there was hepatic expression of the murine mRNA, as previous Northern analyses failed to detect MRP mRNA in human liver, although very low levels of expression were detected with RNase protection assay (4, 34). Expression of an mRNA of a size indistinguishable from that present in other tissues was detectable in mouse liver when relatively large quantities of poly(A)$^+$ mRNA were used (Fig. 2B). The

levels were lower than those in any other tissue examined, with the possible exception of intestine (Fig. 2A). Thus, the amount of MRP in hepatocanalicular membranes, if this is its location in the liver, appears likely to be low compared with other transporters, such as murine mdr2 (36).

High levels of MRP mRNA were detected in testis. Other members of the ABC transporter superfamily, including P-gp and CFTR, are known to be expressed during spermatogenesis in rodents. Consequently, we analyzed the spatial distribution of MRP mRNA with the use of *in situ* hybridization (Fig. 3, a–c). These experiments revealed that the mRNA was present in only a subset of seminiferous tubules and that levels of expression within this subset were highly variable. The cells involved were identified as haploid spermatids on the basis of their location within the seminiferous epithelium (Fig. 3, a and b). In rodent testis, cross sections of seminiferous tubules typically show associations of germ cells at different stages of maturation, from spermatogonial stem cells at the base of the epithelium to differentiated spermatozoa adluminally (37, 38). In a given cross section, different seminiferous tubules will show different cell associations, representing the various phases of spermatogenesis. Thus, the pattern of localization observed is consistent with regulated expression and stage-specific accumulation of MRP mRNA. In mice, CFTR mRNA can be detected in most of the seminiferous tubules in a given cross section and is present in both spermatids and primary spermatocytes (39, 40). In contrast, the pattern of CFTR mRNA expression in rat testis is similar to that seen in the current study (39). *MDR1* mRNA expression in rat testis is also observed in only two or three seminiferous tubules in each cross section and is restricted to spermatogonia or primary spermatocytes (39).

The physiological functions of members of the ABC superfamily during spermiogenesis have not been characterized. Accumulation of the murine MRP mRNA in spermatids occurs during a period of major morphogenic transformation, one aspect of which is a reduction in nuclear and cytoplasmic volume. It has been suggested that loss of water from the cell, caused by active pumping of ions to the extracellular space, contributes to the reduction in volume (41). Increases in the level and degree of phosphorylation of P-gp have been shown to result in changes in activity of outwardly rectifying Cl^- channels (42). Overexpression of MRP in the human small-cell lung cancer cell line H69AR was also accompanied by changes in activity of both Cl^- and K^+ channels (43). Consequently, it is possible that ABC transporters contribute to the process of volume reduction.

MRP mRNA has been shown to be expressed at relatively high levels in normal human lung as well as unselected non-small-cell lung cancer cell lines (4). To date, one study, based on *in situ* hybridization with an antisense MRP oligonucleotide, indicated that in normal human lung MRP, mRNA is expressed in bronchiolar epithelial cells (44). The physiological role of MRP in the lung has not been established. However, the protein has been shown to transport cysteinyl leukotrienes, which in primates and some other species, notably the guinea pig, are potent constrictors of peripheral and, to a lesser extent, central airways (for a review, see Ref. 46). In contrast to the response in primates and guinea pigs, these compounds have little effect on airway smooth muscle contraction in some other rodents. In view of these interspecies differences, we examined the spatial expression of mouse MRP mRNA. A pattern of expression was found that was similar to that reported for human lung, with high levels of MRP mRNA being detectable only in the epithelial lining of bronchi and bronchioles (Fig. 3, d and f) (44).

Multiple-sequence alignment. The relationship of the deduced amino acid sequence of the murine mRNA to human MRP, as well as to other members of the ABC transporter superfamily, was examined further by carrying out a multiple-sequence alignment of all mouse and human ABC trans-

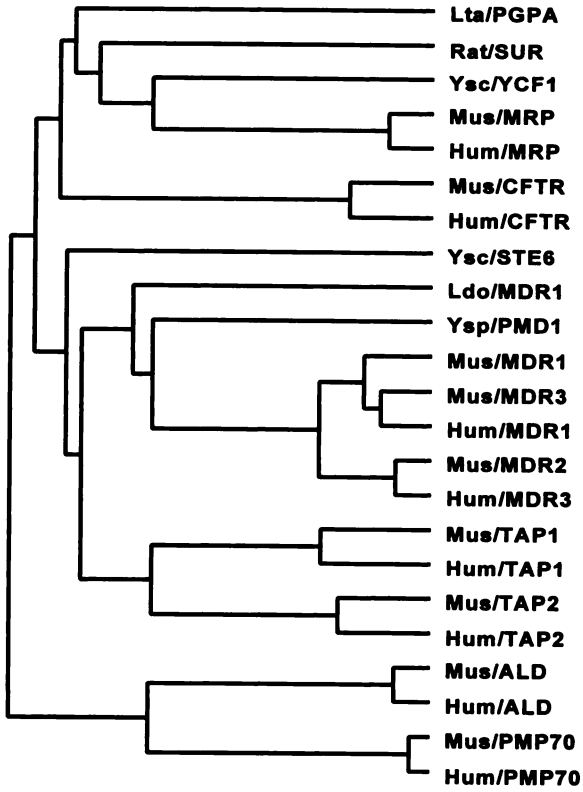

Fig. 4. Cluster analysis of the relative similarity of the open reading frame of the murine MRP mRNA to human MRP and other members of the ABC transporter superfamily. The dendrogram illustrates the relative similarity between all mouse and human ABC transporters for which complete amino acid sequences are available, as well as several other eukaryotic members selected for their relationship to either human MRP or human *MDR1*. A multiple sequence alignment was created by the PILEUP program from the Genetics Computer Group package (version 8.0) using a modified version of the progressive alignment method of Feng and Doolittle (45) with a gap weight of 3.0 and a gap length weight of 0.1. *Horizontal branch lengths* are inversely proportional to the degree of similarity between the sequences. The following protein sequences [with their Swiss Protein (*SP*), GenBank (*GB*), or EMBL (*EM*) accession numbers]) were selected for alignment with the murine mRNA (*Mus/MRP*):human MRP (*Hum/MRP*, SP P33527); the CFTRs from human (*Hum/CFTR*, SP P13569) and mouse (*Mus/CFTR*, SP P26361); P-gps from human (*Hum/MDR1*, SP P08183; *Hum/MDR3*, SP P21439), mouse (*Mus/MDR1*, SP P06795; *Mus/MDR2*, SP P21440; *Mus/MDR3*, SP P21447), *Leishmania tarentolae* (*Lta/PGPA*, SP P21441), and *Leishmania donovani* (*Ldo/MDR1*, GB L01572); peptide antigen transporters from human (*Hum/TAP1*, SP Q03518; *Hum/TAP2*, SP Q03519) and mouse (*Mus/TAP1*, SP P21958; *Mus/TAP2*, SP P36371); the adrenoleukodystrophy proteins from human (*Hum/ALD*, SP P33897) and mouse (*Mus/ALD*, EM Z33637); the 70-kDa peroxisome membrane proteins from human (*Hum/PMP70*, SP P28288) and mouse (*Mus/PMP70*, GB L28836); the *Saccharomyces cerevisiae* mating factor A secretion protein (*Ysc/STE6*, SP P12866); the *Schizosaccharomyces pombe* leptomycin B resistance protein (*Ysp/PMD1*, SP P36619); the *S. cerevisiae* cadmium resistance protein (*Ysc/YCF1*, SP P39109); and the rat SUR (*Rat/SUR*, GB L40624).

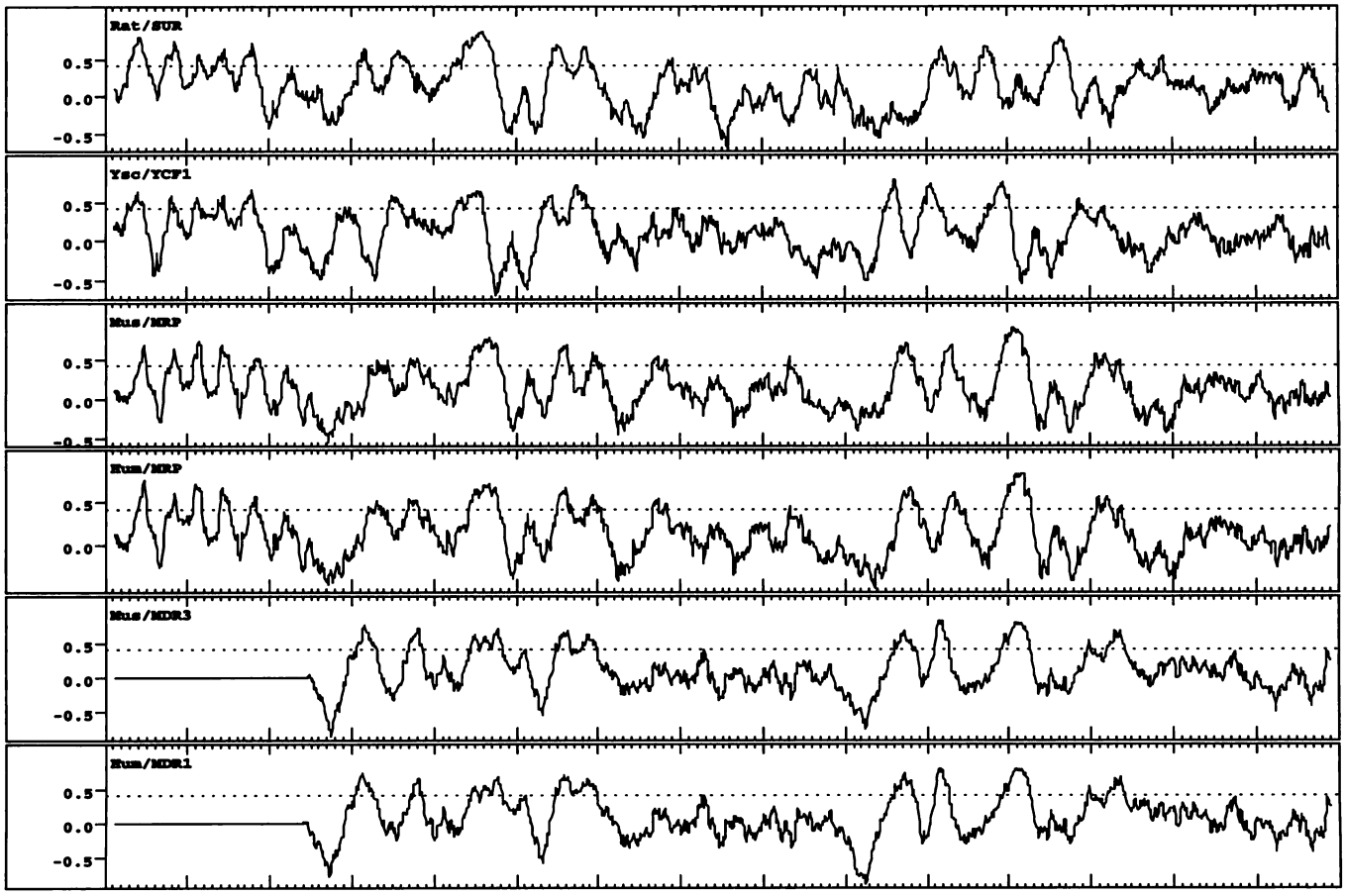

Fig. 5. Hydropathy profiles of human and mouse MRPs and other members of the ABC superfamily of transporters. Plots were generated with the PROfileGRAPH program (version 1.3 by K. O. Hofmann, Institute for Biochemistry, University of Cologne, Germany) using the normalized hydrophobicity values of Eisenberg *et al*. (50) and a window size of 21 amino acids. Vertical axis, mean hydrophobicity. *Horizontal axis*, major divisions correspond to 100 amino acids. *Regions above the dotted line at 0.42*, possible transmembrane α-helical segments. The PROfileGRAPH program has a maximum sequence length of 1500 amino acids. Accordingly, only the analyses for the first 1500 amino acids are displayed for the mouse (*Mus*) MRP (1528 amino acids), human (*Hum*) MRP (1531 amino acids), *Saccharomyces cerevisiae* YCF1 (*Ysc*) (1515 amino acids), and rat SUR (1582 amino acids) sequences. The multiple-sequence alignment generated with the PILEUP program was used to align the COOH-proximal regions of mouse MDR3 (1276 amino acids) and human MDR1 (1280 amino acids) with the corresponding regions of mouse MRP and human MRP. The plots show the first 1249 amino acids of human MDR1 and the first 1245 amino acids of mouse MDR3.

porters using the PILEUP program (Fig. 4). Included in this analysis were other eukaryotic members of the ABC superfamily, which were selected because of their relationship to either human MRP or human *MDR1*. As expected, the amino acid sequence encoded by the cloned murine mRNA clustered with that of the human MRP sequence. The degree of sequence similarity, which is inversely related to the horizontal distance separating the two sequences, was comparable to that found between other mouse and human pairs of ABC superfamily members. This observation provides compelling evidence that the cloned murine mRNA is the mouse homolog of human MRP and not another member of an MRP gene family or a "sister" gene.

Since the cloning of the human MRP mRNA, the sequences of additional members of the ABC superfamily have been deposited in various databases (4). A BLAST search with the mouse MRP sequence identified additional ABC transporters from rat, yeast, and *Leishmania*. In our earlier analysis, the *Leishmania tarentolae* Lt/PGPA protein, which confers resistance to heavy metal oxyanions, had the greatest sequence similarity to human MRP (4, 47). The current PILEUP analysis shows that the *Saccharomyces cerevisiae* YCF1 protein, which confers cadmium resistance, is now the sequence that is most similar to both the human and mouse MRP sequences (23). The degree of similarity is comparable to that between *Schizosaccharomyces pombe* PMD1 protein, which confers leptomycin B resistance, and the mammalian P-gps. This suggests that YCF1 is the yeast homolog of MRP and that PMD1 is the corresponding homolog of the P-gps. Included in the MRP cluster is the recently identified rat SUR protein (22), which has been implicated in the regulation of insulin secretion. Although more similar to MRP than Lt/PGPA, SUR is clearly not as closely related as YCF1. In addition, pairwise comparison indicates that the similarity between YCF1 and rat SUR (score, 0.59) is considerably lower than that between YCF1 and mouse MRP (score, 0.74) and is more comparable to the level of similarity between, for example, mouse MRP and Lt/PGPA (score, 0.56). Thus, the pairwise comparisons are more consistent with YCF1 being a homolog of MRP than being representative of an ancestral gene of both MRP and SUR.

Hydrophobicity analysis. The amphipathic structures present in pore-forming, multispanning transmembrane sequences complicate prediction of membrane topology (48).

This is particularly true of the ABC superfamily because the members tend to be large proteins with ≥12 predicted transmembrane helices, which increases the potential of tertiary structure interactions to influence the membrane topography of the protein. In addition, more recently developed methods of predicting membrane protein structure that rely on comparison with model proteins are limited by a lack of data on members of the ABC superfamily (49). Consequently, rather than proposing a secondary structure model, we simply aligned the hydropathy plots of several of the transporters to illustrate the degree of organizational conservation of potential membrane-spanning domains (Fig. 5). The mouse and human MRP and P-gp sequences, as well as the *S. cerevisiae* YCF1 and the rat SUR sequences, were compared with the use of a hydropathy plot with a window size of 21 amino acids and the normalized amino acid hydropathy values of Eisenberg *et al.* (50). The most striking difference between the members of the MRP cluster and the P-gps was the presence of an extremely hydrophobic NH_2-terminal extension of ~230 amino acids that contained five or six potential transmembrane domains. These closely spaced hydrophobic segments are not found in other members of the ABC superfamily and thus represent a distinguishing structural feature of the MRP cluster. Whether MRP, SUR, and YCF1 also share some functional characteristic attributable to this region of the proteins is unknown.

With the exception of the NH_2-proximal region, the hydropathy profiles of MRP, SUR, and YCF1 profiles are quite similar to those of the P-gps, particularly in the COOH-proximal halves of the molecules. This conserved organization of putative transmembrane domains suggests that previous predictions of the topology of MRP, SUR, and YCF1 may have underestimated the number of membrane-spanning sequences in these regions of the proteins. Examination of the MRP-like profiles suggests there may be as many as 18 membrane-spanning α-helices rather than 12 or 13.

Expression of murine MRP. The ability of the murine protein to confer drug resistance was examined by constructing an episomal expression vector containing the predicted coding region of murine MRP mRNA plus 6 nucleotides of 5′-untranslated region and 305 nucleotides of 3′-untranslated region. The vector was used to transfect HeLa cells that have previously been successfully transfected with human MRP (5, 15). After hygromycin B selection, Northern blot analysis was used to confirm expression of an mRNA of the predicted size in the population of MRP-transfected cells (mB). The murine MRP mRNA produced from the expression vector has a predicted length of 5.1-5.2 kb, and an mRNA of this size was readily detectable, exclusively in the transfected cell population (Fig. 6A). The size of the vector-encoded MRP mRNA allowed it to be distinguished from any endogenous 6.5-kb human MRP mRNA that might cross-hybridize with the murine cDNA probe. However, under the conditions used, we did not detect the endogenous MRP mRNA, which is present at very low levels in HeLa cells. To confirm that the vector-encoded mRNA was being translated, the production of protein was monitored with immunoblotting using an affinity-purified, MRP-specific polyclonal antibody directed against a conserved peptide in the NH_2-proximal NBF. This antibody detected similar amounts of the 190-kDa human MRP in the T14 cell population and a protein with a slightly faster electrophoretic mobility in mB cells (Fig. 6B). Levels of endogenous human MRP protein in the HeLa cells were below the level of detection, as demonstrated by the lack of a signal in the lane loaded with protein from the cell population transfected with parental vector (C6). The predicted polypeptide molecular mass of both human and murine MRPs is 171 kDa. Because the human protein is known to be both phosphorylated and *N*-glycosylated, the observed differences in electrophoretic mobility may be indicative of differences in post-translational modification.

The murine MRP-transfected cell population (mB) was 5-6-fold resistant to vincristine relative to cells transfected with the parental vector (C6) (Fig. 6C), and the cells also displayed a ~1.5-fold increase in resistance to doxorubicin (data not shown). These results confirm that the murine protein can

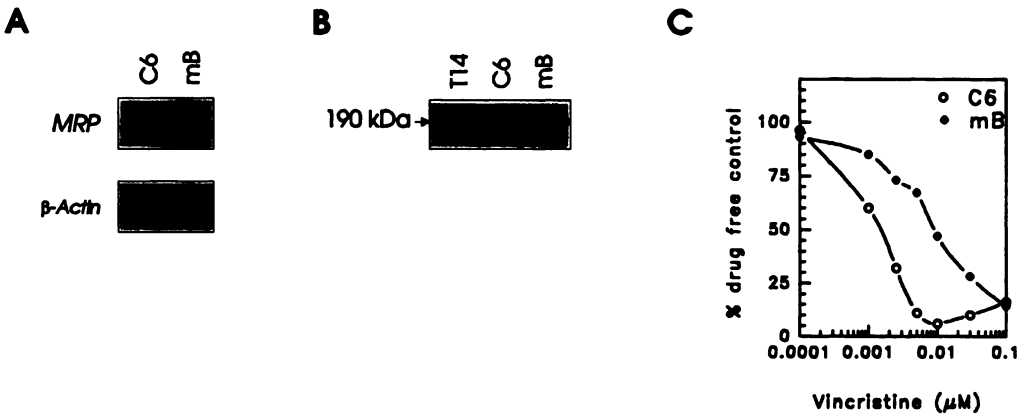

Fig. 6. Levels of MRP mRNA and protein in transfected cell lines and resistance to vincristine. A, Total RNA (10 μg) from the HeLa cells transfected with parental vector (*C6*) or a murine MRP expression vector (*mB*) was analyzed with formaldehyde/agarose gel electrophoresis and blotted as described in Materials and Methods. Hybridization with a ^{32}P-labeled murine MRP cDNA fragment detected an mRNA of ~5.1-5.2 kb exclusively in the mB cell population. The blot was also hybridized with β-actin to provide an indication of RNA loading on the gel. B, Membrane proteins (5 μg) prepared from C6 and mB cell populations, as well as from HeLa cells transfected with a comparable human MRP expression vector (T14), were separated through SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane, as described in Materials and Methods. Blots were probed with an affinity purified polyclonal antiserum, MRP-1, which detected a 190-kDa protein in T14 cells and a protein with slightly faster electrophoretic mobility in mB cells. C, Resistance of the murine MRP-transfected mB and control-transfected C6 cells to vincristine. The results shown are those of a typical chemosensitivity assay, as described in Materials and Methods. Each data point is the mean of quadruplicate determinations, which in all cases had standard deviations of <10%.

confer drug resistance, although the levels of resistance to both vincristine and doxorubicin in the mB population are somewhat lower than those found with the human MRP transfectant T14 population (15). A number of multidrug-resistant sublines of the murine erythroleukemia cell line PC4 have been described that, although clearly vincristine resistant, have very low levels of resistance to doxorubicin (8). These vincristine-selected cell lines overexpress an mRNA of ~6.0 kb that cross-hybridizes with a human MRP cDNA, indicating that they are very likely to overexpress murine MRP. The similarity between the resistance characteristics of these cell lines and the transfectants described here suggests that low resistance to doxorubicin may be an intrinsic property of murine MRP. However, it should be noted that in HeLa cells, human MRP also confers a significantly higher level of resistance to vincristine than to doxorubicin (15). Considerable variability has been observed among the resistance profiles of drug-selected cells overexpressing MRP (7). To what extent this is the result of cell-specific characteristics that influence MRP function or the result of other resistance mechanisms unrelated to MRP is not known. Consequently, comparison of the resistance profiles conferred by the human and murine MRPs in several cell types will be required before conclusions can be drawn regarding possible differences in drug specificity of the two proteins. Cloning of murine MRP mRNA and the establishment of expression systems for the mouse protein represent the first steps toward development of an *in vivo* model with which to study the physiological functions of MRP.

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