MOLECULAR AND CELLULAR BIOLOGY, Sept. 1989, p. 3808–3820
0270-7306/89/093808-13$02.00/0

Vol. 9, No. 9

Structure and Expression of the Human *MDR* (P-Glycoprotein) Gene Family

JANICE E. CHIN, RENA SOFFIR, KEVIN E. NOONAN, KYUNGHEE CHOI, AND IGOR B. RONINSON*

Department of Genetics, University of Illinois at Chicago, Chicago, Illinois 60612

Received 6 March 1989/Accepted 15 June 1989

The human *MDR* (P-glycoprotein) gene family is known to include two members, *MDR1* and *MDR2*. The product of the *MDR1* gene, which is responsible for resistance to different cytotoxic drugs (multidrug resistance), appears to serve as an energy-dependent efflux pump for various lipophilic compounds. The function of the *MDR2* gene remains unknown. We have examined the structure of the human *MDR* gene family by Southern hybridization of DNA from different multidrug-resistant cell lines with subfragments of *MDR1* cDNA and by cloning and sequencing of genomic fragments. We have found no evidence for any other cross-hybridizing *MDR* genes. The sequence of two exons of the *MDR2* gene was determined from genomic clones. Hybridization with single-exon probes showed that the human *MDR1* gene is closely related to two genes in mouse and hamster DNA, whereas *MDR2* corresponds to one rodent gene. The human *MDR* locus was mapped by field-inversion gel electrophoresis, and both *MDR* genes were found to be linked within 330 kilobases. The expression patterns of the human *MDR* genes were examined by enzymatic amplification of cDNA. In multidrug-resistant cell lines, increased expression of *MDR1* mRNA was paralleled by a smaller increase in the levels of *MDR2* mRNA. In normal human tissues, MDR2 was coexpressed with *MDR1* in the liver, kidney, adrenal gland, and spleen. *MDR1* expression was also detected in colon, lung, stomach, esophagus, muscle, breast, and bladder.

The multidrug-resistant phenotype in mammalian cells is defined by cross-resistance to a large group of lipophilic cytotoxic compounds, including plant alkaloids and antitumor antibiotics. Multidrug resistance in human and rodent cell lines is associated with decreased intracellular drug accumulation and correlates with the increased expression of *MDR* genes, which encode membrane glycoproteins (P-glycoproteins) of approximately 170 kilodaltons (19, 29, 34, 46). Expression of a single human gene, designated *MDR1*, or its rodent counterpart is sufficient to confer the multidrug resistance phenotype to drug-sensitive cells (7, 20, 40, 44). The relative levels of resistance to different drugs in multidrug-resistant cells can be altered by spontaneous mutations in the *MDR1* gene (7). cDNA sequence analysis has indicated that the *MDR1*-encoded P-glycoprotein consists of two homologous halves, each half including six transmembrane domains and a nucleotide-binding site (5, 17, 21). P-glycoproteins share homology with a group of membrane-associated bacterial proteins that transport their substrates in an energy-dependent manner (28). P-glycoprotein has been shown in biochemical studies to bind lipophilic drugs (10) and to hydrolyze ATP (26). These observations suggest that P-glycoprotein serves as an active efflux pump, responsible for decreased drug accumulation in multidrug-resistant cells, as originally proposed by Dano (12).

Increased P-glycoprotein expression in many multidrug-resistant cell lines results from amplification of the *MDR1* gene (13, 22, 24, 33, 35, 36, 38, 51). Different patterns of amplification of *MDR*-related genomic sequences observed among multidrug-resistant cell lines have suggested that the mammalian *MDR* genes constitute a small multigene family (22, 24, 31, 33, 36, 38, 51). These studies have indicated the presence of at least two MDR genes in the human genome and three genes in mouse and hamster genomes. This conclusion has been confirmed by cloning and sequencing of different *MDR* cDNA clones (5, 14, 17, 21, 23, 48). Proteins encoded by different members of the *MDR* family share similar structural organization and a high level of sequence homology, suggesting that they all function in active efflux. Only *MDR1* expression, however, has been associated with resistance to lipophilic drugs. The function of the proteins encoded by other members of the *MDR* gene family and the nature of the compounds transported by these proteins remain unknown.

In normal human tissues, relatively high levels of *MDR* mRNA and protein expression have been observed in the adrenal gland, on the luminal surfaces of the kidney, liver, colon, jejunum, and pancreas (16, 42), and in capillary endothelial cells in the brain and testes (9). A somewhat different pattern of tissue-specific expression has been reported for hamster (3) and mouse (11) *mdr* genes. Increased *mdr* gene expression was observed in mouse uterus during pregnancy (2) and in rat liver during regeneration or chemical carcinogenesis (43). *MDR* gene expression has also been observed in many different types of human tumors, which in most cases has not been treated with chemotherapeutic drugs (18). These observations suggest that P-glycoprotein-mediated membrane transport may be involved in a variety of physiological processes.

P-glycoproteins are unique among membrane transport molecules in their ability to carry out active transport of a great variety of compounds that share very little structural similarity. Almost all of the P-glycoprotein substrates now known are plant-derived or chemically synthesized cytotoxic drugs, none of which are likely to play a role in normal physiological processes. The only exception is progesterone, which was recently shown to interact with P-glycoprotein in the gravid mouse uterus (52). Understanding the number, structure, and tissue-specific expression patterns of different P-glycoproteins would help in elucidating their normal physiological functions. Toward this goal, we have analyzed the

* Corresponding author.

structure of the human *MDR* (P-glycoprotein) gene family and the expression of individual members of this family.

MATERIALS AND METHODS

Genomic DNA extraction and Southern hybridization. Genomic DNA was isolated by using a nucleic acid extractor (model 340A; Applied Biosystems, Inc., Fullerton, Calif.) under the conditions recommended by the manufacturer. ^{32}P-labeled probes were prepared by oligolabeling (15) of gel-purified or subcloned fragments of previously described *MDR1* and *MDR2* cDNA and genomic clones (5, 7, 36). Southern hybridization was carried out as previously described (36). For low-stringency hybridization, the filters were washed in 4× SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 65°C after hybridization; high-stringency hybridization conditions included washing in 0.1× SSC at 65°C.

For digestion with infrequently cutting restriction enzymes and analysis by field-inversion gel electrophoresis (FIGE) (4), KB-C4, KB-3-1, or K562 cells were grown to subconfluence and immobilized in agarose plugs (FMC In-Cert Agarose; FMC Bioproducts, Rockland, Maine) at a concentration of approximately 2×10^5 cells per 100 μl by a modification of published procedures (41). The cells were lysed and deproteinized in situ with 1% Sarkosyl (CIBA-GEIGY Corp., Summit, N.J.) and 0.5 mg of proteinase K per ml at 50°C for 48 h; a second sample of proteinase K was added, and the plugs were incubated for an additional 24 h. The plugs were then washed three times for 2 h each in 1 ml of 1 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, Mo.) and rinsed twice for 2 h each in 10 mM Tris (pH 8.0)–1 mM EDTA. DNA in plugs was digested with 25 U of either *Nru*I or *Xho*I at 37°C or *Sfi*I at 50°C overnight. After digestion, the plugs were washed for 2 h at 50°C in 1% Sarkosyl–0.5 M EDTA (pH 9.5) and then for 2 h at 50°C in 1% Sarkosyl–1 mg of proteinase K per ml–0.5 M EDTA (pH 9.5). The plugs were then placed into the wells of a 1.5% agarose gel and electrophoresed by field inversion for 36 h at 325 V. A ramp of pulse times was used, increasing from 3 s in the forward direction and 1 s in the reverse orientation at the beginning of electrophoresis to 60 s in the forward direction and 20 s in the reverse direction at the end of electrophoresis. The buffer temperature was maintained between 10 and 12°C. In some experiments, DNA fragments 50 to 750 kilobases pairs (kbp) in size were resolved in 0.8% agarose gels at 225 V, with the ramp extending in the forward direction from 0.3 to 73.8 s and in the reverse direction from 0.1 to 24.6 s. A modified pulsed-field gel electrophoresis procedure (30) was used in some experiments. Concatamers of bacteriophage λ DNA (Clontech Laboratories, Palo Alto, Calif.) were used as size standards.

Cloning and sequencing procedures. Size-selected genomic DNA libraries were constructed from the DNA of multidrug-resistant KB-C1.5 cells. DNA was digested with *Hin*dIII and fractionated on a sucrose density gradient. Fractions containing *Hin*dIII fragments of the desired size were ligated into the single *Hin*dIII site of the phage vector Charon 28 as previously described (36). About 10^5 plaques from each library were screened by plaque hybridization with gel-purified fragments of *MDR1* cDNA. Inserts from positive phage clones were subsequently recloned into pUC18 and pBR322 plasmid vectors. Highly repeated sequences within the clones were localized by hybridization with ^{32}P-labeled total human genomic DNA. The blots were also hybridized with a full-length *MDR1* cDNA probe (5, 7) in order to map *MDR1*-homologous sequences within each of the clones. Those fragments that hybridized to *MDR1* cDNA were subcloned into pGEM4 (Promega Biotec, Madison, Wis.) and used for sequencing.

For sequence analysis (53), miniscale plasmid DNA preparations were made from 10 ml of overnight bacterial cultures. In dideoxynucleotide DNA chain termination sequencing reactions, 0.5 μg of supercoiled DNA was used as a template, and oligonucleotides corresponding to the sequences of the SP6 or T7 RNA polymerase promoter were used as sequencing primers in the initial reactions. Subsequently, sequence-specific oligonucleotide primers were synthesized by using a DNA synthesizer (model 380A; Applied Biosystems) and used for further sequencing reactions.

RNA analysis by the polymerase chain reaction (PCR). Approximately 100 to 400 mg of ground frozen tissue or 5×10^7 cultured cells were used to isolate total RNA by the guanidinium isothiocyanate procedure (6). RNAs from human lung, stomach, spleen, esophagus, bladder, muscle, kidney, and adrenal gland were a kind gift of A. Fojo (National Cancer Institute); liver RNA was provided by D. K. Weerasinghe.

cDNA was prepared by using 200 U of Moloney leukemia virus reverse transcriptase (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) with 1 μg of total cellular RNA and 200 ng of random hexanucleotide primer (Pharmacia-LKB, Piscataway, N.J.) as described previously (32). The amount of cDNA equivalent to 200 ng of total RNA was combined with 1.5 μM PCR primers and 1.5 U of *Taq* DNA polymerase (Perkin Elmer Cetus, Norwalk, Conn.). PCR was carried out in 50 μl, using the Gene Amp DNA amplification reagent kit and the DNA Thermal Cycler (Perkin Elmer Cetus). An initial 2-min denaturation at 94°C was followed by 30 cycles of extension and denaturation. Each cycle included 1 min of denaturation at 94°C, followed by 5 min of primer annealing and extension at 65°C. In most experiments, the last cycle of PCR included an additional 20-min extension period at 65°C. A 10-μl amount of each reaction was analyzed by electrophoresis on either a 3% agarose or an 8% polyacrylamide gel.

To account for variations in yield and quality of different cDNA preparations, each cDNA sample was initially used to amplify sequences corresponding to the β_2-microglobulin cDNA (positions 317 to 428 in the cDNA sequence) (25). These reactions were carried out with the addition of 0.5 μCi of [α-^{32}P]dCTP. The 112-bp β_2-microglobulin-specific band was detected after gel electrophoresis by ethidium bromide staining and autoradiography. Judging from the autoradiography background, nonspecific incorporation in this reaction was negligible. The yield of each PCR was estimated by liquid scintillation counting of the excised 123-bp band. For the same cDNA preparation, yield of the β_2-microglobulin-specific product varied by <10% in multiple reactions. In the amplification reactions for *MDR1* and *MDR2* cDNA sequences (see Results for description of primers), the initial amounts of template cDNA were adjusted for the efficiency of β_2-microglobulin cDNA amplification. Unlabeled PCR products were analyzed by gel electrophoresis, ethidium bromide staining, and Southern hybridization with *MDR1*- and *MDR2*-specific probes. Each set of reactions was accompanied by negative controls containing the same primer pairs used for PCR in the absence of a cDNA template.

RESULTS

Two homologous *MDR* genes exist in human DNA. Southern hybridization of human genomic DNA with *MDR1* cDNA probes produces a complex pattern of multiple hybridizing bands. To determine whether these bands correspond to different *MDR* genes or to different portions of the same gene, we carried out a series of hybridizations, using different fragments of *MDR1* cDNA as probes. These probes were hybridized with genomic DNA isolated from different cell lines, including drug-sensitive KB-3-1 human epidermoid carcinoma cells and multidrug-resistant cell lines independently derived from KB-3-1 by selection for resistance to colchicine (KB-C4), adriamycin (KB-A1), or vinblastine (KB-V1) (1). We also used DNA from multidrug-resistant mouse NIH 3T3 transfectants that carry the human *MDR1* gene (NIH-T1-C1, a primary transfectant, and NIH-T2-C1, a secondary transfectant) (40). Using genomic probes, we previously showed that the *MDR1* gene was amplified in all multidrug-resistant cell lines used in this study (36, 40). The human *MDR2* gene, however, was not transferred in the NIH 3T3 transfectants and was not amplified in KB-V1 and KB-A1 cells. We have previously described an apparently identical rearrangement of the *MDR2* gene in KB-V1 and KB-A1 cell lines and amplification of the rearranged *MDR2* gene in KB-A1 cells (36). Amplification of these sequences, however, was unstable and was no longer found in the KB-A1 cells used in this study. The rearranged *MDR2* gene was still detectable in KB-A1 (as well as in KB-V1) cells, where it was present at approximately single-copy level (data not shown).

The band patterns of *Hin*dIII-digested DNA from different cell lines, observed after hybridization with full-length *MDR1* cDNA, are shown in a representative blot in Fig. 1A. In this experiment, the 5′ portion of *MDR1* cDNA (positions −424 to 1177; see Chen et al. [5] for numbering of cDNA residues) or the 3′ portion (positions 1178 to 4219) was used as a probe. All of the single-copy fragments hybridizing to *MDR1* cDNA in the drug-sensitive KB-3-1 cells were found to be amplified in the multidrug-resistant KB-C4 cell line. Conversely, all of the bands hybridizing in KB-C4 DNA could be detected at single-copy level in KB-3-1 cells upon prolonged exposure or when shorter segments of cDNA were used as probes (data not shown), indicating that the corresponding amplified sequences were not rearranged in KB-C4 DNA. In contrast, not all of the bands present in KB-3-1 and amplified in KB-C4 cells were amplified in KB-A1 and KB-V1 cells (the latter not shown in Fig. 1A) or present in the NIH 3T3 transfectants, in agreement with our previous results (36, 40).

On the basis of these assays, all but one of the *MDR1*-hybridizing bands could be divided into two groups. Group I consisted of 12 bands that were present in the NIH 3T3 transfectants and amplified in KB-C4, KB-A1, and KB-V1 cells. This group also included a 3.25-kbp band that was rearranged in NIH 3T3 transfectants, where a novel 15-kbp band was observed (marked by an asterisk in Fig. 1A). The 3.25-kbp band in KB cells and the 15-kbp band in the transfectants were found by hybridization with the appropriate cDNA segments to contain the major promoter region of the *MDR1* gene (data not shown). The pattern of amplification and transfer in group I was the same as previously observed with an *MDR1*-specific genomic probe (36, 40), suggesting the tentative identification of these bands as *MDR1*. The second group of five cross-hybridizing bands (arrows in Fig. 1A) was amplified only in KB-C4 cells, not in KB-A1 or KB-V1 cells, and was absent from the NIH 3T3 transfectants. The same pattern was previously observed with an *MDR2*-specific probe (36), suggesting that bands of group II may correspond to the *MDR2* gene. A single 5.1-kbp band, which was detectable only when hybridization was carried out under low-stringency conditions, could not be assigned to either group. Although this fragment was amplified in KB-C4, KB-A1, and KB-V1 cells, it was absent from NIH 3T3 transfectants (Fig. 1A and data not shown). In addition, some but not all isolates of KB-V1 DNA contained several novel bands, most probably reflecting rearrangements of amplified DNA in a subpopulation of cells (Fig. 1B and C).

To further characterize these hybridization patterns, smaller fragments of *MDR1* cDNA were used as probes. More than 30 different overlapping fragments, ranging in size from approximately 140 to 840 bp, were gel purified or subcloned and used as probes (representative blots are shown in Fig. 1B and C). The results of these assays are summarized in Fig. 2. Most bands of group I could be individually associated with specific portions of the *MDR1* cDNA sequence, indicating that they contained different exons of the *MDR1* gene. We have been unable to provide an unambiguous association of bands with specific cDNA sequences in the region corresponding to positions 2700 to 3300 of *MDR1* cDNA. However, recent analysis of a genomic cosmid clone corresponding to this region of *MDR1* cDNA suggested the presence of *Hin*dIII fragments with the sizes corresponding to bands of group I within the *MDR1* gene (C.-J. Chen, J. E. Chin, D. Clark, K. Ueda, M. M. Gottesman, and I. B. Roninson, unpublished data).

All of the bands of group II, tentatively identified as *MDR2*, hybridized to *MDR1* cDNA sequences derived from the protein-coding region of cDNA. The strongest hybridization was observed with the cDNA sequences coding for each of the two nucleotide-binding domains of P-glycoprotein, the most conserved regions of the protein. The rest of the protein-coding sequences of *MDR1* cDNA hybridized either only under low-stringency conditions (sequences hybridizing with the group II bands of 0.9 and 2.4 kbp; Fig. 2) or not at all (the 5′-most one/third of *MDR1* cDNA; Fig. 1A). In contrast, the 5.1-kbp band, which could not be assigned to either group, was found to hybridize with a probe corresponding to the subclone pHDR0.18, containing the first 182 bp of the 5′-untranslated sequence, found in *MDR1* cDNA (Fig. 1B). Ueda et al. (45, 47) have shown that the *MDR1* gene is transcribed from two different promoters (Fig. 3A). The upstream promoter, the exact location of which is unknown, is expressed in some but not all multidrug-resistant cell lines, and even in the cell lines found to express this promoter, only a minority of transcripts were initiated from it. *MDR1* mRNA transcribed from the upstream promoter includes at least two untranslated exons, designated exon −1 and exon 1. The majority of *MDR1* transcripts originate from another, downstream promoter located in the middle of exon 1. Exon 1 can therefore be subdivided into two parts: exon 1a, transcribed from the upstream promoter, and exon 1b, which is the first exon transcribed from the downstream promoter (Fig. 3A). The probe hybridizing to the 5.1-kbp *Hin*dIII fragment includes sequences from exon −1 and exon 1a. Since all of exon 1 is contained within a 3.25-kbp *Hin*dIII fragment (data not shown), it was possible that the 5.1-kbp fragment contained exon −1. The portion of exon −1 present in pHDR0.18 is short (95 bp) and very A+T rich (63%), which could explain why the 5.1-kbp band did not

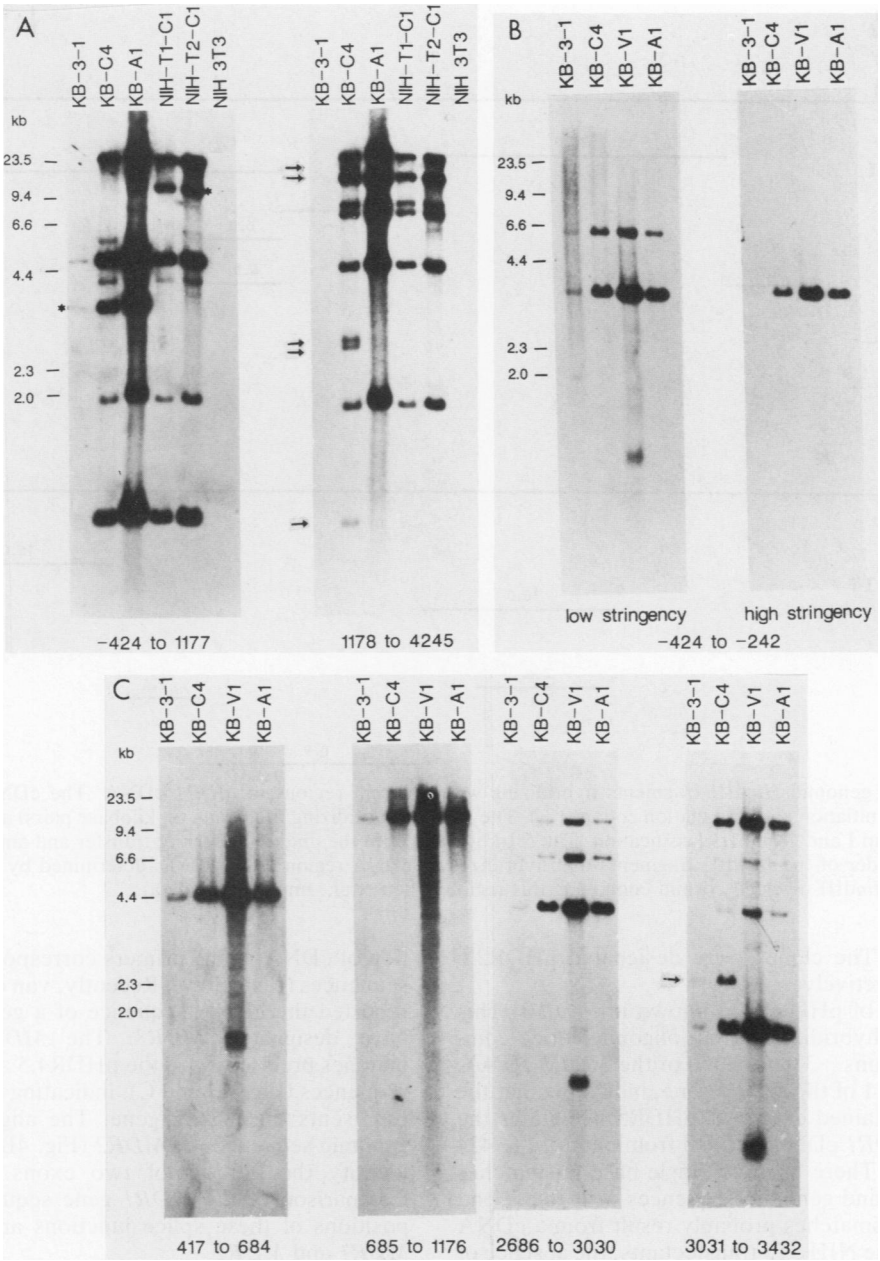

FIG. 1. Southern blot hybridization of *Hin*dIII-digested genomic DNA from different cell lines hybridized with subfragments of *MDR1* cDNA. See text for description of the cell lines. In panel A, each lane contains 5 μg of genomic DNA; in panels B and C, KB-3-1 lanes contain 5 μg of DNA and KB-C4, KB-V1, and KB-A1 lanes contain 1 μg of DNA. The regions of the cDNA sequence present in the probe fragments are indicated at the bottom of each panel. Positions and sizes of *Hin*dIII fragments of phage λ DNA, used as size standards, are indicated on the left of each panel. (A) Hybridization of DNA from multidrug-resistant and transfectant cell lines with full-length *MDR1* cDNA. Symbols: *, band rearranged in the process of gene transfer into NIH 3T3 cells; →, band showing the amplification and transfer patterns of group II (see text). The second arrow from the top designates a fragment of group II that comigrated with a similar-size fragment of group I; this group II band was identified by hybridization with a specific short segment of *MDR1* cDNA (Fig. 2 and data not shown). Hybridization was carried out under low-stringency conditions. (B) Hybridization with the 5′-most 182 bp of the 5′ untranslated sequence of *MDR1* cDNA under conditions of low and high stringency. (C) Representative blots hybridized to the indicated regions of *MDR1* cDNA under conditions of high stringency. →, Group II band.

hybridize with *MDR1* cDNA under high-stringency conditions.

To examine the nature of the bands that did not belong to group I, a representative 2.6-kbp fragment from group II and the unique 5.1-kbp band were cloned from size-selected phage libraries. The libraries were prepared from DNA of the multidrug-resistant KB-C1.5 cell line, which corresponds to an intermediate step of colchicine selection. The 2.6-kbp fragment was isolated by plaque hybridization with an *MDR1* cDNA probe spanning residues 1177 to 1766. The 5.1-kbp clone was isolated by using the pHDR0.18 subclone, containing the 5′ untranslated region of *MDR1* cDNA as a probe. The phage clones were plaque purified and recloned into plasmid vectors pBR322 (2.6-kbp fragment) and pUC18

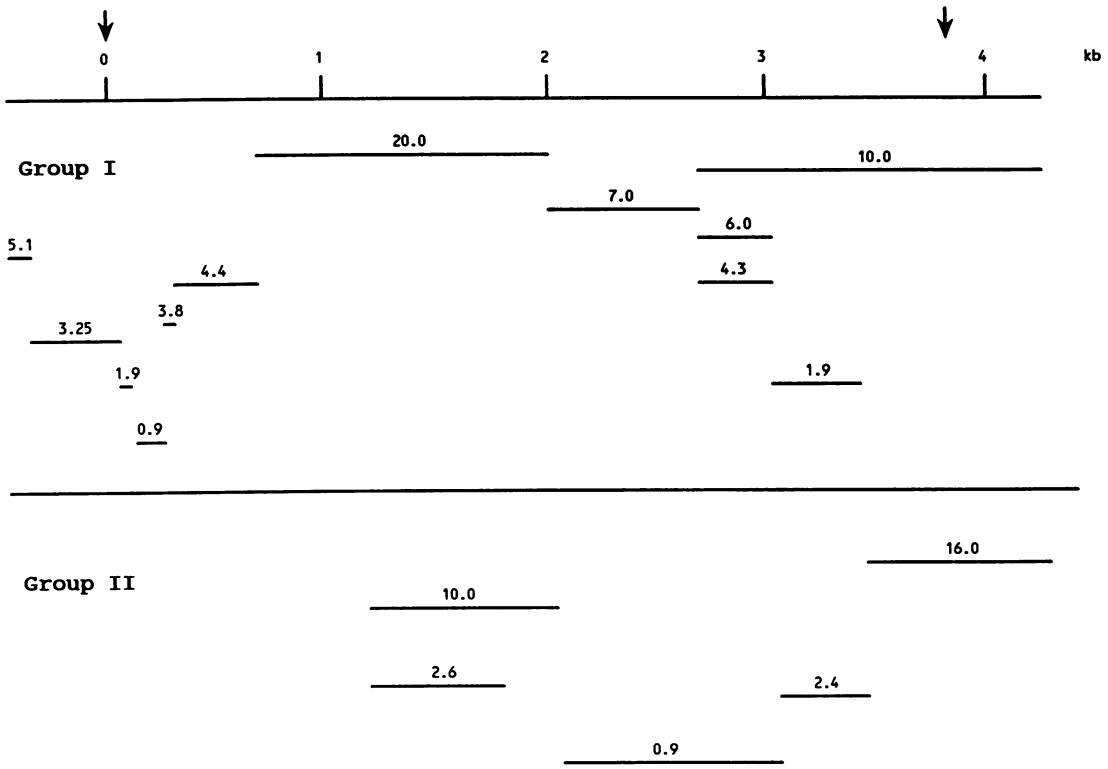

FIG. 2. Diagram of genomic *Hin*dIII fragments hybridizing with different regions of *MDR1* cDNA. The cDNA map (top) shows the position of translation initiation and termination codons (↓). The sizes of hybridizing fragments (in kilobase pairs) are indicated. See text for the explanation of group I and group II classification. The 5.1-kbp band with the unique pattern of transfer and amplification is shown here within group I. The order of the *Hin*dIII fragments that hybridized to cDNA region 0 to 340 was determined by hybridization of specific oligonucleotides to a *Hin*dIII-digested cosmid containing this region (Chen et al., unpublished data).

(5.1-kbp fragment). The clones were designated pHDR2.6 and pHDR5.1, respectively.

A restriction map of pHDR5.1 is shown in Fig. 3B. This clone was found to hybridize with an oligonucleotide complementary to positions −336 to −350 of the *MDR1* cDNA, derived from exon −1 of the *MDR1* gene, indicating that the pHDR5.1 clone contained exon −1. pHDR5.1 contains the entire portion of *MDR1* cDNA derived from exon −1 (−424 to −329) (Fig. 3C). There are two single-base mismatches between the cDNA and genomic sequences near the 5′ end of cDNA. These mismatches probably result from a cDNA cloning artifact. In the NIH 3T3 transfectants, the absence of exon −1 is consistent with the rearrangement of the exon 1-containing 3.25-kbp fragment, indicating that *MDR1* gene transfer in these cells was associated with DNA breakage very close to exon 1 and the downstream promoter.

A restriction map of the pHDR2.6 clone (group II) is shown in Fig. 4A. The region of this clone that hybridized with the *MDR1* cDNA was sequenced. We have also sequenced the *MDR1*-hybridizing region of pHDR4.5, a previously isolated *MDR2* genomic clone (36). Both clones were found to contain exon sequences of a gene homologous to but distinct from *MDR1* (Fig. 4B and C). The exon found in the pHDR4.5 clone has only 77% sequence homology with the corresponding exon of *MDR1*, which apparently explains the lack of hybridization between these sequences on genomic blots (Fig. 2), although the clones carrying these exons were previously shown to cross-hybridize under low-stringency conditions (36). The sequences of both pHDR2.6 and pHDR4.5 are transcribed as parts of the same *MDR2* mRNA molecule, as demonstrated by enzymatic amplification of cDNA, using primers corresponding to each of these sequences (see below). Recently, van der Bliek et al. (48, 50) reported the cDNA sequence of a gene that these authors have designated *MDR3*. The *MDR3* cDNA sequence matches precisely with the pHDR4.5 and pHDR2.6 genomic sequences (Fig. 4B and C), indicating that this cDNA in fact represents the *MDR2* gene. The alignment of cDNA and genomic sequences of *MDR2* (Fig. 4B and C) allows one to identify the borders of two exons of the *MDR2* gene. Comparison of the *MDR1* gene sequences shows that the positions of these splice junctions are conserved between *MDR1* and *MDR2*.

The human *MDR1* gene corresponds to two genes in mouse and hamster DNA. The results described above are consistent with the existence of only two cross-hybridizing *MDR* genes in human DNA. In contrast, analysis of the amplification patterns of cDNA clones of mouse and hamster *mdr* genes indicated that mouse and hamster cells contain at least three different *mdr* genes (13, 14, 31; S. I. Hsu, L. Lothstein, and S. B. Horwitz, personal communication). To resolve this apparent contradiction, genomic DNAs from human KB-3-1, green monkey COS-1, mouse NIH 3T3, and hamster CHO cells were digested with different restriction enzymes and hybridized with fragments of genomic clones, corresponding to single exons derived from homologous regions in the *MDR1* and *MDR2* genes. The *MDR1* probe was a 0.36-kbp *Rsa*I fragment of the genomic clone pMDR1, and the *MDR2* probe was pMDR2, a 1.0-kbp *MDR2* genomic clone (36). The sequences of the exons contained within these probes are shown in Fig. 4B.

The *MDR1* and *MDR2* single-exon probes hybridized to

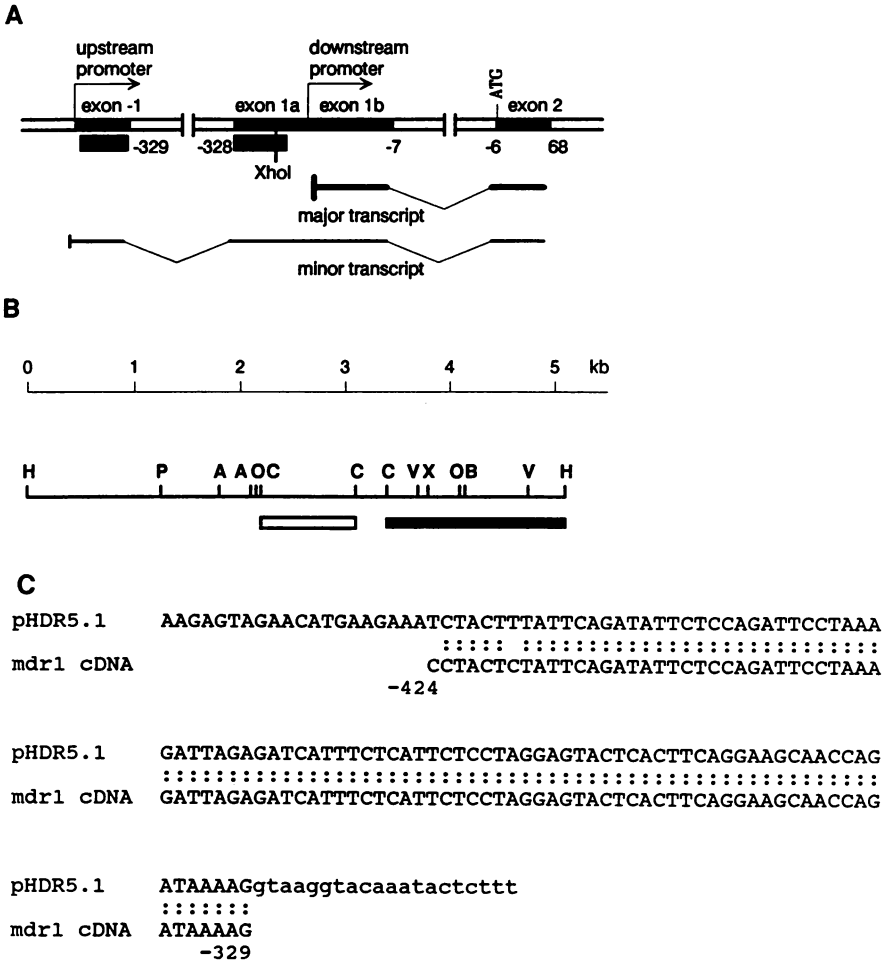

FIG. 3. Characterization of the 5.1-kbp *Hin*dIII fragment. (A) Diagram of transcription initiation in the *MDR1* gene (45, 47). Numbers correspond to the cDNA residues at the exon-intron borders. The striped boxes represent cDNA sequences present in clone pHDR0.18. Positions of the ATG translation initiation codon and the *Xho*I restriction site are indicated. (B) Restriction map of the genomic clone pHDR5.1. Symbols: ■, region containing highly repeated sequences; □, DNA sequence hybridizing to the 5′ end of the *MDR1* cDNA. Restriction sites: A, *Acc*I; B, *Bgl*II; C, *Hin*cII; H, *Hin*dIII; O, *Xho*II; P, *Pst*I; V, *Ava*I; X, *Xho*I. (C) Sequence alignment of the 5′ untranslated region of *MDR1* cDNA (−424 to −329) with the pHDR5.1 genomic clone. Intron sequences are in lowercase letters.

different single bands in each of the human DNA digests (Fig. 5A). Similar results were observed in the monkey COS-1 cells (Fig. 5B). In mouse and hamster DNAs, however, two bands hybridized to the human *MDR1* probe in each of the digests except for the *Eco*RI digest of hamster DNA, which contained three hybridizing bands (Fig. 5C and D). The human *MDR2* probe hybridized to a single band in each of the rodent DNA digests except for the *Xba*I digest of hamster DNA, where two bands were detected. These results suggest that the human *MDR1* gene corresponds to a single gene in the human and monkey genomes but to two genes in the mouse and hamster genomes, whereas *MDR2* corresponds to a single gene in both primates and rodents. The two rodent genes hybridizing with the human *MDR1* probe showed different hybridization intensities, suggesting different levels of sequence conservation between these genes. The presence of additional hybridizing bands in some of the hamster DNA digests most probably reflects the presence of species-specific restriction sites within the hybridizing regions. Thus, the difference in the numbers of *MDR* genes between human and rodent DNAs can be explained by a duplication of the *MDR1* gene in the rodent lineage.

Mapping of the human *MDR* locus by FIGE. Infrequently cutting restriction enzymes and FIGE (4) were used to construct a map of the human *MDR* locus. Multidrug-resistant KB-C4 cells, which contain amplified copies of both the *MDR1* and *MDR2* genes, were immobilized in agarose plugs and deproteinized in situ (41). DNA in plugs was digested with the infrequently cutting restriction enzymes *Nru*I, *Sfi*I, and *Xho*I. The resulting large restriction fragments were then separated by FIGE. After blotting, the filters were hybridized successively to different genomic and cDNA-derived probes (Fig. 6A). These probes included a 1.0-kbp *Hin*cII fragment of the *MDR1* genomic clone pHDR5.1, containing exon −1; pHDR0.18, a cDNA probe containing 5′ untranslated sequences found in exons −1 and 1a (Fig. 3A); pMDR1, which is a 0.8-kbp *MDR1* genomic clone containing two exons from the 5′ half of the *MDR1* gene (5, 36); the entire *MDR1* cDNA (7); pHDR0.44, which corresponds to the last exon of the *MDR1* cDNA; pMDR2, which is a 1.0-kbp *Pst*I subclone of pHDR4.5, an *MDR2* genomic clone (36); and another *MDR2* genomic clone, pHDR2.6 (Fig. 4). Band sizes were estimated relative to those of concatamers of phage λ DNA. The same size

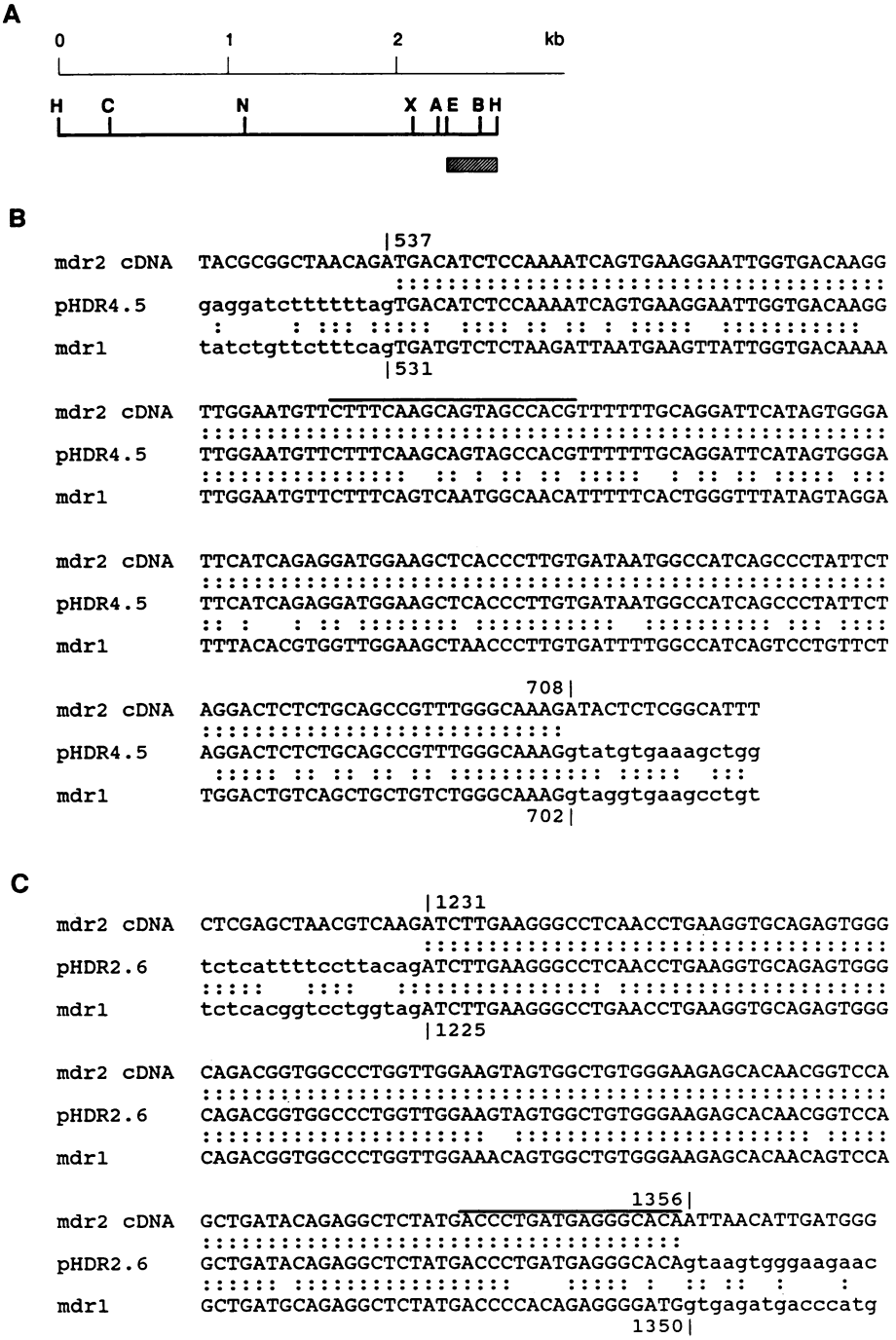

FIG. 4. Characterization of the 2.6-kbp *Hin*dIII fragment (group II). (A) Restriction map of the genomic clone pHDR2.6. Restriction sites: E, *Eco*RI; N, *Nde*I; other restriction site abbreviations are as for Fig. 3B. The striped bar indicates the region that was sequenced and that cross-hybridized with *MDR1* cDNA. (B and C) Sequence alignments of the pHDR4.5 (B) and pHDR2.6 (C) genomic sequences with *MDR2* and *MDR1* cDNAs. Numbering of the cDNA sequences follows that of Van der Bliek et al. (50) and Chen et al. (5). Exon-intron borders are indicated. The genomic sequences of *MDR1* are from reference 5 and Chen et al. (unpublished data). Sequences used as primers for PCR amplification of *MDR2* cDNA are marked by a horizontal line above the sequence (the primer in pHDR2.6 was complementary to the marked sequence).

estimates were obtained by FIGE and by modified pulsed-field gel electrophoresis (30) (data not shown).

All of the *MDR1* and *MDR2* sequences are contained within the 600-kbp *Nru*I fragment (Fig. 6A). Except for pHDR5.1, which corresponds to the 5′-flanking region of *MDR1*, all *MDR1* and *MDR2* sequences are located within the 330-kbp *Sfi*I fragment. pHDR5.1 sequences, however, are linked to the rest of the *MDR* locus within the 450-kbp *Sfi*I band, which represents a product of partial *Sfi*I digestion, judging from the variable representation of this band in different digests (data not shown). In the *Xho*I digest, all of the *MDR1*-derived probes except pHDR5.1 and pHDR0.18

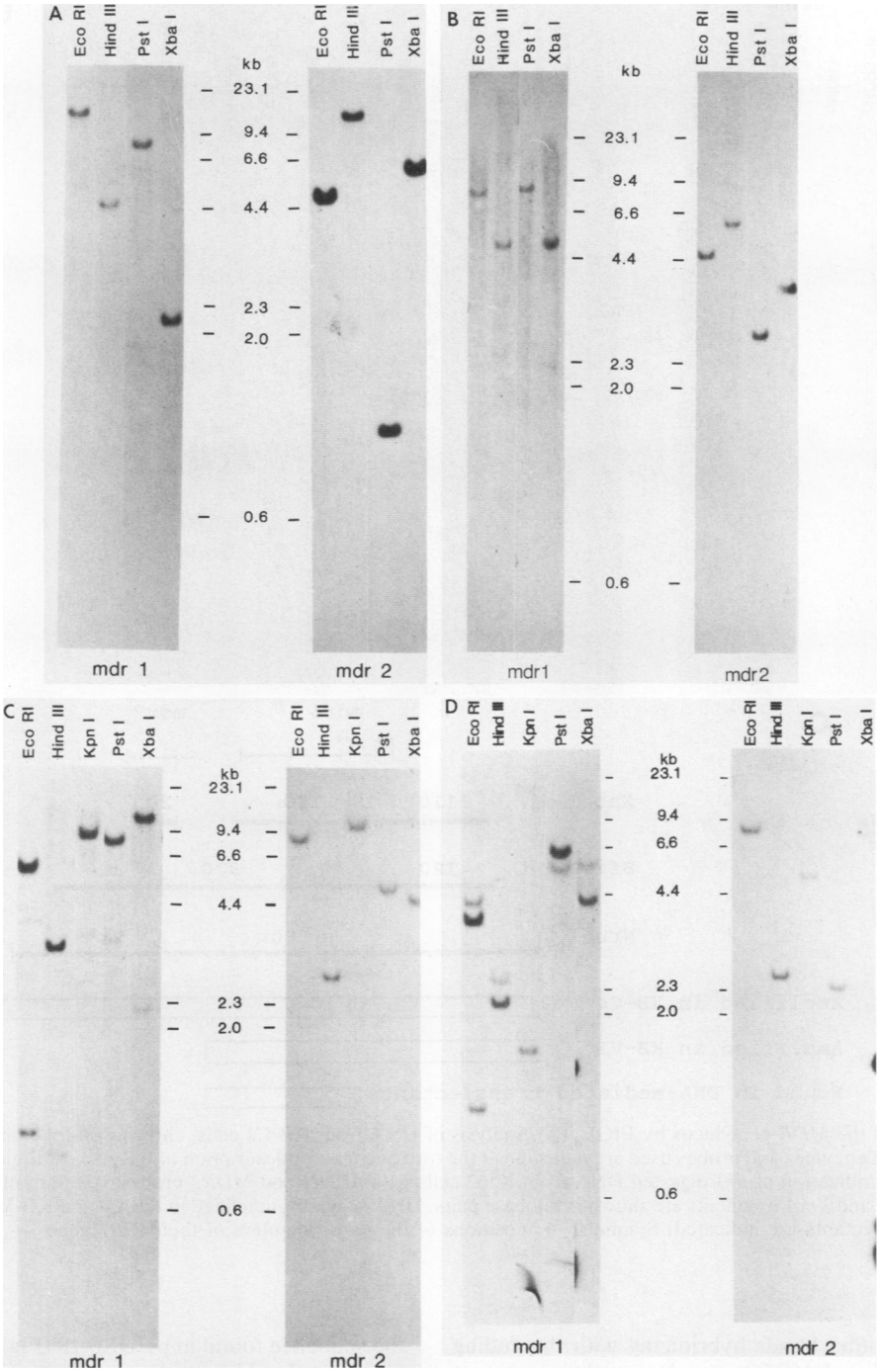

FIG. 5. Hybridization of *MDR1*- and *MDR2*-specific single-exon probes with human (A), monkey (B), mouse (C), and hamster (D) genomic DNAs digested with the restriction enzymes indicated. See text for description of the probes. The blots were hybridized under conditions of low stringency. Weak bands of 2.2 to 2.3 kbp, hybridizing with the *MDR1* probe in panel B, represent nonspecific cross-hybridization with highly repeated sequences.

(which contain 5′ untranslated sequences of *MDR1*) hybridized to a 120-kbp fragment. The *MDR1* cDNA and pHDR0.44 probes also hybridized with less intensity to a 20-kbp fragment. Conversely, the *MDR2*-derived probes, pMDR2 and pHDR2.6, hybridized more intensely to the 20-kbp fragment and less intensely to the 120-kbp fragment. Since the probes that hybridized with both the 120- and 20-kbp fragments also showed cross-hybridization with *MDR1* and *MDR2* sequences on Southern blots of *Hin*dIII-digested DNA (data not shown), it seems most likely that the 120-kbp *Xho*I fragment contains the entire protein-coding sequences of the *MDR1* gene and that the 20-kbp fragment corresponds to the *MDR2* gene, although we do not know whether the entire *MDR2* gene is located within this frag-

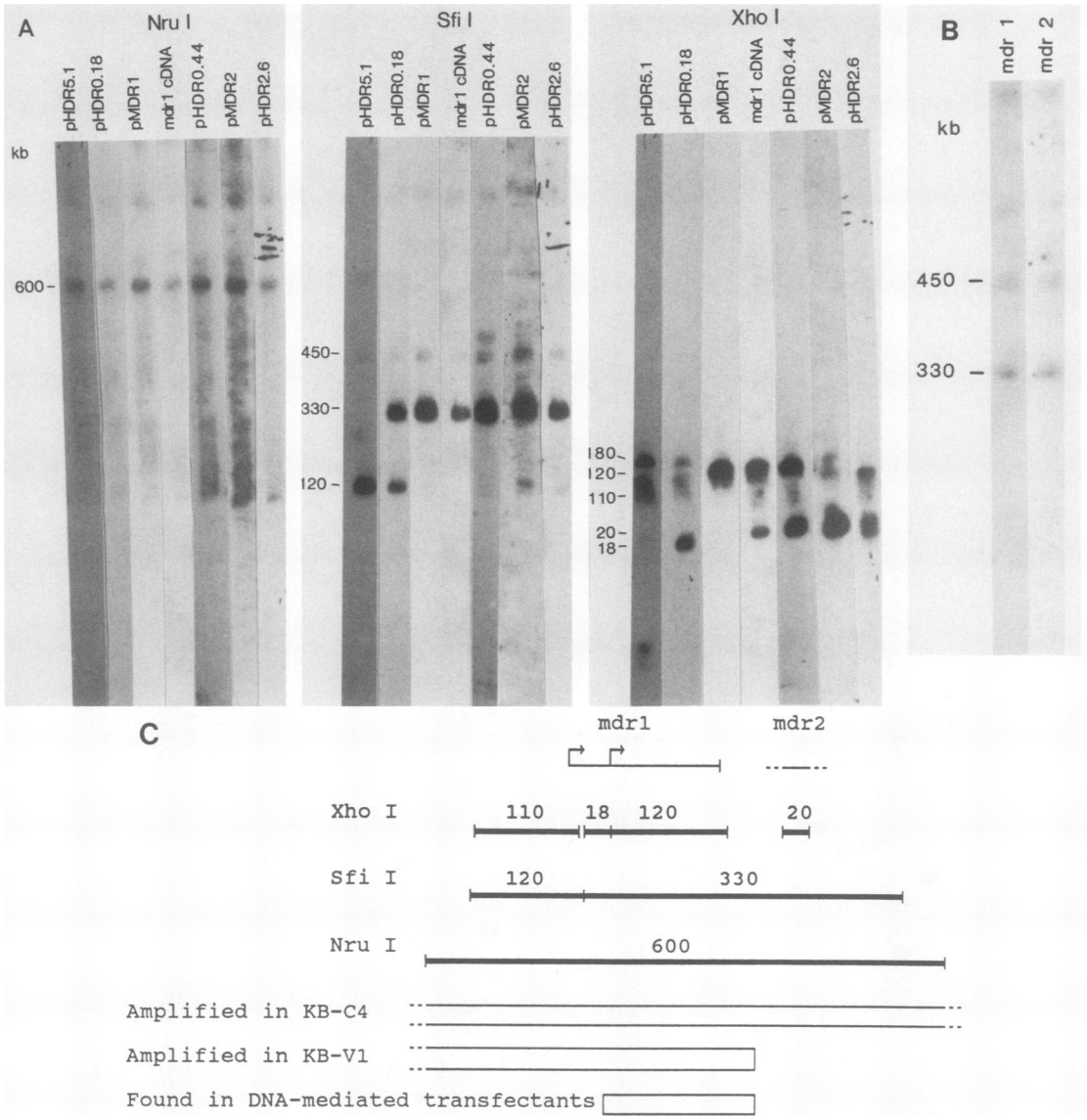

FIG. 6. Analysis of the *MDR* gene locus by FIGE. (A) Analysis of DNA from KB-C4 cells, carrying 30-fold amplification of *MDR1* and *MDR2*. The restriction enzymes and probes used are indicated at the top. See text for description of the probes. Band sizes (in kilobase pairs) are indicated. (B) Hybridization of *Sfi*I-digested DNA from K562 cells with *MDR1* and *MDR2* probes. (C) Map of the human *MDR* locus. Sizes of the *Nru*I, *Sfi*I, and *Xho*I fragments are shown in kilobase pairs. DNA segments amplified in KB-C4 and KB-V1 cells and those present in the NIH 3T3 transfectants are indicated. Symbols: →, positions of the two promoters of the *MDR1* gene. ---, Borders that are as yet undefined.

ment. The lack of other bands hybridizing with the coding sequences of *MDR1* and *MDR2* provides another argument that only two *MDR* genes are present in the human genome.

The probes containing 5′ untranslated sequences of *MDR1*, pHDR5.1 (containing exon −1; Fig. 3A) and pHDR0.18 (a cDNA probe containing portions of exon −1 and exon 1a), hybridized to *Sfi*I and *Xho*I fragments distinct from those that hybridized with the other *MDR1* probes. Both probes hybridized to a 120-kbp *Sfi*I fragment and to *Xho*I fragments of 110 and 180 kbp, indicating that these fragments contained exon −1; the 180-kbp *Xho*I band was most probably a product of partial digestion. pHDR0.18 also hybridized to the same 330-kbp *Sfi*I fragment as did the rest of the *MDR1* cDNA probes and to an 18-kbp *Xho*I fragment. Since an *Xho*I site is present in exon 1a, near the 3′ end of the sequence found in pHDR0.18 (Fig. 3A), the 18-kbp *Xho*I fragment should hybridize to the exon 1a sequences located 5′ from the *Xho*I site (the region of the pHDR0.18 probe 3′ from the *Xho*I site is too short [29 bp] to hybridize under our conditions). The 18-kbp *Xho*I fragment therefore is located between the upstream and downstream promoters of the *MDR1* gene (Fig. 3A), indicating that 18 kbp is the minimal distance separating these promoters.

To confirm that the linkage of *MDR1* and *MDR2* observed in KB-C4 cells does not represent gene amplification-associated DNA rearrangements, DNAs from K562 cells and KB-3-1 cells, in which the *MDR1* and *MDR2* genes are not amplified, were digested with *Sfi*I and separated by FIGE. The digests were hybridized successively with the *MDR1* cDNA probe and the *MDR2*-specific genomic probe,

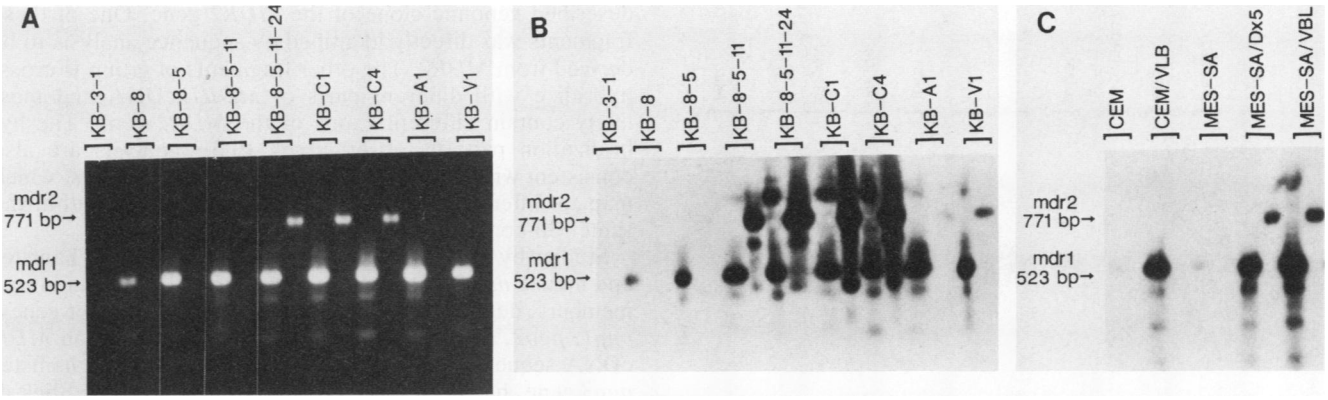

FIG. 7. Analysis of *MDR1* and *MDR2* mRNA expression in multidrug-resistant and -sensitive cell lines by PCR amplification of cDNA sequences. See text for description of the assay. PCR products were separated in 3% agarose gels. →, Positions and sizes of *MDR1*- and *MDR2*-specific PCR products. Cell lines used as the source of RNA are indicated at the top. (A) Ethidium bromide staining of PCR products from RNA of multidrug-resistant KB cell lines. (B) Hybridization of a gel similar to the one shown in panel A with 2×10^6 cpm of the pMDR1 probe and 14×10^6 cpm of the pMDR2 probe under conditions of high stringency. Additional bands, observed after hybridization, most probably correspond to single-stranded products of PCR, judging from their poor staining with ethidium bromide; these bands were largely abolished when an additional 20-min extension period was added to the last cycle of PCR. (C) PCR products from RNA of other multidrug-resistant and -sensitive cell lines. Hybridization was carried out as for panel B. A weak *MDR2*-specific band was seen in the lane with CEM-VLB_{100} RNA upon longer exposure (not shown).

pMDR2. Figure 6B shows the results obtained with K562 DNA. Both probes hybridized to the same 330-kbp *Sfi*I band and the 450-kbp partial digest product, previously observed in KB-C4 cells. Similar results were observed in KB-3-1 cells (data not shown), indicating that there was no major rearrangement of this region associated with gene amplification in KB-C4 cells.

The results of the structural analysis of the human *MDR* locus are summarized in the map shown in Fig. 6C. It appears from this analysis that the size of the amplicon in KB-C4 cells is at least 600 kbp, with the *MDR1* and *MDR2* genes linked within 330 kbp of DNA.

Expression patterns of the *MDR* genes. We have previously demonstrated that steady-state levels of *MDR1* mRNA correlate with the levels of drug resistance in multidrug-resistant cell lines (39). We have been unable, however, to detect expression of the *MDR2* gene in multidrug-resistant KB cells by Northern (RNA) hybridization with *MDR2* genomic clones (36; data not shown). These negative results could be due to the limited sensitivity of these assays, especially in view of the high-stringency hybridization conditions required to avoid cross-hybridization of the *MDR2* probes with *MDR1* mRNA. The isolation and partial sequencing of two *MDR2* genomic clones has now allowed us to design a more sensitive and specific assay for *MDR2* expression, based on enzymatic amplification of mRNA sequences by PCR (37). To avoid amplification of genomic DNA sequences that may contaminate RNA preparations, sequences of *MDR2*-specific PCR primers were selected from different exons of the *MDR2* gene, one present in pHDR4.5 and the other in the pHDR2.6 clone (Fig. 4B and C, respectively). The primer sequences were chosen for their divergence from the homologous sequences of the *MDR1* gene. These primers amplify a 771-bp segment of *MDR2* cDNA. To correlate the patterns of *MDR2* expression with the expression of the *MDR1* gene, a pair of *MDR1*-specific primers derived from different exons in the homologous region of the *MDR1* gene (positions 302 to 321 in the sense orientation and 825 to 804 in the antisense orientation) were selected for amplification of a comparably sized fragment (523 bp) of *MDR1* cDNA. A pair of β_2-microglobulin-specific primers, amplifying 123 bp of β_2-microglobulin cDNA (25), was used as an internal standard to normalize the amounts of cDNA used in different PCR reactions (see Materials and Methods). Total RNA, isolated from a variety of human tissues and from multidrug-sensitive and -resistant cell lines, was reverse transcribed by using a random hexanucleotide primer (32), and gene-specific sequences were amplified by PCR. After gel electrophoresis, the specific PCR products were visualized by ethidium bromide staining and by Southern hybridization with gene-specific probes. The use of random primer for reverse transcription and an internal standard for normalization of the signal allows for reliable semiquantitative comparison of mRNA levels for the same gene in different RNA preparations (K. Noonan, C. Beck, J. E. Chin, and I. B. Roninson, manuscript in preparation).

The results of these assays for different parental drug-sensitive and multidrug-resistant cell lines are shown in Fig. 7. The parental cell lines KB-3-1, CEM, and MES-SA did not show detectable expression of either *MDR1* or *MDR2*. All of the resistant cell lines, however, showed overexpression of the 523-bp *MDR1*-specific PCR product. In addition to expression of *MDR1*, a small increase in *MDR2* mRNA expression could also be detected in all multidrug-resistant cell lines. Among colchicine-selected KB cell lines, an increase in *MDR2* expression occurred in parallel with increasing levels of multidrug resistance and *MDR1* mRNA expression. The increase in *MDR2* mRNA in colchicine-selected cell lines KB-8-5-11 to KB-C4 was associated with amplification of the *MDR2* gene (36). In contrast, the KB-A1 and KB-V1 cell lines, in which only the *MDR1* gene is amplified (36), expressed *MDR2* at a much lower level. Low levels of *MDR2* expression were also detectable in colchicine-selected lines KB-8 and KB-8-5, which did not show *MDR* gene amplification. In other multidrug-resistant cell lines, CEM VLB_{100} (8), MES-SA VBL, and MES-SA DX5 (27), *MDR2* was also expressed at a lower level than was MDR1. The relative level of *MDR2* expression was higher in MES-SA DX5 and MES-SA VBL cells, in which both *MDR1* and *MDR2* genes are amplified at a low level (S. Scudder, M. Fukumoto, I. B. Roninson, and B. I. Sikic, manuscript in

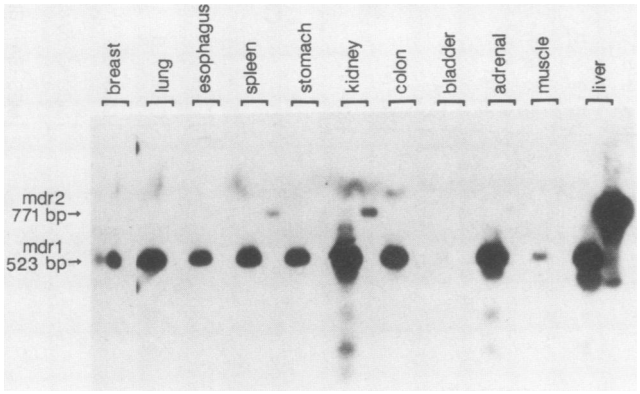

FIG. 8. PCR analysis of *MDR1* and *MDR2* mRNA expression in normal human tissues. PCR products were analyzed as described for Fig. 7B.

preparation), than in CEM-VLB_{100} cells, in which only *MDR1* is amplified (data not shown).

Figure 8 shows the results of the analysis of *MDR1* and *MDR2* mRNA expression in normal human tissues. In agreement with the results of Fojo et al. (16), *MDR1* expression was observed at a high level in the liver, kidney, adrenal gland, and spleen. *MDR2* mRNA expression was seen in the liver (the highest level), kidney, adrenal gland, and spleen. Whereas *MDR1* expression was detected at lower levels in colon, lung, stomach, esophagus, breast, muscle, and bladder, no *MDR2* expression was seen in these organs. It should be noted that while the yield of the PCR products in these assays correlated with the amount of the mRNA template, there are significant differences in the efficiency of PCR for different primers, and therefore the relative intensities of the *MDR1*- and *MDR2*-specific PCR products shown in Fig. 8 do not represent the relative amounts of these two types of mRNA. Thus, the *MDR2*-specific band showed a higher intensity in normal liver than did the *MDR1*-specific band, whereas Van der Bliek et al. (48) have estimated by Northern hybridization and analysis of cDNA clones that *MDR2* is expressed at a lower level than is *MDR1* in this tissue.

DISCUSSION

Analysis of human DNA sequences hybridizing with *MDR1* cDNA suggests that the human *MDR* gene family is unlikely to include more than two closely related genes. Although *MDR1* cDNA hybridizes with at least 17 different genomic *Hin*dIII fragments, 12 of them (group I) appear to correspond to different parts of the *MDR1* gene, as judged by their hybridization with distinct portions of the cDNA sequence and their common pattern of amplification and transfer in different cell lines. The only fragment of this group not transferred with the rest of the *MDR1* gene in a DNA-mediated transfectant cell line was found to contain the 5′ untranslated sequences of the *MDR1* gene, derived from a secondary (upstream) promoter. The absence of this fragment in DNA-mediated transfectants indicates that the upstream promoter is not necessary for *MDR1* gene expression, at least in some cell types. This conclusion is in agreement with the studies by Ueda et al. (47), who found that the majority of cell lines and normal tissues tested show no detectable transcription from the upstream promoter.

The remaining five fragments (group II) showed the same pattern of amplification and transfer as did a previously described genomic clone of the *MDR2* gene. One of these fragments was directly identified by sequence analysis to be derived from *MDR2*. The other fragments of group II cross-hybridize with different parts of *MDR1* cDNA and most likely contain different exons of the *MDR2* gene. The hybridization patterns observed by FIGE analysis are also consistent with the existence of only two *MDR* genes, which map to different *Xho*I fragments but are linked within the same 330-kbp *Sfi*I and 600-kbp *Nru*I fragments.

Studies by other investigators have indicated that hamster and mouse *mdr* (P-glycoprotein) gene families include three members, designated, in the case of Chinese hamster genes, *pgp1*, *pgp2*, and *pgp3* (11, 14, 17, 20, 23). A human *MDR* cDNA sequence, which shares homology with the hamster *pgp3* gene, has been designated *MDR3* by Van der Bliek et al. (48, 50). Sequence comparison between this cDNA and genomic clones pHDR4.5 and pHDR2.6 showed that the *MDR3* cDNA sequence in fact corresponds to the previously described (36) *MDR2* gene. Furthermore, hybridization of primate and rodent DNAs with single-exon probes corresponding to the human *MDR1* and *MDR2* genes demonstrated that rodent genomes contain two genes corresponding to the human *MDR1* but only one gene corresponding to *MDR2*. Among the two rodent homologs of the human *MDR1* gene, one gene hybridizes with the human *MDR1* probe more strongly than does the other gene. Analysis of sequence similarities among the known *MDR* cDNA sequences indicates that the first gene most probably corresponds to the gene previously designated *pgp1* in hamsters (14) and *mdr3* in mice (11) and that the second gene corresponds to the gene designated *pgp2* in hamsters (14) and *mdr1* in mice (21). Expression of both genes has been detected in multidrug-resistant rodent cells (14; Hsu et al., personal communication). To indicate the structural and functional similarities between these genes and the human *MDR1*, we propose to call the first rodent gene *mdr1a* and the second rodent gene *mdr1b*. The human *MDR2* gene corresponds to the gene previously designated *pgp3* in hamsters (14) and *mdr2* in mice (23); we propose to call these genes, which do not appear to be associated with multidrug resistance, *mdr2*. The existence of two rodent genes related to human *MDR1* can be most readily explained by a relatively recent duplication in the rodent lineage, which occurred after the divergence of mammalian orders. After duplication, the *mdr1b* gene has apparently diverged at a higher rate than *mdr1a*. Alternatively, *mdr1* duplication could have occurred prior to mammalian radiation, with the loss of the primate homolog of the rodent *mdr1b* gene during evolution. Sequence analysis of mouse *mdr1a* and *mdr1b* cDNAs suggests, however, that the latter possibility is less likely, since these mouse genes frequently shared identical residues at the positions where they differed from the human *MDR1* sequence (Hsu et al., personal communication). Similar conclusions about the evolutionary relationship between the rodent and human *mdr* (*pgp*) genes were recently reported by Ng et al. (31).

Although *MDR1* and *MDR2* genes encode highly homologous proteins, suggesting that the *MDR2* gene product also functions as an efflux pump (50), the spectra of substrates transported by these proteins seem to be quite different. Whereas *MDR1* expression was shown to be sufficient for resistance to different lipophilic drugs (7, 20, 40, 44), the *MDR2* gene does not appear to be involved in multidrug resistance, since expression of the human or mouse *MDR2* cDNA inserted into mammalian expression vectors did not result in resistance to drugs associated with the multidrug-

resistant phenotype (23, 50). Using *MDR2*-specific genomic probes, we have previously failed to detect the expression of *MDR2* mRNA in multidrug-resistant human cells by Northern hybridization (36; unpublished data). Other investigators, using *MDR2* cDNA probes, were also unable to detect *MDR2* expression in multidrug-resistant cell lines that had amplified both *MDR1* and *MDR2* (49). By using a more sensitive technique, based on enzymatic amplification of cDNA sequences by PCR, we have now detected the presence of *MDR2* mRNA in several multidrug-resistant cell lines. Whereas no *MDR1* or *MDR2* mRNA was observed in drug-sensitive KB-3-1 cells, an increase in *MDR1* mRNA expression in multidrug-resistant KB cells at the first steps of selection, before the onset of *MDR* gene amplification, was paralleled by the appearance of low levels of *MDR2* mRNA, suggesting that *MDR1* and *MDR2* gene expression in these cells may be regulated by a common mechanism. The levels of *MDR2* mRNA were further increased in the cell lines in which both *MDR1* and *MDR2* genes were amplified, although *MDR2* in these cells was still expressed at much lower levels than was *MDR1*. Because the *MDR2* gene is apparently unable to confer resistance to at least some of the drugs associated with multidrug resistance, it is doubtful that the low levels of *MDR2* expression in multidrug-resistant cells contribute to the resistance phenotype. Among multidrug-resistant KB cell lines, colchicine-selected cells, in which the *MDR2* gene is amplified and expressed at higher levels, are characterized by preferential resistance to colchicine compared with vinblastine- or adriamycin-selected cells, in which *MDR2* is not amplified. This preferential resistance, however, was shown to be a result of spontaneous mutations in the *MDR1* gene (7) rather than amplification of *MDR2*. Since *MDR1* and *MDR2* genes are linked within 330 kbp of DNA, *MDR2* amplification and the associated increased expression in some multidrug-resistant cell lines most likely is a consequence of coamplification of flanking sequences together with the *MDR1* gene, responsible for the resistance.

The results of cDNA-PCR analysis of *MDR1* and *MDR2* mRNA expression in normal tissues are in agreement with the tissue-specific distribution of *MDR* mRNA and protein described by Fojo et al. (16) and Thiebaut et al. (42). As previously suggested (19), high levels of *MDR1* gene expression in kidney, liver, and colon are consistent with a function for this gene in the removal of potentially cytotoxic natural compounds present in the diet, whereas its expression in the adrenal gland may indicate a role in the transport of steroid hormones. We have found that the *MDR2* gene is expressed in some of the same tissues as is *MDR1*, including liver, kidney, spleen, and the adrenal gland, albeit at lower levels. This distribution suggests that *MDR1* and *MDR2* gene products may be involved in some of the same processes or that coexpression of these mRNAs may reflect a common regulatory pathway. We have recently found that the *MDR2* gene is expressed in many tumor-derived cell lines of different origins in which its expression is not correlated with *MDR1* (Noonan et al., in preparation). The nature of the substrates transported by the *MDR2* gene product and the role of this gene in normal and tumor cells remain to be determined.

ACKNOWLEDGMENTS

We thank M. M. Gottesman, I. Pastan, B. I. Sikic, and W. T. Beck for providing cell lines used in this study, A. Fojo and D. K. Weerasinghe for their generous gifts of normal-tissue RNA preparations, S. B. Horwitz and S. I. Hsu for providing results prior to publication, and L. Sha for excellent technical assistance.

This work was supported by Public Health Service grants CA-40333 and CA-39365 from the National Cancer Institute.

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