The Three Mouse Multidrug Resistance (*mdr*) Genes Are Expressed in a Tissue-Specific Manner in Normal Mouse Tissues

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The gene responsible for multidrug resistance (*mdr*), which encodes the P-glycoprotein, is a member of a multigene family. We have identified distinct *mdr* gene transcripts encoded by three separate *mdr* genes in the mouse. Expression levels of each *mdr* gene are dramatically different in various mouse tissues. Specific *mdr* RNA transcripts of approximately 4.5, 5, and 6 kilobases have been detected. Each of the *mdr* genes has a specific RNA transcript pattern. These results should be considered in relation to understanding the normal physiological function of the *mdr* multigene family.

Resistance to multiple chemotherapeutic agents remains a major obstacle in the successful management of human malignancies. Drug-resistant cell lines selected by stepwise selection using a single cytotoxic compound are often cross-resistant to a broad spectrum of structurally and functionally unrelated chemotherapeutic agents (5, 6, 9, 21, 23). Multidrug-resistant cultured cell lines express increased levels of the P-glycoprotein (22), a 170-kilodalton membrane glycoprotein encoded by a gene termed *mdr* (16, 24, 25) or *pgp* (11). P-glycoprotein appears to cause multidrug resistance via an energy-dependent drug efflux mechanism (10, 26). The amino acid sequence of the P-glycoprotein deduced from cloned cDNAs (7, 14, 15) suggests a membrane-spanning protein with consensus sequences for two nucleoside triphosphate (NTP) binding sites (31). The NTP binding sites are in domains which share extensive homology with a series of bacterial transport proteins (1, 20). The P-glycoprotein apparently functions as a transport protein with a broad specificity in multidrug-resistant cell lines.

The physiological function of the P-glycoprotein in normal tissues, however, is unknown. Analysis of the distribution of the P-glycoprotein in normal tissues should provide some insight into this question. Several studies indicate that P-glycoprotein is localized to secretory surfaces in a number of tissues, including the adrenal gland, kidney, liver, intestinal tract, and uterine epithelium (2, 12, 29). However, different molecular probes (4), antisera (13), and experimental conditions (28) have yielded conflicting results regarding the level of expression and the exact tissue distribution.

A central issue in understanding the normal functions of the P-glycoprotein has been the development of data indicating that the gene encoding the P-glycoprotein is a member of a multigene family (11, 15, 18, 30). Probes from the amplified DNA domain in multidrug-resistant cell lines cross-hybridized to multiple genomic sequences (16, 25) and identified two homologous but distinct mouse cDNAs (17). Sequence analysis of the mouse *mdr1* (15) and *mdr2* cDNAs (18) indicates 85% amino acid homology between the products of the two different genes. Further analysis has led to the identification of a third member of the mouse *mdr* gene

family with similar homology to *mdr1* and *mdr2* (M. Raymond and P. Gros, manuscript in preparation).

The development of specific DNA probes for each member of the *mdr* gene family now permits an analysis of *mdr* gene expression which precisely accounts for the expression level of each family member. An in-depth analysis using the *mdr* gene-specific probes in normal mouse tissues provides significant new insights into *mdr* gene expression. The results indicate that there is dramatic differential expression of each member of the *mdr* gene family in tissue. Furthermore, multiple *mdr* RNA transcript sizes have been identified, and the gene-specific probes indicate that each of the three *mdr* genes has a specific RNA transcript pattern. This detailed understanding of the tissue-specific expression of the *mdr* gene family members is clearly relevant to the eventual understanding of the normal physiological role of this multigene family.

Expression levels of total *mdr* RNA. Probe pcDR1.3, derived from the cDNA for *mdr1*, includes a highly conserved NTP binding site (17) (Fig. 1). This probe cross-hybridizes extensively to homologous regions of *mdr2* and *mdr3* transcripts, yielding an estimate of the total level of *mdr* RNA expressed in each tissue. RNA was isolated (3) from the tissues of adult C57BL/6J mice (placenta and pregnant uterus samples from day 16 of gestation) and from NIH 3T3 cells, the 3T3/A1.0 cell line, an adriamycin-selected multidrug-resistant cell line maintained at an adriamycin concentration of 1.0 µg/ml, the L1210 mouse leukemia cell line, and the daunorubicin-selected multidrug-resistant cell line L1210 DN maintained at a daunorubicin concentration of 0.5 µg/ml (27). The RNA (10 µg unless otherwise indicated) was fractionated on formaldehyde gels and electroblotted onto Gene Screen Plus (New England Nuclear Corp.) (8). RNA transfer was assessed by inspection of the membrane and gel after completion of the transfer. RNA-DNA hybridization was carried out in 1 M NaCl-1% sodium dodecyl sulfate-10% dextran sulfate-400 ng of denatured salmon sperm DNA per ml at 65°C with 10⁷ cpm of ³²P-labeled hybridization probe (8). Blots were washed to a final stringency of 30 mM NaCl-3 mM sodium citrate-0.1% sodium dodecyl sulfate at 65°C, and multiple exposures were taken with preflashed film and an intensifying screen. Expression levels were deter-

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FIG. 1. Position of *mdr* gene-specific probes in the respective cDNA clones. The model is a representation of P-glycoprotein with three membrane-spanning loops followed by an intracytoplasmic domain that includes the consensus sequence for an NTP binding site. The entire structure is repeated in the distal half of the molecule (7, 14, 15). Approximate amino acid homologies among the three *mdr* cDNAs are indicated as similarly shaded regions. The probe pcDR1.3, a *Bgl*II fragment of λDR11 (17) from nucleotides 1220 to 2666, includes the highly homologous regions surrounding the NTP binding sites and cross-hybridizes to all three *mdr* genes (data not shown). The *mdr*1-specific probe, probe A, is a *Bst*NI-*Hpa*II fragment of λDR11 (15) including nucleotides 1907 to 2331. The *mdr*2-specific probe, probe B, is a *Hinf*I fragment of λDR29 (18) including nucleotides 1869 to 2276. The *mdr*3-specific probe, probe C, is a *Bgl*II-*Kpn*I fragment of λDR13 (unpublished data) including nucleotides 847 to 1220. Southern blot analysis at high stringency of mouse genomic DNA with each of the *mdr* gene-specific probes identified distinct bands, indicating that there is no cross-hybridization among the probes (data not shown).

mined from autoradiograms of RNA blots probed with pcDR1.3, scanned with an LKB Ultrascan densitometer, and normalized for RNA loading by comparing the intensities of the ribosomal bands in ethidium bromide-stained gels.

An *mdr*-specific RNA was easily detected in each of the tissues examined with pcDR1.3 (Fig. 2 and Table 1). The highest levels of expression of *mdr* RNA were present in the pregnant uterus. We have recently shown that *mdr* RNA is induced 200-fold during pregnancy in the secretory epithelium lining the uterine cavity and endometrial glands (2). The placenta also exhibited very high levels of *mdr* expression

(Fig. 2B), which is localized to the trophoblastic giant cells by *in situ* RNA hybridization (data not shown). In the normal mouse, the adrenal gland and the intestine express the highest levels of *mdr* RNA. Most of the other tissues examined—muscle, liver, kidney, heart, brain, spinal cord, testes, lung, and spleen—displayed similar intermediate amounts of *mdr* RNA. Only the stomach, nonpregnant uterus (data not shown), and lactating breast tissue (data not shown) expressed low levels of *mdr* RNA. However, even these tissues exhibited detectable *mdr* RNA levels upon longer autoradiographic exposure. There has been little

FIG. 2. Northern blots of RNA from mouse tissues hybridized with the generic *mdr* probe, pcDR1.3. The *mdr* RNA transcripts of 6, 5, and 4.5 kb (small arrowheads) were determined relative to the mobilities of the 28S and 18S ribosomal bands (large arrowheads) in the ethidium bromide-stained gels. The exposure time was 18 h (A) or 10 h (B). L1210 DN, 5 μ g of RNA.

TABLE 1. Expression of *mdr* RNA

Tissue	Expression ^a of:		
	<i>mdr1</i>	<i>mdr2</i>	<i>mdr3</i>
Pregnant uterus	+++++		
Uterus	+		
Adrenal	+++++	++	
Kidney	++		+
Liver	+	++	+
Muscle	+	++	+
Spleen	+	++	+
Heart	++	++	++
Brain	+		++
Lung	+		++
Intestine			+++
Spinal cord ^b			++
Testes ^b			++
Stomach ^b			+
Placenta ^b	+++		
3T3 cell line	++		
3T3/A1.0	+++++		
L1210 cell line	++	+	
L1210DN	+++++	++	

^a Symbols represent expression compared with that of the 3T3 cell line: +, less than one-half the expression level; ++, 1- to 10-fold the expression level; +++, 10- to 20-fold the expression level; +++++, 20- to 40-fold the expression level; ++++++, greater than 40-fold the expression level. Gene-specific probes A, B, and C identified *mdr* family members.

^b Expression was determined with pcDR1.3; small amounts of other gene family members may also be present.

variability between samples from different mice and different strains (data not shown).

Sizes of *mdr* transcripts. There are *mdr* RNA species of three different sizes identified by pcDR1.3 in the mouse tissues—approximately 4.5, 5, and 6 kilobases (kb) (Fig. 2A and B). Most tissues, including kidney, heart, brain, spinal cord, intestine, stomach (on longer exposures), testes, lung, placenta, lactating breast (data not shown), and uterus, contain an *mdr* RNA of approximately 5 kb that migrates similarly to the 5-kb species overexpressed in multidrug-resistant cell lines. RNA from the liver, muscle, and spleen contains a 4.5-kb message as the major *mdr* transcript when hybridized with pcDR1.3. The 5-kb *mdr* message in adrenal, heart, and L1210 cell lines extends down to the level of the

4.5-kb *mdr* message (Fig. 2A). Brain, spinal cord, lung, heart, intestine, and testes (Fig. 2A and B), as well as spleen, muscle, kidney, liver, and stomach (on longer exposures), contain an additional *mdr* RNA approximately 6 kb in size. The relative amount of 5-kb *mdr* RNA in the brain and heart tissue increased when increased amounts of supporting tissue were included in the sample preparations. The *mdr* transcript of approximately 5 kb in the intestine migrated slightly slower than the other 5-kb transcripts.

Specific expression of *mdr1*, *mdr2*, and *mdr3*. Hybridization with probe A, specific for *mdr1* (Fig. 1), identified a 5-kb RNA species expressed at high levels in the pregnant uterus and the adrenal gland and expressed at intermediate levels in kidney and heart tissue (Fig. 3A). A 5-kb RNA species was also expressed in muscle, brain, liver, spleen, and lung tissue upon longer exposures (Fig. 3B). This RNA species has the same mobility as the *mdr* RNA overexpressed in the multidrug-resistant cell lines 3T3/A1.0 and L1210 DN.

Hybridization with probe B (Fig. 1), specific for *mdr2*, identified a 4.5-kb RNA species in liver, muscle, and spleen which represents the major *mdr* RNA species in these tissues (Fig. 4). Probe B also identified a 4.5-kb RNA species in heart (Fig. 4) and adrenal gland (data not shown) that represents a minor component of the *mdr* RNA species in these tissues. The L1210 leukemia cell line and its daunorubicin-selected L1210 DN multidrug-resistant cell line also contain the 4.5-kb *mdr2*-specific RNA (Fig. 4).

Hybridization with probe C (Fig. 1), specific for *mdr3*, identifies both a 6- and a 5-kb RNA species expressed at high levels in the intestine and a 6-kb RNA species expressed at intermediate levels in the brain, heart, and lung and at low levels in muscle and kidney (Fig. 5) and in liver and spleen (longer exposures). Probe C does not hybridize to the 5-kb *mdr* RNA in pregnant uterus, kidney, adrenal, or muscle tissue or to the multidrug-resistant cell lines 3T3/A1.0 and L1210 DN.

The analysis of the *mdr* multigene family in mouse tissues indicates a distinct pattern of tissue-specific expression. Specific molecular probes for each of the three mouse *mdr* genes clearly show differential expression of the *mdr* genes in the mouse tissues. This observation, along with the close homology among the three mouse *mdr* cDNAs and their ancestral relationship to bacterial transport proteins, suggests that each *mdr* family member has a specific transport

FIG. 3. Northern blots of RNA from mouse tissues hybridized with the *mdr1*-specific probe, probe A. (A) Exposure time, 18 h. L1210 DN, 5 μ g of RNA. (B) Exposure time, 72 h. Pregnant uterus, 5 μ g of RNA. Specific hybridization of a 5-kb (small arrowhead) transcript was detected in RNA from pregnant uterus, adrenal gland, kidney, and heart and in RNA from the multidrug-resistant cell lines 3T3/A1.0 and L1210 DN. On longer exposures (panel B), a 5-kb *mdr1*-specific transcript was also detected in liver, muscle, spleen, brain, and lung (small arrowhead). Large arrowheads indicate the positions of the 28S and 18S ribosomal bands.

FIG. 4. Northern blot of RNA from mouse tissues hybridized with the *mdr2*-specific probe, probe B. Exposure time, 18 h. Specific hybridization of a 4.5-kb transcript (small arrowhead) was detected in RNA from muscle, liver, spleen, and heart and in RNA from the L1210 cell lines. Large arrowheads indicate the positions of the 28S and 18S ribosomal bands.

function. However, the exact relationship between the ability to transport cytotoxic agents out of cells and the normal transport function of the *mdr* genes is not yet clear. Although the *mdr1* cDNA is capable of conveying the multidrug-resistant phenotype to drug-sensitive cells (8, 17, 19), the *mdr2* cDNA is not, despite considerable homology to the *mdr1* cDNA (18). When expressed in multidrug-resistant cell lines, the *mdr2* RNA transcript is present at only slightly increased levels (Raymond and Gros, in preparation). The potential of *mdr3* to convey drug resistance has not yet been tested.

mdr RNA transcripts of at least three sizes are expressed in patterns characteristic for each of the mouse *mdr* genes. The *mdr1*-specific transcript is a 5-kb RNA, which is the major *mdr* species detected in the pregnant uterus, adrenal gland, kidney, and heart as well as in the 3T3/A1.0 and

FIG. 5. Northern blot of RNA from mouse tissues hybridized with the *mdr3*-specific probe, probe C. Exposure time, 120 h. L1210 DN and pregnant uterus, 5 μ g of RNA. Specific hybridization of both a 6- and a 5-kb transcript (small arrowheads) was detected in RNA from intestine. A 6-kb RNA transcript was detected in RNA from heart, brain, lung, kidney, and muscle. The large arrowhead indicates the position of the 28S ribosomal band. The 18S ribosomal band is just below the bottom of the figure.

L1210 DN multidrug-resistant cell lines. The *mdr1*-specific 5-kb RNA is a minor component of muscle, liver, brain, lung, and spleen. The *mdr2*-specific RNA transcript is a 4.5-kb RNA representing the major *mdr* RNA expressed in muscle, liver, and spleen and a minor component in the heart and adrenal gland. The *mdr3*-specific probe identifies both a 6- and a 5-kb RNA species in the intestine and a 6-kb RNA in lung, brain, and heart as the major *mdr* transcript. The *mdr3*-specific probe also identifies a 6-kb *mdr* transcript at low levels in the spleen, muscle, liver, and kidney. The 5-kb *mdr3* RNA transcript is distinct from the 5-kb *mdr1* RNA transcript, as indicated by the lack of hybridization of probe C, the *mdr3*-specific probe, with the 5-kb *mdr1* RNA in the pregnant uterus, adrenal gland, kidney, and multidrug-resistant cell lines. This is also confirmed by the lack of hybridization of probe A, the *mdr1*-specific probe, with the 5-kb *mdr3* RNA species in the intestine. Independent cDNA clones suggest that the 5- and 6-kb *mdr3* transcripts are produced by alternative polyadenylation signals from a unique *mdr3* gene (Gros and Raymond, unpublished data).

Previous analysis of the distribution of the *mdr* gene in human tissue that used RNA slot blots indicated a wide range of expression, with the adrenal gland having extremely high levels of expression and the colon, kidney, and liver having moderately high levels while most other tissues exhibited low levels (12). The analysis of mouse tissues by Northern (RNA) blotting with gene-specific probes also indicates high levels of expression of the *mdr* gene in the adrenal gland and intestine, while the pregnant uterus expresses the greatest amount of *mdr* message (2). However, most mouse tissues express readily detectable levels of *mdr* RNA, and the range of *mdr* expression in most mouse tissues does not appear to be as extreme as that found in human tissues. Although it has been suggested that the P-glycoprotein acts as a mechanism of protection against a variety of toxic natural products (12), its distribution in a wide variety of tissues indicates that specific cellular products may be the substrates normally transported. Whether each family member transports a single substrate from each cell type, a variety of substrates from each cell type, or different substrates in different cell types is unknown. We are currently analyzing tissues by in situ hybridization to delineate the cell types expressing each of the *mdr* genes. This approach is also useful for analyzing gene expression in cell types representing a small component of the tissue and hence below the level of detection by Northern blot analysis. However, it is clear that each of the *mdr* genes is regulated in a tissue-specific manner.

Understanding the differential regulation of the *mdr* gene family may be important in cancer chemotherapy strategies. On the basis of these studies, however, analysis of tissue samples for expression of the *mdr* gene must take into account the specific gene family member being expressed. Only tissues which express high levels of *mdr1* would appear to have the potential to be resistant to the wide range of compounds in the multidrug-resistant phenotype. Furthermore, physiologic responses, such as in the secretory endometrial epithelium during pregnancy (2), may be able to increase *mdr* gene expression in addition to induction by chemotherapeutic agents. Understanding the factors which control expression of this gene family may ultimately play a role in defining therapeutic options for the treatment of human malignancy.

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