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BIOCHEMICAL AND GENETIC CHARACTERIZATION OF THE MULTIDRUG RESISTANCE PHENOTYPE IN MURINE MACROPHAGE-LIKE J774.2 CELLS

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Abstract—The development of multidrug resistance (MDR) in malignant tumors is a major obstacle to the treatment of many cancers. MDR sublines have been derived from the J774.2 mouse macrophage-like cell line and utilized to characterize the phenotype at the biochemical and genetic level. Two isoforms of the drug resistance-associated P-glycoprotein are present and distinguishable both electrophoretically and pharmacologically. Genetic analysis has revealed the presence of a three-member gene family; expression of two of these genes, *mdr*1a and *mdr*1b, is associated with MDR whereas the expression of the third, *mdr*2, is not. Studies of these three genes have revealed similarities and differences in the manner in which they are regulated at the transcriptional level, and have suggested that post-transcriptional effects may also be important.

One of the major problems facing physicians treating malignancies is the development of acquired drug resistance (see Ref. 1). Drugs that induce tumor remission at the time of initial treatment often lose their effectiveness when the tumor recurs. When chemotherapeutic regimens that include hydrophobic drugs of natural product origin such as vincristine or doxorubicin are utilized as first line treatment, recurrent tumors often appear that are not only resistant to these compounds but also to a variety of structurally and functionally unrelated chemotherapeutic agents.

This multidrug resistance (MDR†) phenotype can be modeled using tissue culture cells. In our laboratory, we have used stepwise selection to generate sublines of the murine macrophage-like cell line J774.2 that are highly resistant to cytotoxic drugs. To understand the spectrum of MDR in murine cells, a number of independently-derived MDR cell lines were generated, including one colchicine-resistant line (J7.C1-100), three vinblastine-resistant lines, (J7.V1-1, J7.V2-1 and J7.V3-1) and two taxol-resistant lines (J7.T1-50) and J7.T2-50) [2–3]. Like recurrent tumors in the clinical setting, these cell lines are not only highly resistant to the drug with which they were selected but also exhibit cross-resistance to other agents that induce the MDR phenotype (e.g. vinblastine, colchicine, taxol, doxorubicin and actinomycin D). This cross-resistance is specific to this general class of cytotoxic compounds since sensitivity to drugs such as bleomycin that do not induce the phenotype is unaffected (Table 1).

Initial recognition of the MDR phenotype and its characterization at the cellular level revealed that the resistance is due mainly to a reduced intracellular steady-state accumulation of drug. Experiments to determine the mechanism of this decreased accumulation demonstrated that the resistant cells had an enhanced efflux of drug, suggesting the possible involvement of a pumping mechanism. This was further shown to be an energy-dependent process since the differences in drug accumulation between MDR and sensitive cells was abolished in the presence of metabolic inhibitors [4–6].

Studies on MDR have been reviewed extensively in recent years [7–11]. In this laboratory we are investigating the murine MDR phenotype on a variety of levels [12]. Our initial interest in MDR came from cellular studies of resistance to the drug taxol. We first focused on studying the biochemical aspects of this phenomenon, an analysis which is not yet complete. We have since extended our interests to include characterization of the phenotype at the molecular biological level with studies of the *mdr* genes. This review will cover progress made to date in studying both of these aspects in the J774.2 cell line and will also introduce our ongoing work concerning the regulation of the *mdr* genes in MDR cells and in the mouse.

Characterization of MDR proteins (P-glycoproteins)

Common features of P-glycoproteins. Early studies of MDR in cells led to the suggestion that the alterations present in these cells resided in the membrane [4, 13]. Analysis of the membranes from MDR cells led to the observation of a large protein species of ~170 kDa molecular weight, which was termed P-glycoprotein, for its effect on cell membrane permeability [14].

Coomassie-stained protein gels of membrane extracts of MDR cells revealed the presence of a major band of 130–140 kDa in all of the highly drug-resistant sublines of J774.2. This protein was barely visible in the parental drug-sensitive cells [2, 15–17].

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† Abbreviation: MDR, multidrug resistance; R3, polyclonal antibody raised against P-glycoprotein from J7.VI-1; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

Table 1. Resistance profile of J774.2 MDR cells

	Fold-resistance*						
	VBL	CLC	DXR	TX	PUR	ACT	BLM
J774.2	1	1	1	1	1	1	1
J7.V1-1	1083	382	234	643	147	8	1.2
J7.V3-1	1017	268	166	163	126	21	0.5
J7.C1-20	17	550	81	65	88	61	.15
J7.T1-50	43	58	128	833	97	88	.30

* Relative ED_{50} value of experimental cell line compared to drug-sensitive cell line. See Ref. 22 for ED_{50} values of drug-sensitive cells.

VBL, vinblastine; CLC, colchicine; DXR, doxorubicin; TX, taxol, PUR, puromycin; BLM, bleomycin.

J774.2 is the drug-sensitive parental cell line. MDR cell line code is as in the following example: "J7.V1-1" where "V1" indicates the first independently selected cell line selected with vinblastine and "1" is the micromolar drug concentration used to maintain the cells; V, vinblastine; C, colchicine; T, taxol.

In the J7.T1-1 (T1) cell line, there were two major proteins presenting as a doublet of closely migrating species. R3 antiserum [18], prepared against the protein from J7.V1-1 (V1), was used to verify that the proteins observed in all of the resistant cell lines were immunologically cross-reactive with each other and with the previously described P-glycoprotein present in MDR Chinese hamster ovary cells [16].

Characterization of these proteins revealed a number of common features in P-glycoproteins from all of the different cell lines. First, as had been shown previously for the Chinese hamster ovary P-glycoproteins, the J774.2-derived proteins were glycosylated and phosphorylated in intact cells [2]. *In vitro* studies with partially purified membranes from drug-resistant cells showed that phosphorylation of P-glycoprotein was enhanced 2-fold by cAMP, suggesting that one of the enzymes responsible for P-glycoprotein phosphorylation was a membrane-associated protein kinase A [19].

Diversity of P-glycoproteins. A comparison of the P-glycoproteins present in the various MDR sublines revealed apparent size heterogeneity in SDS-polyacrylamide gels, with proteins ranging in size from 130 to 140 kDa [15]. To resolve this discrepancy, the proteins, which had been shown previously to contain carbohydrate, were subjected to treatment with different glycosidases to remove N-linked sugars. Pulse-chase analysis of the proteins both in the glycosylated and deglycosylated state revealed the presence of two distinct precursor forms of P-glycoprotein, one at 120 kDa, and the other at 125 kDa [15]. Careful analysis of these precursors showed that the 120-kDa precursor, present mainly in V3 and T1 cells, chased to a 130-kDa mature protein product. The 125-kDa precursor, present in the remaining cell lines (C1, V1, V2) as well as in T1, matured into a 135–140-kDa mature protein with variations in size seen among the cell lines. The heterogeneity in the size of the 125-kDa precursor products was abolished by treatment with the non-specific *N*-glycosidase PNGaseF and was therefore ascribed to the presence of heterogeneous glycosylation of the mature protein.

This analysis suggested that there might be two distinct P-glycoproteins, a hypothesis that was subsequently corroborated utilizing the technique of Cleveland mapping [3]. Isolation of P-glycoprotein from polyacrylamide gels in which membranes from V1 or V3 cells had been resolved followed by further digestion of the protein with V8 protease revealed two distinct peptide maps giving further proof that these two proteins have distinct peptide backbones.

The presence of the two distinct P-glycoprotein isoforms was further verified by the generation of site-directed antipeptide polyclonal antibodies (Fig. 1) [17]. This work was carried out using information gleaned from sequence analysis of the cDNAs (*vide infra*) and selecting peptide sequences from the highly divergent linker region of the proteins. Comparison of the results of immunoblots using the specific antisera as well as the non-isoform specific R3 antisera corroborated our results, and verified the presence of two distinct P-glycoproteins.

Functional differences between P-glycoprotein isoforms. Once the presence of two distinct P-glycoprotein isoforms was established, the focus of this project shifted to the functional differences between the two proteins. The system established in our laboratory seemed uniquely qualified to begin to address this question since we had a cell line producing predominantly the 120-kDa precursor product (V3), other cell lines producing predominantly the 125-kDa precursor product (C1, V1, V2, T2) and one cell line producing both, in approximately equal amounts (T1). To examine the functional differences between the isoforms a comparison was done between the V1 and V3 cell lines, two independent cell lines selected for growth in 1 μM vinblastine each of which produced predominantly one single precursor (see Table 1).

Careful inspection of stained gels or immunoblots from V1 and V3 suggested that there was approximately half as much P-glycoprotein in V3 membranes as in V1 membranes. More quantitative work ensued with the observation that the photoactive calcium channel blocker azidopine could specifically label P-glycoprotein in membrane vesicles [20, 21]. Use of this technique allowed confirmation of the data indicating that there was approximately

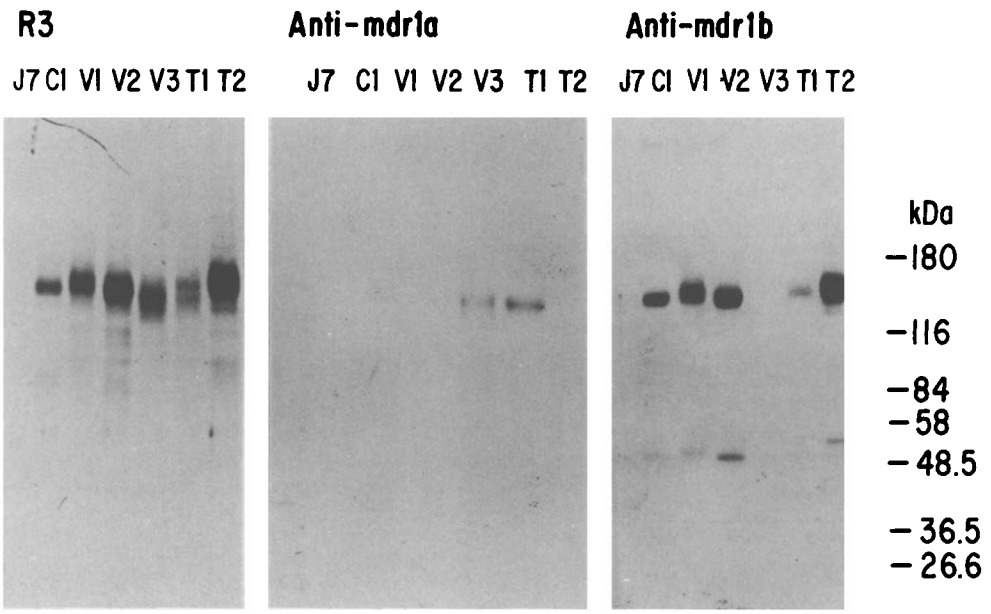

Fig. 1. Immunoblot analysis of P-glycoproteins with general and isoform-specific polyclonal antibodies. Plasma membrane-enriched subcellular fractions (15 μg) were resolved by SDS–PAGE and transferred to nitrocellulose. Blots were probed with antibodies indicated in each panel. Anti-*mdr*1a and anti-*mdr*1b are affinity-purified peptide-directed antibodies specific for each isoform. Reproduced from *J Biol Chem* **264**: 12053–12062, 1989 [17] with permission.

half as much P-glycoprotein in V3 cells as in V1 [22], suggesting that the product of one 120-kDa precursor was somehow twice as "efficient". Studies of the processing times [15] and half-lives [23] of the two P-glycoprotein isoforms revealed essentially no differences, indicating that other mechanisms must be responsible for the inequality of protein levels.

Additional data to address this question came during studies of the development of the V3 cell line [24]. It was observed that cells in the early stages of selection for this cell line expressed the 125-kDa precursor but that, at one point in selection, production switched to that of the 120-kDa precursor. A study of this phenomenon revealed that this switch occurred during a narrow time or drug concentration window, and that the total amount of immunoreactive P-glycoprotein did not change appreciably during this switch. Careful comparison of resistance characteristics before, during and after the switch revealed that for the same amount of P-glycoprotein, the cells producing the 120-kDa precursor product were nearly four times as resistant (Fig. 2), confirming the hypothesis that this protein is more efficient than the 125-kDa precursor product.

Another functional difference between the two proteins was detected pharmacologically. Studies of photolabelling with azidopine [21], or with another photoaffinity labeled compound, 125-iodoaryl azidoprazosin [25], revealed that both protein backbones were labelled with approximately equal affinity by the two photolabels. Inhibition of this photolabelling could then be used as an indirect measure of the binding affinities of other compounds for the P-glycoprotein. In general, for the two proteins, the order of potency of equal molar concentrations of MDR drugs in terms of inhibition of azidopine photolabelling was vinblastine > actinomycin D > doxorubicin > colchicine (see Ref. 26).

The calcium channel blocker verapamil, which had been shown previously to be an effective agent at reversing drug resistance *in vivo*, [27] was also shown to be an effective inhibitor of azidopine photolabelling [21]. This compound, as well as a variety of other calcium channel blockers, in general, inhibited the photolabelling of both P-glycoprotein isoforms equally, although the compounds prenylamine and perhexiline showed slight but repeatable differences. Attempts were made to use this assay to identify potential physiological substrates for P-glycoprotein. It was reasoned that compounds that interfere with the binding of azidopine might bind to P-glycoprotein in a physiological setting. For this analysis, the ability of a variety of steroids was tested in the photolabelling inhibition assay using V1 membranes [28].

It was observed that progesterone was an efficient inhibitor of photolabelling. This was especially interesting, in view of the fact that high levels of the 125-kDa precursor product were shown to be present in the pregnant uterus [29] and that this induction required progesterone, in combination with estrogen [30]. More in-depth analysis of the interaction of progesterone with P-glycoproteins revealed that the steroid was able to distinguish between the two isoforms of P-glycoprotein (Fig. 3) [22]. Despite the fact that it appears to bind equally well to the two P-glycoproteins, it does not seem to be transported and, furthermore, it is able to block the action of

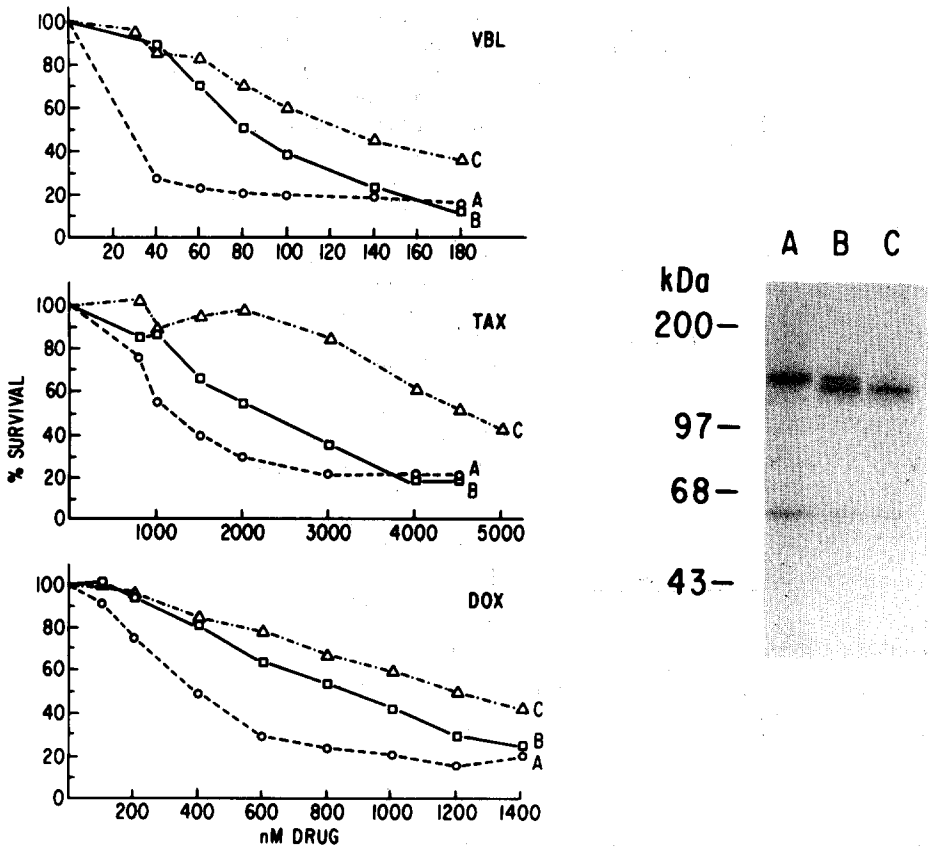

Fig. 2. Relationship between drug resistance and a switch in *mdr* gene product. Right panel: quantitation and analysis of *mdr* gene product in V3 sublines (A, B, and C) that were maintained in 40 nM vinblastine. Plasma membrane-enriched fractions were resolved by SDS-PAGE, transferred to nitrocellulose and probed with R3. The decrease in P-glycoprotein size is known to be associated with a switch from the *mdr*1b to the *mdr*1a gene product. Left panel: drug resistance profiles of the three V3 sublines. Reproduced from *J Biol Chem* **264**: 16054–16058, 1989 [24] with permission.

the 125-kDa precursor product in a number of *in vitro* and *in vivo* assays without affecting to the same degree that of the 120-kDa precursor product.

Characterization of MDR genes

Identification and nomenclature of mdr *gene family members.* Initial attempts to characterize the genes responsible for encoding MDR proteins relied on the fact that these genes are amplified and overexpressed in MDR cells. cDNA fragments from MDR genes were obtained either by screening an expression library with an antibody [31] or by the in-gel renaturation technique [32]. Use of these probes for analysis of genomic DNA suggested these cDNA sequences originated from a multigene family. Screening of cDNA libraries prepared from the MDR V1 cell line yielded three classes of cDNAs, consistent with previous work carried out in hamster cells [33]. In contrast, only two classes of cDNAs were obtained from extensive screening of human libraries. When full length cDNAs became available for the three mouse cDNAs and the two human cDNAs, it became clear that two of the mouse genes were quite similar, and also similar to one of the human genes. The third mouse gene was also highly homologous to the second human gene ([11], see ref. 34).

Functional analysis based on circumstantial evidence and later proved by transfection studies, demonstrated that only one of the human genes, as well as the two homologous mouse genes, was able to confer the MDR phenotype upon transfection into drug-sensitive cells [35–37]. The remaining human gene and its mouse homolog were not able to confer MDR [38, 39]. Based on this functional analysis, the MDR genes have been divided into two classes: Class I which is able to confer drug resistance upon transfection and Class II which is not (Table 2).

The two murine Class I MDR genes have been designated [40] as *mdr*1a (also referred to as *mdr*3) [37] and *mdr*1b (also referred to as *mdr*1) [41]. The *mdr*1a gene is the most homologous to human MDR1 (68% at the nucleotide level in the coding region and in the 3′-untranslated region), although *mdr*1b is also quite homologous to human MDR1 (68% in the coding region, but only 48% in the 3′-untranslated region). The mouse homolog of human

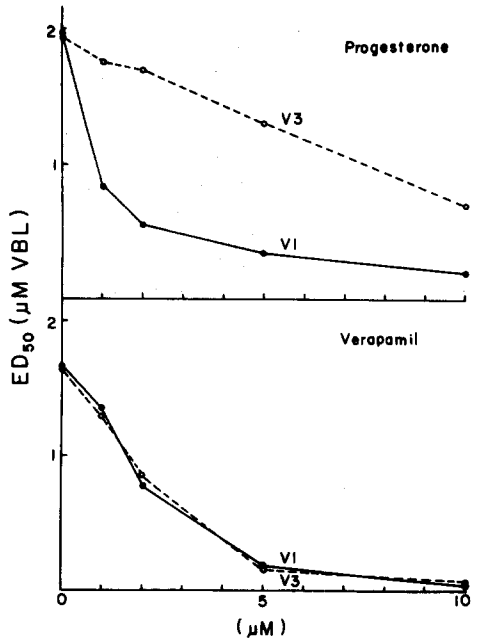

Fig. 3. Effect of progesterone and verapamil on drug sensitivity. ED_{50} values for vinblastine in V1 and V3 were determined in the absence or presence of 1, 2, 5 and 10 μM verapamil or progesterone (see Ref. 22).

Table 2. MDR genes in mouse, hamster and human

	Gene class I		II
Mouse	*mdr*1a*	*mdr*1b*	*mdr*2
Hamster	*pgp*1	*pgp*2	*pgp*3
Human	*mdr*1	—	*mdr*2†

* Mouse *mdr*1a and *mdr*1b are also known as *mdr*3 and *mdr*1, respectively [36, 39].

† Human *mdr*2 is also known as human *mdr*3 [38].

MDR2 is designated *mdr*2 and belongs to Class II. In the hamster system, the nomenclature is different for historical reasons and the three genes are known as *pgp*1, *pgp*2 and *pgp*3 [33].

Structural studies of the coding regions of the mdr *cDNAs.* Analysis of the sequence of the three full-length mouse cDNAs reveals extensive homology in the coding region (*mdr*1a [37, 40]; *mdr*1b [40]; *mdr*2 [39]). *mdr*1a and *mdr*1b are approximately 93% identical and *mdr*2, which is the most divergent, is approximately 85% identical. The three cDNAs predict proteins of identical structure containing twelve hydrophobic, membrane-spanning domains organized into two sets of six (Fig. 4). At the C-terminus of each of these putative six-loop motifs are consensus ATP binding sites. The N- and C-terminal halves are joined by a so-called "linker" region, a stretch of 50–60 hydrophilic amino acids that is highly divergent among the three predicted proteins. The predicted structure of the protein is such that the N- and C-termini as well as the two nucleotide binding folds and the linker region are cytoplasmic [34, 41].

As mentioned above, transfection of either the *mdr*1a or *mdr*1b cDNA is able to convey the MDR phenotype in drug-sensitive cells [37] whereas transfection of the *mdr*2 cDNA is not [39]. This observation has led to the suggestion that those regions that are conserved between *mdr*1a and *mdr*1b but are divergent from *mdr*2 may be the areas that are important for drug resistance [42].

Identification of P-glycoproteins associated with each mdr *message.* Since three cDNAs were known, an important question became the identification of their gene products. As described above, two P-glycoprotein isoforms were detected in our cell lines, distinguished by the distinct mobilities of both the precursors and the mature proteins. Comparison of expression of the genes by Northern blot analysis and the presence of protein precursors suggested that the *mdr*1a gene encoded the 120-kDa protein precursor, and that the *mdr*1b encoded the 125-kDa form [17]. This hypothesis was further corroborated by a study of the V3 cell line which switched percursor from the 125-kDa to the 120-kDa form during selection (Fig. 2). This switch correlated well with the time of appearance of the *mdr*1a message [24].

A comparison of the predicted protein sequences of these two cDNAs does not reveal the basis for the apparent difference in the sizes of the protein precursors. It has been demonstrated previously that the gel system used for the analysis of P-glycoproteins can have a significant effect on their mobility [16]. Both sequences predict proteins of approximately 1280 residues with a size of 140 kDa. The reason for the size discrepancy is still unclear, although the most likely explanation is aberrant migration of one or both of the proteins.

The question of a protein associated with the *mdr*2 transcript is more problematic. Although this message appears to be overexpressed in V1 and V2 cells, the level of overexpression appears to be much lower relative to levels of the *mdr*1a and *mdr*1b message [17]. Western blotting with a non-isoform specific P-glycoprotein polyclonal antibody shows a single band in all cell lines except T1. However, based on relative message levels, it is likely that any *mdr*2 protein signal would be overwhelmed by the *mdr*1a or *mdr*1b signal. Attempts to raise peptide-directed polyclonal antibodies against the *mdr*2 gene product have so far failed to produce satisfactory results. It does appear likely, however, that there is an *mdr*2 protein in the mouse, based on the indirect evidence from studies carried out in Chinese hamsters [43, 44]. These studies demonstrated the presence of an immunoreactive P-glycoprotein in muscle cells that failed to react with monoclonal antibodies specific for the class I isoforms of P-glycoprotein. The biochemical nature of this protein is not known. Interestingly, earlier studies in humans revealed an immunoreactive species of approximately 200 kDA present in skeletal and cardiac muscle [45]. This finding was, however, dismissed as an artifact based on apparent cross-reactivity of this band with antibodies to muscle myosin. In view of the more

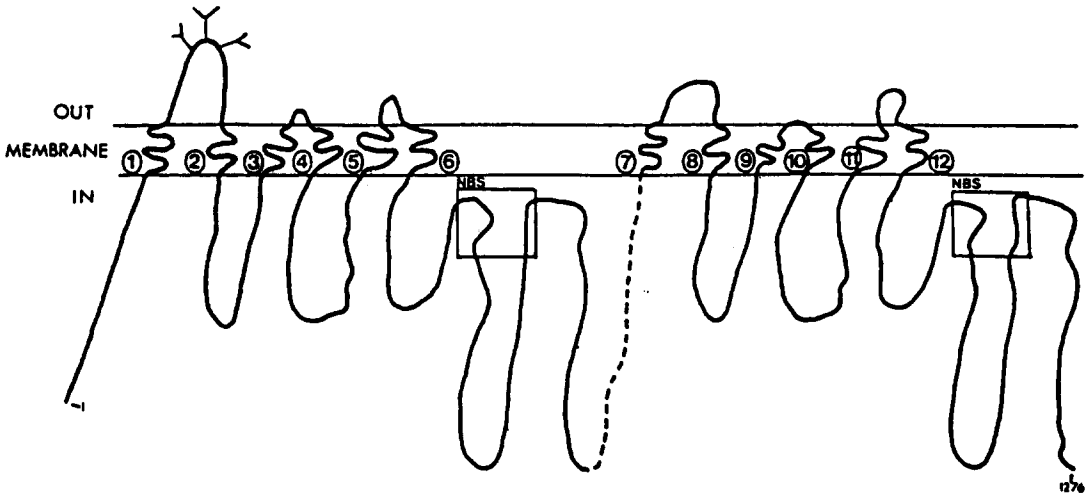

Fig. 4. Proposed structure of P-glycoprotein. The twelve transmembrane domains are labelled with the first extracellular loop containing the only consensus N-linked glycosylation sites located outside the cell. NBS, nucleotide-binding sequence consensus region. Dashed line indicates the linker region, the most divergent domain among the P-glycoproteins (Ref. 42).

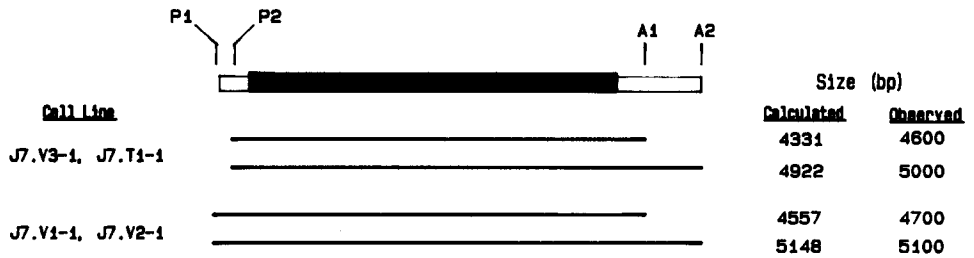

Fig. 5. The basis for *mdr*1a transcript size heterogeneity resides in the 5′ and 3′ non-coding regions. Generation of *mdr*1a transcript heterogeneity by differential initiation from two promoters (P1 and P2) and alternative usage of polyadenylation sites (A1 and A2) is illustrated schematically. The cell lines in which each type of transcript is found are indicated. The protein coding region is shown as a solid bar. Calculated transcript sizes are based on primer extension mapping of the 5′-end and 3′-end cDNA sequence analysis. Reproduced from *Mol Cell Biol* **10**: 3596–3606, 1990 [40] with permission.

recent work in hamsters, it may be worthwhile to review this data and to reconsider the identity of this 200-kDa protein.

Characterization of MDR *gene regulation*

Structure and function of the mdr1a *transcription unit.* Analysis of *mdr*1a gene expression revealed the presence of two distinct bands on Northern blots. One of these was approximately 4.6 kb, the same size as the *mdr*1b message. There was also a second, larger message, which appeared to be 5.1 kb in the V1 and V2 cell lines and 5.0 kb in V3 and T1 [17]. Subsequent analysis of this phenomenon revealed that the basis of the 500-bp difference between the 4.6 and 5 kb messages lay in the presence of two polydenylation signals at the 3′ end of the gene separated by 591 bp [40]. Quantitation of the two forms of the message in any given cell line suggested that the two polyadenylation signals were utilized with approximately equal efficiency.

To understand the difference between the 5.0- and 5.1-kb messages, an investigation of the 5′ end of the message was also undertaken [40]. Primer extension analysis of the message using an oligonucleotide from the first exon revealed the presence of two different sizes at the 5′ end with a difference of 227 nucleotides between them. The shorter form of the transcript was observed in V3 and T1, the cell lines that produce the 5.0-kb message. The longer form was found in V1 and V2, the cell lines that produce the 5.1-kb message (Fig. 5). In the human MDR1 gene, the gene to which *mdr*1a is most homologous, the presence of multiple 5′-ends of the message has been documented previously [46]. In this case, the different ends are generated by two distinct promoters, believed to be separated by approximately 10 kb in the genome [47]. We believe that a similar mechanism is most likely to account for the heterogeneity at the 5′-end of the murine *mdr*1a transcript and therefore

Table 3. Comparison of the *mdr*1a steady-state protein/transcript ratio in MDR J774.2 cell lines

	Relative mRNA level*	Relative P-glycoprotein level†	Relative protein/transcript ratio‡
J774.2	0	0	—
J7.V1-1	49	15 ± 2.4	30 ± 4.9
J7.V2-1	114	18 ± 5.5	16 ± 4.7
J7.V3-1	100	100	100

* Values obtained by mRNA slot blot analysis with an *mdr*1a gene-specific probe were adjusted for the backgroud signal in parental J774.2 cells in which *mdr*1a is known to be silent, normalized to β-actin and expressed relative to the value for J7.V3-1 which was set arbitrarily at 100.

† Values obtained by quantitative immunoblot analysis with a site-directed polyclonal antibody specific for the *mdr*1a gene product are expressed relative to J7.V3-1 which was set arbitrarily at 100. Standard deviation reflects the results of two independent experiments.

‡ Values are expressed relative to J7-V3-1 which was set arbitrarily at 100. Reproduced from *Mol Cell Biol* **10**: 3596–3606, 1990 [40] with permission.

postulate the presence of an additional promoter for this gene, although experimental evidence for this hypothesis has yet to be obtained.

To understand the significance of this heterogeneity, a comparison of the levels of *mdr*1a protein and message was undertaken [40]. Quantitation of the message levels in V1, V2 and V3, three of the four cell lines expressing *mdr*1a, showed that they all expressed *mdr*1a message at similar levels. Use of the *mdr*1a-specific polyclonal antibody revealed the presence of low levels of the protein in V1 and V2, the cell lines expressing the 5.1-kb message, whereas there was much more of the protein in V3, the cell line expressing the 5.0-kb message (Table 3). These data suggest that post-transcriptional effects play a role in regulating the levels of *mdr*1a protein in the cell.

Characterization of the mdr1*b and* mdr2 *transcription units.* The *mdr*1b gene seems likely to be the least complex of the three genes. It appears to produce a single 4.6-kb transcript from its promoter. Also, there is a good correlation between the level of transcript and the level of protein in all of the cell lines.

As mentioned above, the levels of the *mdr*2 gene product are not known, due to the inability to specifically detect this gene product. Although the data is preliminary at this time, it appears as though this transcription unit may also contain interesting features. It appears that there may be complexity at the 5′ end of the *mdr*2 message (LSK and SBH, unpublished data).

Comparison of the promoter regions of the mdr *genes.* Studies of the expression of the three murine *mdr* genes in tissues have shown that each of the genes exhibits a unique pattern of expression in mouse [48, 49] and hamster [43, 44]. The *mdr*1a gene is most highly expressed in the intestinal lining and the gene product of the hamster homolog has been shown to be present in small arterioles and capillaries, as well as the lining of the large intestine. The *mdr*1b gene is expressed at high levels in the adrenal gland, although studies in hamster suggest that this may be true only in male rodents. Interestingly, it has been shown in mice that the pregnant uterus contains extremely high levels of *mdr*1b gene product in the lumenal lining at a specific time in gestation [29]. These levels can be mimicked either by pseudopregnancy or by artificial hormone treatments with estrogen and progesterone that mirror the natural changes in these hormones during pregnancy [30]. *mdr*2 gene transcription is relatively high in liver and skeletal muscle. Immunohistochemical studies of the hamster suggest that cardiac, as well as skeletal muscle, may be a major site of *mdr*2 protein production.

To understand the basis for the differential expression of the three genes in tissues, the promoter regions for the three genes have been cloned. In accordance with the identification of the murine *mdr*1a gene as the most homologous to human MDR1, the *mdr*1a gene promoter region is most similar to the human MDR1 gene promoter [40].

At this time, only the *mdr*1b gene promoter has been characterized at the level of *cis*-acting elements [50]. Fusion of the *mdr*1b promoter from −540 to +97 to the chloramphenicol acetyltransferase structural gene was carried out and 5′-end deletion mutants of the promoter were subsequently constructed. These plasmids were then transfected into Cos-1 cells and the ability of each of the deletions to drive CAT expression was assayed. As can be seen in Fig. 6, the full-length construct gave maximal CAT activity. The change in activity upon successive deletions suggested that there are positive as well as negative elements operating in this promoter. Similar work by Raymond and Gros [51] also offers the suggestion that some of these elements may function in a tissue-specific fashion.

Preliminary experiments with the *mdr*1a and *mdr*2 promoters suggest that they too are able to drive CAT expression in a similar assay system. A comparison of the 5′-ends of the three genes reveals a familiar pattern: the *mdr*1a and *mdr*1b genes are

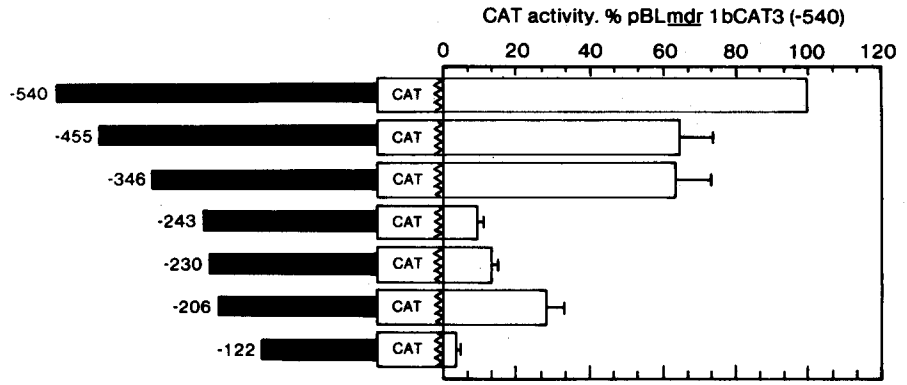

Fig. 6. Expression of *mdr*1b-CAT constructs in a transient transfection assay. Plasmids containing *mdr*1b promoter sequences from −540 to +97 (1bCAT3-540) or 5′-end deletions (as marked) were transfected into COS-1 cells by a DEAE-dextran mediated method. Cells were harvested after 48 hr and CAT activity was determined from 150 μg of cell lysate. Data are expressed as means ± SE from four separate experiments. Reproduced from *J Biol Chem* **266**: 2239–2244, 1991 [50] with permission.

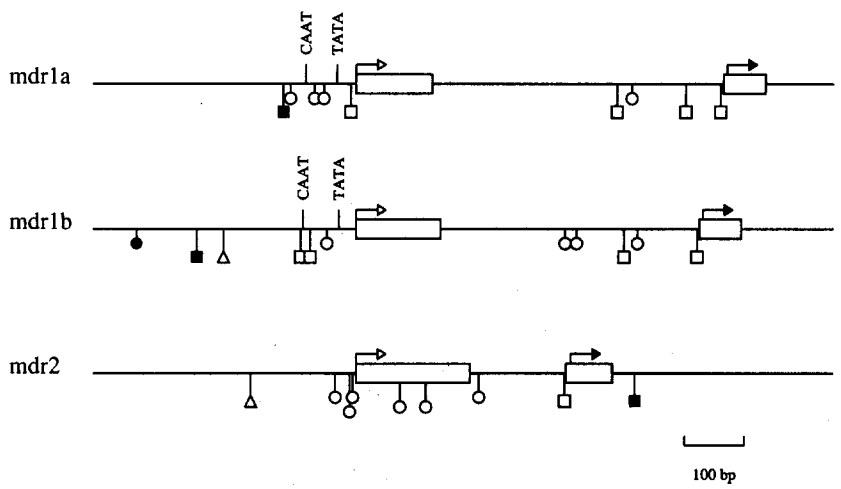

Fig. 7. Comparison of the promoter regions of the three murine *mdr* genes. Promoter regions for the three genes were cloned and sequenced. The diagrams are aligned at the transcription initiation sites (open arrows), which were determined by primer extension. Translation is believed to initiate near the 5′ of the second exon (filled arrow). TATA and CAAT sequences in *mdr*1a and *mdr*1b are shown. Putative binding sites for other transcription factors, as determined by homology to consensus sequences, are denoted as follows: (○) SP-1; (□) AP-2; (△), glucocorticoid receptor; (●), CREB; (■), AP-1.

quite similar whereas the *mdr*2 gene is different. Each gene contains a similar set of consensus binding sites for transcription factors, such as SP-1, AP-1 and AP-2. *mdr*1a and *mdr*1b appear to contain TATA boxes whereas this feature is lacking for *mdr*2. As shown in Fig. 7, the exon and intron sizes are also divergent among the three genes. *mdr*2 has a longer first exon and shorter first intron than the other two genes which are similar in length. A similar situation has been shown for the human genes where MDR1 has a first intron of 500 bases [52] and MDR2 has a first intron of 160 bases [53].

Conclusions and future directions

Work in this laboratory has demonstrated that the two P-glycoprotein isoforms which have been observed in MDR cells are the products of the *mdr*1a and *mdr*1b genes, and are functionally different. Aside from the observation that the *mdr*1a gene product is apparently more efficient as a drug-efflux pump, we have demonstrated the ability of the steroid hormone progesterone to pharmacologically differentiate the two proteins. These data imply that the two gene products are likely to have distinct physiological substrates. Because progesterone is able to interact specifically with the *mdr*1b gene product and to regulate in some fashion the level of its message, it is tantalizing to think that this interaction may have physiological relevance. Progesterone does not appear to be transported by

P-glycoproteins, although the assay is complicated by the extreme hydrophobicity of the steroid. Despite this difficulty, it appears that progesterone may be the first example of a modulator of P-glycoprotein function. Significant work still needs to be done in characterizing the interaction between progesterone and the protein.

Indeed, further studies with other synthetic and natural drugs may help to narrow the range of compounds that are able to differentiate between the two proteins. Such studies may allow the development of a "differential pharmacophore" that will give a theoretical picture of the similarities and differences between the binding pockets of the two P-glycoproteins. The ultimate goal of this direction of research is 2-fold. First is to identify the substrates transported by P-glycoprotein in the physiological setting. This would allow resolution of the question of whether or not P-glycoprotein has a specific substrate in the body, or whether its physiological role is the pumping of toxic substances out of cells, as some authors have postulated [34]. Second is to understand the pharmacological requirements of P-glycoprotein in order to aid the development of drugs that are able to reverse the MDR phenotype by inhibiting the action of the protein. Ideal compounds are those that have this ability without significant toxicity of their own.

Such research would be greatly expedited by the development of a good *in vitro* assay system for P-glycoprotein function. The inability to reconstitute P-glycoprotein into lipid bilayers has been a major obstacle. The development of a good reconstitution system would be a great aid. We have already shown that a functional P-glycoprotein can be expressed in *Xenopus* oocytes [54]. Further use of this system may also prove a valuable tool.

At this time, the *mdr*2 gene product is still uncharacterized at even a basic level. A direct demonstration of a protein product of this gene has yet to be obtained, although a re-examination of the data obtained from studies of muscle cells would seem appropriate. At the biochemical level, it will be necessary to determine if the product of this gene is similar to the other P-glycoproteins. If this proves to be the case, a study of the small differences between the class I and class II P-glycoproteins may help to determine the areas in this *mdr* gene product that prevent its functioning in drug resistance. Like the other gene products, the ultimate goal in studying *mdr*2 is the determination of is physiological substrate.

Studies of the regulation of the *mdr* genes to determine the basis for their differential cell-type specificity are already underway. The data that have been obtained from transient transfection assays into cell lines need to be verified by checking the function of any identified tissue-specific elements in the mouse, perhaps by creating transgenic animals.

In analogy with the protein work, an important goal of these studies is to understand the mechanisms governing regulation of this gene so that agents that inhibit *mdr* gene transcription, thus enhancing drug sensitivity of tumor cells, can be found. In this regard, studies in the mouse of the *mdr*1a gene promoter are the most relevant since this gene is most similar to the human MDR1 promoter.

In conclusion, the J774.2 cell line and its multidrug resistant sublines have provided an excellent model system for studying the clinical multidrug resistant phenotype. We have been able to use this system for the investigation of both the biochemical and genetic aspects of this phenomenon. Clinical workers in the MDR field are just now beginning to examine treatments predicated on the information gained from the MDR research in the laboratory [55, 56]. Although much progress has been made in studying the phenomenon of MDR both in the laboratory and in the clinic, our knowledge about this interesting and important subject is still woefully incomplete.

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