

新 制
農
609
京大附図

**STUDIES ON THE MECHANISM OF ACQUIRING  
RESISTANCE BY HUMAN MULTIDRUG-  
RESISTANCE GENE *MDR1***

**NORIYUKI KIOKA**

**1991**

**STUDIES ON THE MECHANISM OF ACQUIRING  
RESISTANCE BY HUMAN MULTIDRUG-  
RESISTANCE GENE *MDR1***

NORIYUKI KIOKA

1991

To my family and to my wife Miki

## Abbreviations

ATP	adenosine 5'-triphosphate
b p	base pair(s)
cDNA	complementary DNA
cpm	counts per minute
CTP	cytidine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EMS	ethyl methanesulfonate
GTP	guanine 5'-triphosphate
k b	kilobase pair
mRNA	messenger RNA
PAGE	polyacrylamide gel electrophoresis
RNA	ribonucleic acid
RNase	ribonuclease
SDS	sodium laurylsulfate
Tris	Tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
UTP	uridine 5'-triphosphate

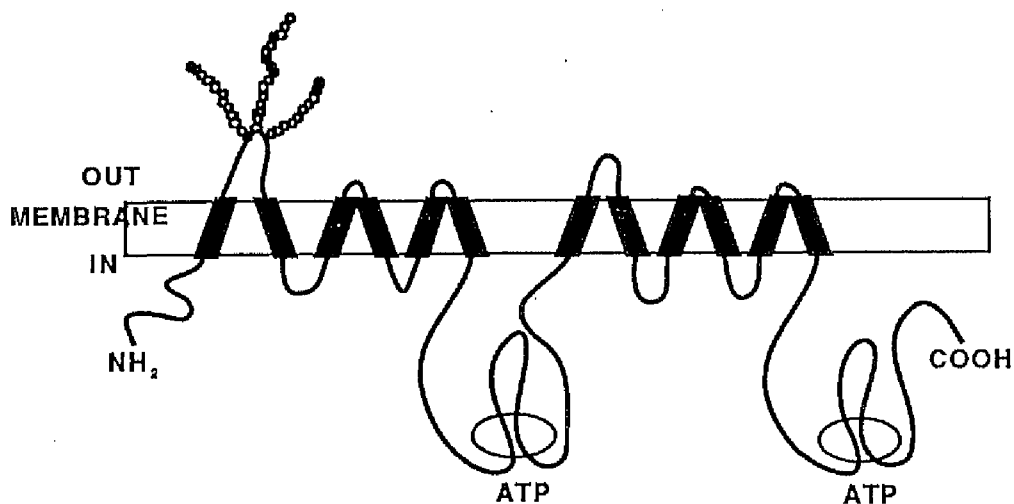
## Contents

Introduction	---	1	
Chapter 1	<i>MDR1</i> cDNA from Human Adrenal: Normal P-Glycoprotein Carries Gly <sup>185</sup> with an Altered Pattern of Multidrug Resistance	---	5
Chapter 2	Detection of <i>MDR1</i> Gene mRNA Expression in Human Tumors by a Sensitive Ribonuclease Protection Assay	---	19
Chapter 3	Transcriptional Activation of <i>MDR1</i> Gene in Response to Arsenite Treatment	---	31
Chapter 4	Quercetin, a Bioflavonoid, Inhibits the Increase of Human Multidrug Resistance Gene ( <i>MDR1</i> ) Expression Caused by Arsenite	---	49
Summary		---	60
List of publications		---	63
Acknowledgements		---	65

## Introduction

Resistance of tumor cells to anticancer agents is a major clinical problem in cancer chemotherapy. Tumors that are sensitive to anticancer agents often recur and become resistant to multiple chemically and functionally unrelated drugs such as colchicine, adriamycin, vinblastine, vincristine, and actinomycin D. Mammalian cultured cells selected for resistance to an anticancer agent have been studying these phenomenon as *in vitro* models because these cells are also cross-resistant to multiple drugs (1, 2). The characteristics of this phenotype include (a) a net decrease of the intracellular concentration of drugs resulted from an increased rate of an energy-dependent drug efflux (3-5), (b) overproduction of a 170 kilodalton glycoprotein called P-glycoprotein or P-gp in the plasma membrane, (c) karyotypic abnormalities such as homogenous staining region or double minute chromosomes (6, 7), suggesting that a part of chromosome is amplified, (d) reversing multidrug resistance by calcium channel blockers such as verapamil (4, 8).

The *MDR1* gene, which encodes P-glycoprotein, was isolated from a multidrug-resistant cell line KB-C2.5 using amplified DNA fragments as a probe (9, 10). Overexpression of *MDR1* gene can confer multidrug resistance on drug-sensitive cell lines after transfection (10). P-glycoprotein has been proved to bind colchicine (11), vincristine (12), and verapamil (13) and to have an ATPase activity (14), indicating that P-glycoprotein plays as an energy-dependent drug efflux pump. P-glycoprotein is composed of two homologous halves (Figure 1). Each half has six transmembrane domains and an ATP binding domain, and shares high homology with bacterial transport protein (ex: HlyB: hemolysin transport protein) (9, 15), yeast STE6 (a factor transport protein) (16, 17), and human



**Figure 1.** Schematic representation of the putative structure of P-glycoprotein

CFTR (cystic fibrosis transmembrane conductance regulator) (18). These proteins form a superfamily of membrane-associated transport proteins.

It has been reported that some multidrug-resistant cell lines are more resistant to the drugs used for the selection than other drugs (1, 19), and in some cases EMS treatments are used during the selection to obtain multidrug-resistant cell lines (1). These results indicate that some genetic mutations, such as the mutations that change the P-glycoprotein structure and function, and that increase the *MDR1* gene expression, might be involved in acquiring multidrug resistance. Some physiological stresses might affect the P-glycoprotein function or the *MDR1* gene expression.

In this thesis, the author intends to clarify the mechanism of acquiring the multidrug resistance by *MDR1* gene and describes: differences between *MDR1* genes expressed in normal tissues and in colchicine-selected multidrug-resistant cell line KB-C2.5 (Chapter 1):

development of a RNase protection assay using *MDR1* promoter region isolated from human normal tissue to detect *MDR1* mRNA accurately (Chapter 2): induction of *MDR1* gene expression after exposure to sodium arsenite (Chapter 3): and inhibition of the increase of *MDR1* mRNA by quercetin, a bioflavonoid (Chapter 4).

## REFERENCES

1. Shen, D.-w., Cardarelli, C., Hwang, J., Cornwell, M., Richert, N., Ishii, S., Pastan, I. and Gottesman, M. M. (1986) *J. Biol. Chem.* **261**, 7762-7770
2. Juliano, R. L. and Ling, V. (1976) *Biochim. Biophys. Acta* **455**, 152-162
3. Inaba, M., Kobayashi, H., Sakurai, Y. and Johnson, R. K. (1979) *Cancer Res.* **39**, 2200-2203
4. Fojo, A., Akiyama, S.-i., Gottesman, M. M. and Pastan, I. (1985) *Cancer Res.* **45**, 3002-3007
5. Marsh, W., Sicheri, D. and Center, M. S. (1986) *Cancer Res.* **46**, 4053
6. Baskin, F., Rosenberg, R. N. and Dev, V. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 3654-3658
7. Grund, S. H., Patil, S. R., Shah, H. O., Pauw, P. G. and Stadler, J. K. (1983) *Mol. Cell. Biol.* **3**, 1634-1647
8. Tsuruo, T., Iida, H., Tsukagoshi, S. and Sakurai, Y. (1981) *Cancer Res.* **41**, 1967-1972
9. Chen, C.-j., Chin, J. E., Ueda, K., Clark, D. P., Pastan, I., Gottesman, M. M. and Roninson, I. B. (1986) *Cell* **47**, 381-389
10. Ueda, K., Cardarelli, C., Gottesman, M. M. and Pastan, I. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 3004-3008
11. Safa, A. R., Mehta, N. D. and Agresti, M. (1989) *Biochem. Biophys. Res. Commun.* **162**, 1402-1408



12. Naito, M., Hamada, H. and Tsuruo, T. (1988) *J. Biol. Chem.* **263**, 11887-11891
13. Safa, A. R. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 7287-7191
14. Hamada, H. and Tsuruo, T. (1988) *J. Biol. Chem.* **263**, 1454-1458
15. Gros, P., Croop, J. and Housman, D. (1986) *Cell* **47**, 371-380
16. McGrath, J. P. and Varshavsky, A. (1989) *Nature (London)* **340**, 400-404
17. Kuchler, K., Sterne, R. E. and Thorner, J. (1989) *EMBO J.* **8**, 3973-3984
18. Riordan, J. R., Rommens, J. M., Kerem, B.-s., Alon, N., Plavsic, R., Chou, J.-L., Drumm, M. L., Iannuzzi, M. C., Collins, F. S. and Tsui, L.-C. (1989) **245**, 1066-1073
19. Scotto, K. W., Biedler, J. L. and Melera, P. W. (1986) **232**, 751-755

## Chapter 1 *MDR1* cDNA from Human Adrenal:

Normal P-glycoprotein carries Gly<sup>185</sup> with an altered pattern  
of multidrug resistance

The *MDR1* mRNA is found at substantial level in the liver, kidney, colon, and small intestine and at high level in the adrenal (1). Immunohistochemical analysis has demonstrated the specific localization of P-glycoprotein at the apical surface of biliary hepatocytes, columnar epithelial cells of the colon and small intestine and on the brush border of proximal tubule cells in the kidney (2). P-glycoprotein is also found in specialized capillary endothelial cells of the brain and testis (3, 4). These results suggest that P-glycoprotein helps excrete metabolites and natural cytotoxic substances in the diet (5) or act as part of the blood-brain or blood-testis barrier. In the adrenal, however, P-glycoprotein was found to be diffusely distributed (2), suggesting that it might have a role different from in other organs.

Recent studies revealed high levels of *MDR1* expression in tumors derived from adrenal, colon and kidney which are known to be intrinsically drug resistant (1, 6, 7). These results indicate that high level of expression of P-glycoprotein can be associated with multidrug resistance in human tumors.

One strategy to circumvent multidrug resistance in tumors is to inhibit the function of P-glycoprotein. It is very important to clarify the structural and functional difference between the *MDR1* genes expressed in normal tissues and in multidrug-resistant cell line. In order to foresee the possible side effects caused by preventing its activity, it is necessary to elucidate the physiological function of P-glycoprotein in human organs. As a first step the author isolated a full-length *MDR1* cDNA from a normal human

adrenal.

## MATERIALS AND METHODS

### *Materials and cells*

[ $\alpha$ -<sup>32</sup>P]dCTP was purchased from Amersham. All cell lines used in this chapter were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum. KB-3-1, a HeLa subline, is the drug-sensitive human carcinoma cell line. KB-C1.5 and KB-C3 were multidrug-resistant cell lines selected in successive steps from KB-3-1 by colchicine. Multidrug-resistant cell lines KB-V1 was isolated from KB-3-1 by vinblastine. The details of isolation and characterization of the cell lines have been previously described (8, 9).

### *cDNA cloning and sequencing*

Poly(A)<sup>+</sup> RNA from human adrenal was isolated by the standard procedure. cDNA was synthesized essentially as described by Gubler and Hoffman (10) except for using an oligo(dT) primer with a *Not*I site at its 5'-end. After blunt-end synthesis, cDNA was digested with *Not*I, and inserted into *Sma*I and *Not*I digested and dephosphorylated Bluescript vector (Stratagene). The library was screened with the *MDR1* cDNA clones pMDR5A and pMDR10 (11). Sequencing of cDNA clones was done by the dideoxy sequencing procedure (12) after subcloning into M13mp18 and M13mp19.

### *Dried-gel Southern hybridization*

To detect single copy genes with oligonucleotide probes, the author used dried-gel hybridization (13). *Eco*RI digested genomic DNA was denatured in 0.5% agarose after electrophoresis. The dried-

gel was hybridized in 0.9 M NaCl, 6 mM EDTA, 0.5% SDS, 90 mM Tris-HCl, pH 7.5, 200 µg/ml denatured salmon testis DNA, and  $2 \times 10^6$  cpm/ml  $^{32}\text{P}$ -labeled oligonucleotide at 37°C. Oligonucleotide probes were labeled by synthesizing complementary strands from a primer with Klenow polymerase and [ $\alpha$ - $^{32}\text{P}$ ]dCTP (111TBq/mmol). The gel was then washed in a solution containing 3M tetramethylammonium chloride at 2°C-5°C below the postulated  $T_m$  for the oligonucleotides (14).

#### *Construction of chimera plasmids and drug resistance assay of transfectants*

The plasmid pHaMDR1/A contains an *MDR1* cDNA derived from the colchicine-selected KB cell line KB-C2.5, carrying valine at codon 185 and alanine at codon 893 (15). The plasmid pHaMDRA1 carrying glycine at codon 185 and serine at codon 893 was constructed by replacing the 3.5-kb *SacII-PstI* fragment of pHaMDR1/A with the equivalent fragment of pMDRA1. The plasmid pHaMDRGA, encoding glycine at codon 185 and alanine at codon 893, was obtained by replacing the 2.2-kb *SacII-HindIII* fragment of pHaMDR1/A with the equivalent fragment of pMDRA1.

Plasmids were cotransfected into drug-sensitive NIH3T3 cells with pSV2neo by the calcium phosphate coprecipitation method as described (11). Transfected cells were first enriched by selection in medium containing G418 at 0.8 mg/ml; then pooled G418-resistant cells (about 800 colonies each) were selected in medium containing colchicine at 60 ng/ml. After two weeks of selection, about 500 colonies per 10 µg of pHaMDR1/A and 200 colonies per 10 µg of pHaMDRA1 or pHaMDRGA were obtained. Five independent clones from each transfection were picked and expanded for further analysis.

The colony-forming ability of transfectants was measured by

plating aliquots of 300 cells in duplicate or triplicate into 60 mm dishes. After 10 days of incubation in 4 ml medium containing different concentration of the drugs, colonies were stained with methylene blue and counted.

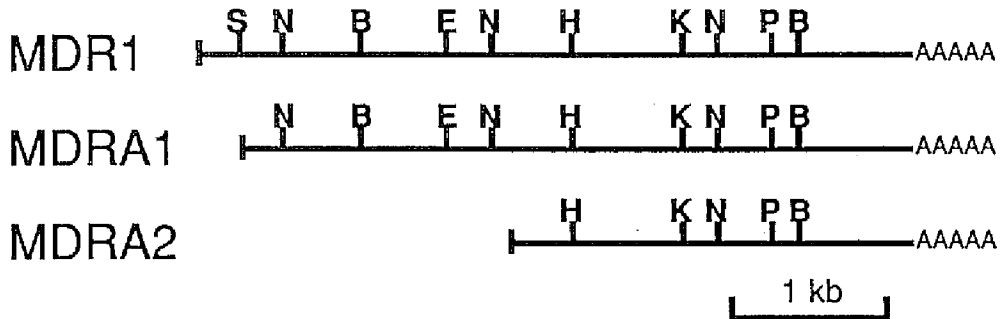
## RESULTS

### *Cloning and sequencing of full-length cDNA from human adrenal*

A cDNA library from human normal adrenal was constructed and screened as described in MATERIALS AND METHODS. Two positive clones pMDRA1 and pMDRA2 were isolated (Fig. 1A). Sequence analysis revealed that the larger clone, pMDRA1, contained the entire coding region. The 5'-noncoding region of pMDRA1 was 137 bases long and was shorter than the previously isolated *MDR1* cDNA. The *MDR1* cDNA was initially isolated from the colchicine-selected multidrug-resistant KB carcinoma cell line, KB-C2.5, and appeared to represent a transcript from the upstream promoter of the *MDR1* gene (16). The downstream promoter is mainly used in human organs including adrenal (17). The 5'-end of pMDRA1 corresponded to the transcription start site of the downstream promoter of *MDR1* gene. These results indicate that pMDRA1 is a full-length *MDR1* cDNA from the human adrenal.

**Figure 1. Structural analysis of *MDR1* cDNA.** (A) Restriction maps of KB-C2.5 derived *MDR1* cDNA and the *MDR* cDNA clones isolated from human adrenal. B, *Bal*I; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; N, *Nco*I; P, *Pst*I; S, *Sac*I. (B) The different nucleotides and deduced amino acid sequences between *MDR1* and pMDRA1. The different nucleotides and amino acids are underlined. Nucleotides are numbered from the putative translation initiation site.

(A)



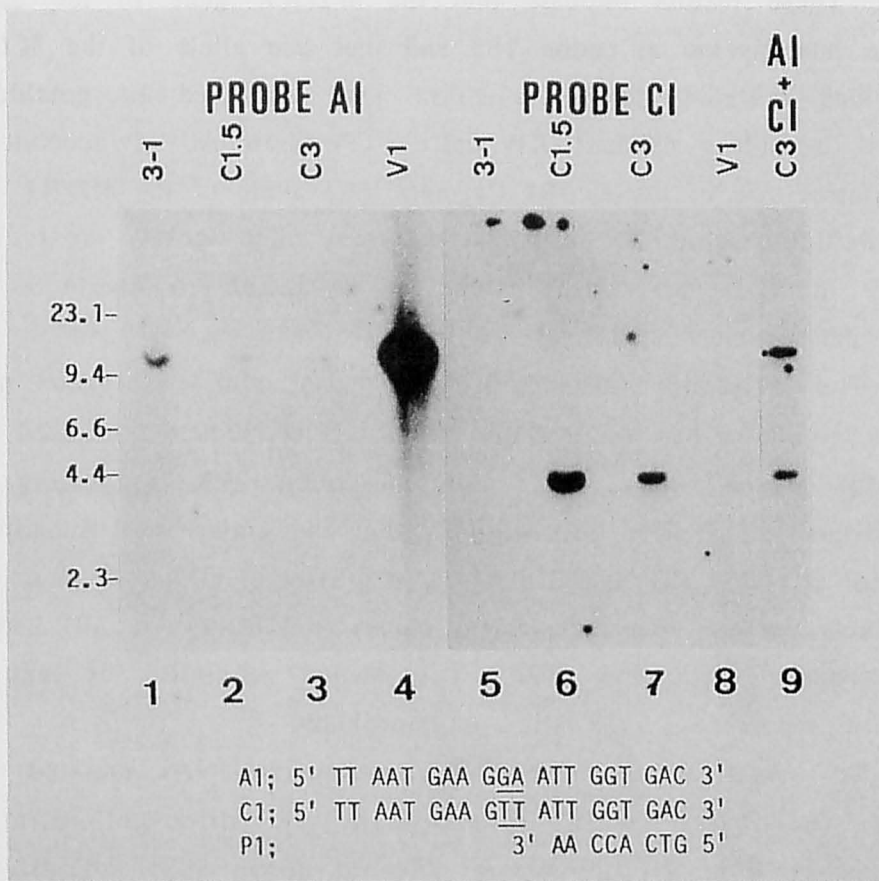
(B)

MDR1	MDRA1
554	554
AAT GAA <u>GTT</u> ATT GGT	AAT GAA <u>GGA</u> ATT GGT
Val (185)	Gly (185)
2677	2677
GAA GGT <u>GCT</u> GGG AAG	GAA GGT <u>TCT</u> GGG AAG
Ala (893)	Ser (893)
-43	-43
CTTCCAAGATTTCAC	CTTCCAA <u>A</u> ATTTCAC
540	540
ATGTCTC <u>T</u> AAGATTA	ATGTCTC <u>C</u> AAGATTA
1236	1236
TGAAGGG <u>C</u> CTGAACC	TGAAGGG <u>T</u> CTGAACC
1641	1641
TGGTTCG <u>C</u> AACCCCA	TGGTTCG <u>T</u> AACCCCA
3435	3435
AAGAGAT <u>C</u> GTGAGGG	AAGAGAT <u>T</u> GTGAGGG
4036	4036

There were, however, nine nucleotide differences between the sequence of KB-C2.5 derived cDNA and pMDRA1 (Fig. 1B). Recently a full-length *MDR1* cDNA was isolated from the vinblastine-selected KB cell line, KB-V1, and was found to have three nucleotide differences compared to the sequence of KB-C2.5 derived cDNA (18). These three nucleotide differences were found also in pMDRA1 at positions 540, 554 and 555 and these nucleotides in pMDRA1 were identical to those in KB-V1 derived cDNA. The differences at positions 554 and 555 resulted in a single amino acid substitution (Val→Gly) at codon 185. The nucleotide difference at position 2677 also resulted in an amino acid substitution (Ala→Ser) at codon 893. One of nine differences was found in the 5'-noncoding region at position -43. This sequence was confirmed by analyzing the first exon of the *MDR1* gene in genomic DNA (see chapter 2). Other nucleotide differences were in the third position of each codon or in the 3'-noncoding region and did not result in amino acid substitutions.

#### *Analysis of two amino acid substitutions in adrenal derived cDNA*

As noted above, there were two amino acid substitutions between adrenal derived and KB-C2.5 derived *MDR1* cDNAs. Dried-gel Southern hybridization analyses were performed using oligonucleotide probes (Fig. 2) specific to each sequence to determine which amino acids are found in the human genome and in DNA from multidrug-resistant KB cells. The probe A1 (Gly at codon 185) hybridized to a fragment of approximately 10-kb DNA from multidrug-resistant cells (Fig. 2) and human kidney (data not shown). This fragment was amplified in vinblastine-selected multidrug-resistant KB cells, KB-V1 (lane 4). The probe C1 (Val at codon 185) hybridized to DNAs only from colchicine-selected multidrug-resistant KB cells (KB-C1.5 and -C3) (lanes 6 and 7). The



**Figure 2.** Dried-gel Southern hybridization of DNA from KB cell lines. The oligonucleotides used for preparing the  $^{32}\text{P}$ -labeled probes are shown at the bottom. A1 and C1 correspond to positions 545 to 564 of the adrenal derived *MDR1* cDNA and the KB-C2.5 derived *MDR1* cDNA, respectively. P1 was used as a primer for preparing the  $^{32}\text{P}$ -labeled antisense probes. 10  $\mu\text{g}$  of *EcoRI* digested DNA from KB3-1 (lanes 1 and 5), 2.5  $\mu\text{g}$  of *EcoRI* digested DNA from KB-C1.5 (lanes 2 and 6), KB-C3 (lanes 3, 7 and 9), and KB-V1 (lanes 4 and 8) were analyzed by dried-gel Southern hybridization with probes A1 (lanes 1-4), C1 (lanes 5-8), and mixed probes of A1 and C1 (lane 9). The upper signal in lane 9 looks strong because the A1 probe contains three cytidine sites which can incorporate [ $\alpha$ - $^{32}\text{P}$ ]dCTP, but the probe C1 has only two.



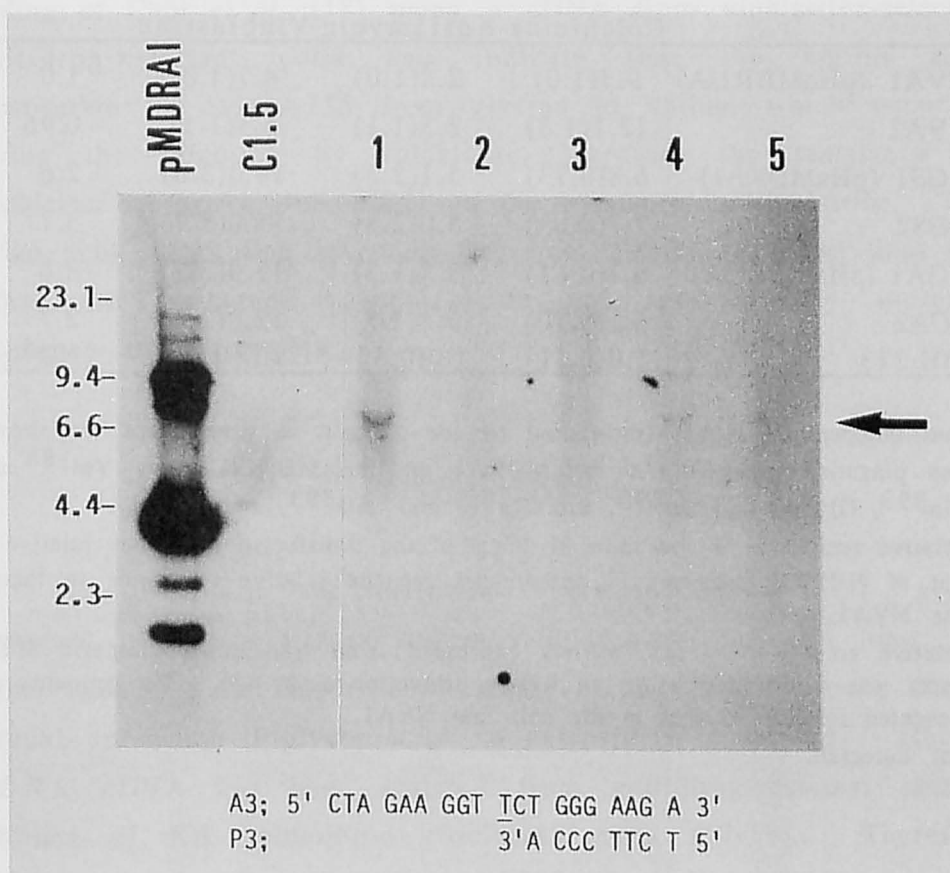
hybridized fragment was about 4.4 kb and was amplified in these cells. These results indicate that the *MDR1* gene in the human genome has glycine at codon 185 and that one allele of the *MDR1* gene was mutated during selection and amplified in colchicine-selected multidrug-resistant KB cells. Furthermore, to account for the different size of the *EcoRI* fragment, one allele of the *MDR1* gene in colchicine-selected multidrug-resistant KB cells must have another mutation or rearrangement in an intron to create a new *EcoRI* site.

The sequence at codon 893 was also analyzed using oligonucleotide probes A3. DNAs from KB cell lines hybridized only with the probe which codes Ala at codon 893, suggesting two possibilities. The first possibility is that the amino acid substitution Ala→Ser at codon 893 in pMDRA1 is an artifact of cDNA cloning. This is unlikely because two independent clones (pMDRA1 and A2) had the same sequence at codon 893. The second possibility is that this mutation represents a genetic polymorphism.

To investigate this possibility, genomic DNAs isolated from human leukocytes were investigated by dried-gel Southern hybridization (Fig. 3). Probe A3 (Ser at codon 893) hybridized to leukocyte DNA from three (lanes 1, 4, and 5) out of five persons. Under these conditions, probe A3 did not hybridized to the amplified *MDR1* gene from KB-C1.5, but did hybridized to the plasmid pMDRA1 (Fig. 3). These results indicate that the differences at position 2677 could result from genetic polymorphism.

#### *Functional analysis of two amino acid substitutions*

To determine whether the amino acids at codons 185 and 893 have functional roles, the author constructed three expression vectors. These expression plasmids were cotransfected with pSV2neo into NIH3T3 cells. Transfectants were first selected with G418 and



**Figure 3.** Detection of the sequence at position 2677 of human leukocyte DNA. A3 corresponds to the position 2668 to 2686 of the adrenal derived *MDR1* cDNA. P3 was used as a primer for preparing the  $^{32}\text{P}$ -labeled antisense probe. 10 ng of pMDRA1, 2.5  $\mu\text{g}$  of *EcoRI* digested DNA from KB-C1.5, and 10  $\mu\text{g}$  of *EcoRI* digested DNA from human leukocytes (lanes 1-5) were hybridized with probe A3. The position of the hybridized band is shown by an arrow.

Table 1 Relative resistance of transfectants

Transfected cell line <sup>a</sup>	Relative resistance <sup>b</sup>			MDR mRNA <sup>c</sup>
	Colchicine	Adriamycin	Vinblastine	
NVA1 (pHaMDR1/A)	9.3(1.0)	2.2(1.0)	4.7(1.0)	1.0
NVA2	12.1(1.3)	2.5(1.1)	5.3(1.1)	0.96
NGS1 (pHaMDRA1)	6.8(0.73)	5.1(2.3)	14.0(3.0)	2.6
NGS2	7.7(0.83)	5.0(2.3)	15.0(3.2)	3.7
NGA1 (pHaMDRGA)	5.7(0.61)	3.2(1.5)	11.0(2.3)	3.6
NGA2	6.5(0.70)	3.2(1.5)	12.0(2.6)	2.7
NIH 3T3	1.0(0.11)	1.0(0.45)	1.0(0.21)	ND <sup>d</sup>

<sup>a</sup>Two independent clones transfected by the plasmid in parentheses are shown. The plasmids pHaMDR1/A, pHaMDRA1 and pHaMDRGA carry Val<sup>185</sup> and Ala<sup>893</sup>, Gly<sup>185</sup> and Ser<sup>893</sup>, and Gly<sup>185</sup> and Ala<sup>893</sup>, respectively.

<sup>b</sup>Relative resistance is the ratio of IC<sub>50</sub> of the transfected cell lines relative to that of NIH3T3. Values in parentheses are the relative resistance to the cell line NVA1.

<sup>c</sup>Relative amount of *MDR* mRNA expressed from transfected chimeric *MDR* genes was determined using an RNase protection assay (5). The amounts are presented relative to that in the cell line NVA1.

<sup>d</sup>Not detected.

then with colchicine.

The relative resistance of transfectants to colchicine, vinblastine and adriamycin were analyzed by a quantitative colony formation assay and compared to the expression levels of the transfected *MDR* gene (Table 1). The expression levels of *MDR* mRNA in transfectants corresponded well to the relative resistance to vinblastine. The resistance to colchicine relative to the *MDR* mRNA expression level, however, drastically decreased in cells transfected

with pHaMDRA1 (NGS1 and NGS2) and pHaMDRGA (NGA1 and NGA2) which contain wild type *MDR1* cDNAs compared to cells transfected with pHaMDR1/A (NVA1 and NVA2). These results confirm previous finding of Choi *et al.* (18) using a cDNA from vinblastine-selected multidrug-resistant cells and indicate that the amino acid substitution at codon 185 from glycine to valine, which occurred during the selection by colchicine, increases the resistance to colchicine but has a little effect on the resistance to vinblastine. The amino acid substitution at codon 893 from alanine to serine does not affect the resistance to colchicine but may slightly increase resistance to adriamycin.

## DISCUSSION

To determine the difference between the *MDR1* genes expressed in normal tissues and in multidrug-resistant cell lines, the author isolated a full-length cDNA of the *MDR1* gene from the human adrenal in which P-glycoprotein is extensively expressed. Human *MDR1* cDNA has been isolated from multidrug-resistant mutant sublines of KB epidermoid carcinoma cells (17-19). Therefore pMDRA1 is the first full-length cDNA clone isolated from human normal tissue and analyzed. The author indicated that the expression of the normal adrenal derived *MDR1* gene can confer the multidrug resistance (MDR) phenotype.

Sequence analysis revealed that the cDNA clone isolated from the human adrenal has nine nucleotide differences compared to the sequence of a previously isolated KB-C2.5 derived cDNA. These nucleotide differences result in two amino acid substitutions. One of them is identical to the amino acid substitution found in KB-V1 derived cDNA (18) and has been reported to alter the cross-resistance

patterns. This was confirmed by the results in this chapter.

The nucleotide difference which causes the amino acid substitution at codon 893 resulted from genetic polymorphism. Recently, another polymorphism has been reported in the *MDR1* gene affecting a *HindIII* site (20). The author also found a mutation or rearrangement in an intron of the *MDR1* gene from colchicine-selected multidrug-resistant KB cells (Fig. 2). Mutations in KB-C2.5 derived cDNA has been presumed to occur spontaneously during exposure to the selecting drug (18). It might be important to investigate whether exposure to antitumor agents during chemotherapy causes spontaneous mutations in this gene. These could result in the alteration of sensitivity of cancer cells to antitumor agents.

The RNase protection analysis (unpublished results) revealed that the *MDR1* gene is the major member of the P-glycoprotein gene family expressed in human adrenal and its expression level is at least 10 times higher than that of *MDR2* (*MDR3*), another member of this gene family. The *MDR2* gene is expressed in human liver but not in colon or multidrug-resistant KB cells. These results suggest that the products of *MDR1* gene and *MDR2* gene may have different physiological functions and that the product of the *MDR1* gene plays the important role in the human adrenal and multidrug-resistant cells.

The author reported in this chapter the deduced amino acid sequence of the wild type *MDR1* cDNA from the normal adrenal. The author believes that this will facilitate the studies on the physiological function of P-glycoprotein in the adrenal.

## REFERENCES

1. Fojo, A. T., Ueda, K., Slamon, D. J., Poplack, D. G., Gottesman, M. M. and Pastan, I. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 265-269
2. Thiebaut, F., Tsuruo, T., Hamada, H., Gottesman, M. M., Pastan, I. and Willingham, M. C. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 7735-7738
3. Thiebaut, F., Tsuruo, T., Hamada, H., Gottesman, M. M., Pastan, I. and Willingham, M. C. (1989) *J. Histochem. Cytochem.* **37**, 159-164
4. Cordon-Cardo, C., O'Brien, J. P., Casals, D., Rittman-Grauer, L., Biedler, J. L., Melamed, M. R. and Bertino, J. R. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 695-698
5. Gottesman, M. M. and Pastan, I. (1988) *J. Biol. Chem.* **263**, 12163-12166
6. Kakehi, Y., Kanamaru, H., Yoshida, O., Ohkubo, H., Nakanishi, S., Gottesman, M. M. and Pastan, I. (1988) *J. Urol.* **139**, 862-865
7. Goldstein, L. J., Galski, H., Fojo, A., Willingham, M., Lai, S.-L., Gazdar, A., Pirker, R., Green, A., Crist, W., Brodeur, G. M., Lieber, M., Cossman, J., Gottesman, M. M. and Pastan, I. (1989) *J. Natl. Cancer Inst.* **81**, 116-124
8. Akiyama, S., Fojo, A., Hanover, J. A., Pastan, I. and Gottesman, M. M. (1985) *Somat. Cell Genet.* **11**, 117-126
9. Shen, D.-w., Cardarelli, C., Hwang, J., Cornwell, M., Richert, N., Ishii, S., Pastan, I. and Gottesman, M. M. (1986) *J. Biol. Chem.* **261**, 7762-7770
10. Gubler, U. and Hoffman, B. J. (1983) *Gene* **25**, 263-269
11. Ueda, K., Cardarelli, C., Gottesman, M. M. and Pastan, I. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 3004-3008
12. Sanger, F., Nicklen, S. and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467
13. Miyada, C. G. and Wallace, R. B. (1987) *Methods in Enzymol.* **154**, 94-1107

14. Wood, W. I., Gitschier, J., Lasky, L. A. and Lawn, R. M. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 1585-1588
15. Pastan, I., Gottesman, M. M., Ueda, K., Lovelace, E., Rutherford, A. V. and Willingham, M. C. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 4486-4490
16. Ueda, K., Clark, D. P., Chen, C.-j., Roninson, I. B., Gottesman, M. M. and Pastan, I. (1987) *J. Biol. Chem.* **262**, 505-508
17. Ueda, K., Pastan, I. and Gottesman, M. M. (1987) *J. Biol. Chem.* **262**, 17432-17436
18. Choi, K., Chen, C.-j., Kriegler, M. and Roninson, I. B. (1988) *Cell* **53**, 519-529
19. Chen, C.-j., Chin, J. E., Ueda, K., Clark, D. P., Pastan, I., Gottesman, M. M. and Roninson, I. B. (1986) *Cell* **47**, 381-389
20. Yoshimoto, K., Iwahana, H., Yokogoshi, Y., Sato, S., Shiraishi, M., Sekiya, T., Gottesman, M. M. and Pastan, I. (1988) *Nucl. Acids Res.* **16**, 11850

## Chapter 2 Detection of *MDR1* Gene mRNA Expression in Human Tumors by a Sensitive Ribonuclease Protection Assay

Recently, a 1-kb genomic fragment (PV) containing the major transcription initiation sites for the human *MDR1* gene has been isolated from the vinblastine-selected multidrug-resistant human KB carcinoma mutant cell line KB-V1 (1). This fragment linked to the chloramphenicol acetyltransferase gene showed promoter activity, although its activity was low (1). No differences were detected in the promoter activity of the cloned fragment measured as chloramphenicol acetyltransferase activity in drug-resistant and drug-sensitive KB cell lines. These results suggest that the cloned promoter sequence was insufficient to allow regulation of transcription after transfection or that the promoter itself might be altered in the drug-resistant cell lines.

To study the sequence of the promoter in the native *MDR1* gene, the author isolated a 3-kb genomic fragment from a phage library made from human normal tissue by Maniatis *et al.* (2). The sequence of the PV fragment was identical to that of the native *MDR1* gene except for a point mutation in the first exon. In this chapter, the author shows that a ribonuclease protection assay using the promoter sequence isolated from normal tissue is very sensitive and reliable for studying *MDR1* mRNA expression in tumor samples.

### MATERIALS AND METHODS

#### *Materials and cells*

[ $\alpha$ -<sup>32</sup>P]CTP (29.6TBq/mmol) was purchased from Amersham.



SP6 RNA polymerase was from Promega Biotec. Ribonucleases A and T1 were from Boehringer Mannheim and Bethesda Research Laboratories, respectively. Multidrug-resistant cell lines KB-C4 and KB-A1 were selected in successive steps from KB-3-1 by colchicine and adriamycin (doxorubicin), respectively. All cell lines used in this chapter were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum.

### *Specimens*

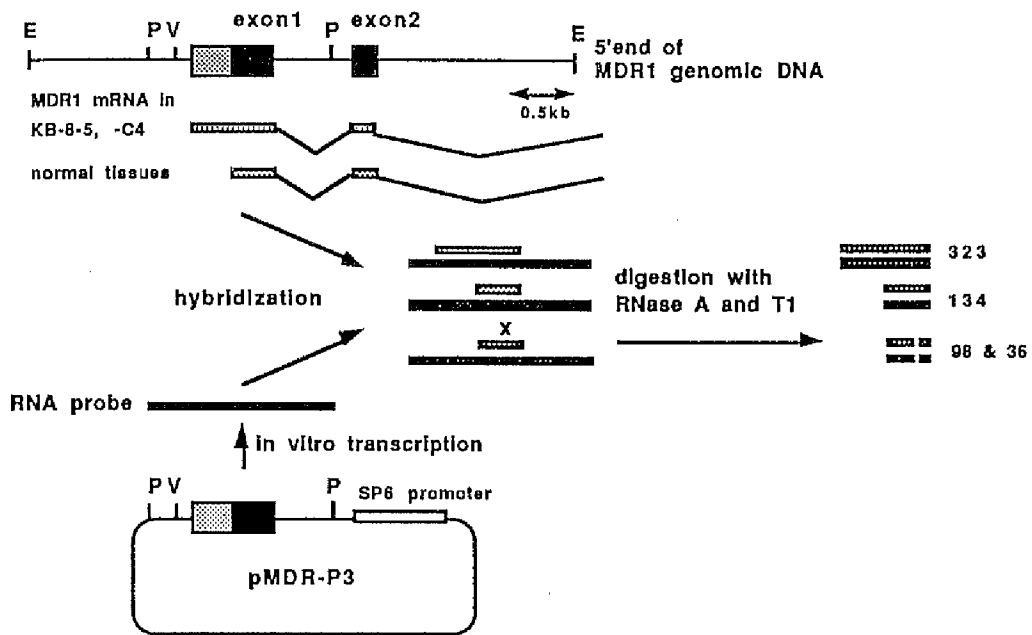
Twelve renal cell carcinoma (RCC) samples (9 primary and 3 metastatic lesions) and 4 colon adenocarcinomas were surgically obtained at Kyoto University Hospital and its satellite hospitals, Shiga University of Medical Science Hospital and Mie University Hospital. These patients had not been treated with any anti-cancer drugs prior to surgery.

### *Isolation of MDR1 genomic fragment*

A human genomic library in a Charon 4A vector made by Maniatis *et al.* (2) was screened with the 5'-region of human *MDR1* cDNA as a probe. A phage clone was identified. A 3-kb *EcoRI* fragment was subcloned into pUC19. The nucleotide sequence of this fragment was determined by the dideoxy method of Sanger (3).

### *Ribonuclease protection assay*

The 1-kb *PstI* fragment in the cloned fragment was inserted into pGEM3 (pMDR-P3). The uniformly labeled antisense RNA probe (785 nucleotides) was transcribed with SP6 RNA polymerase from *PvuII* digested plasmid pMDR-P3 according to the protocol supplied by Promega Biotec. Total RNA (10  $\mu$ g or 20  $\mu$ g) was hybridized with  $2 \times 10^6$  cpm of probe at 45°C in hybridization buffer (80% formamide, 0.4 M NaCl, 40 mM PIPES, 1 mM EDTA) for 14 hr. The probe was digested



**Figure 1.** Schematic diagram of ribonuclease protection assay for *MDR1* mRNA. The uniformly labeled antisense RNA probe was transcribed with SP6 RNA polymerase from *Pvu*II digested plasmid pMDRR-P3. Total RNA from KB cells, normal tissues and clinical samples were hybridized with the RNA probe and were digested with ribonucleases A and T1. The protected probe was analyzed as described in MATERIALS AND METHODS and in the text. The single base change at +98 in PV probe is indicated by X. Because of this base change, a fraction of the RNA probe was digested and produces two extra bands of about 98 nucleotides and 36 nucleotides.

by ribonucleases A (49  $\mu$ g/ml) and T1 (28 units/ml) at 30°C for 1 hr. After proteinase K treatment, phenol/chloroform extraction and ethanol precipitation, the protected probe was analyzed on a 6% or 8% polyacrylamide, 7 M urea gel.

## RESULTS

The 3-kb genomic fragment was isolated from the phage library made from human normal tissue by Maniatis *et al.* (2). This fragment contained the promoter region, the 134-bp first exon, the 562-bp first intron, the 74-bp second exon and a part of the second intron of the *MDR1* gene (Fig. 1). The nucleotide sequence analysis revealed that the genomic fragment (PV) previously isolated from vinblastine-selected multidrug-resistant KB cell line (4) had a point mutation at +98 in the first exon which is a non-coding region (Fig. 2). The nucleotide sequence of the first and second exons determined from the normal human genomic *MDR1* gene fragment was identical with the nucleotide sequence of the full-length *MDR1* cDNA isolated from normal human adrenal (Chapter 1).

It has been reported that normal human adrenal, colon and liver cells, the human hepatoma cell line HepG2, and vinblastine-selected human KB multidrug-resistant cells initiate transcription of the *MDR1* gene at the same site as detected by a ribonuclease protection assay using the PV fragment (1). The author compared the ribonuclease protection assay using the 1-kb PstI fragment isolated in this work (PN) with that using the PV fragment (Fig. 3). Using the PV RNA probe, human adrenal RNA and kidney RNA produced three bands (arrows 1, 2 and 3) but RNAs from KB mutant cell lines did not produce band 2 or 3 (Fig. 3). Using the PN RNA probe, all RNA samples produced a clear major band (arrow 1) but did not produce band 2 or 3. In both cases, RNA from KB-8-5 and KB-C4 produced a fourth band, indicating the presence of a transcript from an upstream promoter. These results indicate that i) because of the single base change at +98, a fraction of the PV RNA probe was digested by ribonuclease A and produced two extra bands of about 98 nucleotides (arrow 2) and 36 nucleotides (arrow 3) as schematically

```

PstI
CTGC AGAAAAATT CTCCTAGCCT TTCAAAGGT GTTAGGAAGC -391

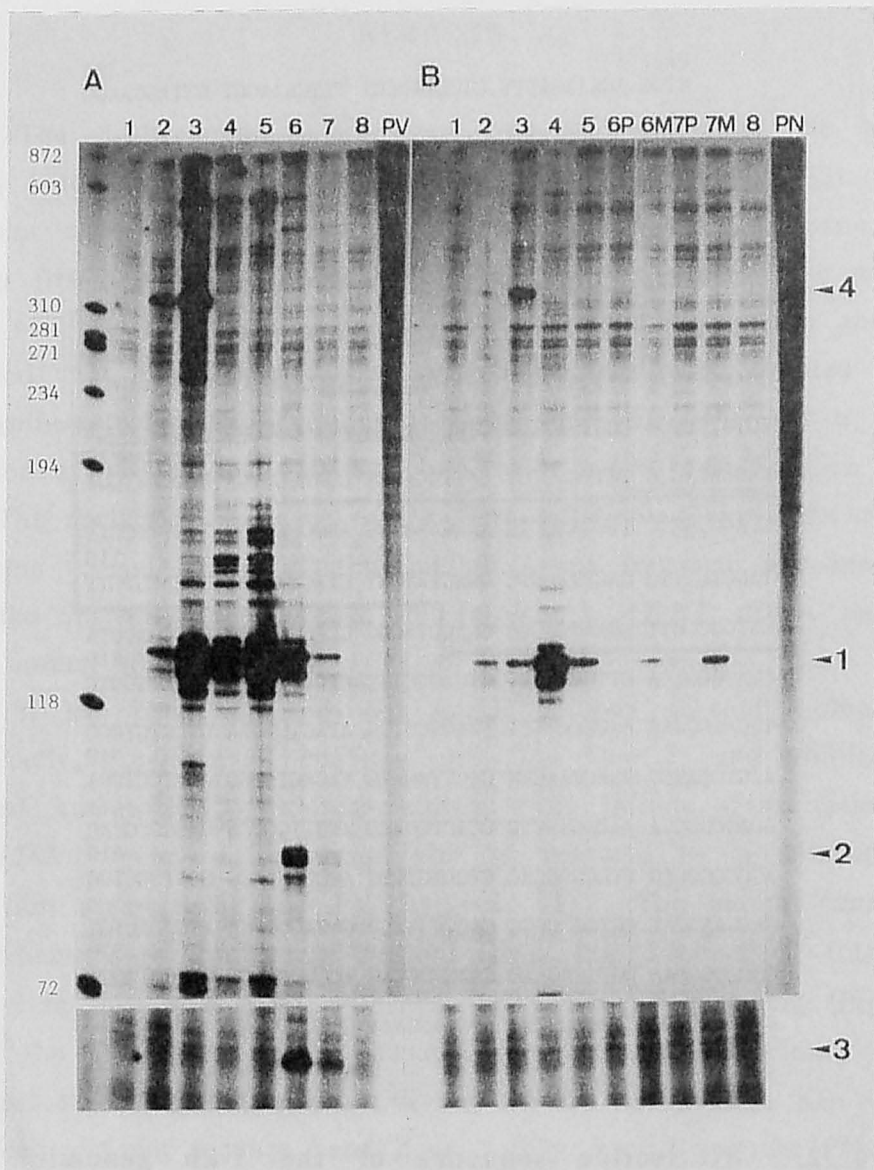
AGAAAAGTGA TACAGAATTG GAGAGGTCGG AGTTTTTGTA TAACTGTAT
-291
TAAATGCGAA TCCCGAGAAA ATTTCCCTTA ACTACGTCCT GTAGTTATAT

GGATATGAAG ACTTATGTGA ACITTGAAAG ACGTGTCTAC ATAAGTGA
PvuII -191
ATGTCCCAA TGATTGAGCT GATGCGCGTT TCTCTACTTG CCCTTCTAG

AGAGGTGCAA CGGAAGCCAG AACATTCCCTC CTGGAATTC AACCTGTTTC
-91
GCAGTTTCTC GAGGAATCAG CATTCAGTCA ATCCGGGCCG GGAGCAGTCA
TCTGTGGTGA GGCTGATTGG CTGGGCAGGA ACAGCGCCGG GGCCTGGGCT
GAGCACAGCC GCTTCGCTCT CTTTGCACA GGAAGCCTGA GCTCATTCCA 10
GTAGCGGCTC TTCCAAGCTC AAAGAAGCAG AGGCCGCTGT TCGTTTCCTT
110
TAGGTCTTTC CACTAAAGTC GGAGTATCTT CTCCAAAAT TTCACGTCCT
GGTGGCGGTT CCAAGGAGCG CGAGGTAGGG GCACGCAAAG CTGGGAGCTA
210
CTATGGGACA GTTCCAAGT GTCAGGCTTT CAGATTTCTT GAACCTGGTC
TTCACGGGAG AAGGCTTCT TGAGGCGTGG ATAGTGTAAG GTCCTCTGGC
310
AAGTCCATGG GGACCAAGTG GGGTTAGATC TAGACTCAGG AGCTCCTGGA
GCAGCGCCCA AACCGTAGTG GCACTGGACC ATGTTGCCCG GAGCGCGCAC
410
AGCCCGGGCG GTGCGGGGAC CTGCTCTCTG AGCCCGGGG CGGTGGGTGG
GAGGAAGCAT CGTCCGCGGC GACTGGAACC GGGAGGAGA ATCCCACTGG
510
CGCGGGCAA AGTCCAGAAC GCGCTGCCAG ACCCCAACT CTGCCITCGT
PstI
GGAGATGCTG GAGACCCCGC GCACAGGAAA GCCCTGCAG

```

Figure 2. Nucleotide sequence of the 1-kb genomic *Pst*I fragment isolated from human tissue. Exon 1 is enclosed with a bold line. Transcripts present in colchicine-selected multidrug-resistant KB cells initiating from the upstream promoter contain the exon enclosed by a thin line linked to exon 1. A CAAT box (double underlined) and GC box-like sequence (underlined) are shown. The A residue indicated by ● at +98 is the nucleotide which is changed to G in KB cells. The C residue at -32 and the CGCGC sequence from +356 to +369 indicated by Δ's were mistakenly deleted or presented as G, respectively, in the previously published sequence (1). The numbers are presented with the major transcription initiation site as +1.



**Figure 3. Ribonuclease protection assay.** The PV RNA probe (A) transcribed from pMDR-P2 (1) or the PN RNA probe (B) transcribed from pMDR-P3 was hybridized with total RNA and analyzed as described in MATERIALS AND METHODS. A1 and B1, KB-3-1 (20  $\mu$ g); A2 and B3, KB-8-5 (20  $\mu$ g); B2, KB-8-5 (10  $\mu$ g); A3, KB-C4 (5  $\mu$ g); A4, KB-V1 (5  $\mu$ g); A5, KB-A1 (5  $\mu$ g); A6 and B4, adrenal (10  $\mu$ g); A7 and B5, kidney (10  $\mu$ g); B6 and B7, renal cell carcinomas (P, primary lesions; M, metastatic lesions). B6 and B7 correspond to B5 and B7 in Fig. 5. A8 and B8, tRNA (20  $\mu$ g); PV, PV RNA probe; PN, PN RNA probe. Arrows 1 to 4 indicate protected RNA probes. The numbers indicate size markers in nucleotides.

shown in Fig. 1; ii) the uridine residue at +98 in the PN RNA probe base paired with the guanine residue in RNAs from KB mutant cell lines (Fig. 4) and was not digested by ribonuclease A or T1; and iii) RNAs from the KB mutant cell lines independently isolated by colchicine (KB-8-5 and KB-C4), vinblastine (KB-V1) and adriamycin (KB-A1) did not produce band 2 or 3, suggesting that they have a point mutation at the same site, and that this point mutation was probably derived from the parent cell line KB-3-1.

The mismatch at the point mutation in the PV probe resulted in the reduction of the strength of the major signal (arrow 1) produced with RNAs from human adrenal and kidney (Fig. 3, A6 and B4) compared with the signal using the PN probe (Fig. 3, A7 and B5), whereas the strength of the band produced with RNA from KB-8-5 did not change (Fig. 3, A2 and B3). The ribonuclease protection assay using the PN RNA probe is 1.5 to 2-fold more sensitive for RNAs from human tissues than using the PV RNA probe and would be, therefore, sensitive and reliable for studying *MDR1* mRNA expression level in clinical samples.

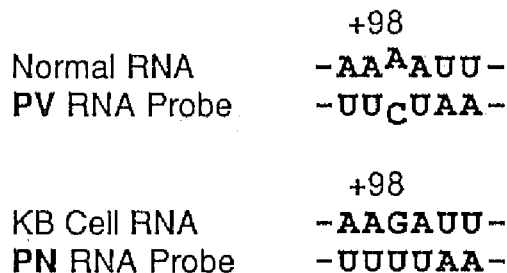


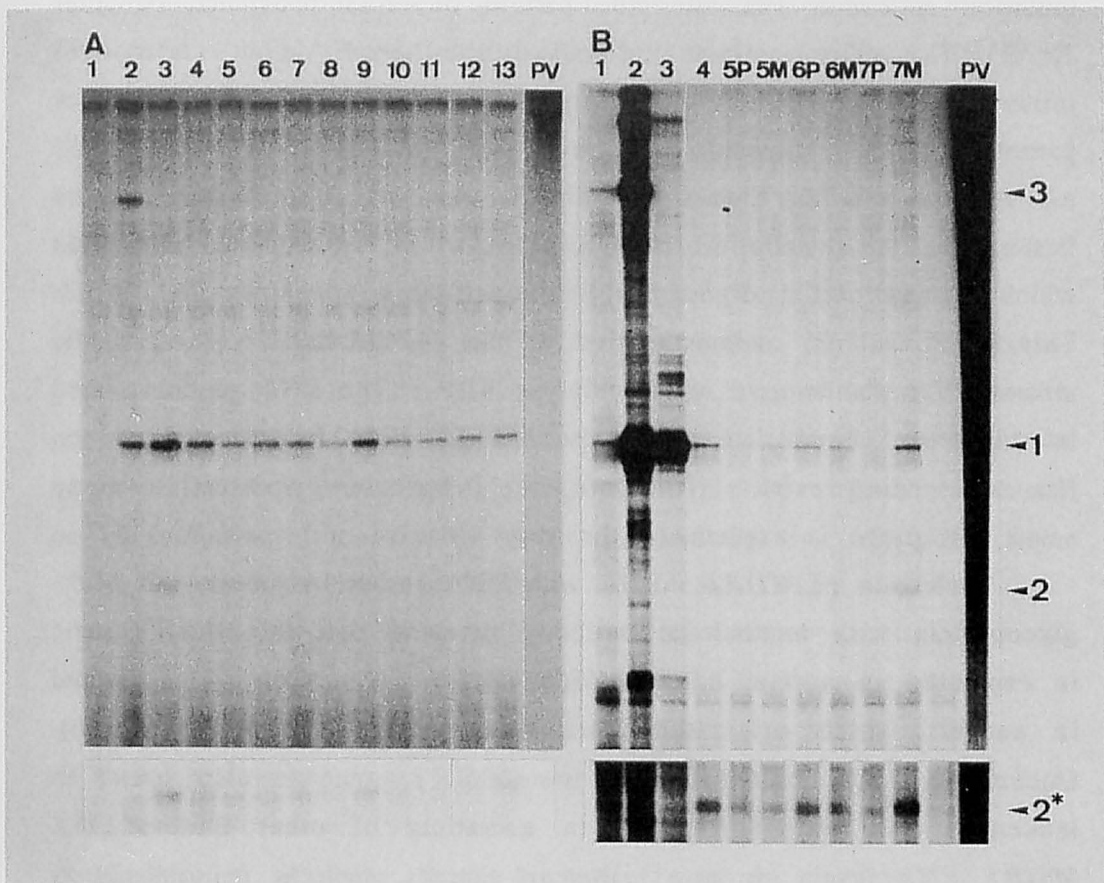
Figure 4. Nucleotide sequence (+96 to +101) of RNAs from human normal tissue and KB mutant cells hybridized with PV RNA probe and PN RNA probe, respectively.

The author measured *MDR1* mRNA levels in 12 renal cell carcinoma samples (9 primary and 3 metastatic lesions) and 4 colon adenocarcinomas (Fig. 5) using the PV RNase protection probe. The RNA levels in many of these carcinoma cells were as high as the levels in the multidrug-resistant KB-8-5 cells. Elevated *MDR1* mRNA levels were also observed in 2 out of the 3 metastatic lesions. These two cases were confirmed by using the PN probe (Fig. 3). All of these samples produced band 2 when using the PV RNA probe (Fig. 5), indicating that the nucleotide at position +98 in these RNA samples was adenosine (wild-type).

## DISCUSSION

The nucleotide sequence analysis of the 3-kb genomic fragment containing the promoter region, the first and second exons, and the first intron of the human *MDR1* gene isolated from human normal tissue revealed that this gene from the multidrug-resistant KB mutant cells has a point mutation in the first exon. A ribonuclease protection assay of several independently isolated KB multidrug-resistant mutants suggested that this point mutation was derived from the drug-sensitive KB-3-1 parent cell, and that all the tested RNAs from human tissues and carcinoma cells were "wild type". It remains to be determined if this point mutation affects the regulation of *MDR1* gene expression.

The author has isolated a full-length cDNA of the *MDR1* gene from normal human adrenal and found that the *MDR1* cDNA previously isolated from a colchicine-selected multidrug-resistant KB cell has 9 point mutations (Chapter 1). These mutations include one in the first exon noted in this work, as well as those in the coding sequence. It has previously been reported that mutations occur in the



**Figure 5. Ribonuclease protection assay.** The PV RNA probe was hybridized with 10  $\mu$ g of total RNA from clinical samples. A: 1, tRNA; 2, KB-8-5; 3, normal kidney; 4-9, renal cell carcinomas; 10-13, colon adenocarcinomas. B: 1, KB-8-5; 2, KB-C4; 3, KB-V1 (5  $\mu$ g); 4, normal kidney; 5-7, renal cell carcinomas (P, primary lesions; M, metastatic lesions); PV, PV RNA probe. Arrows 1-3 indicate protected RNA probes. Arrow 2\* indicates the band 2 in a long exposed autoradiograph.



coding sequence of the *MDR1* gene during the selection of resistant cells in colchicine (5). Two point mutations resulting in a Gly $\rightarrow$ Val mutation at codon 185 alter the pattern of cross resistance (Chapter 1) (5). The other amino acid substitution Ser $\rightarrow$ Ala at codon 893 probably reflects genetic polymorphism and may slightly alter the pattern of cross resistance (Chapter 1).

Whatever the functional significance of a mutation in the first exon, the nucleotide change produced a mismatched nucleotide which caused partial digestion by ribonucleases as shown in Fig. 3. This may result in underestimation of the *MDR1* mRNA level if the mutant PV probe is used with wild type RNA. The RNA probe isolated in this work contains the wild-type *MDR1* RNA sequence from the first non-coding exon. Therefore, the ribonuclease protection assay using this probe is expected to be very sensitive and specific.

Detection of *MDR1* mRNA with DNA and RNA probes and of P-glycoprotein with monoclonal antibody revealed that the *MDR1* gene is expressed in normal adrenal, kidney, liver and colon (4, 6-8) and in cancers which originated from these organs (4, 6, 7, 9, 10). Occasional high expression of the *MDR1* gene is also found in leukemias and lymphomas, and in a variety of other tumors (11). *MDR1* RNA levels are also higher in cancers, such as neuroblastomas and leukemias, relapsing after chemotherapy (11).

It has been reported that in colchicine-selected multidrug-resistant KB cell lines, transcription started also from another promoter upstream of the promoter used in normal tissues (1). In all the clinical samples studied in this work including two metastatic RCC samples of high RNA levels, only the normal promoter was active. Although some clinical samples appear to use the upstream as well as downstream promoters (11), further investigations are necessary to clarify the role of the upstream promoter in clinical samples.

In the study of *MDR1* expression level in clinical samples,

overestimation of *MDR1* RNA levels may result from the cross-hybridization of DNA probes to the closely related *MDR2* (*MDR3*) gene (12) which has not been shown to be associated with multidrug resistance. The ribonuclease protection assay using the PN RNA probe is specific for *MDR1* mRNA, since the detection of the specific 134 nucleotide band eliminates problems of cross-hybridization and background. The degradation of RNA may also result in underestimation of *MDR1* mRNA. Because the probe is at the 5'-end of the *MDR1* mRNA, the 134 nucleotide band may be the last to be affected by degradation of RNA samples. This ribonuclease protection assay using the PN probe will be a sensitive and specific method to study *MDR1* mRNA levels in clinical samples, and can be employed to show the threshold level of the *MDR1* mRNA expression as an indicator for sensitivity to the standard *in vivo* chemotherapy.

## REFERENCES

1. Ueda, K., Pastan, I. and Gottesman, M. M. (1987) *J. Biol. Chem.* **262**, 17432-17436
2. Maniatis, T., Hardison, R. C., Lacy, E., Lauer, J., O'Connell, C., Quon, D., Sim, D. K. and Efstratiadis, A. (1978) *Cell* **15**, 687-701
3. Sanger, F., Nicklen, S. and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467
4. Fojo, A. T., Ueda, K., Slamon, D. J., Poplack, D. G., Gottesman, M. M. and Pastan, I. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 265-269
5. Choi, K., Chen, C.-j., Kriegler, M. and Roninson, I. B. (1988) *Cell* **53**, 519-529
6. Fojo, A. T., Shen, D.-W., Mickley, L. A., Pastan, I. and Gottesman, M. M. (1987) *J. Clin. Oncol.* **5**, 1922-1927

7. Thiebaut, F., Tsuruo, T., Hamada, H., Gottesman, M. M., Pastan, I. and Willingham, M. C. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 7735-7738
8. Sugawara, I., Kataoka, I., Morishita, Y., Hamada, H., Tsuruo, T., Itoyama, S. and Mori, S. (1988) *Cancer Res.* **48**, 1926-1929
9. Kakehi, Y., Kanamaru, H., Yoshida, O., Ohkubo, H., Nakanishi, S., Gottesman, M. M. and Pastan, I. (1988) *J. Urol.* **139**, 862-865
10. Kanamaru, H., Kakehi, Y., Yoshida, O., Nakanishi, S., Gottesman, M. M. and Patan, I. (1988) *J. Natl. Cancer Inst.* **81**, 844-849
11. Goldstein, L. J., Galski, H., Fojo, A., Willingham, M., Lai, S.-L., Gazdar, A., Pirker, R., Green, A., Crist, W., Brodeur, G. M., Lieber, M., Cossman, J., Gottesman, M. M. and Pastan, I. (1989) *J. Natl. Cancer Inst.* **81**, 116-124
12. Van der Blicq, A. M., Baas, F., Ten Houte de Lange, T., Kooiman, P. M., Van der Velde-Koerts, T. and Borst, P. (1987) *EMBO J.* **6**, 3325-3331

### Chater 3    Transcriptional Activation of *MDR1* Gene in Response to Arsenite Treatment

The development of multidrug resistance (two- to severalfold) in cultured cells is initially accompanied by elevated expression of the *MDR1* gene without gene amplification (1). During further selection for increased levels of resistance, expression of *MDR1* mRNA is extraordinarily increased simultaneously with amplification of *MDR1* gene. However because no amplification of the *MDR1* gene has been reported in clinical samples, the mechanism of acquiring low levels of resistance accompanied by a moderate increase of *MDR1* expression without gene amplification is not known.

The *MDR1* gene is expressed in normal tissues such as adrenal, liver, kidney, colon, small intestine, and the blood-brain barrier (2-4). These localization of P-glycoprotein suggests that this protein helps excrete metabolites and natural cytotoxic substances in the diet. Interestingly the expression of P-glycoprotein mRNA is elevated in chemically induced preneoplastic and neoplastic liver nodules and in regenerating rat liver after partial hepatectomy (5, 6), but it is not clear how *MDR1* gene expression is regulated in human liver.

Recently Chin *et. al.* reported that exposure of a renal adenocarcinoma cell line to heat shock and sodium arsenite increased the *MDR1* mRNA level (7). In this chapter, the author shows that the expression of *MDR1* gene in a hepatocarcinoma cell line HepG2 is increased by exposure to sodium arsenite and that this response is dependent on a promoter region containing a tandem repeat of heat-shock responsive elements (HSEs).

## MATERIALS AND METHODS

### *Materials, Plasmids and Cells*

[<sup>35</sup>S]methionine and [<sup>14</sup>C]butyryl coenzyme A were purchased from NEN Research Products. [ $\alpha$ -<sup>32</sup>P]CTP was from Amersham. Anti-P-glycoprotein monoclonal antibody was from Centocor. Plasmid pSV00CAT (8), pBLCAT2 (9), p $\alpha$ -gal, pGAD28 (10) and LSNWT (11) were provided by Dr. Y. Ebina, Dr. B. Luckow, Dr. S. Ishii, Dr. M. Hatanaka and Dr. R.I. Morimoto, respectively. All cells in this chapter were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum. HeLa cells were provided by Japanese Cancer Research Resources Bank.

### *Slot blot hybridization and RNase protection*

A part of *MDR1* cDNA, pMDR5A, encoding the nucleotide binding region (12) was used as a probe for slot blot hybridization. Total RNAs extracted from cells were transferred to GeneScreen Plus (Du Pont). Hybridizations were done at 60°C for 16 hr in 1% SDS, 1 M NaCl, 10% dextran sulfate, and 200  $\mu$ g/ml of denatured salmon sperm DNA. Filters were washed to a final stringency of 2 x SSC and 1% SDS at 60°C. Comparable RNA loading was confirmed using a human  $\beta$ -actin probe (13). RNase protections were performed as previously described using probe PN (14). Autoradiography was performed at -70°C with intensifying screens.

### *Immunoprecipitation of P-glycoprotein*

Cells in 35-mm dishes were labeled with [<sup>35</sup>S]methionine (>37 TBq/mmol) for 1 hr. Cells were harvested by adding the lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 2 mM N-ethylmaleimide, 2 mM (p-aminophenyl)methanesulfonyl fluoride hydrochloride, 1  $\mu$ g/ml leupeptin, and 1  $\mu$ g/ml pepstatin).

Lysates with the same amount of radioactivity were reacted with anti-P-glycoprotein monoclonal antibody C219 for 1 hr at 4°C. The P-glycoprotein-C219 complexes were precipitated using protein A/Sepharose. Electrophoresis was done with 7% PAGE as described by Ling *et al.* (15). The gel was treated with 1 M sodium salicylate and dried. Fluorography was done at -70°C.

#### *Nuclear run-on assay*

Plasmid templates (30 µg) were denatured and bound to nitrocellulose filter. Approximately  $2 \times 10^7$  cells in two 100 mm dishes were harvested with a rubber policeman. Cell pellets were incubated on ice in 0.5 ml of NP-40 buffer (0.5% Nonidet P-40, 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, and 3 mM MgCl<sub>2</sub>) for 10 min and centrifuged at 3000 rpm. Nuclei were rinsed twice with 1 ml of NP-40 buffer and resuspended in 200 µl of suspension buffer (50 mM Tris-HCl, pH 8.3, 40 % glycerol, 5 mM MgCl<sub>2</sub>, 0.1 mM EDTA). Then nuclei were mixed with 100 µl of transcription buffer (15 mM Tris-HCl, pH 8.0, 7.5 mM MgCl<sub>2</sub>, 450 mM KCl, 0.75 mM each ATP, GTP, and UTP, 0.18 µM CTP, and 3.7 MBq of [ $\alpha$ -<sup>32</sup>P]CTP) and incubated at 30°C for 30 min. Labeled RNAs ( $3 \times 10^6$  cpm) extracted from nuclei were hybridized in 1 ml of hybridization solution (50% formamide, 3xSSC, 10 mM NaPO<sub>4</sub>, pH 7.0, 10xDenhardt's, 0.1% SDS, 100 µg/ml denatured salmon sperm DNA, and 100 µg/ml yeast tRNA) at 50°C for 48 hr. Filters were washed to a final stringency of 2xSSC and 0.1% SDS at 65°C.

#### *Plasmid construction and CAT assay*

A three kilobasepair fragment containing the *MDR1* promoter region, first exon, first intron, second exon, and a part of the second intron (14) was excised from the 3'-terminal using exonuclease III and mungbean nuclease. The deleted promoter from the 3'-terminal to the sixth nucleotide of the second exon (Fig. 5) was cloned into

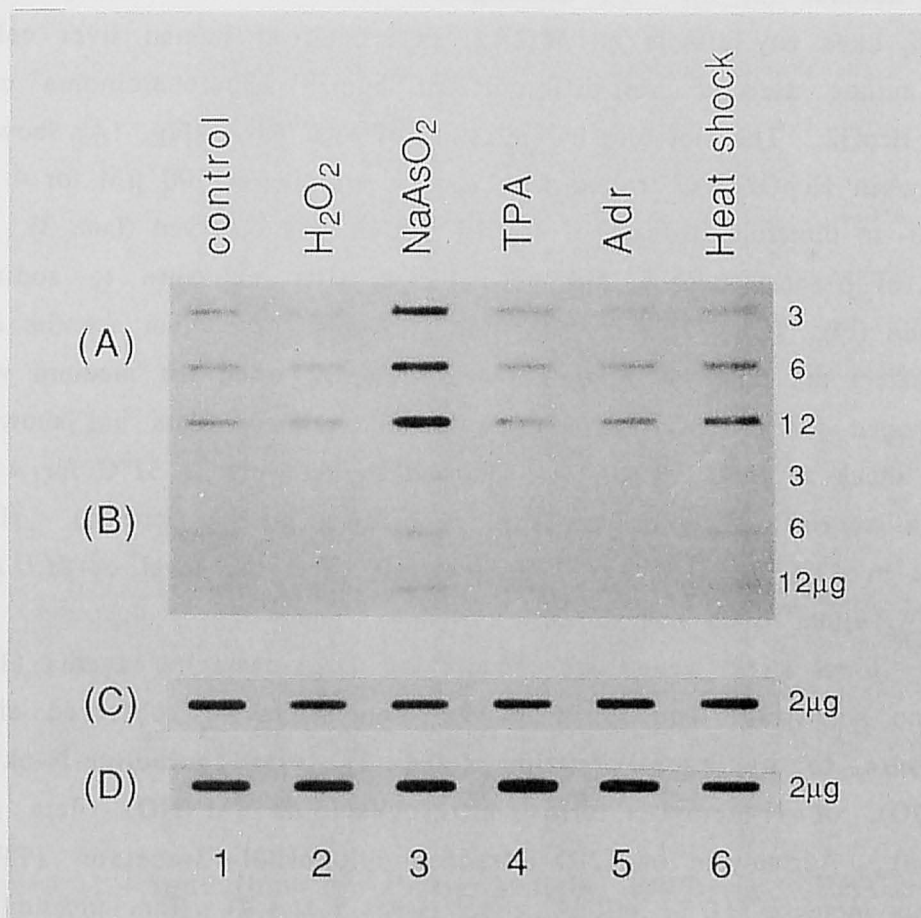
pSV00CAT (pMP1CAT). pMP1CAT was excised from the 5-terminal to construct pMP5CAT, pMP6CAT, and pMP8CAT. The exon-intron region of pMP1CAT was deleted using the SacI site to construct pPICAT (Fig. 6).

Ten micrograms of plasmid DNA was transfected into HepG2 cells with five micrograms of the internal control plasmid, pact- $\beta$ -gal, which carries the  $\beta$ -galactosidase gene under the control of the chicken  $\beta$ -actin promoter (16), using the calcium phosphate coprecipitation method as previously described (17). Cells were exposed to 100  $\mu$ M of sodium arsenite 96 hr after the transfection because DNA transfection and glycerol shock may cause some stresses. When cells were exposed to sodium arsenite 48 hr after the transfection of LSNWT containing the CAT gene under the control of the *hsp70* promoter, no significant increase in CAT activity was detected (data not shown). Cells were treated with sodium arsenite for 4 hr. Cell extracts which showed the same  $\beta$ -galactosidase activity were added to the assay solution containing 100 mM Tris-HCl, pH 7.8, 1 mM chloramphenicol, and 1.85 kBq of [ $^{14}$ C]butyryl coenzyme A. The reaction mixture was gently overlaid with 5 ml of Econofluor and then incubated at 37°C. At 30 min intervals, individual vials were counted for 0.1 min. The CAT activities were compared at the time where all the samples show the linearity over time.

## Results

### *Effects of stresses on MDRI expression*

*MDRI* is expressed in human normal liver and is believed to act as a secretory pump for physiological metabolites and natural toxic substances in the diet (4), and the expression of the rodent *mdr* gene is elevated in chemically induced preneoplastic and neoplastic



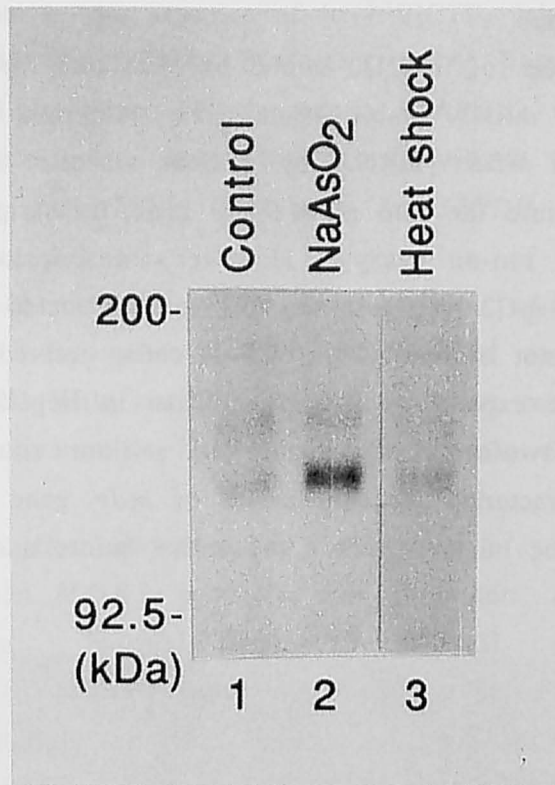
**Figure 1. Induction of *MDR1* mRNA expression in HepG2 (A) and HeLa cells (B).** Twelve micrograms of total cellular RNA and twofold serial dilutions were applied to Gene Screenplus and hybridized with probe 5A as described in MATERIALS AND METHODS. Two micrograms of RNA was hybridized with human  $\beta$ -actin probe as a control (C, D). Panel (A, C): Total RNA from HepG2 cells (lane 1), treated with 300  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 1 hr (lane 2), 100  $\mu$ M sodium arsenite for 4 hr (lane 3), 30 ng/ml TPA for 30 min (lane 4), 12.5 ng/ml adriamycin for 24 hr (lane 5), heat shock at 45°C for 10 min (lane 6). Panel (B, D): Total RNA from HeLa cells (lane 1), treated with 200  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 1 hr (lane 2), 50  $\mu$ M sodium arsenite for 4 hr (lane 3), 30 ng/ml TPA for 30 min (lane 4), 6.25 ng/ml adriamycin for 24 hr (lane 5), heat shock at 45°C for 10 min (lane 6). Treatment with drugs were followed by recovery in fresh medium for 3 hr. The heat shock was followed by recovery at 37°C for 4 hr.



liver nodules (5, 6). To investigate whether stresses and cytotoxic agents have any effects on *MDR1* expression in human liver cells, the author used a well-differentiated human hepatocarcinoma cell line, HepG2. The slot blot hybridization of total RNA (Fig. 1A) showed that when HepG2 was treated with sodium arsenite at 100  $\mu$ M for 4 hr, a two- to threefold increase of *MDR1* mRNA was observed (lane 3). A level of  $\beta$ -actin mRNA did not change after exposure to sodium arsenite (Fig. 1C). This concentration (100  $\mu$ M) of sodium arsenite did not affect the colony-forming ability of HepG2 when the medium was exchanged for a fresh one after 4 hr of treatment (data not shown). Heat shock at 45°C for 10 min followed by recovery at 37°C for 4 hr had a marginal effect on the level of *MDR1* mRNA (lane 6). Heat shock at 42°C for 1 hr or 2 hr scarcely affect the level of *MDR1* mRNA, either (data not shown).

Some stress genes are induced by DNA-damaging agents (18), but no significant increase of *MDR1* expression was observed after exposure to hydrogen peroxide (lane 2), 4-nitroquinoline-N-oxide (4NQO), or N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (data not shown). Adriamycin or 12-*O*-tetradecanoylphorbol-13-acetate (TPA) did not increase *MDR1* mRNA, either (lanes 5 and 4). The induction of *MDR1* mRNA by sodium arsenite was also observed in HeLa cells (Fig. 1B), in which the basal level of *MDR1* mRNA expression is very low.

To see whether the increased expression of *MDR1* mRNA caused by exposure to sodium arsenite caused the increase of the P-glycoprotein synthesis, after HepG2 cells were exposed to 100  $\mu$ M of arsenite for 4 hr and had recovered for 2 hr in the fresh medium these cells were labeled with [<sup>35</sup>S]methionine for 1 hr. The labeled proteins were immunoprecipitated with monoclonal antibody C219, and analyzed by 7% SDS-PAGE (Fig. 2). The exposure to arsenite caused a two- to threefold increase in the P-glycoprotein synthesis (lane 2), while heat shock (42°C for 2 hr) had a marginal effect (lane

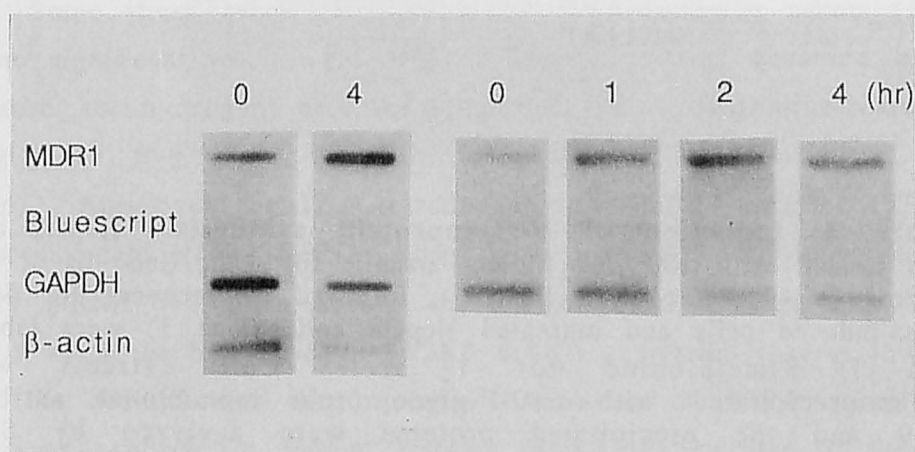


**Figure 2. Induction of P-glycoprotein synthesis.** HepG2 cells were treated with 100  $\mu$ M sodium arsenite for 4 hr (lane 2) or heat shocked at 42°C for 2 hr (lane 3), followed by recovery for 4 hr. Stress-induced cells and untreated HepG2 cells (lane 1) were labeled with [<sup>35</sup>S]methionine for 1 hr. Cell extracts were immunoprecipitated with anti-P-glycoprotein monoclonal antibody C219 and the precipitated proteins were analyzed by SDS-polyacrylamide gel electrophoresis as described in MATERIALS AND METHODS. The numbers on the left indicate the position of size markers in kilodaltons.

3). These changes corresponded well with the increase of *MDR1* mRNA after exposure to arsenite and heat-shock.

### *Mechanism of the increase of MDR1 gene expression by sodium arsenite*

The increase of mRNA could be mediated by transcriptional activation and/or mRNA stabilization. To determine the mechanism of the increase of *MDR1* mRNA by sodium arsenite, the author first attempted to measure the rate of *MDR1* gene transcription in HepG2 cells by nuclear run-on assay. However transcriptional activity of *MDR1* gene in HepG2 cells was too low to be detected by nuclear run-on assay (data not shown). In CV-1 cells derived from monkey kidney, *mdr* gene expression was higher than in HepG2 cells and was increased about twofold by exposure to sodium arsenite (data not shown). To characterize the mechanism of *mdr* gene expression, the author isolated the nuclei from CV-1 cells before and after exposure



**Figure 3.** Transcriptional activation of *mdr* gene in CV-1 cells. CV-1 cells were treated with sodium arsenite for indicated hours and then nuclei were isolated. Nuclear run-on assay was performed as described in MATERIALS AND METHODS. Plasmid templates used for hybridization were as follows: MDR1, full-length *MDR1* cDNA cloned into pBluescript KS (Chapter 1) ; Bluescript, pBluescript KS without insert; GAPDH, pGAD28 containing GAPDH cDNA ; β-actin, human β-actin cDNA

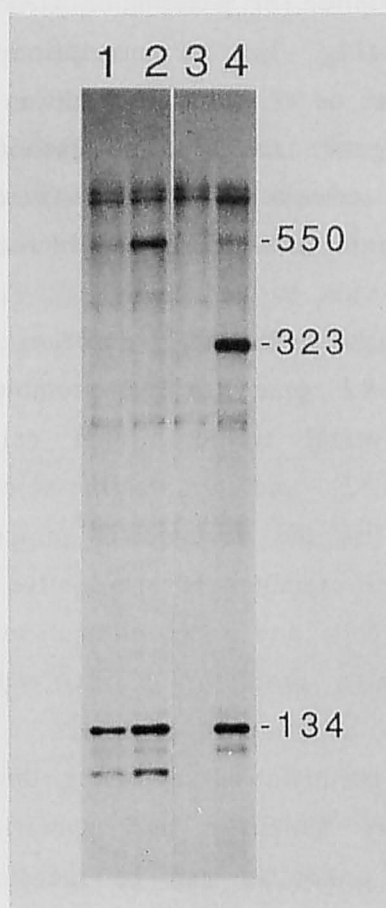
to 100  $\mu$ M sodium arsenite for various hours. After extension of nascent transcripts in the presence of [ $\alpha$ - $^{32}$ P]CTP, labeled RNAs were hybridized to *MDR1* cDNA, Bluescript DNA,  $\beta$ -actin cDNA, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA. Sodium arsenite significantly increased the transcriptional activity of *mdr* gene, while the transcription of  $\beta$ -actin or GAPDH did not increase but rather decreased (Fig. 3). Transcription of *mdr* gene was increased twofold 1 hr or 4 hr after addition of sodium arsenite. Stimulation of *mdr* gene transcription reached to threefold when cells were treated with arsenite for 2 hr. These results indicate that transcriptional activation is involved in the increase of *mdr* mRNA.

#### *Activation of the downstream promoter*

The human *MDR1* gene has two promoters, the upstream and the downstream. Normal tissues, renal cell carcinomas, colon adenocarcinomas, HepG2, and vinblastine-selected KB multidrug-resistant cells mainly use the downstream promoter (Chapter 2) (12, 17). But the upstream promoter is also active in colchicine-selected KB multidrug-resistant cells and some clinical samples (17, 19). The author investigated which promoter is involved in the increase of *MDR1* expression after exposure to arsenite.

A ribonuclease protection assay using the first exon from the normal tissue is very sensitive and specific (Chapter 2), and transcripts from both promoters can be measured accurately. The upstream promoter is as active as the downstream promoter in colchicine-selected multidrug-resistant cell KB8-5. Therefore, the 323-nucleotide fragment (transcript from upstream promoter) as well as the 134-nucleotide fragment (transcript from downstream promoter) was protected from digestion by RNase (Fig. 4, lane 4). Exposure of HepG2 cells to sodium arsenite led to a two- to threefold increase of the 134-nucleotide fragment (lane 2). However, no

transcript from the upstream promoter was detected even after exposure to arsenite. The 550-nucleotide fragment clearly shown in lane 2 is considered to be the nascent transcript (splicing intermediate) from the downstream promoter.



**Figure 4.** RNase protection analysis of *MDR1* mRNA from arsenite-treated HepG2 cells. Total RNA (10  $\mu$ g) extracted from HepG2 cells before (lane 1), and after arsenite treatment at 100  $\mu$ M for 4 hr (lane 2), from drug-sensitive KB3-1 cells (lane 3), and from drug-resistant KB8-5 cells (lane 4) analyzed by RNase protection as described in MATERIALS AND METHODS. The numbers on the right indicate nucleotide numbers of the protected fragments.

### Involvement of HSE sequences in the induction

To identify the sequence involved in the increase of *MDR1* mRNA after exposure to sodium arsenite, the author first isolated a 3-kb fragment from normal human tissue (Chapter 2), which contains the downstream promoter, and sequenced it (Fig. 5). This fragment spanned the sequence from -1073 to +1859 compared to the major

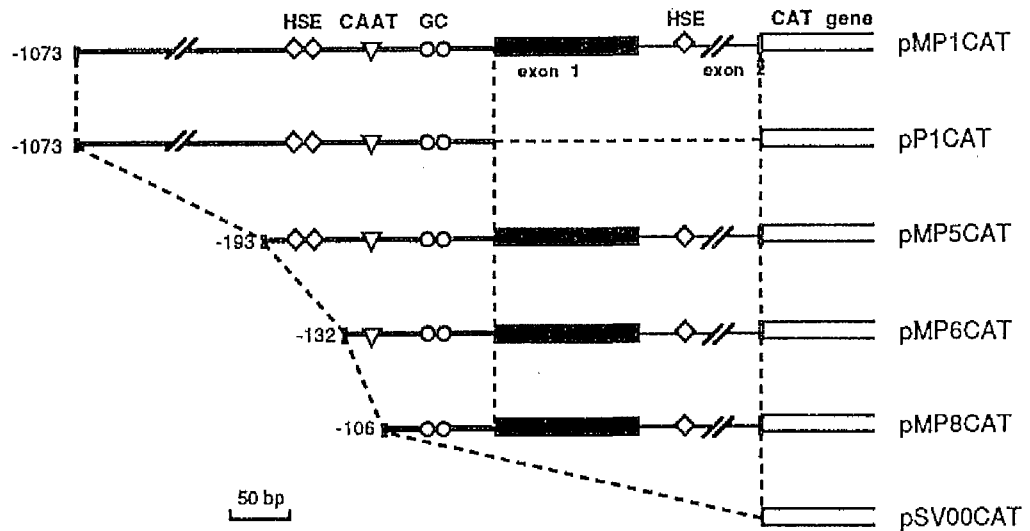
```

-1073   GCC AAGAACAAAA TATATCAAGA TAAGGAAAAAT TTGTAGTCAA GAATAGAAAA AAATTATGGC TTTGAAGTAT -1001
GAGTATTATTA AAGAAAGTGG AAACATCCTC AGACTATGCA GTAAAAAACA AAGTGATTTT CTTCTTCTAA ACTTATGCCA TAAACTGATA GGTAATATGT -991
GAAAGTGCATA GAATGTAGAC TAGAGGATAC AACAAGGCTA TTTCTCTAT GTTCATAGA AGTAAGAAAA GCTCTGATGT GAGTTAGCAT TCGTTTAGAA -981
TTTTGAATTC TGCAGATTGC ACCTACTTTT CCTCAGTTTG AAGTAAATAG TGGACAGGAA AAAATATTTAA ATGTTCGGAG TAAATATGGA AGGAAATTAC -701
AACATAATGA ATATGCTAAA ACATGCTATG TTTATTTTAC TAAATTTGAT TAAAAATGTA GAAATTTAAA TGCCCTGGAA AAACAGGGGC ATTGATCTGA -601
CGTCTGAAGT TTTAAAAATAT TACACACTTT GAAATAGCAT TTGTAGCTTG AAATAGCTGT CTCTATATAT TTTTAAAAAC TCGCTTTTTC TTTCAATCCA -501
TTTATGATCA AATAAAGGAT GAACAGATGT AACTCAGAAA CTGTCAAGCA TGCTGAAGAA AGACCACATG AGAAAAATTT CTCTAGCCTT TTTCAAAAGGT -401
GTTAGGAAGC AGAAAGGTGA TACAGAATTC GAGAGGTCTG AGTTTTYGT TAAACTGTAT TAAATGCCAA TGCCGAGAAA ATTTCCTTAA ACTAGCTGCT -301
GTAGTTATAT GGATATGAAG ACTTATGTGA ACTTTGAAAG ACCTGTCTAC ATAAGTTGAA ATGTCCGCAA TGAATTAGCT GATCCGCTTT TCTCTACTTG -201
CCCTTTCTAG AGAGGTGCAA CGGAAGCCAG AACATTGCTC CTGAAAAATC AACCTGTTTG GCAGTTTCTC GAGGAAATCA GATTCAATCA ATCCGGGGCC -101
GGAGCAGTGA TCTGTGGTGA GGCTGATTCG CTRGGCCAGGA ACAGGCGCCG GCGCTTGGCT GAGCACAGCC GCTTCGCTCT CTTTGGCCAA GAAAGCCCTGA -1

GGTCATTCGA GTAGCGGCTC TCCAAAGCTC AAAGAACCA GAGCTCTCT TCGTTTTCTT TAAATCTTTC CAGTAAAGTC GGAATATGCT CTTTCCAAAA 100
TTACCGTCTT GGTGCGGCTT CGAAGGAGCC CGAGCTAGGG GCACGCAAG CTGGGAGCTA CTATGGGAGA GTTCCCAAGT GTCAGGCTTT CAGATTTCC 200
GAACTTGGTC TTCACGGGAG AAGGCGTTCT TGAGGCGTGG ATAGTGTGAA CTCTCTGCGG AAGTCCATGG GAGCCAAAGT GCGTTAGATC TAGACTCAG 300
AGCTCGTGGG GCAGCGGCCA AACCGTAGTG GCAGTGGAGC ATGTGCCCCG GAGCGCGCAG AGCCCGGGCC GTGCGGGGAG CTCTCTCTG AGCCCGCGGG 400
CGGTGGGTGG GAGGAAGGAT CGTCCGCGGC GACTGGAAGC GGGAGGGAGA ATCCACTGCG CGGCGGGGCA AGTCCGAGAA GCGCTCCGAG ACCCCCAACT 500
CTGCGCTTCT GGAGATGCTG GAGACCCCGC GCACAGGAAA GCGCCCTGAG TGCCCATCGG GCGCAGAGCA GCTGGGGCAT CAACGGCGGG GCGTCCCTCT 600
TAGTGGCTCT TGGCTTCCAG GGGGACTAG AGGTTAGTCT CACCTCCAGC GGGCCTGAGG CTGATGCATT TGGCTAATGA GCTGCGGTTT CTCTTCAG 700
CGGATGCA TCTGAGGGG ACCGCAATG AGGAGGAAA AGAAGAAGT TTTTAAAG GAACAATAAA AGTAACTAG CTGTGTTTAT TTTTCAATG 800
TACATAGTTG CGAGATTGA GTAATTTATT TCTAGCCTCC AGCTGTGAAA TAAATGACAT GTTGTGTTT TTAATTTAT TTAAGAAACG CAAGCTAGCC 900
TTTGGAAATGA ATATCCCTGC TTAGAGCAGA AGTTTGTGG CTGAGTGGAG CACAGCATAT GCATTTTCCC TGTCTTTTTT GTTCTTTCT TTAATGATAC 1000
ATAATATTT ACATATTTT GAAATGGGDT ACATGGAAGC GTTTTTTACA TGCCCGGAAAT GTGTAATGAT CAACTCCGGG TATTTGAAAG ATACATCACC 1100
TTAGGTTAT TGCATTTTGA TGTGTTGATA ACATTTTAAQ TCTTCTAGCT ACTTTGAAAT ATACAATATA TGTCTAAGT TACCTACCCCT CCTCTGGTAT 1200
CGAACATTTG AACATTTTTC TGCTATCCAA CCGTTCTTAG TCATTCCACA ACCCTGTTTC ATTTACACTT TTTACCGCTC GCGGCGCTCT TCCCTTAGTC 1300
TTGGTGTGCC TCTTTCTGAG CTTCCTGCC CCAGCAGCCG GATGCTCAT ATGTGTCTT GTCTTATGAA CTCTGCTTT TCAAGTGGTG TGGTCCGCC 1400
ACAGGTGAGC CATATGCTGC TGGTGATCTG CTCTGTGGTC CAGGCTCTTG CTTCGGTAA ATGGCTATGT AAACATGCGG TTTGTGGCCT GGCTGATGAG 1500
ACAGAAGGTC AAAAGTACAT TTAGGTTGTT AACTGGCAAT AAATATCTGT ATATAATATY GGTAAATGTA TCAATATAGG AAAATAATTA TTTAAAGTAA 1600
ATTTTATGCA TGGTGTCTG CCTTTATAGA ATATTTAAAA GTTCACTAAA TAGATTCATT GTTAGTAGTA AATTGTAAAA TAGACTAGTA AGTTTAAATA 1700
TATTAGAAAC TGAATGTAAA ATTTAAGAT AATTTAGCTA AACACATAAA TATFATAAGA AACCAAGCTT TTGAGTGA TA GAGAAAAAAT ACAAATGTGG 1800
AAATCAATA CATTITTTAA AATAATGTTA AGTTTGAAT AGAAAAATTA ATATGAAT 1859

```

Figure 5. Nucleotide sequence of the 3-kb fragment containing the *MDR1* downstream promoter. Exon 1 and 2 are enclosed by solid lines. An arrow head indicates the site at which the chloramphenicol acetyl transferase gene is fused in pMPICAT. GC boxes, a CAAT box, and heat-shock responsive elements are underlined by a solid line, a dotted line, and two solid lines, respectively. The first ATG is indicated by asterisks. The numbers are presented with the major transcription initiation site as +1.



**Figure 6. Schematic representation of *MDR1* promoter deletions.** Open box indicates the CAT gene in pSV00CAT and filled boxes indicate the first exon and a part of the second exon of *MDR1* gene. HSE, heat-shock responsive element; CAAT, CAAT box; GC, GC box.

transcriptional initiation site and consisted of 1073 bp of the upstream sequence, 134 bp of the first exon, 562 bp of the first intron, 74 bp of the second exon where the first ATG exists, and a part of the second intron. To examine the roles of sequences in the upstream region, the first exon, and the first intron, the second exon was excised from 3' to one nucleotide upstream from the first ATG and the excised fragment was fused to the *E. coli* chloramphenicol acetyl transferase (CAT) gene in pSV00CAT (Fig. 6). The resulting plasmid was named pMP1CAT.

When HepG2 cells were treated with 100  $\mu$ M of sodium arsenite 96 hr after the transfection of pMP1CAT, the CAT activity increased to 2.6-fold that without arsenite treatment (Table 1). The CAT activity of an extract from cells transfected with pBLCAT2, which contains CAT

Table 1

Promoter activity of deletion constructs in HepG2

	CAT activity <sup>a)</sup>		Fold increase
	-arsenite	+arsenite	
pMP1CAT	26	68	2.6
pP1CAT	23	35	1.5
pMP5CAT	33	66	2.0
pMP6CAT	59	65	1.1
pMP8CAT	34	31	0.9
pSV00CAT	0	0	-
pBLCAT2( <i>tk</i> )	100	80	0.8
LSNWT( <i>hsp70</i> )	63	132	2.1

<sup>a)</sup>CAT activity is the percent ratio relative to the CAT activity of pBLCAT2 without arsenite treatment. Each value is average of three experiments. Plasmid carrying the  $\beta$ -galactosidase gene cDNA under the control of the  $\beta$ -actin promoter was cotransfected, and the  $\beta$ -galactosidase activity of cell extracts were used as an internal control.

gene under the control of *tk* promoter, did not change after exposure to arsenite. The 2.6-fold increase in the promoter activity of *MDR1* was significant because the *hsp70* promoter in LSNWT showed no more than a 2.1-fold increase after the exposure to arsenite under these conditions.

To identify the functionally important region for the activation by arsenite treatment, the author constructed a series of deletion mutants from pMP1CAT (Fig. 6). Because pMP8CAT had a similar promoter activity to pMP1CAT (Table 1), sequences from -1073 to -106 including HSEs and the CAAT box may not be significant for the basal promoter activity. The promoter activity of pMP5CAT (-193



to +704) was increased twofold after exposure to arsenite, but that of pMP6CAT (-132 to +704) or pMP8CAT (-106 to +704) was not. Also in CV-1 cells, the promoter activity of pMP1CAT was increased 2.0-fold after exposure to sodium arsenite but that of pMP8CAT was not (data not shown). These results suggest that the nucleotide sequences from -193 to -133, which contains a tandem repeat of HSEs (-174 CCAGAACATTCCIC -161 and -161 CCTGGAAATTCAAC -148), are necessary for the induction by arsenite treatment. pP1CAT, in which the first exon, the first intron, and the second exon were deleted showed similar basal promoter activity to pMP1CAT and a 1.5-fold increase by arsenite treatment, indicating that the exon-intron region (+1 to +704) that contains a HSE sequence (195 TTTCCTGAAAC 204) could modify the induction by arsenite, although it did not affect the basal promoter activity.

## DISCUSSION

The author has shown in this chapter that sodium arsenite treatment increases the *MDR1* mRNA expression in two cell lines, HeLa and HepG2, which are derived from human uterus and liver, respectively. In CV-1 cells derived from monkey kidney, the *mdr* gene was also increased after exposure to sodium arsenite (data not shown). The author could not detect a significant increase of *MDR1* gene expression in these cell lines after heat shock. Recently, Chin *et al.* reported that *MDR1* mRNAs in two human renal carcinoma cell lines, HTB-44 and HTB-46, were increased by arsenite treatment and heat shock (7). The reason why heat shock induces *MDR1* gene expression only in these two renal carcinoma cell lines is unclear. These results, however, indicate that *MDR1* is one of the stress genes.

The increase of *MDR1* mRNA could be mediated by either

transcriptional activation or mRNA stabilization or both. Nuclear run-on assay using CV-1 cells and CAT assay using HepG2 cells and CV-1 cells indicated that sodium arsenite activated the transcription of *MDR1* gene. Induction ratio of nuclear run-on and CAT assays (two- to threefold) corresponded well to the increase of *MDR1* mRNA level in these cells. The 2.6-fold increase in the promoter activity of *MDR1* in the CAT assay was significant because the *hsp70* promoter showed no more than a 2.1-fold increase under these conditions (Table 1). These results suggested that at least a part of, probably most of, the increase of mRNA in HepG2 and CV-1 cells after exposure to arsenite is mediated by transcriptional activation.

Deletion analysis indicated that sequences between -193 to -133 are necessary for the induction by arsenite treatment (Fig. 6, Table 1). There are two classical HSEs (C--GAA--TTC--G) tandemly repeated in this region. One (-174 to -161) is a 7/8 match and the other (-161 to -148) a 6/8 match. Recently Peisic *et al.* reported that HSE is composed of contiguous arrays of 5-bp units, -GAA- (20). Head-to-head (-GAA--TTC-) and tail-to-tail (-TTC--GAA-) repeats of this unit can bind HSF and show stress-induced expression. In this respect, one tail-to-tail HSE exists in the first intron (+195 to +204). CAT assay analysis using a deleted *MDR1* promoter suggested that this internal HSE may slightly modify the promoter activity after exposure to arsenite.

Acquisition of multidrug resistance could involve two phenomena, induction of *MDR1* gene expression and selection. *MDR1* mRNA level in HepG2, HeLa, or CV-1 cells was not changed after exposure to chemotherapeutic agents or DNA damaging agents under these experimental conditions. But the data shown here indicate that *MDR1* gene expression can be induced under certain conditions of stress. This induction mechanism might be involved in the initial response in the acquisition of multidrug resistance *in vivo*.

## REFERENCES

1. Shen, D.-w., Fojo, A., Chin, J. E., Roninson, I. B., Richert, N., Pastan, I. and Gottesman, M. M. (1986) *Science* **232**, 643-645
2. Fojo, A. T., Ueda, K., Slamon, D. J., Poplack, D. G., Gottesman, M. M. and Pastan, I. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 265-269
3. Cordon-Cardo, C., O'Brien, J. P., Casals, D., Rittman-Grauer, L., Biedler, J. L., Melamed, M. R. and Bertino, J. R. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 695-698
4. Thiebaut, F., Tsuruo, T., Hamada, H., Gottesman, M. M., Pastan, I. and Willingham, M. C. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 7735-7738
5. Fairchild, C. R., Ivy, S. P., Rushmore, T., Lee, G., Koo, P., Goldsmith, M. E., Myers, C. E., Farber, E. and Cowan, K. H. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 7701-7705
6. Thorgeirsson, S. S., Huber, B. E., Sorrell, S., Fojo, A., Pastan, I. and Gottesman, M. M. (1987) *Science* **236**, 1120-1122
7. Chin, K.-V., Tanaka, S., Darlington, G., Pastan, I. and Gottesman, M. M. (1990) *J. Biol. Chem.* **265**, 221-226
8. Araki, E., Shimada, F., Shichiri, M., Mori, M. and Ebina, Y. (1988) *Nucl. Acids Res.* **16**, 1627
9. Luckow, B. and Schutz, G. (1987) *Nucl. Acids Res.* **15**, 5490
10. Dugaiczky, A., Haron, J. A., Stone, E. M., Dennison, O. E., Rothblum, K. N. and Schwartz, R. J. (1983) *Biochemistry* **22**, 1605-0613
11. Williams, G. T., McClanahan, T. K. and Morimoto, R. I. (1989) *Mol. Cell. Biol.* **9**, 2574-2587
12. Ueda, K., Cardarelli, C., Gottesman, M. M. and Pastan, I. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 3004-3008
13. Nakajima-Iijima, S., Hamada, H., Reddy, P. and Kakunaga, T. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 6133-6137
14. Ueda, K., Yamano, Y., Kioka, N., Kakehi, Y., Yoshida, O. and Gottesman, M. M. (1989) *Jpn. J. Cancer Res.* **80**, 1127-1132

15. Debenham, P. G., Kartner, N., Siminovitch, L., Riordan, J. R. and Ling, V. (1982) *Mol. Cell. Biol.* **2**, 881-889
16. Seiler-Tuyns, A., Eldridge, J. D. and Paterson, B. M. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 2980-2984
17. Ueda, K., Pastan, I. and Gottesman, M. M. (1987) *J. Biol. Chem.* **262**, 17432-17436
18. Hollander, M. C. and Fornace, A. J., Jr. (1989) *Cancer Res.* **49**, 1687-1692
19. Goldstein, L. J., Galski, H., Fojo, A., Willingham, M., Lai, S.-L., Gazdar, A., Pirker, R., Green, A., Crist, W., Brodeur, G. M., Lieber, M., Cossman, J., Gottesman, M. M. and Pastan, I. (1989) *J. Natl. Cancer Inst.* **81**, 116-124
20. Perisic, O., Xiao, H. and Lis, J. T. (1989) *Cell* **59**, 797-806



## Chapter 4    Quercetin, a Bioflavonoid, Inhibits the Increase of Human Multidrug Resistance Gene (*MDR1*) Expression Caused by Arsenite

The development of low levels (two- to severalfold) of multidrug resistance in cultured cells is initially accompanied by elevated expression of the *MDR1* gene without gene amplification (1). It has been reported that the expression of *MDR1* is induced in human renal adenocarcinoma cell lines (HTB-44 and HTB-46) (2) and a human hepatocarcinoma cell line (HepG2) (Chapter 3) after exposure to some stresses. Transcriptional activation was involved in the increase of *MDR1* expression in HepG2 (Chapter 3) and the increased expression of *MDR1* caused a transient increase in resistance to vinblastine in HTB-46 (2). Because the emergence of cancer cells that have acquired two- to threefold resistance should be a serious problem in chemotherapy, reagents that inhibit the increase of *MDR1* expression might be useful to suppress the emergence of resistant cells.

Quercetin, a bioflavonoid widely distributed in plants, inhibits the synthesis of heat-shock proteins induced by heat-shock and other stresses (3). Quercetin inhibited the induction of *hsp70* at the level of mRNA accumulation. In this chapter the author shows that quercetin inhibits the increase of P-glycoprotein synthesis and *MDR1* mRNA in HepG2 cells after exposure to sodium arsenite. The CAT assay analysis indicated that quercetin affected the transcriptional activity of *MDR1*.

## MATERIALS AND METHODS

### *Materials and Cells*

[<sup>35</sup>S]methionine and [<sup>14</sup>C]butyryl coenzyme A were purchased from NEN Research Products. [ $\alpha$ -<sup>32</sup>P]CTP was from Amersham. Anti-P-glycoprotein monoclonal antibody was from Centocor. HepG2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum.

### *Immunoprecipitation*

Cells were treated with 100  $\mu$ M quercetin for 4 hr, and then treated with 100  $\mu$ M sodium arsenite for 4 hr or heat shock at 42°C for 2 hr in the presence of quercetin. After recovering for 2 hr in fresh medium containing quercetin, cells were labeled with [<sup>35</sup>S]methionine for 1 hr and harvested. Immunoprecipitation was done as described in chapter 3.

### *RNA isolation, slot blot hybridization, and RNase protection*

A part of *MDR1* cDNA, pMDR5A, encoding the nucleotide binding region (4) and *hsp70* cDNA in the plasmid pH2.3 (5) were used as probes for slot blot hybridization. HepG2 cells were treated with 100  $\mu$ M quercetin for 4 hr, then exposed to 100  $\mu$ M sodium arsenite for 4 hr in the presence of quercetin. Total RNA was extracted from HepG2 cells and transferred to GeneScreen Plus (Du Pont). Hybridizations were done at 60°C for 16 hr in hybridization solution (1% SDS, 1 M NaCl, 10% dextran sulfate, and 200  $\mu$ g/ml of denatured salmon sperm DNA) containing each probe ( $5 \times 10^5$  cpm/ml). Filters were washed to a final stringency of 2xSSC and 1% SDS at 60°C. Comparable RNA loading was confirmed using a human  $\beta$ -actin probe (6). RNase protections were done as previously described using the probe PN (Chapter 2).

### *CAT assay*

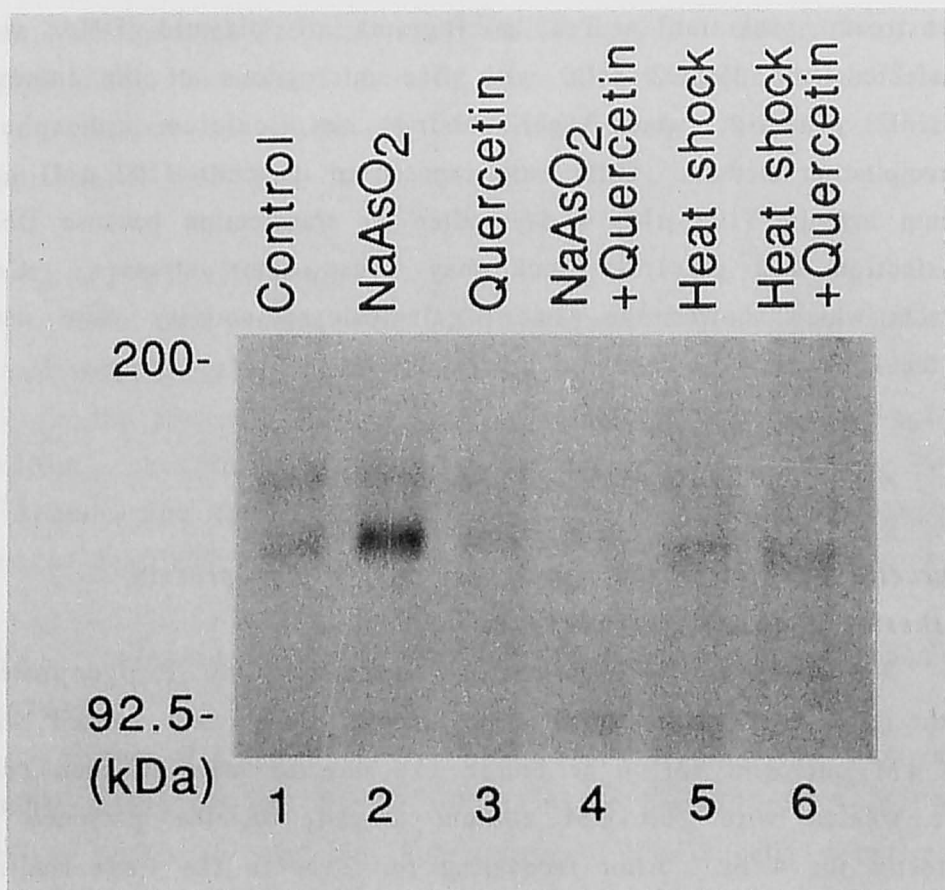
Plasmid pMP1CAT contains the chloramphenicol acetyltransferase (CAT) gene under the control of the *MDR1* downstream promoter. Ten micrograms of plasmid DNA was transfected into HepG2 cells with five micrograms of the internal control plasmid, pact- $\beta$ -gal, using the calcium phosphate coprecipitation method. Cells were exposed to quercetin (100  $\mu$ M) and sodium arsenite (100  $\mu$ M) 4 days after the transfection because DNA transfection and glycerol shock may cause some stresses. Cell extracts which showed the same  $\beta$ -galactosidase activity were used for the CAT assay as described in chapter 3.

## RESULTS

### *Quercetin inhibits the increase of P-glycoprotein synthesis induced by arsenite*

To analyze the effects of quercetin on P-glycoprotein synthesis after exposure to arsenite, HepG2 cells were treated with 100  $\mu$ M quercetin for 4 hr before exposure to arsenite, then cells were treated with 100  $\mu$ M sodium arsenite in the presence of quercetin for 4 hr. After recovering for 2 hr in the fresh medium containing quercetin, HepG2 cells were labeled with [ $^{35}$ S]methionine for 1 hr. The labeled proteins were immunoprecipitated with anti-P-glycoprotein monoclonal antibody C219, and analyzed by 7% SDS-PAGE. The exposure to arsenite caused a two- to threefold increase in the P-glycoprotein synthesis, as described in chapter 3 (Fig. 1, lane 2). Quercetin slightly inhibited the constitutive synthesis of P-glycoprotein (lane 3). Quercetin completely inhibited the increase of the P-glycoprotein synthesis caused by exposure to arsenite (lane 4). Heat shock at 42°C for 2 hr did not increase the synthesis of P-





**Figure 1.** Inhibition of the increase of P-glycoprotein synthesis caused by exposure to arsenite. HepG2 cells treated with or without 100  $\mu$ M sodium arsenite or heat-shocked (42°C, 2 hr) in the presence or absence of quercetin were labeled with [ $^{35}$ S]methionine and cell extracts were immunoprecipitated with the anti-P-glycoprotein monoclonal antibody C219 and analyzed as described in MATERIALS AND METHODS. The numbers on the left indicate the positions of size markers in kilodaltons.

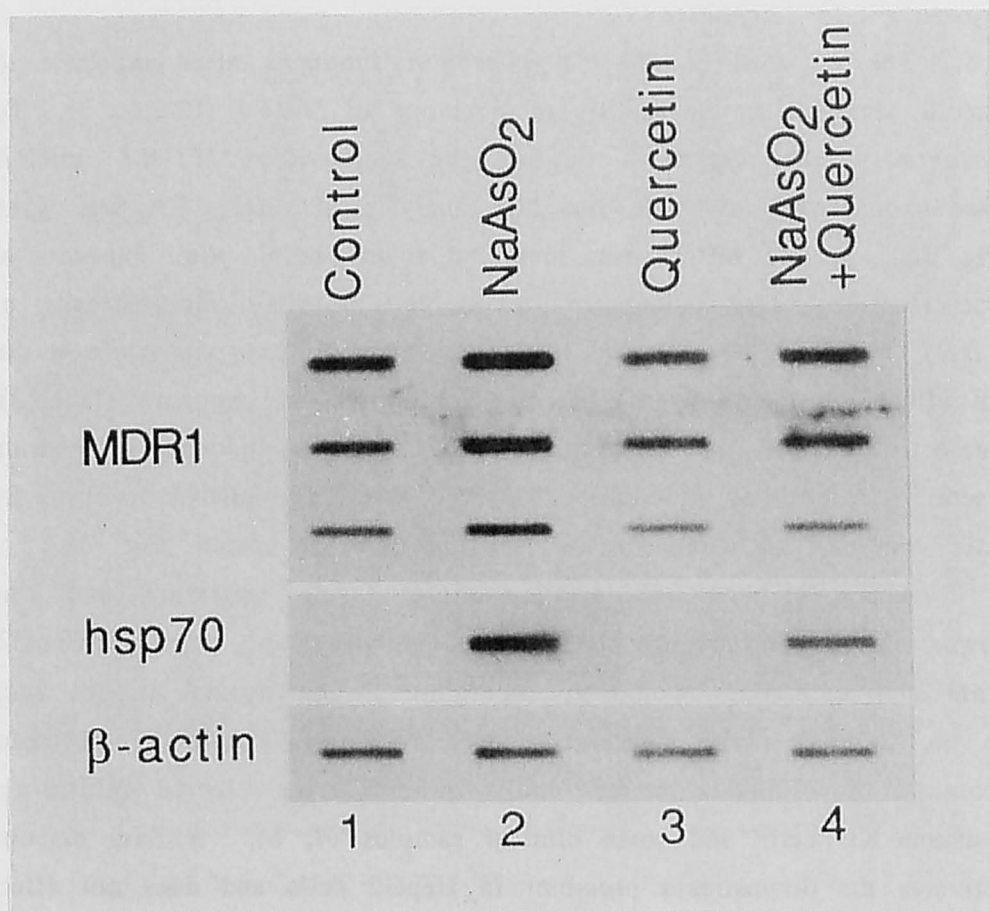
glycoprotein in HepG2 significantly (lane 5) nor did quercetin affect P-glycoprotein synthesis after heat shock (lane 6).

*Quercetin inhibits the increase of MDR1 mRNA after exposure to arsenite*

The increase in the P-glycoprotein synthesis after exposure to arsenite was due to the mRNA accumulation of *MDR1* (Chapter 3). To discover whether quercetin inhibits the increase of *MDR1* mRNA after exposure to arsenite, slot blot analysis of total RNA was done (Fig. 2A). *MDR1* mRNA was increased about twofold after exposure to arsenite (lane 2). Quercetin completely inhibited the increase of *MDR1* mRNA after exposure to sodium arsenite (lane 4), while it did not affect the level of *MDR1* mRNA before the exposure (lane 3). Quercetin reduced the increase of *hsp70* mRNA induced by sodium arsenite as described previously (3) (Fig. 2A). The mRNA level of  $\beta$ -actin gene did not change after exposure to these agents (Fig. 2A).

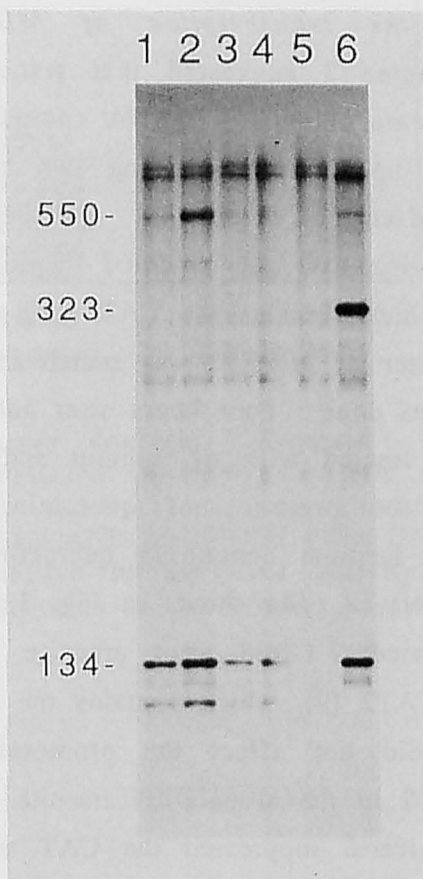
*MDR1* gene has two promoters, one upstream and one downstream (7). The downstream promoter is mainly used in HepG2, renal cell carcinoma, colon adenocarcinoma, and normal tissues such as the adrenal, liver, and intestine. Transcripts from the upstream promoter have been detected only in colchicine-selected multidrug-resistant KB cells and some clinical samples (7, 8). Sodium arsenite activates the downstream promoter in HepG2 cells and does not affect the activity of the upstream promoter (Chapter 3) (Fig. 2B). To confirm that quercetin affected the transcripts from the downstream promoter, total RNA from HepG2 cells treated with sodium arsenite and quercetin was analyzed by a ribonuclease protection assay (Fig. 2B). In colchicine-selected multidrug-resistant cells KB8-5 both promoters are active, therefore both the 323 nucleotide fragment (transcript from the upstream promoter) and the 134 nucleotide fragment (transcript from the downstream promoter) were detected

(A)



**Figure 2. Inhibition of the increase of *MDR1* mRNA accumulation.** (A) Slot blot hybridization. HepG2 cells treated with or without sodium arsenite in the presence or absence of quercetin. Twelve micrograms of total RNA and twofold serial dilutions were applied to GeneScreen plus and hybridized with *MDR1* probe. Two micrograms of RNA was hybridized with *hsp70* probe or a human  $\beta$ -actin probe as a control.

(B)



(B) RNase protection analysis. Total RNA (10  $\mu$ g) was extracted from untreated HepG2 cells (lane 1), treated with sodium arsenite (lane 2), quercetin (lane 3), and both reagents (lane 4), from drug-sensitive KB3-1 cells (lane 5), and from drug-resistant KB8-5 cells (lane 6). The RNase protection assay was done as described in MATERIALS AND METHODS. The numbers on the right indicate nucleotide numbers of the protected fragments. The 550-nucleotide fragment in lane 2 is considered to be the nascent transcript (splicing intermediate) from the downstream promoter.

(lane 6). In HepG2 cells the exposure to sodium arsenite led to a two- to threefold increase of transcript from the downstream promoter (lane 2), and quercetin inhibited this increase (lane 4).

### *Quercetin inhibits the transcription of MDR1*

Results in chapter 3 suggested that transcriptional activation is involved in the increase of *MDR1* mRNA caused by sodium arsenite and depends on a 60-bp region containing two heat-shock responsive elements. To discover whether quercetin inhibits the transcriptional activation of the *MDR1* gene after exposure to arsenite, pMP1CAT, which contains the CAT gene under the control of the downstream promoter of *MDR1*, was transfected into HepG2 cells, and the CAT assay was done. Four hours after addition of 100  $\mu$ M of quercetin, cells were treated with or without sodium arsenite for an additional 4 hr in the presence of quercetin. After 2 hr of incubation with fresh medium containing quercetin, the CAT activity in cell extracts was assayed. As shown in Fig. 3, the CAT activity of pMP1CAT was increased 2.1-fold after arsenite treatment while the CAT activity of pBLCAT2 (9), which contains the *tk* promoter, did not change. Quercetin did not affect the promoter activity of either pMP1CAT or pBLCAT2 in the absence of arsenite. In the presence of arsenite, however, quercetin suppressed the CAT activity of pMP1CAT to the level without arsenite treatment. The CAT activity of pBLCAT2 was not changed in the presence of these agents. These results suggested that quercetin specifically inhibited the increase of transcriptional activity of the *MDR1* gene induced by arsenite.

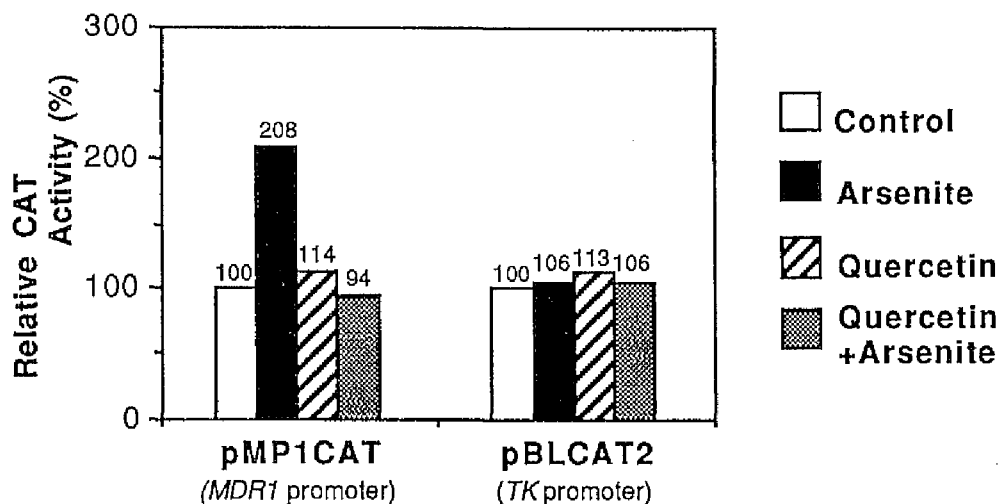


Figure 3. CAT assay analysis. Plasmid DNAs (pMP1CAT, pBLCAT2) were transfected to HepG2. Cells were treated with or without arsenite in the presence or absence of quercetin as described in MATERIALS AND METHODS. The CAT activity is shown by the percent ratio relative to the CAT activity of cell extracts of untreated cells. Each value is the average of five experiments.

## DISCUSSION

Quercetin is distributed widely in plants and has many biological effects (10-12) and is recently reported to inhibit the induction of heat shock proteins (3). In this chapter, the author has shown that quercetin inhibited the increase of P-glycoprotein synthesis and *MDR1* mRNA accumulation. Because the author showed that the transcriptional activation of *MDR1* gene after exposure to arsenite depends on a 60-bp region containing tandemly repeated heat-shock responsive elements (Chapter 3), quercetin might affect heat-shock factors. However it is possible that other trans-acting

factors or RNA polymerases are involved in the inhibition by quercetin.

Many reagents have been screened for reversing multidrug resistance and some calcium channel blockers such as verapamil and quinidine have been reported to have this property. These reversing agents interact with P-glycoprotein and inhibit its function competitively. However the most promising way to conquer multidrug resistance should be to suppress the emergence of cancer cells with acquired resistance. This is the first report to describe a reagent that inhibits the increase of the *MDR1* gene expression.

## REFERENCES

1. Shen, D.-w., Fojo, A., Chin, J. E., Roninson, I. B., Richert, N., Pastan, I. and Gottesman, M. M. (1986) *Science* **232**, 643-645
2. Chin, K.-V., Tanaka, S., Darlington, G., Pastan, I. and Gottesman, M. M. (1990) *J. Biol. Chem.* **265**, 221-226
3. Hosokawa, N., Hirayoshi, K., Nakai, A., Hosokawa, Y., Marui, N., Yoshida, M., Sakai, T., Nishino, H., Aoike, A., Kawai, K. and Nagata, K. (1990) *Cell Struct. Funct.* **15**, 393-401
4. Ueda, K., Cardarelli, C., Gottesman, M. M. and Pastan, I. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 3004-3008
5. Wu, B., Hunt, C. and Morimoto, R. (1985) *Mol. Cell. Biol.* **5**, 330-341
6. Nakajima-Iijima, S., Hamada, H., Reddy, P. and Kakunaga, T. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 6133-6137
7. Ueda, K., Pastan, I. and Gottesman, M. M. (1987) *J. Biol. Chem.* **262**, 17432-17436

8. Goldstein, L. J., Galski, H., Fojo, A., Willingham, M., Lai, S.-L., Gazdar, A., Pirker, R., Green, A., Crist, W., Brodeur, G. M., Lieber, M., Cossman, J., Gottesman, M. M. and Pastan, I. (1989) *J. Natl. Cancer Inst.* **81**, 116-124
9. Luckow, B. and Schutz, G. (1987) *Nucl. Acids Res.* **15**, 5490
10. Kim, J. H., Kim, S. H., Alfieri, A. A. and Young, C. W. (1984) *Cancer Res.* **44**, 102-106
11. Graziani, Y., Chayoth, R., Karny, N., Feldman, B. and Levy, J. (1981) *Biochim. Biophys. Acta* **714**, 415-421
12. Graziani, Y. and Chayoth, R. (1979) *Biochem. Pharmacol.* **28**, 397-403



## Summary

### Chapter 1

The author isolated a full-length *MDR1* cDNA from human adrenal where P-glycoprotein is expressed at high level. The deduced amino acid sequence shows two amino acid differences from the sequence of P-glycoprotein obtained from colchicine-selected multidrug-resistant cultured cells. The amino acid substitution Gly→Val at codon 185 in P-glycoprotein from colchicine resistant cells occurred during selection of cells in colchicine. As previously reported, cells transfected with the *MDR1* cDNA carrying Val<sup>185</sup> acquire increased resistance to colchicine compared to other drugs. The other amino acid substitution Ser→Ala at codon 893 probably reflects genetic polymorphism. The *MDR1* gene, the major member of the P-glycoprotein gene family expressed in human adrenal, is sufficient to confer multidrug resistance on culture cells.

### Chapter 2

The human *MDR1* gene encoding P-glycoprotein, an energy-dependent drug-efflux pump, was initially isolated from a multidrug-resistant KB carcinoma cell. When a 3-kb genomic sequence isolated from normal human tissue including the major downstream promoter and the first and second exons of the *MDR1* gene was compared to the equivalent fragment from KB cells, the *MDR1* gene from KB carcinoma cells was found to have a point mutation in the first exon. Although this mutation does not affect the downstream promoter sequence or the coding sequence of the *MDR1* gene, it

creates a single base mismatch between the 5' KB genomic fragment previously used for RNase protection analysis of *MDR1* RNA expression in normal tissues and thereby reduces the sensitivity of this assay. Using the DNA fragment from normal tissues rather than KB cells, the author has reanalyzed *MDR1* mRNA levels in 12 renal carcinomas and 4 colon adenocarcinomas. By this RNase protection assay, *MDR1* RNA levels are as high in these tumors as in the multidrug-resistant cell line, KB-8-5. The ribonuclease protection assay indicated that the major downstream promoter was mainly used in these clinical samples including two samples of RNA from metastatic renal cancer. This assay appears to be a very sensitive and specific assay for detecting *MDR1* mRNA levels and mRNA initiation sites in clinical samples.

### Chapter 3

The *MDR1* gene, which confers a MDR phenotype, is expressed in liver, kidney, small intestine, and the blood-brain barrier, and is considered to help excrete metabolites and natural cytotoxic substances in the diet. The author investigated *MDR1* gene expression in the well-differentiated hepatoma cell line HepG2 after exposure to several stresses and found that sodium arsenite treatment led to a two- to threefold increase of *MDR1* gene expression and P-glycoprotein synthesis. Nuclear run-on assay indicated that transcriptional activation was involved in the increase of *MDR1* mRNA caused by sodium arsenite. RNase protection assay showed that transcription started from the downstream promoter. Deletion analysis of the downstream promoter fused to the chloramphenicol acetyltransferase gene indicated that the transcriptional activation after exposure to arsenite depends on a 60-bp region containing two

heat-shock responsive elements.

#### Chapter 4

Expression of the *MDR1* gene, which encodes P-glycoprotein, is involved in multidrug resistance of cancer cells. *MDR1* gene expression is increased under some stress conditions. It has reported that quercetin, a bioflavonoid, inhibits the expression of heat shock proteins. The author has identified the effects of quercetin on the *MDR1* gene expression in the human hepatocarcinoma cell line HepG2. The increase of P-glycoprotein synthesis and *MDR1* mRNA accumulation caused by exposure to arsenite was inhibited by quercetin. The CAT assay suggested that quercetin suppressed the transcriptional activation of *MDR1* gene after exposure to arsenite. Although many drugs that prevent the P-glycoprotein function have been reported, this is the first report to describe the inhibition of *MDR1* expression by a reagent.

## List of publications

- A. Y. Yamano, K. Oyabayashi, M. Okuno, M. Yato, N. Kioka, E. Manabe, H. Hashi, H. Sakai, T. Komano, K. Utsumi and A. Iritani (1988) Cloning and sequencing of cDNA that encodes goat growth hormone. *FEBS lett.* **228**, 301-304
- B. J.-D. Bahk, N. Kioka, H. Sakai, and T. Komano (1988) A runaway-replication plasmid pSY343 contains two *ssi* signals. *PLASMID* **20**, 266-270
- C. M. Yato, Y. Yamano, K. Oyabayashi, M. Okuno, N. Kioka, E. Manabe, H. Hashi, H. Sakai, T. Komano, K. Utsumi, and A. Iritani (1988) Nucleotide sequence of the growth hormone gene cDNA from goat *Capra hirus* L. (Tokara). *Nucl. Acids Res.* **16**, 3578
- D. N. Kioka, E. Manabe, M. Abe, H. Hashi, M. Yato, M. Okuno, Y. Yamano, H. Sakai, T. Komano, K. Utsumi, and A. Iritani (1989) Cloning and sequencing of goat growth hormone gene. *Agric. Biol. Chem.* **53**, 1583-1587
- E. N. Kioka, J. Tsubota, Y. Kakehi, T. Komano, M. M. Gottesman, I. Pastan, and K. Ueda (1989) P-glycoprotein gene (*MDR1*) cDNA from human adrenal: normal P-glycoprotein carries Gly<sup>185</sup> with an altered pattern of multidrug resistance. *Biochem. Biophys. Res. Commun.* **162**, 224-231

- F. K. Ueda, Y. Yamano, N. Kioka, Y. Kakehi, O. Yoshida, M. M. Gottesman, I. Pastan, and T. Komano (1989) Detection of multidrug resistance (*MDR1*) gene RNA expression in human tumors by a sensitive ribonuclease protection assay. *Jpn. J. Cancer Res.* **80**, 1127-1132
- G. Y. Yamano, M. Abe, S. Mikawa, N. Kioka, E. Manabe, H. Sakai, T. Komano, K. Utsumi, and A. Iritani (1991) Structural analysis of repetitive DNA sequences in the goat growth hormone gene region. *Agric. Biol. Chem.* **55**, 633-639
- H. N. Kioka, Y. Yamano, T. Komano, and K. Ueda (1991) Transcriptional regulation of multidrug resistance gene (*MDR1*) expression in response to arsenite treatment. (submitted)
- I. N. Kioka, N. Hosokawa, T. Komano, K. Hirayoshi, K. Nagata, and K. Ueda (1991) Quercetin, a bioflavonoid, inhibits the increase of human multidrug resistance gene (*MDR1*) expression caused by arsenite. (submitted)

Chapter 1 is described in reference E.

Chapter 2 is described in reference F.

Chapter 3 is described in reference H.

Chapter 4 is described in reference I.

## Acknowledgements

The author wishes to express his sincere gratitude to Dr. Tohru Komano, professor of Kyoto University, for his valuable suggestion and encouragement through the course of this study.

The author wishes to express his grateful acknowledgment to Dr. Kazumitsu Ueda, Kyoto University for his valuable suggestion, discussion, and kind encouragement in carrying out this study.

Special thanks must be given to Dr. Hiroshi Sakai, associate professor of Kyoto University, and Dr. Yoshiaki Yamano, associate professor of Tottori University for their useful discussions and technical advices.

The author also appreciates to Dr. Ira Pastan and Dr. Michael M. Gottesman, National Institutes of Health, National Cancer Institute for their valuable suggestions.

The author expresses his sincere thanks to Dr. Kazuhiro Nagata, Dr. Osamu Yoshida, professors of Kyoto University for their useful discussions. The author also thanks to Dr. Yoshiyuki Kakehi, Dr. Nobuko Hosokawa, and Dr. Kazunori Hirayoshi, Kyoto University for their valuable suggestions and technical advices.

Finally the author is indebted to Mr. Jun Tsubota, Mr. Tsuguteru Ohtsubo, and other members of Laboratory of Biochemistry, Department of Agricultural Chemistry, Kyoto University.



Noriyuki Kioka