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Heat-shock responsive elements in the induction of the multidrug resistance gene (*MDR1*)

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The *MDR1* gene, considered to be involved in multidrug resistance of cancer cells, is expressed in liver, kidney, small intestine and the blood–brain barrier. We investigated *MDR1* gene expression in the well-differentiated hepatoma cell line HepG2 after exposure to several stresses and found that sodium arsenite treatment increased *MDR1* gene expression 2.6-fold. Deletion analysis of the *MDR1* promoter indicated that the transcriptional activation after exposure to arsenite depends on a 60-bp region containing two heat-shock responsive elements.

Multidrug resistance; *MDR1*; *P*-Glycoprotein; Heat-shock protein

1. INTRODUCTION

Acquisition of multidrug resistance of cancer cells is a big obstacle in chemotherapy. The development of multidrug resistance (two- to severalfold) in cultured cells is initially accompanied by elevated expression of the *MDR1* gene, which codes for *P*-glycoprotein, without gene amplification [1]. During further selection for increased levels of resistance, expression of *MDR1* mRNA is extraordinarily increased simultaneously with amplification of the *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. Recently Chin et al. reported that exposure of a renal adenocarcinoma cell line to heat shock or sodium arsenite increased *MDR1* mRNA levels [2]. In this report, we show that the expression of the *MDR1* gene in a hepatocarcinoma cell line, HepG2, was increased by exposure to sodium arsenite and that this response dependend on a promoter region containing a tandem repeat of heat-shock responsive elements (HSEs).

Abbreviations: HSE, heat-shock responsive element; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; CAT, chloramphenicol acetyltransferase

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2. MATERIALS AND METHODS

2.1. *Plasmids and cells*

Plasmid pSV00CAT [3], pBLCAT2 [4], pact-β-gal, and LSNWT [5] were provided by Dr. E. Araki, Dr. B. Luckow, Dr. S. Ishii, and Dr. R.I. Morimoto, respectively.

2.2. *Slot blot hybridization and ribonuclease protection assay*

A part of *MDR1* cDNA, pMDR5A [6], was used as a probe. Total RNAs extracted from cells were transferred to GeneScreen Plus (Du Pont). Hybridizations were done at 60°C for 16 h in 1% SDS, 1 M NaCl, 10% dextran sulfate, and 200 μg/ml of denatured salmon sperm DNA. Filters were washed to a final stringency of 2×SSC and 1% SDS at 60°C. Comparable RNA loading was confirmed using a human β-actin probe [7]. Ribonuclease protection assays were done as described using probe PN [8].

2.3. *Plasmid construction and CAT assay*

A three-kilobasepair fragment containing the *MDR1* promoter region, the first exon, first intron, second exon, and a part of the second intron [8] 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 (the first ATG codon starting from the seventh nucleotide was excised) was cloned into pSV00CAT (pMP1CAT). pMP1CAT was excised from the 5′ terminal to construct pMP5CAT, pMP6CAT and pMP8CAT (Fig. 2).

10 μg of plasmid DNA was transfected into HepG2 with 5 μg of the internal control plasmid, pact-β-gal, which carries the β-galactosidase gene under the control of the chicken β-actin promoter [9], using the calcium phosphate coprecipitation method as previously described [10]. Cells were exposed to 100 μM of sodium arsenite 96 h after the transfection because DNA transfection and glycerol shock may cause some stress. Actually, when cells were exposed to sodium arsenite 48 h 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 h. Cell extracts which showed the same β-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 linearity over time.

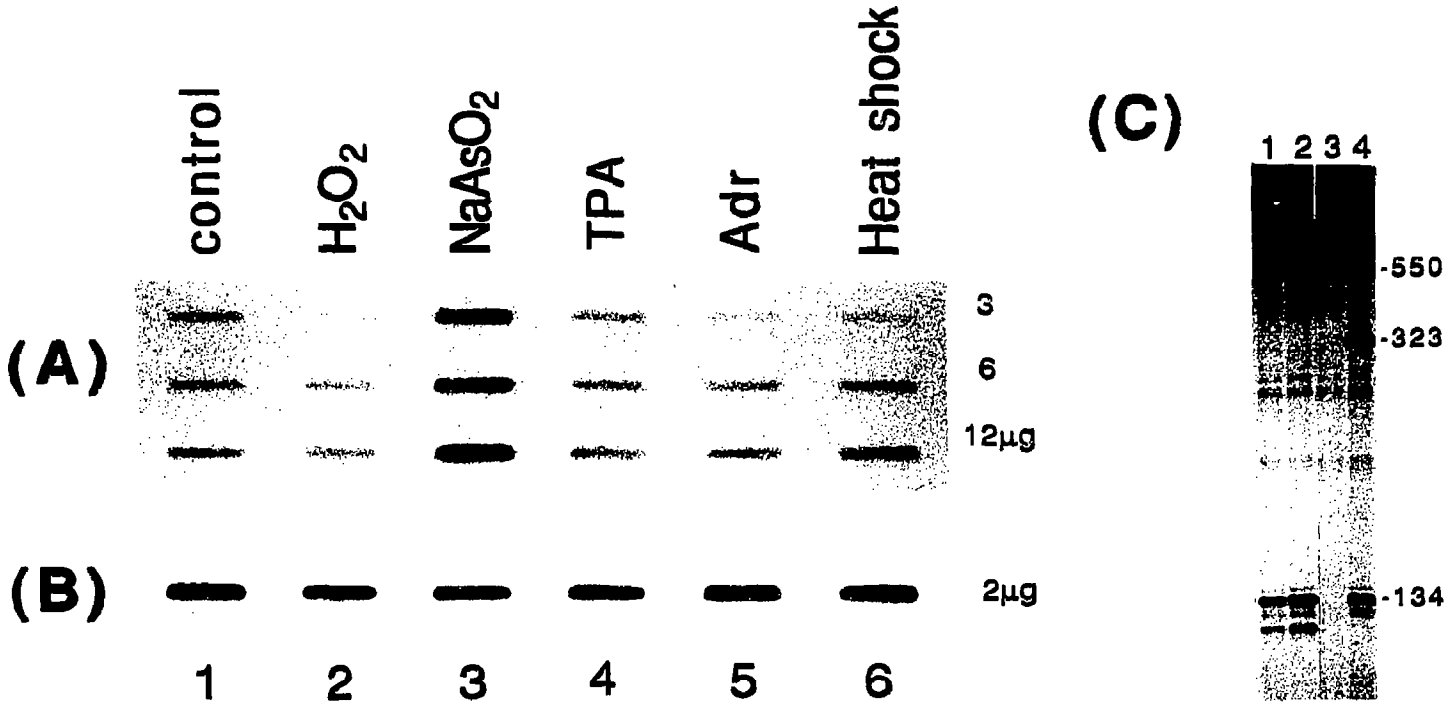

Fig. 1. Induction of *MDR1* mRNA expression in HepG2. Panel (A,B): slot blot hybridization. 12 μg of total cellular RNA and twofold serial dilutions were put on GeneScreen plus and hybridized with probe 5A as described in Materials and Methods (A). 2 μg of RNA were hybridized with a human β-actin probe as a control (B). Total RNA from HepG2 (lane 1), treated with 300 μM H_2O_2 for 1 h (lane 2), 100 μM sodium arsenite for 4 h (lane 3), 30 ng/ml TPA for 30 min (lane 4), 12.5 ng/ml adriamycin for 24 h (lane 5), heat shock at 45°C for 10 min (lane 6). Treatment with drugs were followed by recovery in fresh medium for 3 h. The heat shock was followed by recovery at 37°C for 4 h. Panel (C): ribonuclease protection assay. Total RNAs (10 μg) extracted from HepG2 before (lane 1), and after arsenite treatment at 100 μM for 4 h (lane 2), from drug sensitive KB3-1 (lane 3), and from drug-resistant KB8-5 (lane 4) were analyzed by ribonuclease protection as described in Materials and Methods. The 134-nucleotide fragment is the transcript from the major initiation site of the downstream promoter [8]. The 550-nucleotide fragment in lane 2 is considered to be the nascent transcript (splicing intermediate) from the downstream promoter. The 323-nucleotide fragment in lane 4 is the transcript from the upstream promoter [8]. The numbers on the right indicate nucleotide numbers of the protected fragments. Slot blot hybridization and ribonuclease protection assay were done twice.

3. RESULTS

3.1. *Effects of stress on MDR1 expression*

MDR1 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 [11], and the expression of the rodent *mdr* gene is elevated in chemically induced preneoplastic and neoplastic liver nodules [12,13]. To investigate whether stress and cytotoxic agents have any effects on *MDR1* expression in human liver cells, we used a well-differentiated human hepatocarcinoma cell line, HepG2. The slot blot hybridization of total RNA showed that when HepG2 was treated with sodium arsenite at 100 μM for 4 h, about a twofold increase of *MDR1* mRNA was observed (Fig. 1A, lane 3). A ribonuclease protection assay (Fig. 1C) and densitometric scanning indicated that *MDR1* mRNA increased 2,6-fold. The level of β-actin mRNA did not change after exposure to sodium arsenite (Fig. 1B). Heat shock at 45°C for 10 min followed by recovery at 37°C for 4 h had a marginal effect on the level of *MDR1* mRNA (Fig. 1A, lane 6). Heat shock at 42°C for 1 h or 2 h scarcely affected it, either (data not shown). Some stress proteins are induced by DNA-damaging agents [14], but no significant increase of *MDR1* expression was observed after exposure to hydrogen peroxide (lane 2), 4-nitroquinoline-*N*-oxide, or *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (data not shown). Adriamycin or TPA did not increase *MDR1* mRNA, either (lanes 5 and 4).

3.2. *Involvement of HSE sequences in the induction*

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 [6,8,10]. But the upstream promoter is also active in colchicine-selected KB multidrug-resistant cells and some clinical samples [10,15]. A ribonuclease protection assay using the first exon indicated that the transcript from the downstream promoter increased when HepG2 cells were treated with sodium arsenite (Fig. 1C). To identify the sequence involved in the increase of *MDR1* mRNA after exposure to sodium arsenite, CAT assay analysis was done using pMP1CAT, which contains the downstream promoter

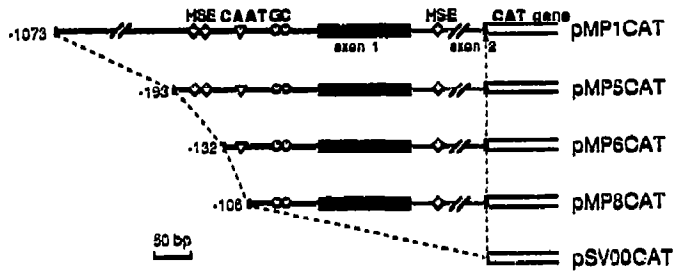

Fig. 2. Schematic representation of *MDR1* promoter deletions. pMP1CAT contains the *MDR1* genomic fragment spanning from −1073 to +703 compared to the major transcription initiation site, which consists of 1073 bp of the upstream sequence, 134 bp of the non-coding first exon, 562 bp of the first intron, and a part (6 bp) of the second exon. Open boxes indicate the CAT gene in pSV00CAT and filled boxes indicate the first exon and a part of the second exon of the *MDR1* gene. The numbers are presented with the major transcription initiation site as +1. HSE, heat-shock responsive element; CAAT, CAAT box; GC, GC box.

of the *MDR1* gene isolated from normal human tissue [8] (Fig. 2). When HepG2 cells were treated with 100 μM of sodium arsenite 96 h after the transfection of pMP1CAT, the CAT activity increased 2.3-fold (Table I). The CAT activity of an extract from cells transfected with pBLCAT2, which contains the CAT gene under the control of the *tk* promoter, did not change after exposure to arsenite. The 2.3-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 exposure to arsenite under our conditions.

A series of deletion mutants from pMP1CAT (Fig. 2) was examined by the CAT assay. Because pMP8CAT had a similar promoter activity to pMP1CAT (Table I), sequences from −1073 to −106 including HSEs and the CAAT box may not be significant for the basal promoter activity in HepG2. The promoter activity of pMP5CAT (−193 to +704) was increased 1.8-fold after exposure to arsenite, but that of pMP6CAT (−132 to +704) or pMP8CAT (−106 to +704) was not. These results suggest that the nucleotide sequence from −193 to −133, which contains a tandem repeat of HSEs (−174 CCAGAACATTCCTC −161 and −161 CCTGGAAATTCAAC −148), is necessary for the induction by arsenite treatment.

Table I

Promoter activity of deletion constructs in HepG2

	CAT activity[a]		Fold[b] increase
	−arsenite	+arsenite	
pMP1CAT	28	65	2.3 (0.59)
pMP5CAT	33	61	1.8 (0.35)
pMP6CAT	59	65	1.1 (0.27)
pMP8CAT	34	32	0.94 (0.28)
pSV00CAT	0	0	–
pBLCAT2(*tk*)	100	96	0.96 (0.16)
LSNWT(*hsp70*)	63	132	2.1 (0.45)

[a] CAT activity is the percent ratio relative to the CAT activity of pBLCAT2 without arsenite treatment. Each value is the average of at least four experiments. Plasmic carrying the β-galactosidase gene cDNA under the control of the β-actin promoter was cotransfected, and the β-galactosidase activity of cell extracts were used as an internal control.

[b] Values in parentheses are the standard deviations.

4. DISCUSSION

We have shown here that sodium arsenite treatment increases the *MDR1* mRNA expression in the hepatocarcinoma cell line HepG2. We also observed that *MDR1* gene expression was increased after exposure to sodium arsenite in HeLa and monkey CV-1 cells (data not shown). The increase of *MDR1* mRNA could be mediated by either transcriptional activation or mRNA stabilization or both. A CAT assay using pMP1CAT indicated that sodium arsenite activated the *MDR1* promoter. A nuclear run-on assay using CV-1 indicated that arsenite treatment activated the transcription of the monkey *mdr1* gene (data not shown). These results suggested that at least a part of the increase of mRNA is mediated by transcriptional activation.

Deletion analysis of the *MDR1* promoter indicated that the transcriptional activation depended on a 60-bp region (−193 to −133) containing two HSEs, one (−174 to −161) is a 7/8 match and the other (−161 to −148) a 6/8 match. In addition, the accumulation of *MDR1* mRNA after exposure to arsenite was inhibited by quercetin (Kioka, N., submitted). This result also supports the involvement of HSEs in the induction of the *MDR1* gene expression, because quercetin interacts with heat shock factor to inhibit the induction of the heat shock response (Nagata, K., personal communication).

We could not detect a significant increase of the *MDR1* gene expression in HepG2 after heat shock. Recently, Chin et al. reported that *MDR1* mRNA in two human renal carcinoma cell lines, HTB44 and HTB46, was increased by arsenite treatment or heat shock [2]. The reason that the induction of the *MDR1* gene by heat shock was detected only in these two renal carcinoma cell lines is unclear. The basal expression of the *MDR1* gene was quite low in HTB44 and HTB46 compared to KB8-5 [2]. The *MDR1* gene expression in HepG2 is comparable to that in KB8-5 (Fig. 2). Because of the substantial expression in HepG2, the induction of *MDR1* expression by heat shock might not be detected under our conditions. Alternatively the *MDR1* gene might be regulated in a cell-type specific manner.

Although the conditions under which the *MDR1* gene expression is regulated are to be examined in more detail, the *MDR1* gene is clearly induced under certain stress conditions. A twofold increase of *P*-glycoprotein expression in cancer cells is supposed to be critical in

chemotherapy. The identification of the sequence involved in the induction of *MDR1* expression will facilitate the approach to modify P-glycoprotein expression to conquer multidrug resistance.

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