Volume 326, number 1,2,3, 11–16 FEBS 12647 July 1993

Involvement of protein kinase in environmental stress-induced activation of human multidrug resistance 1 (MDR1) gene promoter

Takeshi Uchiumi[a], Kimitoshi Kohno[a], Hideyuki Tanimura[a], Katsuhiko Hidaka[b], Keizo Asakuno[a], Hiroko Abe[a], Yuzo Uchida[c] and Michihiko Kuwano[a,d]

Departments of [a]Biochemistry and [c]Surgery, Oita Medical University, Hasama-machi, Oita 879-55, Japan, [b]Department of Surgery, Saga Medical School, Nabeshima, Saga 849, Japan and *[d]Department of Biochemistry, Kyushu University School of Medicine, Maidashi, Fukuoka 812, Japan*

Received 7 April 1993; revised version received 10 May 1993

The human MDR1 gene can be induced in response to various environmental stimuli. To examine whether such stress-induced activation of the MDR1 gene can be modulated by protein kinase, we employed a stable human cancer KB cell line which contained the bacterial chloramphenicol acetyltransferase (CAT) gene directed by the MDR1 gene promoter. H-7, a protein kinase C inhibitor, at more than 40 μM inhibited activation of the MDR1 promoter that was induced by ethylmethane sulfonate, 5-fluorouracil or UV irradiation. DNA binding activity of nuclear factors recognizing the MDR1 promoter was augmented in KB cells treated with UV, but decreased in cells treated concomitantly with H-7. Okadaic acid alone was able to induce the promoter activation, and this induction was dependent on specific promoter sequences. Okadaic acid also enhanced the DNA binding activity of nuclear factors recognizing the MDR1 promoter. The phosphorylation of transacting factors may modulate the MDR1 gene promoter activity.

Multidrug resistance 1 gene; H-7; Okadaic acid; Ultraviolet light

1. INTRODUCTION

The multidrug resistance (MDR) 1 gene encodes a membrane glycoprotein of 170 kDa, termed P-glycoprotein, that is expressed in various normal tissues such as adrenal gland, colon, kidney and pregnant uterus. The MDR1 gene is often overproduced in multidrug-resistant cell lines [1,2]. The regulatory mechanisms of MDR1 gene expression in MDR cell lines appear to be different from that in normal tissue. We have previously characterized the tissue-specific enhancer, which is responsible for the expression in normal tissues but not in multidrug-resistant cell lines [3]. Expression of the MDR gene is enhanced in response to environmental stimuli, such as heat shock, hepatectomy and arsenite treatment in rodent and human cells [4–8]. In our laboratory, using transient expression systems, we demonstrated that the MDR1 promoter was activated directly by anticancer agents [9]. We then established various stable transfectants which stably expressed the bacterial chloramphenicol acetyltransferase (CAT) gene driven by the MDR1 gene promoter [10,11]. Using these cell lines, we have previously reported that the expression of the CAT gene driven by the MDR1 promoter increases in response to various environmental stimuli, such as anticancer agents, DNA damaging agents, heat shock, serum starvation and ultraviolet light (UV) irradiation [9–13]. These environmental insults cause induction of other genes in mammalian cells [14]. Under such environmental stress, transcriptional machinery for collagenase genes, HIV, and several oncogenes, is activated, and the promoters of these genes contain specific DNA sequence motifs that can be recognized by AP-1 and NF-kB [15,16]. DNA binding activity of these transcriptional factors in the nucleus is enhanced following irradiated with UV, and this UV-induced activation of relevant genes is blocked by inhibition of protein kinases. These reports suggest that protein kinases may play an important role in such stress-induced gene expression. In this study, we examined whether protein kinases were involved in MDR1 gene expression in response to environmental stimuli.

Correspondence address: K. Kohno, Department of Biochemistry, Oita Medical University, Hasama-machi, Oita 879-55, Japan. Fax: (81) (975) 491 706.

Abbreviations: MDR1, multidrug resistance 1; UV, ultraviolet light; EMS, ethyl methane sulfonate; CAT, chloramphenicol acetyltransferase; 5-FU, 5-fluorouracil; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; RT-PCR, reverse transcription-polymerase chain reaction.

2. MATERIALS AND METHODS

2.1. *Materials*

Restriction and modification enzymes were obtained from either Takara Shuzo Co. (Kyoto, Japan) or New England Biolabs. Acethyl-CoA was obtained from PL-Pharmacia. Silicagel plate (60F254) was purchased from Merk (Germany). [^{14}C]Chloramphenicol, [γ-^{32}P]ATP and [α-^{32}P]dCTP from New England Nuclear. Hybrisol I was pur-

chased from Oncor (Gaithersburg, MD). Poly (dI–dC) and poly (dA–dT) were obtained from Boehringer-Mannheim GmbH (West Germany) and Pharmacia LKB Biotechnology Inc., respectively. Bacterial CAT was purchased from PL-Pharmacia.

2.2. *Drug and chemicals*

5-Fluorouracil (5-FU) and ethylmethane sulfonate (EMS) were obtained from Sigma Chemicals (St. Louis, MO). Okadaic acid and H-7 were from Seikagaku Kogyo Co., Tokyo, Japan.

2.3. *CAT assay*

The cell monolayers were washed with sterile isotonic saline buffer and harvested in 40 mM Tris-HCl, pH 7.5, 1 mM EDTA and 150 mM NaCl. The cell pellet was resuspended and sonicated in 0.25 M Tris-HCl, pH 7.9. Each CAT assay was performed with identical amounts of protein as described before [11].

2.4. *Nuclear extract preparation and gel shift assay*

Nuclear extracts were prepared from 1×10^7–5×10^7 cells as described previously [11,12,17]. In the gel shift assay, nuclear extract (4 μg of protein) was incubated in 20 μl of reaction mixture containing 25 mM HEPES, pH 7.9, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol and 3 μg of non-specific DNA, 1 μg of each poly (dI–dC), poly (dA–dT) and *E. coli* DNA. The ^{32}P-labeled DNA fragment (1–5 ng) was added last to the reaction mixture and incubated for 30 min at 30°C. Then 2 μl of 10 × agarose dye loading buffer (50% glycerol, 0.25% Bromophenol blue, 0.25% xylene cyanol) was added at the end of incubation. The samples were subjected to electrophoresis on a 4% polyacrylamide gel (polyacrylaminde/bisacrylamide, 80:1) at 4°C with 1 × TGE (25 mM Tris-base, 190 mM glycine, 1 mM EDTA, pH 8.5) The gel was analysed directly by autoradiography.

2.5. *Cell culture and transfection*

KB cells were grown in modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics [9,18]. Reporter plasmids were constructed as described previously [3,11]. KB cells were transfected using Lipofectin (BRL, Bethesda, MD, USA), then a mixture of reporter plasmid (10 μg) and pRSV-neo (0.5 μg) were added. After 8 h, the medium containing DNA and Lipofectin was replaced with fresh medium. After overnight incubation with medium, the cells were incubated in selection medium containing 0.6 mg/ml Geneticin (G418) (Gibco). Growing colonies (20–30 per 10^6 cell) were cloned, expanded and tested for CAT activity. Cloned transfectants were grown in modified Eagle's medium supplemented with 10% fetal calf serum, antibiotics and 0.6 mg/ml Geneticin (G418, Gibco).

3. RESULTS

3.1. *Effect of H-7, a protein kinase C (PKC) inhibitor, on MDR1 gene promoter activity*

To examine how the MDR1 gene promoter is regulated in correlation with protein kinase activity, we employed Kac-7, a human cancer KB cell line which was stably transfected with a MDR1 promoter-driven CAT gene construct, pMDRCAT5 (Fig. 1). On this promoter, two specific regions, up-stream at −258 to −198 and down-stream at −136 to −76, are essential for promoter activation in response to serum starvation, heat-shock stress and UV irradiation [11–13]: MDR-Nf1 recognizing −136 to −76 bp responds to heat-shock stress or UV irradiation, and MDR-Nf2 recognizing −258 to −198 responds to serum starvation [10] (see Fig. 1).

As seen in Fig. 2A, treatment of Kac-7cells with UV light induced CAT activity, consistent with our previous report [11,19]. Co-administration of H-7, a protein kinase C inhibitor [20], at 40 μM almost completely inhibited the UV-induced activation of CAT activity, but H-7 at 10 and 20 μM did not. H-7 at 40 μM alone did not affect the CAT activity. We have demonstrated that the sequences between the −136 to −76 bp promoter region were sufficient to induce CAT activity by UV irradiation [11]. We examined the effect of H-7 on UV irradiation-induced CAT activity by using Kxh-14 and Khp-2, which were transfected with pMDRCAT7 and pMDRCAT8, respectively (Fig. 2B). Consistent with our previous data, UV irradiation enhanced CAT activity of Kxh-14 but not that of Khp-2. Co-administration of H-7 at 40 μM inhibited the UV-induced activation of CAT activity of Kxh-14. H-7 alone did not affect the basal CAT activity of either cell line. We have demonstrated that a wide variety of cytotoxic agents increase CAT activity in a dose-dependent manner in Kac-7 cells, including 5-FU, EMS and UV irradiation [11]. Consistent with our previous reports, 5-FU, EMS and UV light were found to enhance CAT activity (Fig. 2C). We then examined whether H-7 could affect the activation of the MDR1 promoter by 5-FU, EMS or UV irradiation. H-7 at 40 μM inhibited the CAT activity induced by 5-FU and EMS, as well as UV (Fig. 2B).

3.2. *Nuclear factors binding to the MDR1 promoter sequence when cells were treated with UV and H-7*

It has been previously demonstrated that one major shifted band with the MDR1 promoter region was augmented in nuclear extract from KB cells treated with

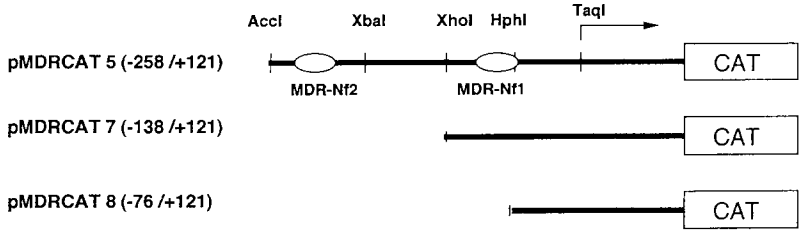

Fig. 1. The deletion constructs of the MDR1 promoter–CAT hybrid plasmid. The arrow indicates the position of the transcription initiation site. Relevant restriction enzyme cleavage sites are indicated on top. MDR-Nf1 and MDR-Nf2 are the names of the proteins that bound to the indicated regions (see [10]).

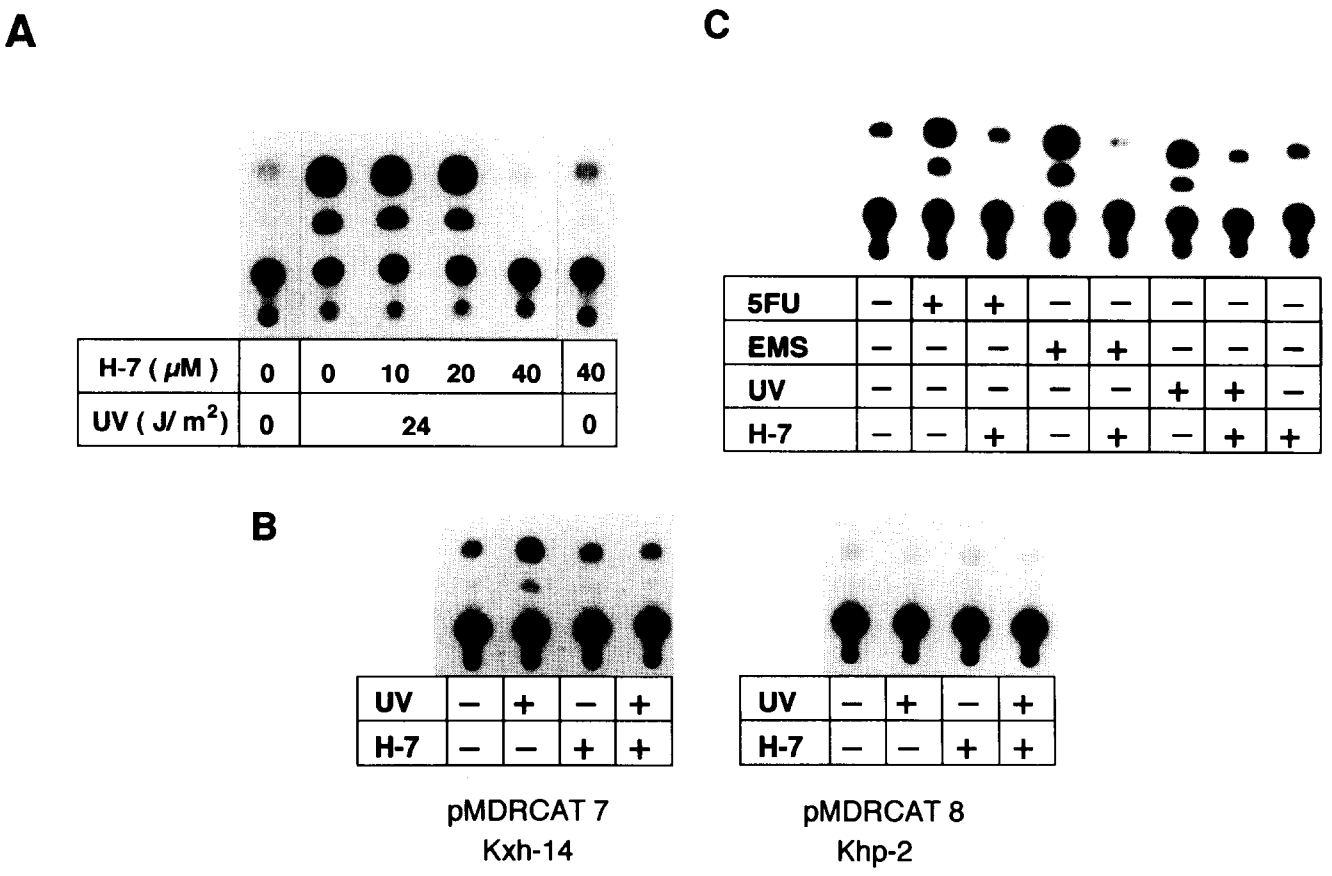

Fig. 2. (A) Effect of H-7 on the activation of the MDR1 promoter. Kac-7 cells were treated with 24 J/m^2 of UV irradiation in the presence of indicated doses of H-7 for 48 h, and equal amounts of cytoplasmic fractions were assayed as described in section 2. (B) Effect of H-7 on the activation of the MDR1 promoter in two stable cell lines. Kxh-14 and Khp-2 cells were treated with 24 J/m^2 of UV irradiation in the presence (+) or absence (−) of 40 μM of H-7 for 48h and assayed. (C) Effect of H-7 on the activation of the MDR1 promoter in response to 5-FU, EMS or UV irradiation. Kac-7 cells were treated with 24 J/m^2 of UV irradiation, 80 μg/ml of 5-FU or 1 mg/ml of EMS in the presence of 40 μM of H-7, as indicated.

anticancer agents and various DNA damaging agents [10,11]. Gel shift analysis and methylation interference analysis of the MDR1 promoter showed that a DNA sequence between −99 and −62, especially the inverted CCAAT box 5′-CTGATTGG-3′, was identified as a specific binding site for MDR-Nf1 (see Fig. 1). When incubated with nuclear extracts of KB cells treated with UV irradiation, apparent complex formation of the MDR1 promoter was remarkably increased [11,21]. Using this oligonucleotide, we performed gel shift assays (Fig. 3). Nuclear extracts were prepared from KB cells that had been treated for the specified periods of time with 24 J/m^2 of UV, in the absence or presence of 40 μM H-7. DNA binding of a protein, MDR-Nf1, increased when KB cells were treated with UV irradiation (Fig. 3). By contrast, DNA binding decreased when UV-irradiated cells were treated with H-7. These data suggest that H-7 inhibits the UV-induced activation of the MDR1 promoter by decreased binding of MDR-Nf1.

3.3. *Effect of okadaic acid on MDR1 promoter activation*

H-7 at 40 μM was found to inhibit UV- or drug-induced MDR1 promoter activation (Fig. 2), but H-7 inhibits other protein kinases as well as protein kinase C [20]. In further studies we used other modulators of protein kinases, namely okadaic acid and phorbol ester (TPA). Okadaic acid inhibits phosphatase, resulting in modulation of various cellular signal transduction [22,23], and TPA is rather a specific activator of protein kinase C [24]. Okadaic acid at 9–12 ng/ml reduced the cell-surviving fraction of Kac-7 cells to about 50% of the initial fraction (data not shown). Okadaic acid at 9–12 ng/ml enhanced CAT activity (Fig. 4). Fig. 4 shows the induction of CAT activity in the two independent transfectants of each construct, pMDRCAT5, pMDRCAT7 and pMDRCAT8, after exposure to various doses of okadaic acid. Okadaic acid at more than 6 ng/ml enhanced CAT activity of Kac-7, Kac-12, Kxh-14 and Kxh-28, but not that of Khp-2 and Khp-6. The se-

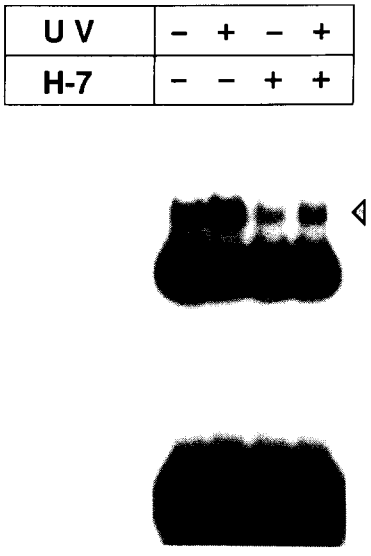

Fig. 3. Analysis of the transacting nuclear factor binding to the MDR1 promoter. KB cells were treated with 24 J/m^2 of UV irradiation in the absence or presence of 40 μM H-7. The nuclear extracts were further incubated with the labeled oligonucleotides (−99 to ~−68) and separated by gel electrophoresis. The arrowhead indicates a band corresponding to MDR-Nf1.

quences between −136 to −76 bp in the promoter region were sufficient to induce CAT activity by okadaic acid. By contrast, TPA alone at 100 μM did not induce CAT activity, and co-administration of TPA with EMS or UV irradiation also did not inhibit the MDR1 promoter activation by EMS or UV irradiation (data not shown).

3.4. *Nuclear factors binding to the MDR1 promoter sequence when cells were treated with okadaic acid*

To characterize transacting factors for the MDR1 promoter, gel shift assays were performed using the nuclear fractions from KB cells treated with okadaic acid. The nuclear extract treated with okadaic acid was found to bind to the oligonucleotide between −99 and −62. This sequence between −99 and −62 bp contains an inverted CCAAT box [11,21]. To determine the binding specificity to the oligonucleotides, extracts were prepared from cells harvested at 0, 6 and 12 h after exposure to okadaic acid (Fig. 5). This complex was significantly increased with time when incubated with nuclear extract after exposure. These results suggest that increased binding might be involved in the induction of MDR1 promoter-driven CAT activity by okadaic acid.

4. DISCUSSION

We have previously demonstrated that the MDR1 gene promoter is activated in response to various environmental stimuli, such as many anticancer agents [9,25], serum starvation [13], heat-shock stress [12], and UV irradiation [11]. These studies propose the hypothesis that the MDR1 gene is a stress-inducible SOS gene

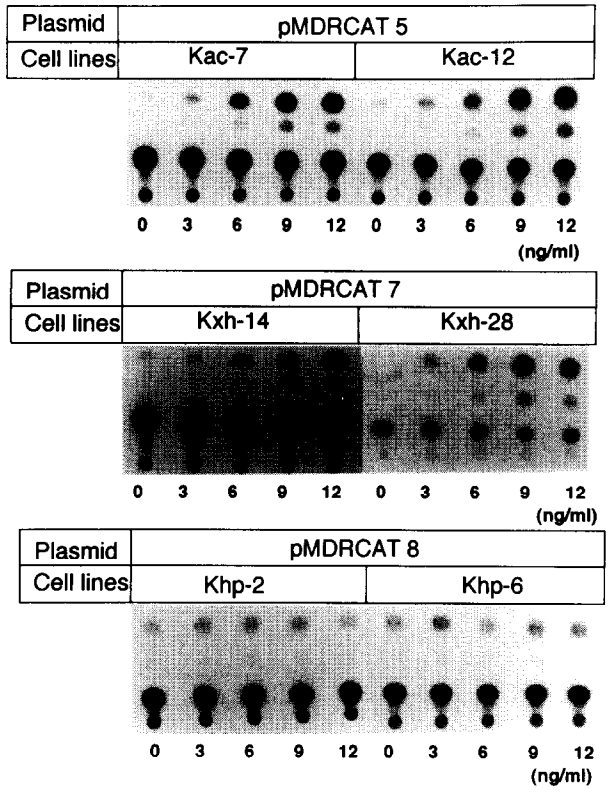

Fig. 4. Induction of CAT by okadaic acid in various stable cell lines (see Fig. 1). Two independent stable cell lines for each MDR1 promoter–CAT construct were treated with the indicated doses of okadaic acid. Autoradiograms of CAT assays are shown.

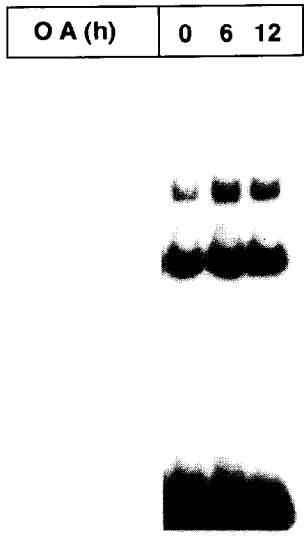

Fig. 5. Analysis of transacting nuclear factor binding to the MDR1 promoter. Nuclear extracts from KB cells treated with or without 12 ng/ml of okadaic acid for the indicated periods. The nuclear extracts were further incubated with the labeled oligonucleotides (−99 to ~−68) and separated by gel electrophoresis. The arrow head indicates the specific major retarded DNA–protein complexes.

[10,19]. This activation appears to be mediated through either the growth arrest-responsible element (GARE) at position −256 to −198 bp, or the drug-responsible element (DRE) at position −136 to −76 bp, on the MDR1 gene promoter, and also through two DNA binding proteins, MDR-Nf2 and MDR-Nf1 (see Fig. 1) [10,21,26]. GARE at position −256 to −198 is regulated negatively by serum starvation [13], and DRE at position −136 to −76 bp, containing an inverted CCAAT box, is regulated positively by anticancer agents, heat-shock stress and UV irradiation [11,12,25]. Okadaic acid-induced MDR1 promoter activation appears to be mediated through DRE (Fig. 4). H-7 inhibits the binding of nuclear protein, probably MDR-Nf1, to the sequence between −99 and −62 bp containing the inverted CCAAT motif (Fig. 3). H-7 inhibits the activation of the MDR1 promoter by drugs or UV irradiation through DRE.

In this study, we employed a human cancer KB cell line which had been permanently transfected with a chimeric construct containing the CAT gene driven by the regulatory region of the MDR1 gene [11,19]. This cell line provided very sensitive probes to evaluate the function of specific DNA sequences of the MDR1 promoter in response to environmental stimuli. Endogenous MDR mRNA levels are often increased in one human kidney cancer cell line or one human liver cancer cell line after heat shock or arsenite treatment [7,27]. In our human cancer KB cell line, we were unable to detect any apparent increase of endogenous MDR1 mRNA by Northern blot analysis after heat shock or drug treatment. RT-PCR analysis, however, demonstrated an apparent increase in MDR1 mRNA levels in KB cells under various environmental stimuli, including UV irradiation and various anticancer agents ([28] and Kohno and Uchiumi, unpublished data). These results again support the hypothesis that the endogenous MDR1 gene is a stress-induced SOS gene.

A high dose (40 μM) of H-7 blocked activation of the MDR1 promoter-driven CAT gene by drugs or UV irradiation. H-7 is known as a protein kinase C inhibitor, but it also inhibits other protein kinases [20]. H-7 also blocks induction of several other genes such as HIV, collagenase and *gadd*45 [29]. One could ask whether protein kinase C or other protein kinases might be involved in the stress-induced activation of the MDR1 promoter. A phosphatase inhibitor, okadaic acid, itself activates the MDR1 promoter (Fig. 4), suggesting an involvement of protein kinases in the activation pathway. TPA, a specific protein kinase C activator, however, could not mimic the stress-induced activation of the MDR1 promoter by EMS or UV irradiation. The MDR1 promoter has no TPA-responsible element [3], and our present results favor the idea that other unknown protein kinases rather than protein kinase C are likely to be involved in the stress-induced activation of the MDR1 promoter. Two DNA binding proteins, MDR-Nf1 and MDR-Nf2, are involved in the stress-induced activation of the MDR1 promoter [10,21,26]. H-7 inhibits the binding of MDR-Nf1 to the promoter, and okadaic acid enhances the binding of MDR-Nf1 (Figs. 3 and 5). A transacting factor, such as MDR-Nf1, might be modulated through the phosphorylation state by protein kinases. A relevant study has proposed that oncogene families, such as *jun*, *rel*, and *fos*, encode nuclear proteins that are found to be associated in a number of transcriptional complexes [30]. These proteins are usually located in the cytosol with their inhibitors, and are activated in response to environmental stress through protein kinase-mediated signal transduction [30]. The phosphorylation state of these proteins determine the expression of various genes. Among these proteins, AP-1 and NF-kB have been well characterized, and their expression of DNA binding activity was induced by several environmental stimuli, such as DNA damaging agents, H_2O_2, cytokines and okadaic acid [31]. There appears to be no such motifs in the MDR1 gene promoter, however [3]. Further study should determine how such transacting factors for the MDR1 promoter are modulated through protein kinase-induced phosphorylation in order to understand the underlying signaling for the SOS-induced promoter activation of the MDR1 gene.

Acknowledgments: This study was supported by a grant-in-aid for cancer research from the Ministry of Education, Science, and Culture of Japan.

REFERENCES

[1] Bradley, G., Juranka, P.F. and Ling, V. (1988) Biochim. Biophys. Acta. 948, 87–128.
[2] Gottesman, M.M. and Pastan, I. (1988) J. Biol. Chem. 263, 12163–12166.
[3] Kohno, K., Sato, S., Uchiumi, T., Takano, H., Kato, S. and Kuwano, M. (1990) J. Biol. Chem. 265, 19690–19696.
[4] Chin, K.V., Chauhan, S.S., Pastan, I. and Gottesman, M.M. (1990) Cell Growth Differ. 1, 361–365.
[5] Marino, P.A., Gottesman, M.M. and Pastan, I. (1990) Cell Growth Differ. 1, 57–62.
[6] Mickley, L.A., Bates, S.E., Richert, N.D., Currier, S., Tanaka, S., Foss, F., Rosen, N. and Fojo, A.T. (1989) J. Biol. Chem. 264, 18031–18040.
[7] Kioka, N., Yamano, Y., Komano, T. and Ueda, K. (1992) FEBS Lett. 301, 37–40.
[8] Kioka, N., Hosokawa, N., Komano, T., Hirayoshi, K., Nagata, K. and Ueda, K. (1992) FEBS Lett. 301, 307–309.
[9] Kohno, K., Sato, S., Takano, H., Matsuo, K. and Kuwano, M. (1989) Biochem. Biophys. Res. Commun. 165, 1415–1421.
[10] Kohno, K. and Kuwano, M. (1993) in: The Mechanism and New Approch on Drug-resistance of Cancer Cells (Miyazaki, T. eds.) Elseviver, Amsterdam (in press).
[11] Uchiumi, T., Kohno, K., Tanimura, H., Matsuo, K., Sato, S., Uchida, U. and Kuwano, M. (1993) Cell Growth Differ. 4, 147–157.
[12] Miyazaki, M., Kohno, K., Uchiumi, T., Tanimura, H., Matsuo, K., Nasu, M. and Kuwano, M. (1992) Biochem. Biophys. Res. Commun. 187, 677–684.
[13] Tanimura, H., Kohno, K., Sato, S., Uchiumi, T., Miyazaki, M.,

Kobayashi, M. and Kuwano, M. (1992) Biochem. Biophys. Res. Commun. 183, 917–924.
[14] Mai, S., Stein, B., van, d.B.S., Kaina, B., Lucke, H.C., Ponta, H., Rahmsdorf, H.J., Kraemer, M., Gebel, S. and Herrlich, P. (1989) J. Cell Sci. 94, 609–615.
[15] Stein, B., Rahmsdorf, H.J., Steffen, A., Litfin, M. and Herrlich, P. (1989) Mol. Cell. Biol. 9, 5169–5181.
[16] Devary, Y., Gottlieb, R.A., Lau, L.F. and Karin, M. (1991) Mol.Cell.Biol. 11, 2804–2811.
[17] Hamanaka, R., Kohno,K., Seguchi, T., Okamura, K., Ono,M., Ogata, J. and Kuwano, M. (1992) J. Biol. Chem. 267, 13160–13165
[18] Takano, H., Kohno, K., Ono, M., Uchida, Y. and Kuwano, M. (1991) Cancer Res. 51, 3951–3957.
[19] Kohno, K., Takano, H., Matsuo, K., Kiue, A., Sato, S., Uchiumi, T., Miyazaki, M., Tanimura, H., Sato, S. and Kuwano, M. (1992) in: Molecular Biology of DNA Topoisomerases (Andoh, T., Ikeda, H. and Oguro, H. eds.) pp. 247–256, CRC Press.
[20] Hidaka, H., Inagaki, M., Kawamoto, S. and Sasaki, Y. (1984) Biochemistry 23, 5036–5041.
[21] Uchiumi, T., Kohno, K., Tanimura, H., Matsuo, K., Sato, S., Asakuno, K., Abe, H. and Kuwano, M. (1993) in: The Mechanism and New Approch on Drug-resistance of Cancer Cells (Miyazaki, T. eds.) Elseviver, Amsterdam (in press).
[22] Bialojan, C. and Takai, A. (1988) Biochem. J. 256, 283–290.
[23] Holladay, K., Fujiki, H. and Bowden, G.T. (1992) Mol. Carcinogen. 5, 16–24.
[24] Nishizuka, Y. (1984) Nature 308, 693–698
[25] Kohno, K., Sato, S., Uchiumi, T., Takano, H., Tanimura, H., Miyazaki, M., Matsuo, K., Hidaka, K. and Kuwano, M. (1992) Int. J. Oncol. 1, 73–77.
[26] Tanimura, H., Kohno, K., Uchiumi, T., Matsuo, K., Sato, S., Asakuno, K., Abe, H. and Kuwano, M. (1993) in: The Mechanism and New Approch on Drug-resistance of Cancer Cells (Miyazaki, T. eds.) Elseviver, Amsterdam (in press).
[27] Chin, K.V., Tanaka, S., Darlington, G., Pastan, I. and Gottesman, M.M. (1990) J. Biol. Chem. 265, 221–226.
[28] Chaudhary, P.M. and Roninson, I.B. (1993) J. Natl. Cancer Inst. (in press).
[29] Papathanasiou, M.A., Kerr, N.C., Robbins, J.H., McBride, O.W., Alamo, I.J., Barrett, S.F., Hickson, I.D. and Fornace, A.J. (1991) Mol. Cell. Biol. 11, 1009–1016.
[30] Hunter, T. and Karin, M. (1992) Cell 70, 375–387.
[31] Mahadevan, L.C., Willis, A.C. and Barratt, M.J. (1991) Cell 65,