SPANDIDOS PUBLICATIONS

Direct activation of the human major vault protein gene by DNA-damaging agents

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Abstract. Vaults are barrel-shaped cytoplasmic ribonucleoprotein particles composed of three proteins. One of the components, the major vault protein (MVP) initially named the lung resistance-related protein (LRP), was found to be overexpressed in various multidrug resistant cancer cell lines and clinical samples. In this study, we investigated whether anticancer drugs could directly induce MVP protein or gene expression in the SW-620 human colorectal cancer cell line, in which MVP has been shown to be induced by the differentiation-inducing agent, sodium butyrate (NaB). MVP protein levels were enhanced in SW-620 cells after a 72 h treatment with doxorubicin (Adr), etoposide (VP-16), *cis*-platinum (II) diammine dichloride (CDDP) or SN-38, but not vincristine (VCR) or paclitaxel (Taxol) at their IC_{50} concentration. Treatment for 48 h with Adr, VP-16 and SN-38 at their IC_{50} concentration also enhanced the expression of MVP mRNA. Moreover, Adr could directly enhance the transcriptional activity of MVP promoter regions. On the other hand, the Adr treatment did not affect the stability of MVP mRNA. Furthermore, MVP levels were also elevated after treatment with the DNA-damaging agents, ethidium bromide (EtBr) and ultraviolet light (UV) irradiation. Our findings therefore suggest that DNA damage enhances MVP promoter activity. Since the MVP protein and mRNA have low turnover rates, a slight enhancement of MVP promoter activity could lead to a considerable increase in the level of MVP.

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Abbreviations: MDR, multidrug resistance; P-gp, P-glycoprotein; LRP, lung resistance-related protein; MVP, major vault protein; VPARP, vault poly(ADP-ribose) polymerase; TEP1, telomerase-associated protein; Adr, doxorubicin; VP-16, etoposide; VCR, vincristine; CDDP, *cis*-platinum (II) diammine dichloride; Taxol, paclitaxel; YB-1, Y-box binding protein; 5-FU, 5-fluorouracil; NaB, sodium butyrate; TSP-1, thrombospondin-1; EtBr, ethidium bromide; UV, ultraviolet light; BRCT, BRCA 1 C-terminus domain

Key words: major vault protein/lung resistance-related protein, multidrug resistance, anticancer agent, DNA damage, gene expression

Introduction

Multidrug resistance (MDR) in cancer cells is a major obstacle to successful chemotherapy. Some drug transporter proteins, such as P-glycoprotein (P-gp) encoded by *MDR1* and multidrug resistance protein-1, have been found to be associated with MDR. Additional mechanisms have also been implicated in the MDR phenotype of cancer cells. One non-transporter protein that may play a role in MDR is the 110-kDa protein frequently overexpressed in P-glycoprotein-negative multidrug-resistant tumor cell lines, initially termed the lung resistance-related protein (LRP) (1). LRP is now known to be identical to a human protein termed the major vault protein (MVP) (2). Vaults are predominantly cytoplasmic ribonucleoprotein particles originally identified in preparations of coated vesicles (3), and conserved throughout evolution and found in numerous eukaryotic species (4). The vault complex has a barrel-like structure with an invaginated waist and two protruding caps. Mammalian vaults are composed of MVP and two minor vault proteins [vault poly(ADP-ribose) polymerase (VPARP), and telomerase-associated protein (TEP1)], as well as small untranslated RNAs (5-7). The precise cellular function of the vault complex is unknown, although its structural features suggest a role in intracellular transport processes (8).

Several lines of evidence suggest a role for MVP in modulating drug resistance. MVP, as well as the vault particle, are overexpressed in drug-resistant cell lines (9,10). Furthermore, MVP levels are enhanced in cancer cell lines selected with the anticancer drugs doxorubicin (Adr), mitoxantrone, etoposide (VP-16), vincristine (VCR), cytarabine, *cis*-platinum (II) diammine dichloride (CDDP) and bleomycin (11-16). The human *MDR1* promoter can be directly activated by various anticancer agents (17). DNA sequences that contain an inverted CCAAT box and a Y-box constitute *cis*-regulatory elements common to various eukaryotic genes (18). The Y-box binding protein (YB-1) is a member of the cold-shock domain protein family. Most of

the YB-1 molecules are localized in the cytoplasm and translocated to the nuclei when cells are exposed to various stresses, such as anticancer agents, hyperthermia or ultraviolet light (UV) (19-21). A Y-box resides in the promoter region of the *MDR1* gene (22), and the YB-1 protein regulates the activity of the *MDR1* gene (22,23). A Y-box is also present in the human *MVP* gene promoter (24), and YB-1 facilitates basal and 5-fluorouracil (5-FU)-inducible expression of the human *MVP* gene (25).

In the present study, we investigated whether anticancer drugs can induce MVP gene expression in the human colorectal cancer cell line, SW-620, in which MVP can be induced by the differentiation-inducing agent sodium butyrate (NaB) (26). We further investigated potential mechanisms by which anticancer drugs modulate MVP gene expression.

Materials and methods

Materials. RPMI-1640 medium was purchased from Nissui Seiyaku Co. (Tokyo, Japan), fetal calf serum (FCS) was from JRH Biosciences (Lenexa, KS), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Adr, VP-16, CDDP, VCR, paclitaxel (Taxol) and NaB were obtained from Sigma Chemical Co. (St. Louis, MO). SN-38 was kindly provided by Daiichi Pharmaceutical Co. Ltd. and Yakult Pharmaceutical Ind. Co. Ltd. (Tokyo, Japan). The α-amanitin was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Cell culture and cell survival analyses. SW-620, a human colon carcinoma cell line, was grown in RPMI-1640 medium containing 10% FCS in a humidified atmosphere of 5% CO_2 at 37˚C. Sensitivity of the cells to the various agents tested was analyzed by determining cell survival using the MTT colorimetric assay as previously described (27). Briefly, $1x10^4$ SW-620 cells in 180 μl of culture medium were inoculated into each well of a 96-well plate. After an overnight incubation at 37˚C, 20 μl of anticancer drugs at various concentrations were added to each well, and the cultures were incubated for 3 days at 37˚C in 5% CO_2. Thereafter, 50 μl of MTT (2.5 mg/ml PBS) was added to each well, and the cultures were incubated for an additional 4 h. The resulting formazan was dissolved with 100 μl of dimethylsulfoxide after aspiration of the culture medium. Absorbance at 570 nm was measured using a model 550 microplate reader (Bio-Rad, Richmond, CA).

Preparation of total cell lysates and immunoblot analysis of MVP. SW-620 cells were treated with anticancer drugs, ethidium bromide or UV under the indicated conditions. Total cell lysates were separated by 6% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and the expression level of MVP was analyzed by immunoblot analysis using an anti-MVP polyclonal antibody (26,28).

Real-time reverse-transcription PCR quantification. SW-620 cells were plated ($2x10^6$/dish) in 100-mm plastic dishes and treated with or without anticancer drugs at their IC_{50} concentration (the concentration that inhibits cell growth by 50%) for 48 h. Total RNA from the cultured cells was isolated using the Trizol reagent (Invitrogen, Carlsbad, CA) and cDNA was synthesized from 1 μg of total RNA with ReverTra Ace α (Toyobo, Osaka, Japan) using a random primer according to

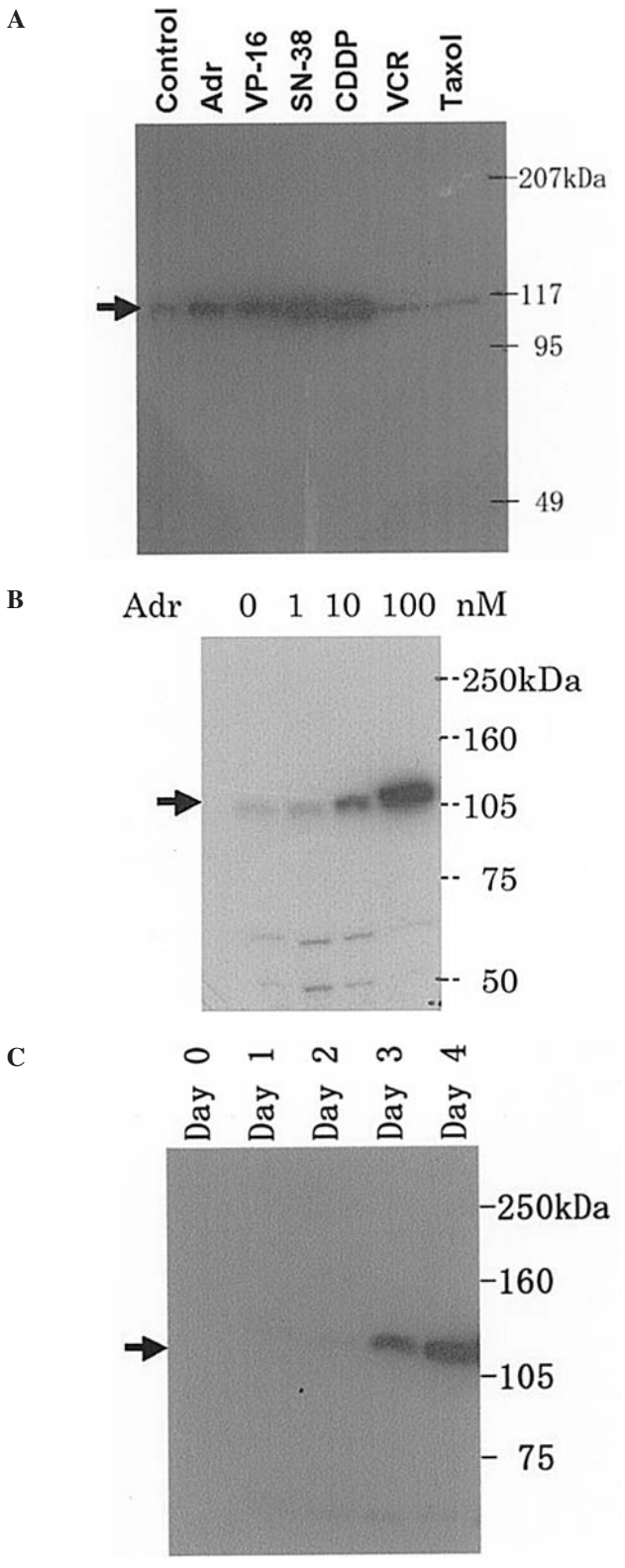

Figure 1. Regulation of MVP protein expression by anticancer agents. MVP protein expression in SW-620 cells was assayed by immunoblot analysis following 72 h continuous exposure to the anticancer agents Adr, VP-16, SN-38, CDDP, VCR or Taxol at their IC_{50} concentration (A); 72 h continuous exposure to 1, 10, and 100 nM Adr (B); or exposure to Adr at its IC_{50} concentration over 4 days (C). MVP is indicated by an arrow at left, and molecular weight markers are on the right.

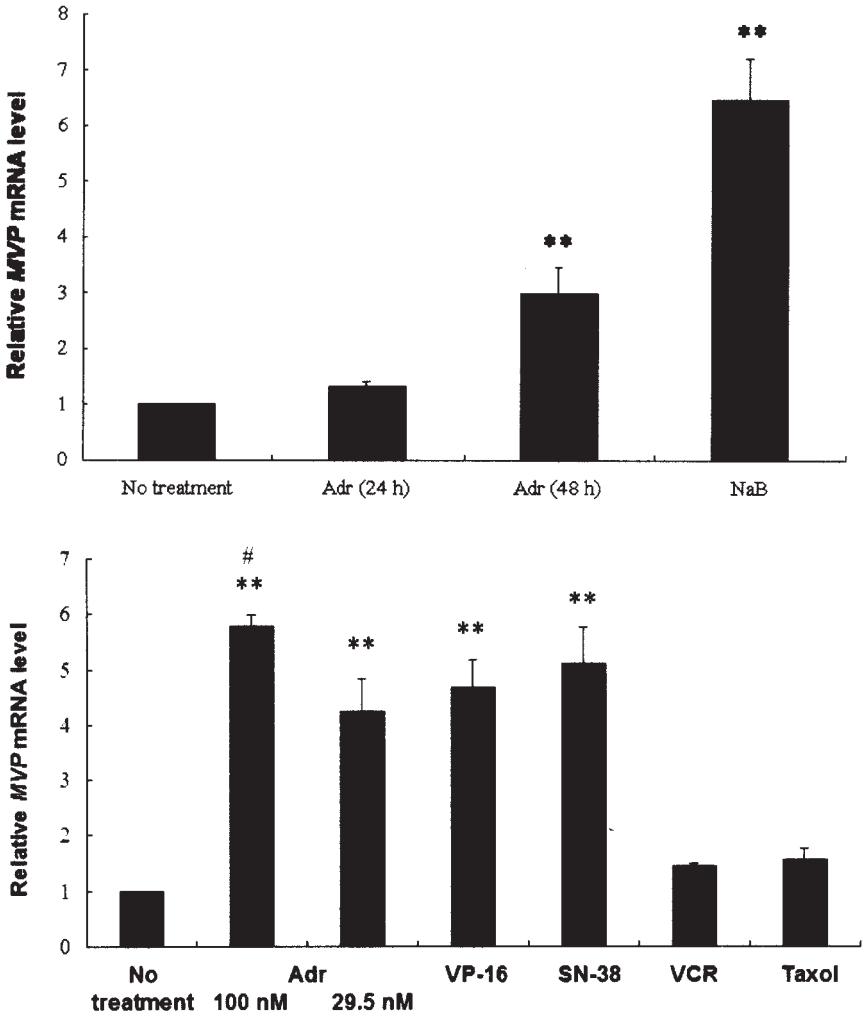

Figure 2. *MVP* mRNA levels are enhanced by anticancer agents. The effect of anticancer drugs on *MVP* mRNA levels was assayed by real-time PCR analyses in SW-620 cells. Relative expression levels of *MVP* mRNA were normalized to *GAPDH* mRNA expression. (A) Cells were cultured with or without 100 nM Adr for 24 or 48 h, or with 2 mM NaB for 24 h as a positive control. (B) Cells were continuously incubated for 48 h with or without 100 nM Adr, or with Adr, VP-16, SN-38, VCR or Taxol at their IC_{50} concentration. Each column and bar represents the mean ± standard deviations (error bars) of three independent experiments. $^{**}p<0.01$ versus no treatment. $^{\#}p<0.05$ versus treatment with Adr at IC_{50}.

the manufacturer's protocol. MVP gene expression levels were assayed by real-time PCR (Prism 7900HT; Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The primers and TaqMan probe used were: forward, 5'-GCGCGCTGTGATTGGAA-3'; reverse, 5'-CCGGGAGGCAGCTCTTTC-3'; and TaqMan probe, 5'-FAM-CATGCTGACCCAGGACGAAGTCCTGT-Tamra-3', which were designed with the primer design software Primer Express version 1.5 (Applied Biosystems). Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization. Relative quantification of GAPDH and MVP mRNA was calculated using a comparative cycle threshold method. Triplicate samples were used for each data point. Data was quantified with a standard curve method (29).

Cloning of the 5'-flanking region of the human MVP promoter and reporter gene constructs. Human MVP promoters expressing a series of 5' deletions ranging from -407, -315, -263, -102 or -78 to +66 were amplified from the genomic DNA of SW-620 cells by PCR with Ex Taq (Takara Bio, Ohtsu, Japan). The upstream PCR forward primers incorporated a 5'-flanking *Kpn*I restriction site (underlined), and the sequences of the primers were 5'-CCGGTACCT GGACTGAATTTTCTGAG-3'; 5'-AGGTACCACTTGAAG GGAGAGGCAGAAT-3'; 5'-TGGTACCAGATGGGTAGA GGTGCCCTCA-3'; 5'-TAGGTACCTGAGAACCAGCTG CACA-3' and 5'-ATGGTACCTTTAACTCCCAAGCCCCA-3' for promoter constructs spanning -407/+66, -315/+66, -263/+66, -102/+66 and -78/+66, respectively. The downstream reverse primer, flanked by the *Sac*I site (underlined), was 5'-AGAGCTCGGGGCTAGTACAATGTACTCACA-3'. The PCR products were cloned into pT7Blue-2 vector (Merck Biosciences, San Diego, CA), then sequenced. Following digestion with *Kpn*I and *Sac*I, 472 bp, 380 bp, 328 bp, 167 bp and 143 bp MVP promoter fragments were gel-purified and ligated into the *Kpn*I and *Sac*I-linearized pGL3-basic vector (Promega, Madison, WI). The resulting constructs were designated pMVP407, pMVP315, pMVP263, pMVP102 and pMVP78, respectively.

Transfection and luciferase assay. Transient transfection of SW-620 cells with a luciferase reporter plasmid was carried

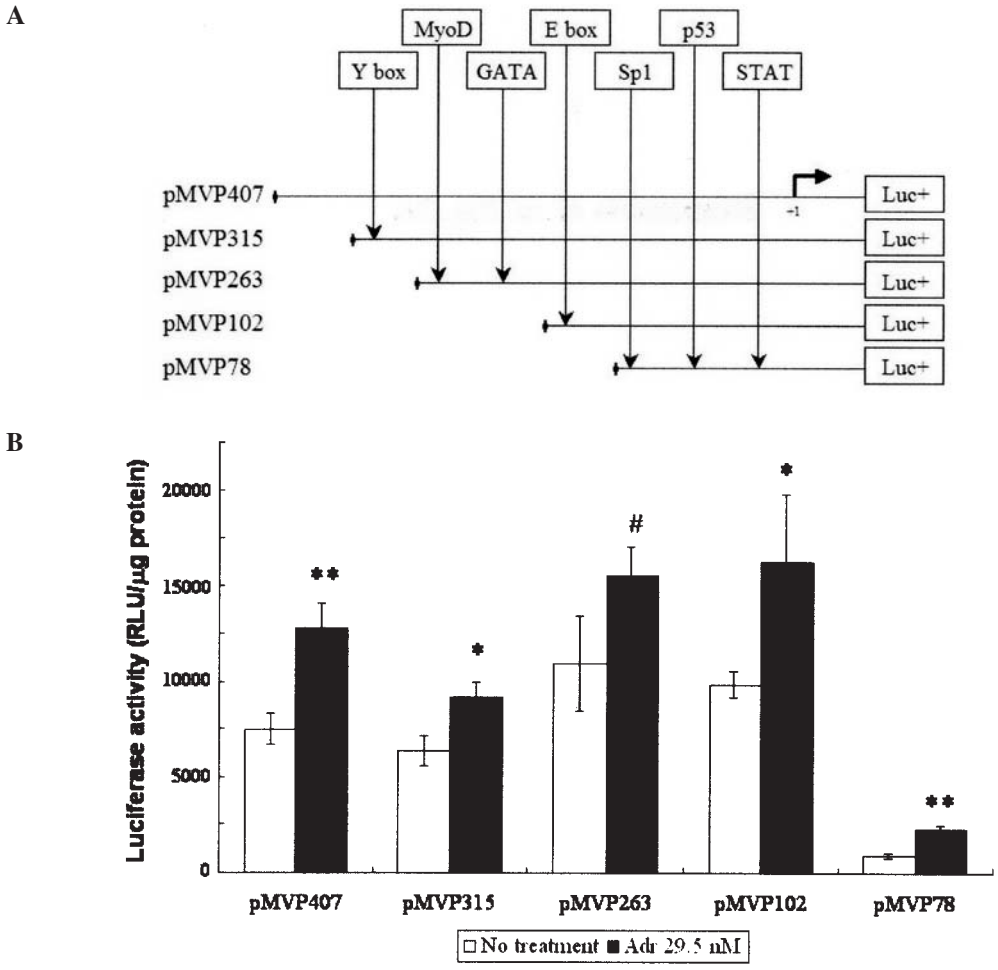

Figure 3. The role of MVP promoter elements for Adr modulation of *MVP* promoter activity. Transcriptional activity was assayed using luciferase (luc+)-based reporter constructs bearing various deletions of the *MVP* promoter region. A schematic representation of the MVP promoter deletion constructs used in this study is shown (A). SW-620 cells, which were transiently transfected with these constructs using lipofectamine reagent, were harvested after 48 h continuous exposure to Adr at its IC_{50} concentration, and their luciferase activities were measured (B). Data were normalized to the protein concentration. Each column and bar represents the mean ± standard errors of three independent experiments. The symbols represent a comparison of the data versus untreated basal luciferase activity measured for each deletion construct ($^{*}p<0.05$, $^{**}p<0.01$ or $^{\#}p=0.0537$).

out using lipofectamine reagent (Invitrogen) according to the manufacturer's protocol. Luciferase assays were performed 48 h after transfection. The determined protein concentration was used for the normalization of luciferase activity.

Measurement of mRNA stability. Cells were plated at a density of $2x10^6$ cells in 6-well plates and incubated in medium without serum for 24 h after which the medium was removed and replaced with medium containing serum. After 4 h incubation, medium was removed and replaced with serum-free medium containing α-amanitin (5 μg/ml) (30) with or without Adr at its IC_{50} concentration. Total RNA was isolated as described above at the indicated times after treatment with α-amanitin. Subsequently, real-time reverse-transcription PCR quantification was performed as mentioned above. Expression levels of *thrombospondin-1* (*TSP-1*) mRNA were used as positive controls. Experiments were performed in triplicate for each data point.

Results

Regulation of MVP protein expression by anticancer agents. We investigated if various anticancer agents could induce MVP expression in SW-620 cells. We first established the cytotoxic effect of the anticancer agents by determining the survival of SW-620 cells following treatment with the various agents using the MTT assay. IC_{50} values for Adr, VP-16, SN-38, CDDP, VCR and Taxol were 29.5 nM, 771 nM, 15.8 nM, 6.10 μM, 8.22 nM and 13.5 nM, respectively (data not shown).

We next examined whether these anticancer drugs could enhance the expression of the MVP protein. SW-620 cells were continuously incubated for 72 h with each anticancer agent at its IC_{50} concentration, and the drug-mediated modulation of MVP expression was investigated by immunoblot analysis (Fig. 1A). MVP protein levels were unchanged, or even decreased, when cells were treated with VCR or Taxol. In contrast, treatment with Adr, VP-16, SN-38 or CDDP dramatically enhanced MVP expression compared to untreated SW-620 cells.

To test whether MVP protein is induced by these agents in a dose-dependent manner, we cultured SW-620 cells for 3 days in the medium containing Adr at doses of 1, 10 or 100 nM. As shown in Fig. 1B, increasing doses of Adr induced increasing levels of MVP protein. Similar results were obtained following treatment with CDDP (data not shown).

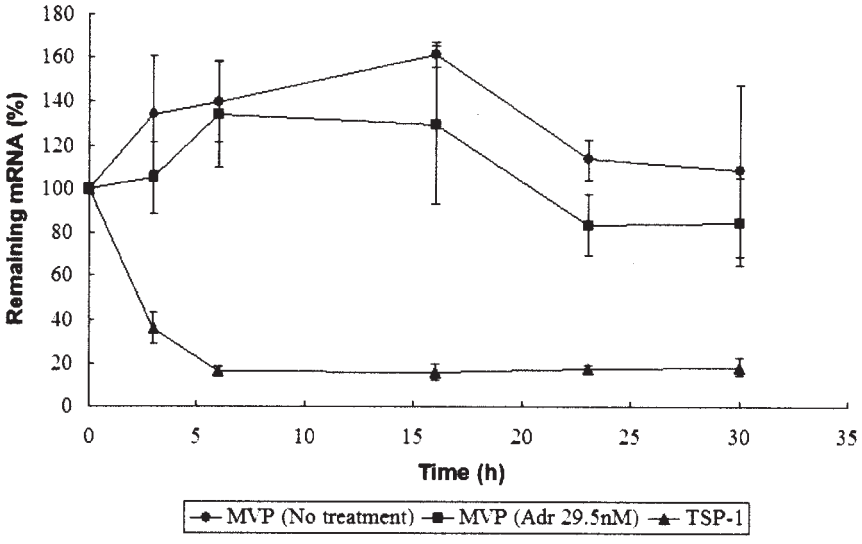

Figure 4. The effect of Adr on the stability of *MVP* mRNA in SW-620 cells. Expression levels of *MVP* mRNA in the absence (circles) or presence (squares) of Adr treatment were analyzed at the indicated periods (h) as described in Fig. 2. The expression of *TSP-1* mRNA (triangles) was used as a positive control. Points represent the means ± standard deviations (error bars) of three independent experiments.

Subsequently, we monitored the time dependence of MVP expression following the addition of Adr at 29.5 nM (IC_{50} concentration). Induction of the MVP protein was detectable on day 2 and enhanced by day 3. This enhanced level of MVP expression was maintained into day 4 (Fig. 1C). Thus, MVP protein is regulated by anticancer drugs in a dose- and time-dependent manner.

Regulation of MVP mRNA levels by anticancer agents. We determined if enhanced *MVP* transcription could contribute to the increased MVP protein levels following Adr treatment. The effect of Adr on *MVP* mRNA expression levels in SW-620 cells was investigated by TaqMan real-time reverse-transcription PCR. Induction of *MVP* mRNA with NaB was used as a positive control (26), and untreated cells were used as a negative control. The 100 nM Adr did not significantly increase *MVP* mRNA in SW-620 cells after 24 h, even though *MVP* mRNA levels were significantly enhanced at this time point by 2 mM NaB compared to non-treated controls ($p<0.0001$). However, Adr did significantly increase the level of *MVP* mRNA in SW-620 cells ($p<0.01$) following treatment for 48 h (Fig. 2A). This delayed induction of *MVP* mRNA by Adr compared to NaB is consistent with the delayed induction of *MVP* protein by Adr (Fig. 1C) compared to that reported for NaB (26).

We then tested if other anti-cancer agents could affect *MVP* mRNA levels following 48 h treatment. SW-620 cells were incubated continuously for 48 h with Adr, VP-16, SN-38, VCR or Taxol at their IC_{50} concentration, and MVP mRNA levels were determined in comparison to non-treated cells (Fig. 2B). In addition to Adr, both VP-16 and SN-38 significantly ($p<0.0001$) enhanced MVP mRNA levels, whereas VCR ($p=0.9236$) and Taxol ($p=0.8085$) had no significant effect.

These findings indicate that a number of anti-cancer drugs, including Adr, VP-16 and SN-38, can induce increased *MVP* mRNA levels. Data for the different Adr concentrations further confirm the dose-dependent effect of Adr on MVP protein expression suggested by Fig. 1B.

Activation of the MVP promoter by Adr. One likely mechanism by which Adr might enhance *MVP* mRNA levels is by the modulation of *MVP* promoter activity. To assess whether Adr can activate *MVP* promoter activity, we generated five luciferase reporter constructs with a series of *MVP* promoter regions containing various promoter elements. The longest constructs, pMVP407 and pMVP315, contain all known conserved promoter elements, i.e. Y-box, MyoD, GATA, E-box, Sp1, p53 and STAT (24). The construct pMVP263 lacks only a Y-box, and MyoD and GATA are further deleted in pMVP102. The shortest construct, pMVP78, lacks all of these elements except Sp1, p53 and STAT (Fig. 3A). Cells individually transfected with these plasmids were then treated with Adr at its IC_{50} concentration, and the ability of Adr treatment to induce luciferase activity of the reporter gene was assayed. Adr treatment increased luciferase activity 1.7-, 1.4-, 1.4-, 1.6- and 2.6-fold compared to the basal luciferase activity in pMVP407, pMVP315, pMVP263, pMVP102 and pMVP78-transfected cells, respectively (Fig. 3B). These data suggest that enhancement of transcriptional activity can contribute to the induction of elevated *MVP* mRNA levels by Adr. The basal luciferase activity of pMVP78 was significantly lower than that of other constructs ($p<0.05$) suggesting that this construct may lack promoter elements essential for basal *MVP* promoter activity (Fig. 3B).

The effect of Adr on the stability of MVP mRNA. Although Adr activates *MVP* promoter activity, the observed level of enhancement could not fully account for the enhanced level of *MVP* mRNA and protein expression measured. We therefore tested if Adr could employ other mechanisms to enhance MVP levels; one possibility was that Adr might also affect the stability of *MVP* mRNA. We treated SW-620 cells with Adr in the presence of the RNA polymerase II inhibitor α-amanitin to prevent further mRNA synthesis and measured *MVP* mRNA levels by real-time reverse-transcription PCR. *MVP* mRNA was stable and not degraded in untreated and Adr-treated SW-620 cells over a period of 30 h (Fig. 4). Adr treatment did not affect the *MVP* mRNA stability. In contrast,

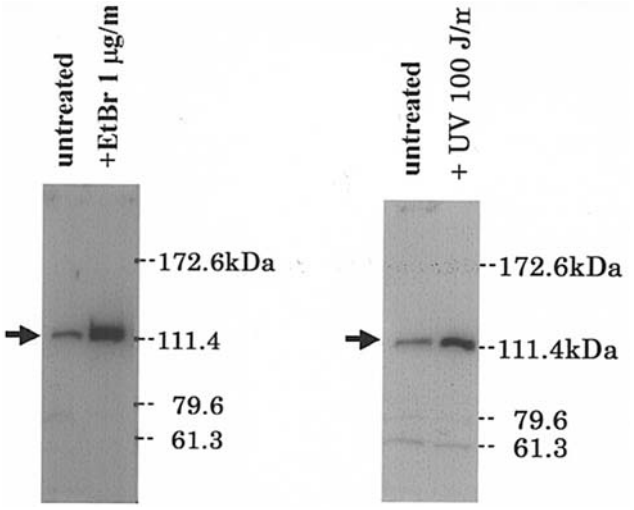

Figure 5. Effect of UV irradiation and EtBr treatment on MVP protein expression. SW-620 cells were continuously incubated with EtBr (1 μg/ml, left) or exposed to UV irradiation (100 J/m^2, right) for 72 h. MVP protein expression was then assessed by immunoblotting. MVP protein is indicated by an arrow at left, and molecular weight markers are on the right.

TSP-1 mRNA, which was assayed as a control, was degraded with a half-life of 2.5 h in untreated cells, and similar results were obtained with *matrix metalloproteinase-9* mRNA (data not shown). Although the data do not indicate a dramatic increase in the stability of *MVP* mRNA by Adr, the unexpected stability of MVP over a period of 30 h suggests that a small increase in *MVP* promoter activity could result in a relatively large increase in MVP expression.

Correlation between MVP expression and DNA damage. We have shown that MVP expression is enhanced by Adr, VP-16, SN-38 or CDDP treatment, but not VCR and Taxol (Fig. 1A). Since all of the agents that elevated MVP expression were DNA damaging agents, we next assessed whether DNA damage induced by UV irradiation or ethidium bromide (EtBr) treatment could also increase MVP expression levels.

SW-620 cells were incubated for 72 h after irradiation with UV at 100 J/m^2 or continuously exposed to EtBr at 1 μg/ml, and MVP expression in the treated cells was compared to that in untreated cells by immunoblot analysis (Fig. 5). Both treatments markedly enhanced MVP expression. These data therefore suggest that MVP expression can be induced by a variety of agents inducing DNA damage.

Discussion

Human MVP (originally termed LRP) was first described as a protein found in a non-P-gp-mediated multidrug-resistant non-small cell lung cancer cell line selected with Adr (1,2). In succeeding years, MVP was found to be overexpressed in several human multidrug-resistant tumor cell lines (9,10,31), suggesting that MVP might play a role in MDR. In this study, we show that several anticancer agents induce MVP expression in the colon cancer cell line, SW-620 (Fig. 1A). This result is consistent with previously published reports of high MVP expression in cells treated with a variety of anticancer agents including Adr, mitoxantrone, methotrexate, VP-16, VCR, cytarabine, 5-FU, bleomycin and CDDP (1,11-13,15,16,25,32-37). We have previously demonstrated that NaB, a histone deacetylase (HDAC) inhibitor, stimulated *MVP* expression and enhanced drug resistance in human colon carcinoma cells (26). However, the sensitivity to anticancer agents of fibroblasts derived from MVP knockout mice is comparable to that of wild-type fibroblasts (38). Thus, the precise role of MVP and the vault particle in MDR, and their physiological role(s), are still undefined. In this study, we demonstrated the activation of *MVP* promoter activity by Adr (Fig. 3B) and the unexpected stability of *MVP* mRNA (Fig. 4). Together with the previous finding that the MVP protein is highly stable (28), these data might explain the elevated MVP levels induced by anticancer agents.

In order to fully understand the regulation of *MVP* expression, it is necessary to identify the mechanism by which *MVP* transcription is regulated. The human *MVP* gene promoter has been cloned and contains an inverted CCAAT-box termed the Y-box, which responds to a number of stresses including anticancer agents, UV, and hyperthermia (20,21,39-41). Nuclear localization of the Y-box binding transcription factor, YB-1, was shown to be correlated with intrinsic expression of the *MDR* gene 1 (*MDR1*) in human carcinomas and osteosarcomas (23,42). Stein *et al* have reported the direct involvement of YB-1 in controlling basal and 5-FU-induced *MVP* promoter activity (25). Our results indicate that a second promoter element, termed the E-box, is essential for basal *MVP* promoter activity. The promoter construct pMVP78, in which the E-box was deleted from the *MVP* promoter region, showed considerably less basal luciferase activity compared to the promoter construct pMVP102 (Fig. 3B). In contrast, deletion of the Y-box did not affect the basal *MVP* promoter activity (Fig. 3A and B). It has also been suggested that the consensus element for Sp1 transcription factor binding is critical for basal *MVP* promoter activity and its stimulation by HDAC inhibitors (43). However, in this study, the *MVP* promoter construct pMVP78 that retained the Sp1 binding site could not maintain basal *MVP* promoter activity. The discrepancy between these data can possibly be attributed to the use of different cell types for these experiments.

We have shown that a number of anticancer agents can enhance *MVP* promoter activity, and examined the possible elements in the *MVP* promoter that might be important for this enhancement. It has previously been shown that anticancer agents can variously enhance the activity of the *MDR1* promoter via Y-box and YB-1. Among several anticancer agents, VCR effectively activated the *MDR1* promoter, whereas Adr only weakly activated it and VP-16 did not activate it at all (17). In contrast to their activation of the *MDR1* promoter, Adr and VP-16 activated the *MVP* gene effectively, but VCR did not (Figs. 1A and 2B). The difference in response to VCR and VP-16 between the *MDR1* and *MVP* promoters may be attributable to differences in the elements in promoter structure, and also suggests that Y-box is not essential for the induction of MVP by Adr in SW-620 cells. It is intriguing that the *MVP* promoter is activated by DNA damaging agents alone.

The fact that deletion of the Y-box in the human *MVP* promoter does not affect basal *MVP* promoter activity in this study, coupled with the fact that the murine *MVP* promoter

-box (44,45), suggests that the Y-box may not be for the basal expression of the *MVP* gene. Since the murine *MVP* promoter does have an Sp1 or E-box, and we have shown that deletion of the E-box dramatically reduces basal *MVP* transcriptional activity, this indicates the E-box, and not the Y-box, may be critical for basal expression of the MVP gene. However, it remains controversial whether YB-1 is directly involved in MVP expression in human tumors.

This study indicated a correlation between MVP expression and DNA damage in SW-620 cells. At present, it is unclear whether high MVP expression modulates DNA damage. It is highly likely that vault is involved in DNA damage repair because VPARP contains a BRCA 1 C-terminus (BRCT) domain that defines a superfamily of DNA damage response proteins (46,47). However, vaults may not be involved in all types of DNA damage, as vaults and VPARP in the cytoplasm are not relocalized to the nucleus in response to DNA damage by UV light irradiation (6). Further investigation is required to determine whether the induction of MVP by DNA damage is common to both human and murine systems and to understand how DNA damage induces MVP expression. Extension of the studies concerning the mechanism by which MVP expression is regulated via promoter binding proteins may also shed light on the cellular function of vaults.

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