PF1070A, a Novel and Potent Inducer of the Synthesis of Metallothionein†

Ikue Asahi,‡ Nobuhiko Miura,§ Yukako Yamabe,‡ Haruka Toyoda,‡ Nobumasa Imura,‡ Masao Koyama,|| and Akira Naganuma*,§

Department of Public Health and Molecular Toxicology, School of Pharmaceutical Sciences, Kitasato University, Minato-ku, Tokyo 108-864, Pharmaceutical Research Center, Meiji Seika Kaisha, Ltd., Yokohama 222-3175, and Department of Molecular and Biochemical Toxicology, Faculty of Pharmaceutical Sciences, Tohoku University, Sendai 980-8578, Japan

Received March 19, 1999; Revised Manuscript Received June 7, 1999

ABSTRACT: Using mouse Ltk$^-$ cells (L13-17 cells) that had been transfected with a plasmid in which the *lacZ* gene had been ligated downstream of 1.4 kbp of the sequence of the promoter of the mouse gene for metallothionein-I (MT-I) as a reporter gene, we examined 268 organic compounds for the ability to activate this promoter. We found that PF1070A, an antibiotic produced by *Humicola* sp., efficiently activated the MT promoter and caused marked enhancement of β-galactosidase activity in L13-17 cells. The extent of activation by PF1070A was almost equivalent to that by of zinc ions, the most effective known inducer of the synthesis of MT. PF1070A also caused marked elevation of the levels of the mRNA for MT and of MT itself in L13-17 cells. A similar result was obtained in human HeLa-S3 cells. When PF1070A was added to the culture medium simultaneously with cadmium ion or dexamethasone, the level of expression of the reporter gene was markedly elevated, compared to the level of expression induced by each agent independently. The effect of PF1070A was reduced considerably by deletion of nucleotides at positions −150 and −149 from the site of initiation of transcription in the promoter region of the MT gene and also by deletion of the seven bases located at positions −49 to −43. Since no known cis element was found in these two regions, PF1070A might be a new type of inducer of MT synthesis that promotes expression of the gene for MT via a mechanism completely different from those exploited by other known agents. These results also suggest the presence of a system for control of transcription of the gene for MT that has not previously been recognized. Both cadmium ions and bismuth ions induce the synthesis of MT by acting on the metal response element (MRE). Bismuth ions had no significant effect on the promoter activity that had already reached a maximum level in response to treatment with the optimal concentration of cadmium ion. By contrast, PF1070A further and markedly increased the promoter activity. This result suggests that it is possible to increase the concentration of MT in tissue using PF1070A as an inducer even in cases where the MRE-mediated activation of the MT promoter has already been induced by the accumulation of cadmium, as is the case in a clinical setting. PF1070A may prove to be an excellent inducer of MT synthesis that is effective and clinically applicable. Moreover, use of PF1070A in combination with salts of heavy metals might be useful in controlling expression of a transfected gene that is regulated by the MT promoter since PF1070A can activate the MT promoter to an extent that cannot be achieved with heavy metal ions alone, when PF1070A is used in combination with zinc ions at a concentration of the latter considerably below the toxic level.

MT[1] is a protein with a molecular weight of approximately 6000. In MT, about one-third of the amino acid residues are cysteine residues (*13*, *18*, *40*). MT interferes with the toxicity of heavy metals by binding heavy metal ions via its SH groups (*13*, *40*). Therefore, cells containing large amounts of MT are highly resistant to heavy metals such as cadmium and inorganic mercury (*2*). In addition to reducing the toxicity of heavy metals, MT reduces the toxicity of anticancer drugs, such as cisplatin (*23*) and adriamycin (*24*), as well as the toxicity of alkylating agents (*6*, *12*). Furthermore, MT can scavenge free radicals (*34*, *39*) and can protect animals against damage due to radiation (*31*) and paraquat (*33*), both of which cause oxidative stress in cells. MT is found in species from fungi to humans (*13*, *40*), and it is considered to be an important biological defense factor.

The synthesis of MT is induced by various factors, such as heavy metal ions (*40*), glucocorticoid (*19*), phorbol ester (*1*), cytokines, and various stressors (*30*). The strongest known inducers of MT synthesis are heavy metal ions such as zinc, cadmium, and bismuth (*13*, *40*). When the concentration of MT in tissues is elevated in mice by treatment with such a heavy metal, the mice exhibit strong subsequent resistance to heavy metals (*40*), anticancer drugs (*23*, *24*, *32*), and radiation (*31*). Moreover, bismuth compounds

† This work was supported by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science, Sports and Culture of Japan.

* To whom correspondence should be addressed.
‡ Kitasato University.
§ Tohoku University.
|| Meiji Seika Kaisha, Ltd.

[1] Abbreviations: MT, metallothionein; MRE, metal response element; MTF-1, metal-regulatory transcription factor-1; GRE, glucocorticoid-responsive element; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-(−)-galactopyranoside; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; bp, base pair(s); kbp, kilobase pair(s); tk, thymidine kinase.

10.1021/bi990655g CCC: $18.00
Published on Web 07/22/1999

promote the synthesis of MT only in normal tissues and do not alter concentrations of MT in tumor tissue (*24*). Thus, adverse effects of anticancer drugs, such as cisplatin and adriamycin, can be selectively reduced without loss of antitumor effects by prior treatment of tumor-bearing mice with bismuth compounds (*23*, *24*). This method for reducing the adverse effects of anticancer drugs using bismuth compounds has been investigated in the clinical setting, but the effects of bismuth in humans are less dramatic than in mice (*25*, *37*, *38*).

The presence of a nucleotide sequence known as a metal response element (MRE) is essential for the metal ion-induced synthesis of MT (*15*, *35*), and zinc, cadmium, and bismuth ions activate the promoter of the gene for MT via the MRE (*27*). In general, relatively large amounts of cadmium accumulate in adult humans (*10*, *36*) and induce the synthesis of MT by the same mechanism exploited by bismuth ions (*27*). This observation suggests that MT synthesis might already be strongly induced in healthy humans and that administration of bismuth to humans does not enhance the synthesis of MT to any further extent. The synthesis of MT is also induced by various nonmetal factors as noted above, and there are nucleotide sequences other than the MRE in the MT promoter that respond to glucocorticoid (*3*, *28*), phorbol ester (*1*), or hydrogen peroxide (*5*). These sequences are termed the glucocorticoid-responsive element (GRE), TPA-responsive element (TRE), and major late transcription factor/antioxidant-responsive element (MLTF/ARE), respectively. Agents that act on cis elements other than the MRE might allow us to raise the tissue concentration of MT in humans above that already induced by cadmium. However, these agents are much less able to induce the synthesis of MT than zinc and bismuth ions, and only minimal effects can be expected. Therefore, in this study, we attempted to identify nonmetal compounds that might activate the MT promoter without involvement of the MRE. As reported herein, we found that PF1070A, an antibiotic produced by *Humicola*, has MT synthesis-inducing activity that is equivalent to that of zinc ions. PF1070A appears to activate the MT promoter by a mechanism different from that involved in Zn^{2+}-induced activation. Furthermore, PF1070A synergistically promoted the synthesis of MT when it was used in combination with zinc ions, a result that suggests that PF1070A might be a novel type of inducer of the synthesis of MT.

MATERIALS AND METHODS

Chemicals. PF1070A was isolated and crystallized from the culture broth of *Humicola* sp. (*43*). 5-Bromo-4-chloro-3-indolyl β-D-(−)-galactopyranoside (X-Gal) was purchased from Wako Pure Chemical Ind. Ltd. (Tokyo, Japan). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Sigma Chemical Co. (St. Louis, MO). Zinquin was purchased from Luminis Pty. Ltd. (Adelaide, South Australia).

Cell Culture. Mouse Ltk$^-$ cells and L13-17 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and kanamycin (60 μg/mL) at 37 °C in an atmosphere of 5% CO_2 in air.

Establishment of Transfectants That Stably Expressed the LacZ Gene under Control of the MT Promoter. Plasmid pMY648 (*20*) carrying a 1.4 kbp region of the promoter of the mouse MT-I gene (*26*) was provided by M. Yamada (National Children's Hospital, Tokyo, Japan). pMPZ-Ika2 was constructed by inserting the *Hin*dIII−*Bam*HI region of pCH110, which contained the entire *lacZ* gene, downstream of the MT-I promoter region (*Hin*dIII−*Hpa*I region) of pMY648. Exponentially growing Ltk$^-$ cells, plated at a density of 10^6 cells/100 mm dish 24 h before transfection, were cotransfected with 20 μg of pMPZ-Ika2 and 2 μg of pSV2-gpt, which contained the gene for xanthine-guanine phosphoribosyl transferase, by the calcium phosphate precipitation method (*22*). After incubation for 36 h, the cells were cultured in selection medium that contained mycophenolic acid (10 μg/mL) for 21 days. Then mycophenolic acid-resistant clones were isolated and screened for expression of *lacZ* by monitoring β-galactosidase activity. Eighteen clones with β-galactosidase activity were obtained. The clone designated L13-17, which had the highest β-galactosidase activity of all these clones, after activation of the MT promoter by treatment with $ZnCl_2$, was selected for use in the study presented here. Southern blotting analysis indicated that the transfected *lacZ* gene had been integrated in the chromosomal DNA of L13-17 cells.

Screening for Compounds That Activate the MT Promoter. Using L13-17 cells, we examined 268 compounds that included natural products and synthetic chemicals for their ability to induce expression of the MT gene. L13-17 cells (1×10^4) in 100 μL of medium were plated in each well of a 96-well plate and cultured at 37 °C. After incubation for 24 h, these cells were further incubated for 24 h in the presence of one of the 268 compounds at various concentrations. Then, the β-galactosidase activity of cells in each well was determined.

Determination of β-Galactosidase Activity. After incubation of the cells with test compounds, the medium in each well was replaced by 50 μL of phosphate-buffered saline (pH 7.4) that contained 0.05% Triton X-100 and 0.08% X-Gal. The β-galactosidase activity of the cells in each well was determined by measuring the absorbance at 650 nm after incubation for 2 h at 37 °C.

Determination of Cell Survival by the MTT Assay. After incubation of the cells with test compounds, we added 10 μL of a solution of MTT (5 mg/mL) to each well and then incubation was continued for 2 h. The resultant formazan product was solubilized by addition of 100 μL of a 20% solution of SDS that contained 50% *N*,*N*-dimethylformamide (pH 4.7), and the concentration of the product was monitored spectrophotometrically at 550 nm.

Northern Blotting Analysis. Five micrograms of total RNA was fractionated on a 1.0% agarose formaldehyde gel and blotted onto a nylon membrane (Nytran, Schleicher & Schuell, Inc., Keene, NH) and UV cross-linked. cDNA fragments of mouse MT-I, mouse heat shock protein 70 (HSP 70), and human GAPDH were obtained by the polymerase chain reaction (PCR), and then these fragments were labeled with digoxigenin (DIG) using the BcaBest DIG Labeling Kit (Takara Shuzo Co. Ltd, Kyoto, Japan) according to the manufacturer's instructions. Hybridization, washing, and detection were performed using the DIG Luminescent Detection Kit and CDP-Star as a substrate (Boehringer Mannheim, Mannheim, Germany), and the membrane was exposed to X-ray film at room temperature.

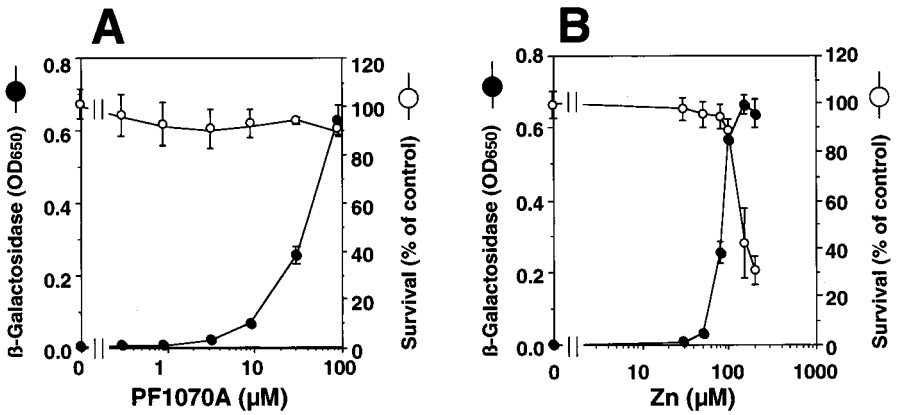

FIGURE 1: Effects of PF1070A (A) and $ZnCl_2$ (B) on β-galactosidase activity and survival of L13-17 cells. L13-17 cells, which stably expressed the *lacZ* gene under control of the mouse MT-I promoter, were incubated with PF1070A or $ZnCl_2$ for 24 h. Survival of cells was monitored by the MTT assay.

Determination of the Cellular Concentration of MT. Cellular concentrations of MT were determined by the ^{203}Hg binding assay (*23*), modified as described below. Cells (2×10^6) were sonicated in 1.8 mL of 1.15% KCl, and the sonicate was incubated with 10 μL of diethyl maleate at 25 °C for 15 min. After the addition of 50 μL of 10 mM $CdCl_2$, particulate matter and high-molecular weight proteins were precipitated by heating at 100 °C for 5 min. Excess $^{203}HgCl_2$ (20 μL of 50 μM $^{203}HgCl_2$, 10^4 cpm/nmol of ^{203}Hg) was added to the aqueous fraction to saturate the metal-binding sites of MT with ^{203}Hg. Non-MT-bound ^{203}Hg was removed by the addition of 0.5 mL of a 60 μM solution of ovalbumin that was followed by acidification with 50 μL of a 100% solution of trichloroacetic acid (TCA). Then the radioactivity of ^{203}Hg bound to MT was measured, and the concentration of MT was expressed as picomoles of Hg bound.

Determination of the Total and Labile Zinc Concentrations in the Cells. L13-17 cells (2×10^6 cells/10 cm dish) were precultured overnight and treated with 90 μM PF1070A or 150 μM $ZnCl_2$ for 6 h at 37 °C. Cells were then washed with Hank's balanced salt solution (HBSS) and incubated with 5 mL of HBSS containing 25 μM Zinquin (*44*) for 40 min at 37 °C, washed with HBSS three times, and scraped with 2 mL of HBSS (*4*). Fluorescence was measured at an excitation wavelength of 370 nm and an emission wavelength of 490 nm. The total zinc concentration in the cells was determined by atomic absorption analysis after incubation with 90 μM PF1070A or 150 μM $ZnCl_2$ for 6 h at 37 °C.

Search for the Cis-Acting Regulatory Element That Responds to PF1070A. Plasmids with deletions in the MT-I promoter linked to the *lacZ* gene were generated by subcloning of products after amplification by PCR that contained various lengths of the mouse MT-I promoter into the *Tth*III−*Hin*dIII site (which contained the early promoter of SV40) that was located upstream of the *lacZ* gene in pCH110. Mouse Ltk$^-$ cells (3×10^5) that had been plated in 6 cm dishes and cultured for 24 h were transfected by the calcium phosphate precipitation method (*22*) with 5 μg of each chimeric plasmid described above and 1 μg of the PicaGene Control Vector (PGV-C, Toyo Ink, Co. Ltd.) which contained the SV40 promoter and a gene for luciferase (*42*). After incubation for 7 h, the cells were washed three times with serum-free medium and cultured for a further 16 h. Then PF1070A (30 μM) or $ZnCl_2$ (80 μM) was added to the medium, and the cells were cultured for 24 h. The promoter activity, determined by monitoring β-galactosidase activity, was normalized by reference to the luciferase activity, which was determined with a PicaGene luminescence kit (Toyo Ink, Co. Ltd.).

RESULTS

Screening for Inducers of MT Synthesis. To facilitate identification of inducers of MT synthesis, we transfected mouse Ltk$^-$ cells with a plasmid in which the *lacZ* gene for β-galactosidase had been ligated downstream of a 1.4 kbp fragment of the mouse MT-I promoter as a reporter gene, and stable transfectants (L13-17 cells) were obtained. Using these L13-17 cells, we examined the ability of 268 compounds to activate the MT promoter, and we found that PF1070A (see Figure 8), an antibiotic produced by *Humicola* sp. (*43*), efficiently activated the MT promoter and increased the β-galactosidase activity in L13-17 cells. PF1070A acted in a dose-dependent manner, and the β-galactosidase activity in L13-17 cells was maximal at 90 μM (Figure 1A). Since the extent of elevation of β-galactosidase activity was almost the same as that induced by $ZnCl_2$ (Figure 1B), PF1070A appeared to have MT synthesis-inducing activity equivalent to that of zinc.

Characteristics of the Activity of PF1070A. The time course of changes in the amount of MT mRNA after the addition of PF1070A to L13-17 cells was investigated by Northern blotting. The level of MT mRNA increased 6 h after the addition of PF1070A and continued to increase for at least an additional 4 h (Figure 2). The level of MT protein also increased with increases in the concentration of PF1070A, and the pattern of increases was almost the same as that of changes in β-galactosidase activity (Figure 3). These observations suggested that PF1070A promoted synthesis of MT mRNA by activating the MT promoter, with resultant increases in the concentration of the MT protein. Levels of MT mRNA and of MT itself were also raised by PF1070A in human HeLa-S3 cells, which gave almost the same results as those obtained with the mouse L13-17 cells (data not shown).

Both MREs and the GRE are present in the promoter of the human MT-IIa gene. This promoter is synergistically activated by simultaneous exposure to Cd^{2+} ions and dexamethasone (*7*), which act on the respective cis elements. Therefore, we investigated the effects of PF1070A on the

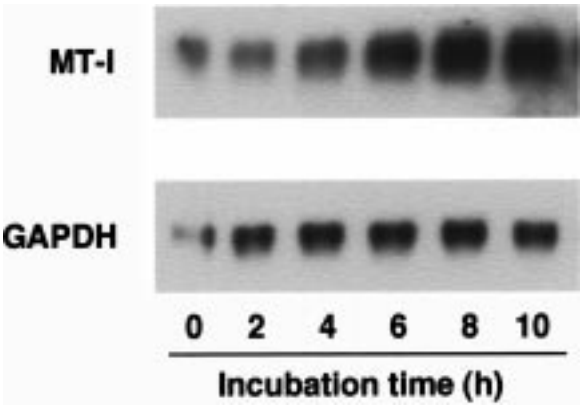

FIGURE 2: Effects of PF1070A on levels of MT-I mRNA in L13-17 cells. Total RNA was isolated from L13-17 cells after incubation with PF1070A (90 μM) and subjected to Northern blotting analysis. GAPDH probe hybridization was used as a control for RNA loading.

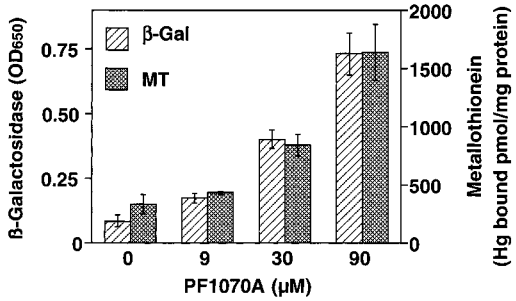

FIGURE 3: β-Galactosidase activity and levels of MT in L13-17 cells after incubation for 24 h with PF1070A.

MT promoter that was activated by metal ions or dexamethasone. When we added PF1070A at a relatively low concentration (9 μM) to the medium simultaneously with $ZnCl_2$ (70 μM), $CdCl_2$ (1 μM), or dexamethasone (50 nM), the β-galactosidase activity was markedly higher than that induced by each compound independently (Figure 4A). There were no significant differences in the rate of cell proliferation, as measured by the MTT method, among all treated and untreated groups. Northern blotting analysis indicated that the level of MT mRNA was also synergistically increased by simultaneous addition of PF1070A and $ZnCl_2$ (Figure 4B).

There was a possibility that the activation of the MT promoter had been due to the enhanced incorporation of zinc in the medium by the cells or displacement of Zn^{2+} from zinc-binding proteins in cells (*27*). Therefore, we added $ZnCl_2$ or PF1070A to the medium, and after L13-17 cells had been cultured for 6 h, we measured the intracellular concentration of total Zn^{2+} by atomic absorption analysis and of labile Zn^{2+} using Zinquin, a specific fluorescent probe for intracellular labile Zn^{2+} (*44*) (data not shown). We found that the intracellular concentration of labile Zn^{2+} increased approximately 7-fold compared to that in controls after incubation of the cells with $ZnCl_2$ (150 μM). However, treatment with PF1070A (90 μM) did not significantly affect the intracellular concentration of labile Zn^{2+}. These results suggest that activation of the MT promoter by PF1070A may not be due to mobilization of labile Zn^{2+}, because the extent of the increase in β-galactosidase activity of L13-17 cells caused by 90 μM PF1070A was almost same as that caused by 150 μM $ZnCl_2$. The intracellular concentration of total Zn^{2+} ions was also increased with increases in the concentration of $ZnCl_2$ added to the medium. By contrast, even in the presence of 90 μM PF1070A, the intracellular concentration of Zn^{2+} ions was almost the same as that in the absence of PF1070A. Furthermore, after simultaneous addition of a lower concentration of PF1070A (9 μM) and $ZnCl_2$ (70 μM), which together synergistically enhanced the promoter activity, the intracellular concentration of Zn^{2+} ions was not significantly different from that after addition of 70 μM $ZnCl_2$ only. No complex formation of PF1070A with Zn^{2+} was also observed when both compoumds were mixed in the culture medium. Moreover, simultaneous addition of PF1070A and $ZnCl_2$ synergistically increased the level of MT mRNA, but not the mRNA level of HSP70 which has been known to be induced by Zn^{2+} (*8*, *9*) (Figure 4B). These results suggest that, at least, the synergistic activation of the MT promoter by simultaneous addition of PF1070A and $ZnCl_2$ was not due to promotion of the incorporation of zinc into the cells.

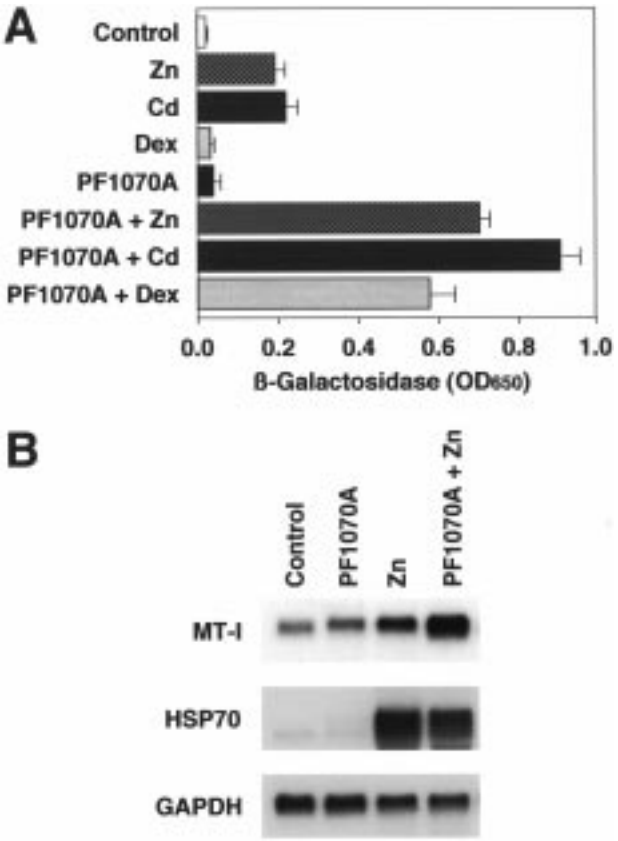

FIGURE 4: Activation of the MT-I promoter by PF1070A, $ZnCl_2$, $CdCl_2$, or dexamethasone (Dex) or their combinations. (A) Expression of the reporter gene. The β-galactosidase activity of L13-17 cells was determined after incubation for 24 h with PF1070A (9 μM), $ZnCl_2$ (70 μM), $CdCl_2$ (1 μM), or dexamethasone (50 nM) or with PF1070A (9 μM) with $ZnCl_2$ (70 μM), $CdCl_2$ (1 μM), or dexamethasone (50 nM). (B) Levels of MT-I mRNA and HSP70 mRNA. Total RNA was isolated from L13-17 cells after incubation for 8 h with PF1070A (9 μM) and/or $ZnCl_2$ (70 μM) and subjected to Northern blotting analysis. GAPDH probe hybridization was used as a control for RNA loading.

Comparison of the Mechanisms of Activation of PF1070A and Zn^{2+} Ions. The synergistic effects of PF1070A and Zn^{2+} ions or dexamethasone (Figure 4) indicated that PF1070A promoted the expression of the promoter by a mechanism different from that exploited by Zn^{2+} ions or dexamethasone. Therefore, we investigated by deletion analysis the domain

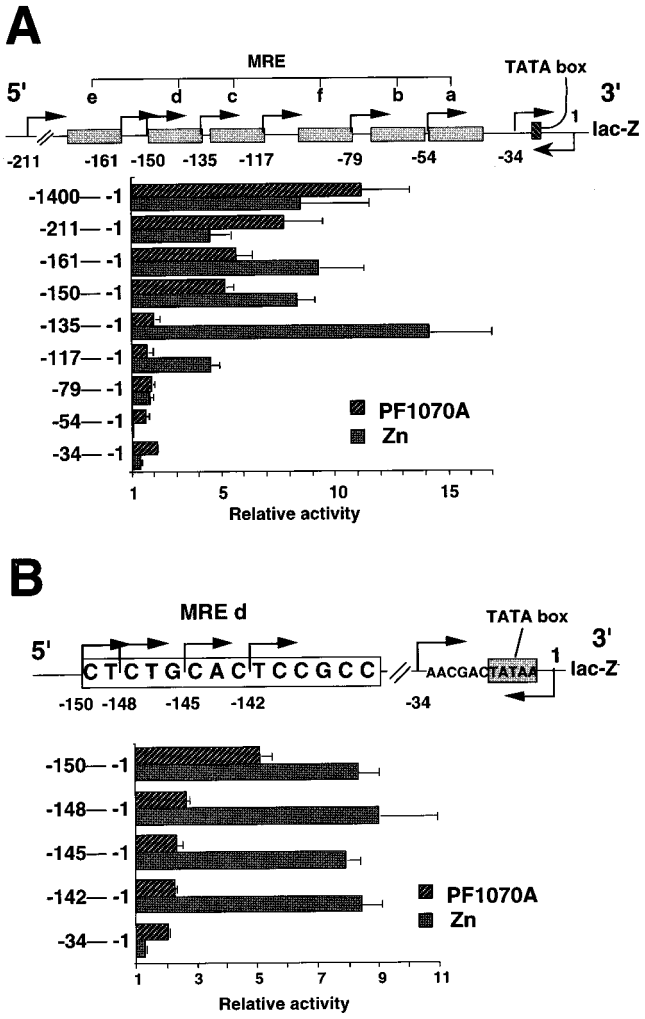

FIGURE 5: Effects of deletions from the 5′ side of the 211 bp (A) and of the 150 bp (B) MT-I promoter on activation by PF1070A and by $ZnCl_2$ in mouse L13-17 cells. The promoter activity was determined by monitoring β-galactosidase activity after incubation for 24 h with PF1070A (30 μM) or $ZnCl_2$ (80 μM). The levels of β-galactosidase activity were normalized by reference to the luciferase activity and are expressed as relative values.

within the MT promoter required for PF1070A-induced activation. We made deletions from the 5′ end of the 1.4 kbp region of the MT-I promoter and examined expression of the reporter gene (*lacZ*). Even after deletion of the region upstream of position −212, β-galactosidase activity was significantly induced by PF1070A and by $ZnCl_2$ (data not shown). The GRE is not present in the region up to 211 bp upstream of the mouse MT-I gene, so these results suggested that the GRE was not involved in the PF1070A-induced activation of the MT promoter. There are six MRE domains (MREa−MREf) present in the mouse MT promoter, and all these MREs are located in the region that extends up to 211 bp upstream of the site of initiation of transcription of the mouse MT-I gene. With deletions up to position −150, PF1070A was still effective, but its activity disappeared when the region up to position −135 was deleted (Figure 5A). By contrast, the Zn^{2+}-induced elevation of β-galactosidase activity was maintained even after the MT promoter had been deleted up to position −118 from the 5′ end (Figure 5A). Stuart et al. (*35*) performed a similar investigation with cadmium and reported that the reporter activity decreased

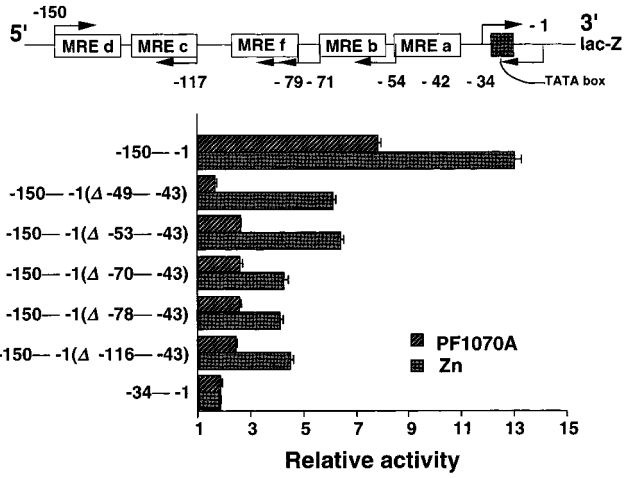

FIGURE 6: Effects of deletion from the 3′ side of the 150 bp MT-I promoter on activation by PF1070A and by $ZnCl_2$ in mouse L13-17 cells. Promoter activity was determined by monitoring β-galactosidase activity after incubation for 24 h with PF1070A (30 μM) or $ZnCl_2$ (80 μM). The levels of β-galactosidase activity were normalized by reference to the luciferase activity and are expressed as relative values.

depending on the length of the MT promoter and that Cd^{2+}-induced activation of the MT promoter disappeared when the promoter was deleted up to about position −60. Thus, even if some of the six MREs in the mouse promoter are deleted, expression of the gene for MT is still promoted via the remaining MREs. We next truncated the 150 bp MT promoter from the 5′ end in steps of 2−3 bp. The PF1070A-induced elevation of β-galactosidase activity disappeared after deletion of only 2 bp (Figure 5B). However, β-galactosidase activity was still significantly enhanced by Zn^{2+} ions even after 8 bp had been deleted from position −150 (Figure 5B). From these results, it appears that, at least, T at position −149, C at position −150, or both are essential for the PF1070A-induced activation of the MT promoter. Figure 6 shows the effects of PF1070A and of Zn^{2+} ions on the expression of the reporter gene when the 150 bp MT promoter was deleted in steps of several base pairs upstream of position −43 on the 3′ side. When the promoter was shortened from the 3′ side, MREs in the promoter were deleted in order starting from MREa, and the extent of Zn^{2+}-induced elevation tended to decrease gradually as the promoter was shortened. The efficiency of the activation of transcription of the MT gene by Zn^{2+} ions seemed, thus, to be dependent on the number of MREs. In contrast, the PF1070A-induced activation of the MT promoter disappeared completely with deletion of 7 bp from position −49 to −43. Thus, PF1070A-induced activation seemed to require the region from position −49 to −43 in addition to the region around position −150. In any case, since the pattern of induction of β-galactosidase activity by PF1070A was completely different from that by $ZnCl_2$, it seemed that PF1070A might activate the promoter and induce the synthesis of MT through a mechanism that is quite different from the mechanism that mediates the induction by heavy metal ions such as Zn^{2+}.

Effects of PF1070A on the Concentration of MT in Cells after Maximal Induction by Cd^{2+} Ions. L13-17 cells were treated with $CdCl_2$, and β-galactosidase activity was measured after 24 and 48 h. The activity was maximal at 3 μM

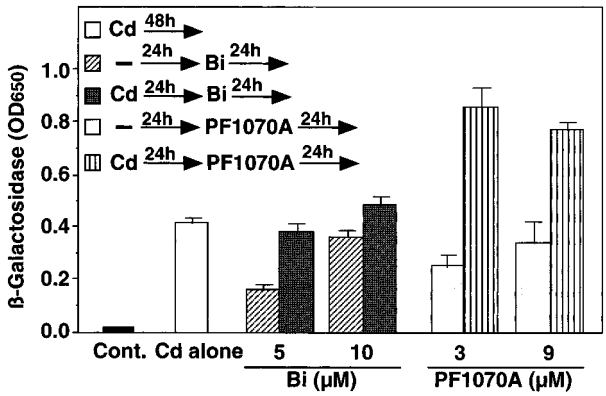

FIGURE 7: Effects of $Bi(NO_3)_3$ and PF1070A on the β-galactosidase activity of L13-17 cells that had been pretreated with $CdCl_2$. L13-17 cells were treated with $Bi(NO_3)_3$ or PF1070A after pretreatment for 24 h with $CdCl_2$ (3 μM). The β-galactosidase activity was determined after incubation for 24 h with $Bi(NO_3)_3$ or PF1070A.

$CdCl_2$ and was almost the same at 5 μM $CdCl_2$ (data not shown). We cultured L13-17 cells for 24 h in the presence of 3 μM $CdCl_2$ and then added $Bi(NO_3)_3$ or PF1070A to the medium and measured the β-galactosidase activity after a further 24 h (Figure 7). Addition of $Bi(NO_3)_3$ induced almost no change in the β-galactosidase activity that had already been maximally induced by $CdCl_2$. However, the activity was further and markedly elevated by PF1070A. This result indicated that, in cells in which the synthesis of MT had already reached a maximum level as a result of the accumulation of Cd^{2+} ions, the addition of Bi^{3+} ions which induce MT synthesis via MREs, as do Cd^{2+} ions, hardly promoted further synthesis of MT. By contrast, the concentration of MT was further elevated by PF1070A.

Relationship between the Chemical Structure of PF1070A and Stimulation of Expression of the Promoter. To investigate the relationship between the chemical structure of PF1070A and its activity, we investigated the effects of various analogues of PF1070A on β-galactosidase activity in L13-17 cells. First, we examined the activity of PF1070B (*43*), in which the piperidine ring of PF1070A is replaced by a pyrrolidine ring, and we found that the MT promoter was activated by PF1070B to almost the same extent as by PF1070A. Then, focusing on the epoxy ring and the carbonyl group on the side chain, we examined synthetic analogues of PF1070A (Figure 8) and PF1070B (Figure 9) for both activity and cytotoxicity. In this experiment, the highest concentrations of PF1070A, PF1070B, and NA1304 were set at 50 μg/mL, $^1/_{10}$ of those of the other compounds examined, because of the respective solubilities. NA1304 and NA1313, which do not have an epoxy ring, enhanced the β-galactosidase activity. Thus, the MT promoter-activating activity of PF1070A might not depend on the epoxy ring. By contrast, NA1311, NA1312, and NA1305, in which the carbonyl group on the side chain of PF1070A, of NA1309, and of PF1070B was replaced by a hydroxyl group, did not elevate the $\beta-$galactosidase activity. Therefore, the carbonyl group on the side chain, represented as R in Figure 8, might be essential for the PF1070A-induced activation of the promoter of the gene for MT.

DISCUSSION

We demonstrated in this study that PF1070A can activate the promoter of the MT gene to almost the same extent as Zn^{2+} ions, the strongest known inducer of MT synthesis. PF1070A is a cyclic peptide that was isolated from the culture fluid of *Humicola* as an antibiotic with antifungal activity (*43*). However, its antifungal activity is relatively weak, and thus, it has not been used for practical purposes. PF1070A significantly increased the levels both of intracellular MT mRNA and of MT itself. The time course of changes in the level of MT mRNA in the cells after the addition of PF1070A to the medium was similar to the pattern previously reported for Zn^{2+} ions (*14*). MT-inducing metal ions other than zinc have been suggested to activate the MT promoter by displacing Zn^{2+} ions from zinc-binging proteins (*27*). However, since PF1070A did not affect the intracellular concentration of total and labile Zn^{2+} ions, it appeared that the activation of the MT promoter by PF1070A was not induced by promotion of the incorporation of Zn^{2+} ions in the medium into cells or mobilization of cellular labile Zn^{2+}.

Partial deletions were made from the 5′ end or the 3′ end of the 1.4 kbp promoter of the mouse MT-I gene, and MT promoter-activating activity was compared between PF1070A and Zn^{2+} ions using β-galactosidase activity as an index. The patterns of induction clearly differed between PF1070A and $ZnCl_2$ (Figures 5 and 6). The above result suggested that PF1070A activates a domain through a mechanism that is different from the zinc-dependent activation. The PF1070A-induced activation of the MT promoter was markedly inhibited when the base pairs at positions -149 and -150 were deleted within the region from the site of initiation of transcription to position -150 in the MT promoter domain and, also, when 7 bp located from position -49 to -43 were deleted (Figures 5 and 6). In general, among cis elements of which the nucleotide sequences are known, regions consisting of several to a dozen or so base pairs are functional. Therefore, it is unlikely that the entire sequence from position -150 through position -43 is responsive to PF1070A. It is more likely that essential sequences are present at sites around position -150 and position -45, and that activation by PF1070A is induced by the simultaneous presence of these two sites in the same promoter. The cis elements that have been demonstrated in the MT promoter include the GRE (*3*, *28*), TRE (*1*), and MLTF/ARE (*5*), in addition to the MREs, and all these elements are located in regions other than the two PF1070A-responsive sites. Thus, PF1070A appears to be a new type of inducer of the synthesis of MT, which promotes expression of the MT gene through a mechanism that is completely different from those that mediate the actions of previously identified inducers.

The two PF1070A-responsive sites found in this study overlap the MREa and MREd, respectively. Metal-regulatory transcription factor 1 (MTF-1) has been known to bind specifically to MRE and activate transcription of the MT genes (*29*, *41*). The binding of MTF-1 to MRE is dependent on Zn^{2+} ions (*17*, *21*, *41*). MTF-1 might be essentialy required for zinc-induced MT gene expression, because MT mRNA was hardly detected in MTF-1 null mutant cells even after treatment with Zn^{2+} (*11*). In this study, deletion of one of the two PF1070A-responsive sites significantly reduced the extent of the transcriptional activation by PF1070A but not by Zn^{2+} ions (Figures 5 and 6). These observations suggested the possibility that PF1070A-induced MT synthesis might result from activation of a transcriptonal factor other

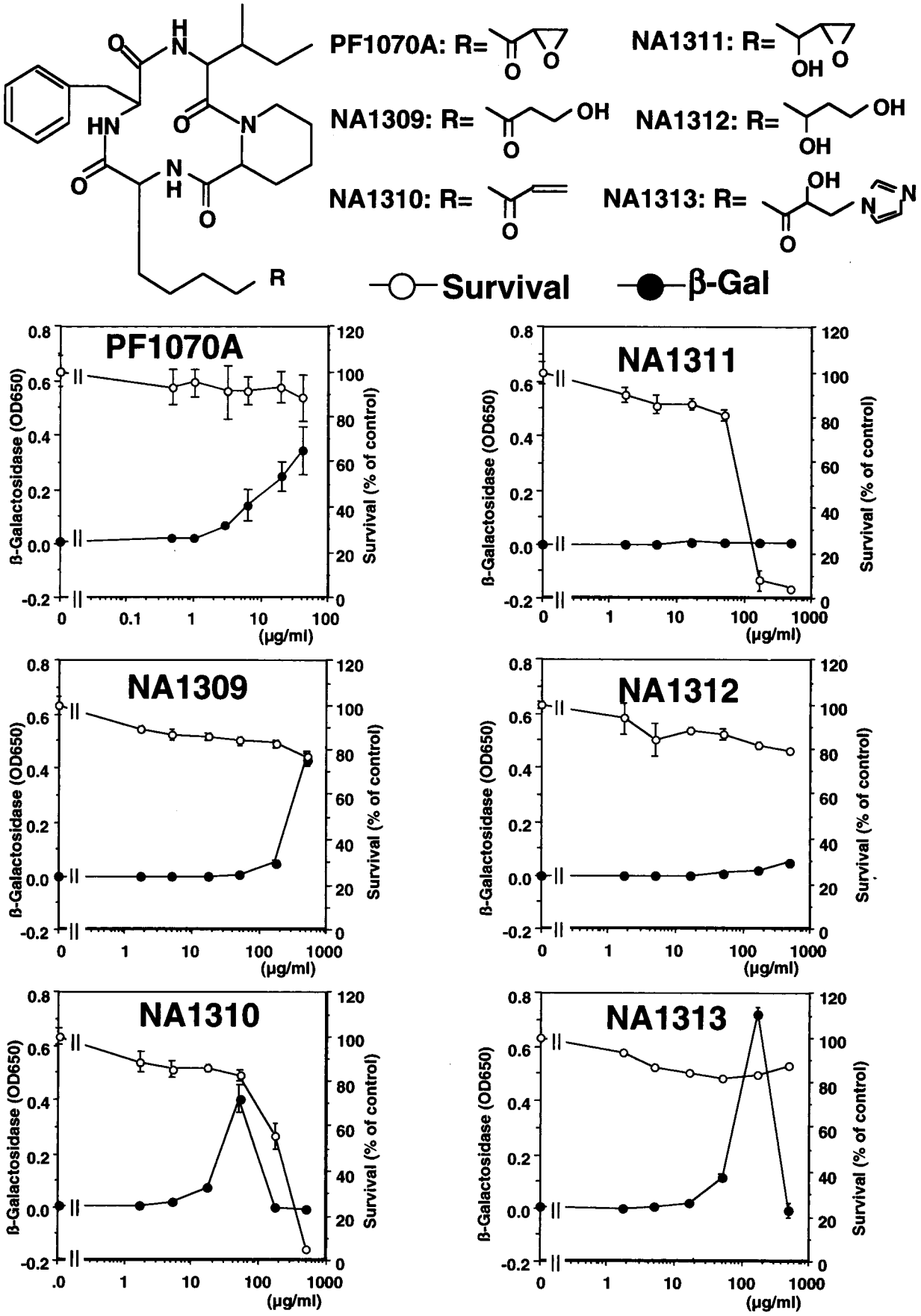

FIGURE 8: Effects of PF1070A and its analogues on β-galactosidase activity and survival of L13-17 cells. β-Galactosidase activity and survival were monitored after incubation for 24 h with each compound.

than MTF-1 or from activation of MTF-1 by a mechanism that is different from the activation mechanism by Zn^{2+} ions. Anyway, detailed investigations of the MT promoter-activating mechanism of PF1070A might reveal a new

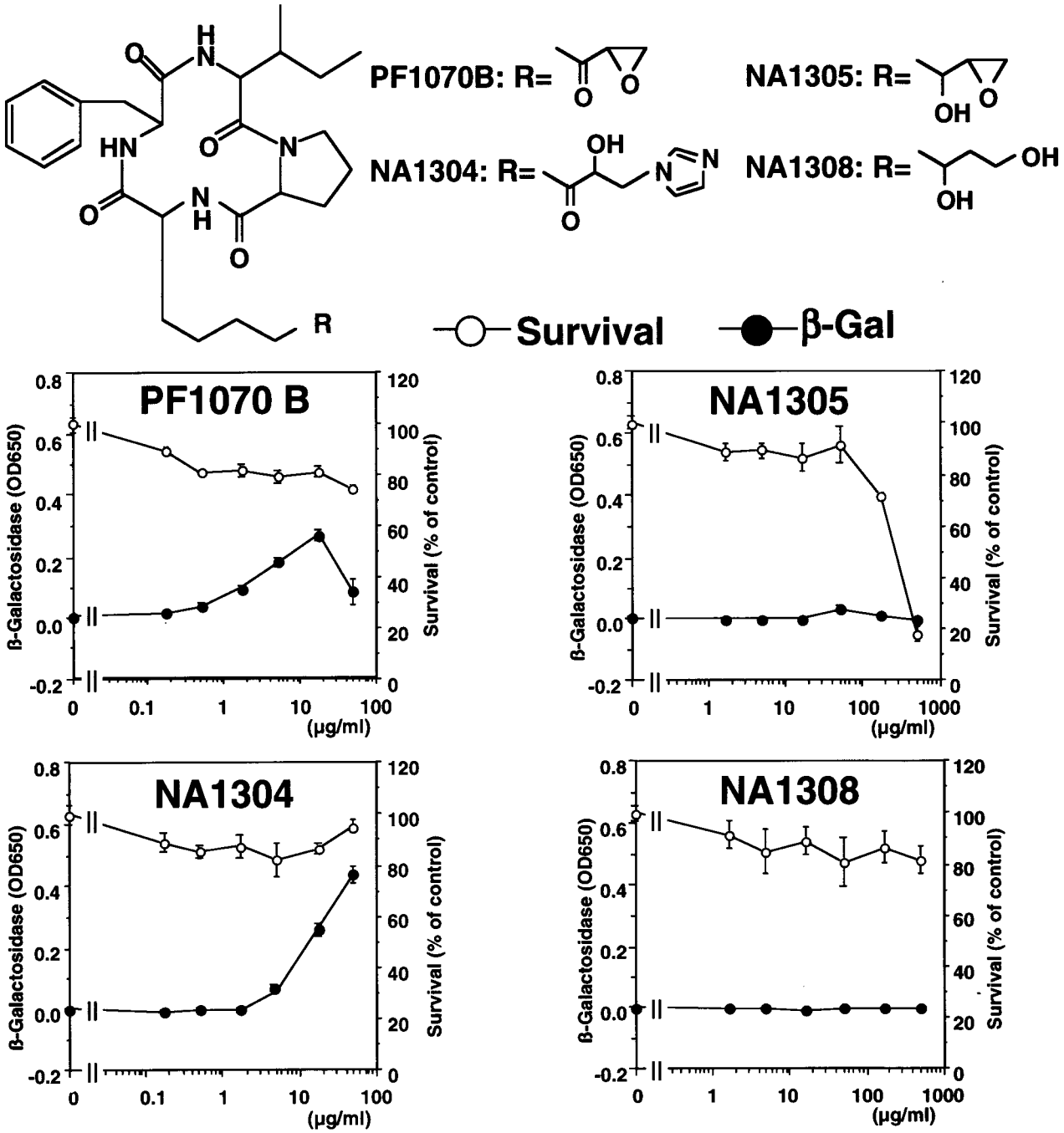

FIGURE 9: Effects of PF1070B and its analogues on β-galactosidase activity and survival of L13-17 cells. β-Galactosidase activity and survival were monitored after incubation for 24 h with each compound.

mechanisms that regulate transcription of the MT gene.

Kelly et al. (*16*) investigated the induction of the synthesis of MT-I and MT-II in mice and reported that there is no GRE in the proximal promoter region of either gene but that a GRE is located approximately 1 kbp upstream of the MT-II gene and approximately 7 kbp upstream of the MT-I gene. In this study with the 1.4 kbp region of the mouse MT-I promoter, expression of the reporter gene was markedly promoted by simultaneous addition of dexamethasone and PF1070A, as compared to that induced independently by each compound (Figure 4). This result suggests that, even though there is no GRE in the 1.4 kbp mouse MT-I promoter, dexamethasone somehow synergistically promotes the PF1070A-induced activation of the promoter. PF1070A might be a useful tool for elucidating the mechanism of action of dexamethasone on the MT promoter.

PF1070A also markedly promoted the expression of the reporter gene when it was added to the medium simultaneously with $ZnCl_2$ or $CdCl_2$ (Figure 4A). Since Zn^{2+} and Cd^{2+} ions promote expression of the MT gene by acting on MREs in the proximal promoter of the MT-I gene, this phenomenon might have been due to the synergistic effect of PF1070A, which acts at regions other than MREs in the proximal promoter. The MT promoter is widely utilized as an inducible promoter in the transfection of cultured cells with specific genes. However, for adequate induction of gene expression, Zn^{2+} or Cd^{2+} ions at concentrations close to toxic levels are required. PF1070A activated the MT promoter to a high level that could not be achieved with heavy metal ions alone, when used in combination with $ZnCl_2$ or $CdCl_2$

at concentrations considerably below toxic levels (Figures 4 and 7). Therefore, a combination of PF1070A and a heavy metal salt might be useful for controlling expression of a transfected gene that is under control of the MT promoter.

As noted above, in a study with a bismuth compound that induces the synthesis of MT, the concentration of MT was markedly increased in normal tissues but not in cancerous tissues of mice, and this phenomenon markedly reduced the adverse effects of anticancer drugs (*23*, *24*). However, in clinical studies in cancer patients, no dramatic reduction in the adverse effects of such drugs was achieved (*25*, *37*, *38*). In normal adults, cadmium ingested via food is accumulated in the body at relatively high concentrations (*10*, *36*). Since the MT promoter appears already to be maximally activated by this high concentration of accumulated cadmium in humans, even when bismuth, which activates the MT promoter by acting on MREs in a manner similar to that of cadmium, is administered, MT synthesis might not be enhanced to any great extent. In our study, $Bi(NO_3)_3$ had no significant effect on MT promoter activity that had already been maximally activated by $CdCl_2$, while PF1070A markedly increased the activity of the MT promoter (Figure 7). This result suggests that, even when the MRE-mediated activation of the MT promoter is considerable as a result of accumulated Cd^{2+} ion in normal adults, it might be possible further to elevate the concentration of MT in tissues using PF1070A as an inducer that acts on a site other than MREs in the promoter region of the gene for MT. However, in a preliminary study in mice, PF1070A did not have a marked effect on the tissue concentration of MT (data not shown). PF1070A might be rapidly converted to an inactive substance in the body. Our investigation of the relationship between the chemical structure of PF1070A and its MT promoter-activating activity showed that the epoxy ring of PF1070A is not necessary for activity and that the carbonyl group on the side chain is essential (Figure 9). Synthesis of analogues with a carbonyl group as a side chain, using PF1070A as the leading compound, and examination of the effects of these analogues in cells and animals might lead to identification of a novel and clinically applicable inducer of the synthesis of MT.

REFERENCES

1. Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R., Rahmsdorf, H., Jonat, D., Herrlich, P., and Karin, M. (1987) *Cell 49*, 729.
2. Beach, L. R., and Palmiter, R. D. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2110.
3. Beato, M., Chalepakis, G., Schauer, M., and Slater, E. P. (1989) *J. Steroid Biochem. 32*, 737.
4. Coyle, P., Zalewski, P. D., Philcox, J. C., Forbes, I. J., Ward, A. D., Lincoln, S. F., Mahadevan, I., and Rofe, A. M. (1994) *Biochem. J. 303*, 781.
5. Dalton, T., Palmiter, R. D., and Andrews, G. K. (1994) *Nucleic Acids Res. 22*, 5016.
6. Endresen, L., Bakka, A., and Rugstad, H. E. (1983) *Cancer Res. 43*, 2918.
7. Filmus, J., Remani, J., and Kerin, M. H. (1992) *Nucleic Acids Res. 20*, 2755.
8. Hatayama, T., Asai, Y., Wakatsuki, T., Kitamura, T., and Imahara, H. (1993) *J. Biochem. 114*, 592.
9. Hatayama, T., Tsukimi, Y., Wakatsuki, T., Kitamura, T., and Imahara, H. (1992) *Mol. Cell. Biochem. 112*, 143.
10. Heilmaier, H. E., Drasch, G. A., Kretschmer, E., and Summer, K. H. (1987) *Toxicol. Lett. 38*, 205.
11. Heuchel, R., Radtke, F., Georgiev, O., Stark, G., Aguet, M., and Schaffner, W. (1994) *EMBO J. 13*, 2870.
12. Hiratuka, A., Kawashima, K., and Watabe, T. (1986) *Eisei Kagaku 32*, P-10.
13. Kägi, J. H. (1991) *Methods Enzymol. 205*, 613.
14. Karin, M., Andersen, R. D., and Herchman, H. R. (1981) *Eur. J. Biochem. 118*, 527.
15. Karin, M., Haslinger, A., Holtgreve, H., Krauter, P., Westphal, M., and Beato, M. (1984) *Nature 308*, 513.
16. Kelly, E. J., Sandgren, E. P., Brinster, R. L., and Palmiter, R. D. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 10045.
17. Koizumi, S., Yamada, H., Suzuki, K., and Otsuka, F. (1992) *Eur. J. Biochem. 210*, 555.
18. Lazo, J. S., and Bahnson, R. R. (1989) *Trends Pharmacol. Sci. 10*, 369.
19. Mayo, K. E., and Palmiter, R. D. (1981) *J. Biol. Chem. 256*, 2621.
20. Moran, E., Grodzicker, T., Roberts, R. J., Mathews, M. B., and Zerler, B. (1986) *J. Virol. 57*, 765.
21. Mueller, P. R., Salser, S. J., and Wold, B. (1988) *Genes Dev. 2*, 412.
22. Mulligan, R. C., and Berg, P. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2072.
23. Naganuma, A., Satoh, M., and Imura, N. (1987) *Cancer Res. 47*, 983.
24. Naganuma, A., Satoh, M., and Imura, N. (1988) *Jpn. J. Cancer Res. 79*, 406.
25. Osumi, M., Iijima, T., Koitabashi, H., and Nagamachi, Y. (1991) *Jpn. J. Cancer Chemother. 18*, 131.
26. Palmiter, R. D. (1987) in *Metallothionein II* (Kägi, J. H. R., and Kojima, Y., Eds.) p 63, Birkahauser-Verlag, Basel, Switzerland.
27. Palmiter, R. D. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 1219.
28. Plisov, S. Y., Nichiporenko, M. G., Shkapenko, A. L., Kumarev, V. P., Baranova, L. V., and Merkulova, T. I. (1994) *FEBS Lett. 352*, 339.
29. Radtke, F., Heuchel, R., Georgiev, O., Hergersberg, M., Gariglio, M., Dembic, Z., and Schaffner, W. (1993) *EMBO J. 12*, 1355.
30. Sato, M., and Bremner, I. (1993) *Free Radical Biol. Med. 14*, 325.
31. Satoh, M., Miura, N., Naganuma, A., Matsuzaki, N., Kawamura, E., and Imura, N. (1989) *Eur. J. Cancer Clin. Oncol. 25*, 1727.
32. Satoh, M., Naganuma, A., and Imura, N. (1988) *Cancer Chemother. Pharmacol. 21*, 176.
33. Satoh, M., Naganuma, A., and Imura, N. (1992) *Arch. Toxicol. 66*, 145.
34. Shiraishi, N., Utsumi, K., and Satoh, M. (1982) *Physiol. Chem. Phys. 14*, 533.
35. Stuart, G. W., Searle, P. F., Chen, H. Y., Brinster, R. L., and Palmiter, R. D. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 7318.
36. Summer, K. H., Drasch, G. A., and Heilmaier, H. E. (1986) *Hum. Toxicol. 5*, 27.
37. Takahashi, A., Takagi, M., Hidaka, H., Sakamoto, Y., Takagi, N., Amano, H., and Ogura, Y. (1989) *Jpn. J. Cancer Chemother. 16*, 2405.
38. Taniwaki, M., Yokota, S., Nishida, K., Tsuda, S., Edagawa, J., Misawa, S., Takino, T., and Abe, T. (1988) *Jpn. J. Cancer Chemother. 15*, 531.
39. Thornalley, P. J., and Vasak, M. (1985) *Biochim. Biophys. Acta 827*, 36.
40. Webb, M. (1979) in *The Chemistry, Biochemistry and Biology of Cadmium* (Webb, M., Ed.) p 195, Elsevier/North-Holland, Amsterdam.
41. Westin, G., and Schaffner, W. (1988) *EMBO J. 7*, 3763.
42. Williams, T. M., Burlein, J. E., Ogden, S., Kricka, L. J., and Kant, J. A. (1989) *Anal. Biochem. 176*, 28.
43. Yoshida, S., Yoshioka, M., Yaguchi, T., Nagasawa, M., and Koyama, M. (1993) *Sci. Rep. Meiji Seika Kaisha 32*, 58.
44. Zalewski, P. D., Forbes, I. J., and Betts, W. H. (1993) *Biochem. J. 296*, 403.

BI990655G