Interdisciplinary Studies on Environmental Chemistry—Biological Responses to Chemical Pollutants, Eds., Y. Murakami, K. Nakayama, S.-I. Kitamura, H. Iwata and S. Tanabe, pp. 225–239.
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Molecular Characterization of Avian Metallothionein (MT) 1 and 2 Isoforms: mRNA Expression, Transactivation Potency, and Detoxification Potential Associated with Element Exposure

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(Received 15 May 2008; accepted 15 July 2008)

Abstract—To assess the inducibility of avian MT genes and potential tolerability of their proteins to element exposure, we investigated the expression profiles of MT isoforms by metal accumulation in wild cormorants (*Phalacrocorax carbo*) and mallards (*Anas platyrhynchos*). We also measured transcriptional activities of avian MT genes by elements and detoxification potencies of their *in vitro*-expressed MT proteins. Expression levels of MT1 and MT2 in cormorant livers showed significantly positive correlations with Cu and Zn concentrations, suggesting a similar mechanism for transcriptional activation of both MT isoforms by these elements. Results of an *in vitro* reporter gene assay that both MT promoter/enhancer regions from cormorant were dose-dependently transactivated by treatments with Cu and Zn agreed well with these observations in the wild population. In contrast, no significant correlation was observed between hepatic Cd levels and MT mRNAs, whereas Cd was the most potent inducer of cormorant MT1/2 promoters by *in vitro* reporter gene assay. This may be due to the masking effect of multiple element exposures, probably by Zn and/or Cu that were highly accumulated in wild cormorants. *In vitro* transactivation potency by co-treatment of Zn with Cd supported that Zn could conceal Cd-induced MT expression in the wild cormorant population. In mallard, MT1 had no correlation with any metal while MT2 expression was positively correlated only with Cu, even though hepatic Cu and Zn concentrations in mallard were much higher than in cormorant. This indicates that cormorant is more susceptible to elemental exposure than mallard in terms of MT induction. MT1 and 2 proteins of cormorant and mallard endowed *E. coli* with significant higher growth rate than control to Cd exposure (500–1000 μM), implying that both avian MTs could be involved in the detoxification of intracellular Cd. This study provides the first evidence on the inducibility of avian MT isoforms by specific elements and functional significance of each avian MT isoform in detoxifying intracellular heavy metals.

Keywords: avian metallothionein (MT), inducibility, tolerability, elements, cormorant, mallard

INTRODUCTION

Induction of metallothionein (MT), a cysteine-rich metal-binding protein, is most likely a compensatory response for detoxification of metals through their binding to the induced MT proteins. In terms of sensitivity/tolerability to metal stress, understanding the regulatory mechanism and the molecular characterization of MTs are necessary to assess the risk of metal exposure to the target species. The metal-induced MT expression is primarily regulated at the transcriptional level through the metal-regulatory transcriptional factor (MTF-1). The core heptanucleotide metal-regulatory element (MRE; TGCRCNC), which is a target DNA sequence of MTF-1, has been identified in the 5′-flanking regions of MT genes. The *cis*-acting MREs are highly conserved in all mammalian MT genes, although the number of copies and distribution of MRE are different among species (Haq *et al.*, 2003).

In avian species, MREs were identified in the promoter region of chicken MT2, indicating that MTF-1 is essential for the transcriptional activation of chicken MT2 by metals (Fernando and Andrews, 1989; Dalton *et al.*, 1997). Except for the sequences of chicken MT2 gene promoter, however, nothing is known about the structure and function of avian MT promoters. Unlike mammals, in which multiple forms of MTs (MT1 to MT4) were characterized, a single avian MT gene and its exclusive expression have been alleged in avian species. As for other avian MT forms, no information on their presence and expression is available. Physiological and toxicological roles of MT proteins in various organisms, especially avian species also remain unclear.

Towards a comprehensive understanding of inducibility of avian MT genes by elements and further assessment of potential tolerability of their proteins to element exposure in avian species, we investigated hepatic expression profiles of MT mRNAs associated with metal accumulation in a wild population of cormorant and mallard. We also examined transactivation potencies of cormorant MT promoter/enhancer regions, and detoxification potential of cormorant and mallard MT proteins to element exposure.

MATERIALS AND METHODS

Sampling and RNA isolation

Eighteen cormorants were collected under appropriate permits from Lake Biwa and Mie Prefecture, and ten mallards were from Izumi coast, Kagoshima, Japan in 2003. The same specimens investigated for metal concentrations in our previous reports (Nam *et al.*, 2005) were used for the quantification of MT mRNA expression. Total RNA from liver tissues were isolated using RNAgents® Total RNA Isolation System (Promega).

Quantification of MT mRNAs

Previously, we identified two distinct MT isoforms in three avian species; novel MT1 and MT2 cDNAs were isolated from chicken, common cormorant,

and mallard (Nam *et al*., 2007). A set of specific primers (forward/reverse) and a TaqMan probe labelled with VIC were designed using the ABI PRISM Primer Express software (Applied Biosystems). Expression levels of MT mRNA levels were quantified by a real time RT-PCR method using TaqMan Two-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems) and ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The mRNA expression levels in each sample were normalized to 18S ribosomal RNA content.

Nucleotide sequencing of the 5′-flanking regions of cormorant MT1 and MT2

Genomic DNAs in cormorant liver samples were extracted using Wizard SV Genomic DNA Purification System (Promega) and digested with a set of four restriction endonucleases, DraI, EcoRV, PvuII, and StuI. For constructing the DNA library, the digested genomic DNA fragments were then ligated to the BD GenomeWalker Adaptor (BD Biosciences Clontech). The primary and secondary PCRs were performed using the adaptor primers (AP1 and AP2) (BD GenomeWalker^{TM} Universal Kit), and an outer (GSP1) and a nested gene-specific primer (GSP2) designed from the sequences of 5′-untranslated regions of common cormorant MT1 (ccMT1) and MT2 (ccMT2) genes (Manuscript in preparation).

Construction of ccMT1 and ccMT2 promoter/enhancer reporter plasmids

The complete sequences of the 5′-untranslated regions of ccMT1 and ccMT2 cDNAs were determined using RACE (rapid amplification of cDNA ends) method (Freedman *et al*., 1993; Nam *et al*., 2007). Transcription initiation sites (+1) of ccMT1 and ccMT2 were deduced to be located at 26 bp- and 42 bp-downstream from a putative TATA box (represented by the variant GATA or CATA), respectively.

To construct a reporter plasmid of ccMT1 promoter, the 5′-flanking sequence of ccMT1 (975 bp), including the putative initiation site (+1) and 55 bp of untranslated sequence as an KpnI-SacI fragment in pGEM-T Easy Vector, was subcloned into KpnI and SacI sites of pGL4.10[luc2] Basic Vector (Promega). The 5′-flanking region of ccMT2 gene inserted into pGEM-T Easy Vector (Promega) was amplified by PCR. Forward (containing ***KpnI*** restriction site) and reverse (***SacI*** restriction site) primers were 5′-ATGGGAGAGG***GGTACC***ATGA-3′ and 5′-CGGCG***GAGCTC***AGCTAGACT-3′, respectively. Amplified PCR products were cloned into pGEM-T Easy Vector and the sequences of fragments were subcloned into KpnI and SacI sites in pGL4.10[luc2] Basic Vector. The pGL4.10[luc2]-ccMT2 promoter reporter plasmid included 1247 bp of 5′-flanking and 24 bp of untranslated regions. The sequences of ccMT1 and ccMT2 fragments inserted in the reporter plasmids were confirmed by ABI PRISM^{TM} 310 Genetic Analyzer.

Cell culture, transient transfection, and ligand treatment

CV-1 cells from African green monkey (*Cercopithecus aethiops*) kidney

were propagated in 24-well plates (5×10^4 cells/well) for 24 h under 5% CO_2 at 37°C, in a phenol red-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS Hyclon). The cells (>90% confluent) were transfected with 200 ng of pGL4.10[luc2]-ccMT1 promoter or -ccMT2 promoter, and pGL4.10[luc2] Basic Vector using the Lipofectamine-2000 (Invitrogen) at 37°C for 5 h. The plasmid (phRL-TK control vector) that constitutively expresses luciferase as an internal standard was also cotransfected to correct transfection efficiencies. Following 5 h of incubation, CV-1 cells were harvested at 37°C for 18 h in 10% charcoal/dextran double treated fetal bovine serum (CDFBS) with each element including $Cd(NO_3)_2$, $PbHNO_3$, $TlNO_3$, $AgNO_3$, CH_3HgOH, $HgNO_3$, $K_2Cr_2O_7$, $Cr(NO_3)_3$, AsO_4H_3, As_6O_3, $NiCl_2$, $Cu(NO_3)_2$, $ZnHNO_3$, $Co(NO_3)_2$, $RbNO_3$, and $Bi(NO_3)_3$. For exposure of mixture compounds, 2 μM Cd were co-treated with 100–300 μM Zn, and also with 0.2–20 μM Cr for 5 h before harvesting.

Reporter gene assay

Transfected cell lysates were harvested, and then analyzed for luciferase activity using a Dual-Luciferase Reporter Assay System (Promega). All firefly luciferase activities were standardized for transfection efficiency by measurement of *Renilla* luciferase activities from phRL-TK control vector. The luciferase activity in CV-1 cells transfected with pGL4.10[luc2]-ccMT1 promoter or -ccMT2 promoter was expressed as relative value (fold induction) to the activity of the pGL4.10[luc2] basic vector.

Construction of an MT expression vector from cormorant and mallard

The open reading frame of MT 1 and 2 from cormorant and mallard was amplified by PCR with a set of attB-adaptor primers and attB-gene specific primers according to Gateway System (Invitrogen). The amplified PCR products were recombined with a donor vector pDONR 221 and the entry clones containing each avian MT gene were transformed into *E. coli* (DH5α) competent cell. The entry clone was then recombined with a destination vector pET-DEST42 to construct high inducible expression clones.

Heterologous expression of avian MT1 and MT2 proteins in E. coli

A constructed expression plasmid, pET-DEST42, in which cormorant and mallard MT1 and MT2 cDNAs were inserted in downstream of a T7*lac* promoter for IPTG-inducible expression, was used to transform BL21 (DE3) *E. coli* strains. The culture medium containing each expression vector was refreshed once to OD_{600} of 0.05, which allowed the cells to quickly return to logarithmic growth and to reach the appropriate cell density. After shaking at 37°C for 1 h, IPTG was added to a final concentration of 1.0 mM, and the cells were cultured for an additional 5 h (OD_{600} = 0.7–0.8). The cell pellet was harvested by centrifugation at 8000 rpm for 10 min at 4°C, and was resuspended in a final concentration of

1 mg/ml lysozyme. The cell suspension was then incubated on ice and a rocking platform for 30 min and 10 min, respectively. The supernatant fractions were separated by centrifugation at 12000 rpm for 20 min at 4°C. Using the supernatant fractions, the recombinant avian MT1 or MT2 proteins were immunochemically detected by Western blot analysis.

Measurement of growth rates of E. coli harboring each avian MT isoform

A portion of *E. coli* harboring each avian MT isoform was refreshed to an OD600 of 0.05 in 5 ml LB medium, and was inoculated for 1 h at 37°C. IPTG was then added to a final concentration of 1.0 mM. Thirty minutes later, the cells were cultured with various concentrations of each heavy metal including Cd (0–1000 μM), Ag (0–500 μM), Zn (0–2000 μM), and Cu (0–2000 μM) at 37°C for 4.5 h. Growth rate (OD_{600}) of *E. coli* was measured by UV/VIS spectrophotometry.

Immunochemical detection of avian MTs

Supernatant fractions from *E. coli* were resolved by sodium deodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A 20 μl of supernatant fractions were placed on 5–20% gradient polyacrylamide gels (ATTO) in duplicate. The fractions were separated by gel electrophoresis at 40 mA for 1.5 h. Protein standards (Bio-Rad) were simultaneously loaded onto the gradient gels as a marker. The resolved fractions were electrophoretically transferred to polyvinylidene fluoride membranes at 43 mA for 75 min using a SemiPhor semidry transfer unit (Amersham Biosciences). The membranes were probed with anti-V5 linked to HRP (Horseradish peroxidase) for avian MT detection. Recombinant avian MT proteins containing a recognition site of epitope derived from the paramyxovirus, SV5, were immunochemically detected with anti-V5-HRP antibody. Detection of the antibody cross-reactive proteins was performed using a highly sensitive ECL Western blotting system (Amersham Biosciences). Protein bands were visualized by an imaging analyzer, ChemiDoc, and quantified using Quantity One (Bio-Rad). Expression levels of the cross-reactive proteins in individual samples were expressed as a relative value to the staining intensity in a certain sample.

Statistical analysis

Relationships between metal concentrations and MT mRNA expression levels were examined using Spearman's rank correlation test. Each *in vitro* experiment was performed in triplicate, and the results were presented as mean ± SD. ANOVA followed by Sheffe's post-hoc test was performed with SPSS package, version 12.0 (SPSS Japan Inc., Tokyo, Japan). $P < 0.05$ was regarded as statistically significant. EC_{50} (50% of effective concentration) and LOEL (lowest-observed-effect-level) for the transactivation of MT promoter/enhancer regions by individual elements were calculated using SigmaPlot, version 9.0 (Systat Inc., Richmond, CA, USA).

Table 1. Correlation coefficients between metal concentrations and MT isoform expression levels in livers of cormorant and mallard.

	Cormorant (n = 18)			Mallard (n = 10)		
	MT1	MT2	Total MTs†	MT1	MT2	Total MTs†
Mn	0.06	0.25	0.22	0.13	0.38	0.35
Co	–0.32	0.16	0.13	–0.08	0.51	0.47
Cu	0.60**	0.65**	0.70**	–0.01	0.70*	0.70*
Zn	0.57*	0.54*	0.57*	–0.10	0.16	0.14
Se	–0.15	0.33	0.21	–0.53	–0.15	–0.29
Rb	–0.43	–0.60**	–0.63**	0.46	0.04	0.33
Mo	0.13	0.32	0.32	–0.38	0.09	0.01
Ag	0.37	0.62**	0.56**	–0.46	0.58	0.25
Cd	–0.13	0.01	0.02	–0.35	0.43	0.33
Hg	–0.18	0.09	0.07	–0.37	0.10	0.00

†Total MTs = MT1 + MT2. Correlation coefficients were analyzed by Spearman's rank correlation test.

*$p < 0.05$, **$p < 0.01$.

RESULTS AND DISCUSSION

Metal- and isoform-specific expression of MT mRNAs in cormorant and mallard

Hepatic expression of both MT isoforms of cormorants were positively correlated with Cu and Zn concentrations (Table 1), suggesting both isoforms are induced by Cu and Zn accumulation and there is a similar mechanism of metal-induced transcription between MT1 and MT2 isoforms. A significant positive correlation of mRNA expression levels between MT1 and MT2 in liver of cormorants ($r = 0.787$, $p < 0.01$) also supports the sharing regulatory mechanism.

In contrast to cormorant, only MT2 isoform showed a positive correlation with Cu concentration in the liver of mallard, and no correlation was detected between MT1 expression and any metal concentration (Table 1). In addition, there was no correlation between MT1 and MT2 in the liver. These findings suggest that two MT isoforms may be differentially regulated in response to metal accumulation at transcriptional level in the liver of mallards.

Transactivation potencies of ccMT promoter/enhancer regions by elements

We have succeeded in isolating 1230 bp and 1278 bp of the 5′-flanking regions of ccMT1 and ccMT2 genes with respect to putative transcription initiation site, respectively (Manuscript in preparation). Sequential analysis of these regions revealed that ccMT promoters lacked a canonical TATAAA motif, but the motif was replaced by GATAAA in ccMT1 and CATAAA in ccMT2. Two potential MREs and a single GC box are present within the promoter/enhancer regions of ccMT1. ccMT2 gene is preceded by two MREs, a single anti-oxidant

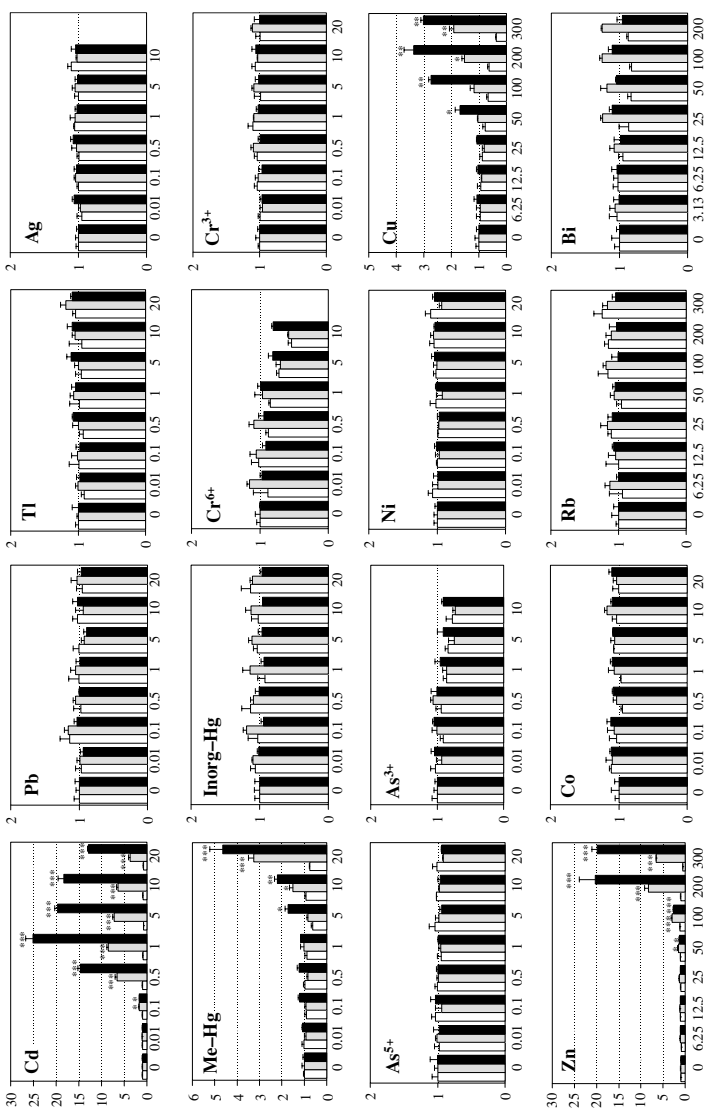

Fig. 1. Transcriptional activities of cormorant MT1 and MT2 promoter/enhancer regions by various elements. The luciferase activity in CV-1 cells transfected with the pGL4.10[luc2]-ccMT1 promoter (□) and pGL4.10[luc2]-ccMT2 promoter (■) are shown as relative (as a fold induction) to the activity in control cells transfected with pGL4.10[luc2] basic vector (□). Asterisks show statistical differences from control cells ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

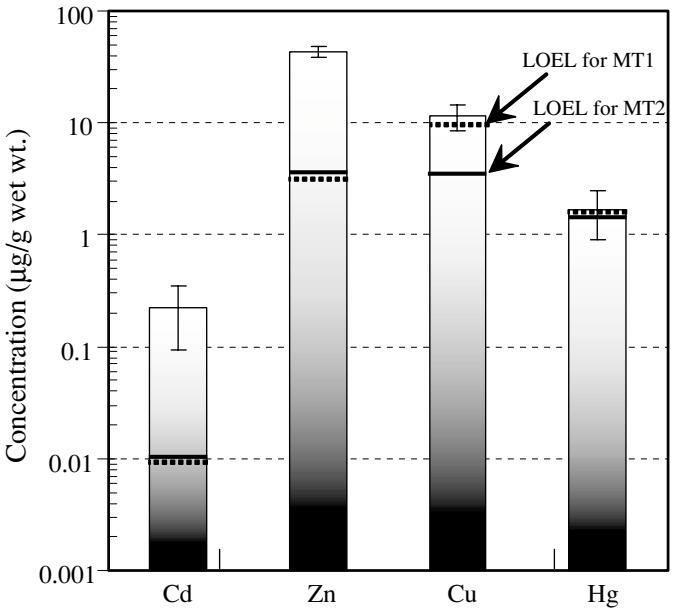

Fig. 2. Comparison of Cd, Zn, Cu, and Hg concentrations (■; mean ± SD) in the liver of wild cormorants and LOELs for cormorant MT1 (dashed line) and MT2 (solid line) promoter/enhancer regions. Mercury concentration represents total Hg (in which methyl Hg was approximately 70–80%), but the LOELs for MT1 and MT2 promoters are indicated on the basis of methyl Hg concentration.

responsive element (ARE), four GC boxes, and two E boxes.

We constructed reporter plasmids containing promoters of ccMT1 (975 bp of the 5′-flanking sequences including a putative cap site (+1) and 55 bp of untranslated sequences) and ccMT2 (1247 bp of 5′-flanking sequences including a putative cap site (+1) and 24 bp of untranslated sequences). Using these reporter plasmids, we investigated transactivation potencies of ccMT1 and ccMT2 promoter/enhancer regions by an *in vitro* reporter gene assay. Both ccMT1 and ccMT2 promoter/enhancer regions were dose-dependently activated by treatment with Cd, methyl Hg, Cu, and Zn (Fig. 1). The comparison of LOELs for individual elements revealed that Cd was the most potent activator for ccMT1 (0.10 μM) and ccMT2 (0.10 μM) promoters, followed by methyl Hg (10 μM for ccMT1 and 5.0 μM for ccMT2), Zn (50 μM for both ccMTs), and Cu (200 μM for ccMT1 and 50 μM for ccMT2) (Fig. 2). The *in vitro* transactivation potency for ccMT2 by each element was greater than that for ccMT1. On the other hand, no transcriptional activation by Pb, Tl, Ag, inorganic Hg, Cr(VI), Cr(III), As(V), As(III), Ni, Co, Rb, and Bi was observed (Fig. 1).

Similar responses of ccMT1 and ccMT2 promoters by Cd, Zn, Cu, and methyl Hg in the present study are consistent with the results that the two MT

mRNAs showed significant positive correlations with Zn and Cu levels in the liver of wild cormorants (Table 1). While the structures of MT gene enhancer/promoter regions are species- and isoform-specific, MT gene generally contains multiple copies of MREs that confer metal-induced transcriptional activity. Assuming that expression of cormorant MT isoforms is regulated by metal-binding regulatory proteins including MTF-1, similar responses of the two MT promoters to Cd, Zn, Cu, and methyl Hg may be due to a sharing mechanism of transcription (e.g. same regulatory protein(s) with MREs and/or other responsive elements in the promoter/enhancer regions) (Searle *et al*., 1984; Yagle and Palmiter, 1985), even if there are variations in the distribution of MRE and other elements in the two promoter/enhancer regions. Although LOELs for the transactivation of ccMT1 and ccMT2 induced by Cd, Zn, Cu, and methyl Hg exposure were similar, transactivation potency of ccMT2 promoter was about 1.5 to 3.0-fold more prominent than that of ccMT1 promoter, reflecting the higher transcriptional efficiency of ccMT2 promoter by these elements.

Since hepatic Ag and Rb levels exhibited significant correlations with ccMT2 mRNA in the liver of wild cormorants (Table 1), we investigated whether both elements can induce transcriptional activation of cormorant MT promoters in our *in vitro* assay system. The results exhibited no transactivation potency of cormorant MT1 and MT2 promoters within the ranges of Ag and Rb levels detected in the wild cormorant population (Fig. 1). Thus, the significant correlations of Ag and Rb with MT2 mRNA expression may be due to the retention of Ag and Rb by MT2 protein induced by other elements (e.g. Zn and/or Cu).

Elements vary in their effectiveness as inducers of different MT isoforms in mammals. Human MT1A and MT2A are induced by heavy metals in different manner (Richards *et al*., 1984; Koizumi *et al*., 1999), while rodent MT homologues are similarly regulated by Zn, Cd, and/or Cu (Searle *et al*., 1984; Yagle and Palmiter, 1985; Lehman-McKeeman *et al*., 2000). When the expression vector for MTF-1 was cotransfected into cells along with a reporter vector containing mouse MT1 promoter, metals including Cd, Zn, Cu, Ag, Bi, Hg, Ni, and Co induced reporter gene activities via MTF-1 that recognizes MRE in the promoter regions (Palmiter, 1994). Induction of MTs in the rat liver by various metals including Pb has also been reported (Arizono *et al*., 1985; Waalkes and Klaassen, 1985). Although there is no conclusive evidence which mammalian MT isoform is orthologous to avian MTs, avian MTs may be evolutionarily close to mammalian MT1 and 2 than MT3 and 4 with respect to proximal DNA sequences including MREs in the promoter/enhancer regions (Manuscript in preparation). The transactivation potencies of ccMT1 and ccMT2 promoters by Zn, Cd, and Cu appeared to be similar with those of rodent MT1s and MT2s, but the responses by other elements including inorganic Hg, Ag, Bi, Ni, Co, and Pb were different between cormorant and rodent MTs. Such dissimilar responses may be associated with their different structures of promoter/enhancer regions.

To examine whether the relationships between MT mRNA expression levels and element accumulation observed in a wild cormorant population could be reproduced by an *in vitro* reporter gene assay, we compared element concentrations

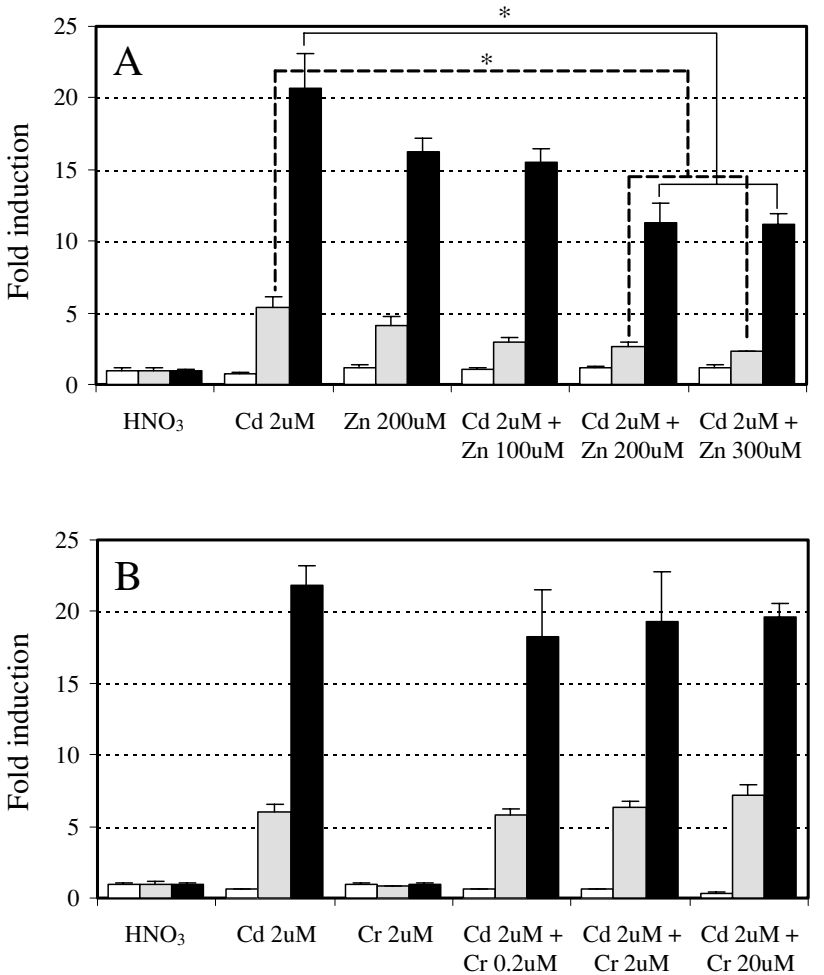

Fig. 3. Transcriptional activities of cormorant MT1 and MT2 promoter/enhancer regions by elements. A, CV-1 cells were transfected with the reporter plasmids (pGL4.10[luc2]-ccMT1 promoter (▒), pGL4.10[luc2]-ccMT2 promoter (■), and pGL4.10[luc2] basic vector (□)) that were left untreated or treated with Zn^{2+}, Cd^{2+}, or Zn^{2+} and Cd^{2+} for 5 h. B. CV-1 cells transfected with reporter plasmids were either left untreated or treated with Cr^{6+} and/or Cd^{2+} before harvest for 5 h. Luciferase activity was determined as relative (as a fold induction) to the activity in control cells transfected with the pGL4.10[luc2] basic vector (□). Asterisks show statistical differences from control cells ($*p < 0.05$).

in the liver of wild cormorants with their LOELs for transactivation of ccMT1 and ccMT2 promoters (Fig. 2). The comparison revealed that Cd was the most potent inducer of ccMT1 and ccMT2 promoters, followed by methyl Hg, Zn, and Cu. As the LOELs of ccMT promoters by Zn and Cu were lower than the levels in liver

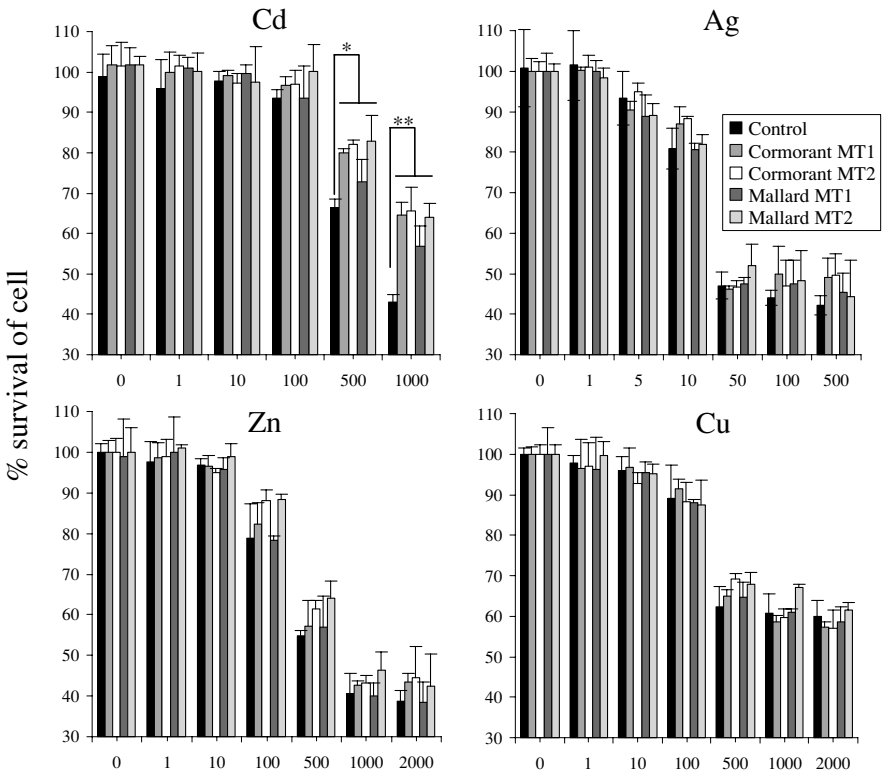

Fig. 4. Effects of heavy metals (Cd, Ag, Zn, and Cu) on growth of *E. coli* harboring without (control) or with (cormorant MT1, cormorant MT2, Mallard MT1 or Mallard MT2) MT genes. Asterisks show statistical difference from control cells ($*p < 0.05$, $**p < 0.01$).

of wild cormorant populations, the significant positive correlations of MT1 and MT2 mRNA levels with Zn and Cu concentrations found in the wild population appear to be reasonable. Since LOELs of methyl Hg for the transactivation of both MT promoters were higher than the levels in the cormorant livers, methyl Hg concentration in the wild population may be considered not to be high enough to induce MT mRNA expression. While LOEL for Cd was much lower than Cd levels in the wild cormorants, no significant correlation of MT mRNAs with Cd levels was observed in the wild population (Table 1). Because ccMT1 and ccMT2 promoters are transactivated mostly by Zn and/or Cu, this could be due to the masking effect of multiple element exposures, especially by Zn in the wild population.

To investigate whether Zn could affect Cd-induced transactivation of MT genes, cells were co-treated with Cd^{2+} (2 μM) and Zn^{2+} (100–300 μM), and the results were compared with those of a single injection of Zn^{2+} (200 μM) or Cd^{2+} (2 μM) (Fig. 3A). After a single injection of 2 μM Cd^{2+} (that corresponds to an

average level in the liver of wild cormorant population) or 200 μM Zn^{2+} (that is less than average level (700 μM) that can induce cellular toxicity in CV-1 cells) into the cells for 5 h, transactivation of ccMT1 and ccMT2 promoters significantly increased when compared to that in non-treated cells. By treatment with a mixture of Cd (2 μM) and Zn (100, 200 or 300 μM), no alteration of transactivation was detected in comparison with that of Zn (200 μM) alone. Co-treatment with Cd (2 μM) and 200 or 300 μM of Zn inhibited the transactivational activity of MT1 and MT2 by 40%, when compared to the result of Cd (2 μM) alone. These results are consistent with previous findings that the expression of MT was not additive by co-treatment with Cd (5.5 μM) and Zn (308 μM), whereas each metal was found to induce MT expression in rat liver (Scheuhammer *et al.*, 1985). However, the mechanism of suppression of transactivation by the mixture of Cd and Zn remains unclear.

Hexavalent chromium (100 μM) preferentially suppresses the induction of mouse MT1 and human MT2A in HepG2 cells by Cd (30 μM) or Zn (100 μM), although Cr(VI) alone does not induce the expression of those MTs (Majumder *et al.*, 2003). Since it is apparent that the inhibitory effect by Cr(VI) occurs at the transcriptional level, following the DNA binding of MTF-1, Cr(VI) might have inhibited the interaction of the MTF-1 transactivation domains with other cofactors (Majumder *et al.*, 2003). To examine whether Cr(VI) affects Cd-induced transactivation of ccMT1 and ccMT2 promoters, we treated the ccMT promoter-transfected cells with Cd^{2+} (2 μM) and Cr^{6+} (0.2, 2, or 20 μM) (Fig. 3B). Hexavalent chromium alone was unable to induce MT gene transactivation at concentrations ranging from 0.01 to 10 μM (that correspond to levels detected in the liver of wild cormorants). No suppressive effect of Cr^{6+} (0.2, 2, or 20 μM) on transactivation by Cd^{2+} was observed. Therefore, it is likely that Zn and/or other elements, but not Cr^{6+} could conceal Cd-induced MT expression in the wild cormorant population.

Effects of heavy metals on growth rate of E. coli harboring each avian MT isoform

For functional analysis of MTs, *E. coli* has been used as a host for the heterologous expression of mammalian MTs (Zheng *et al.*, 2004). Cormorant and mallard MT1 and MT2 isoforms were individually expressed in *E. coli*. By Western blot analysis, a single cross-reactive band with a molecular weight of 10.3 to 10.5 kDa for each avian MT isoform was detected in the *E. coli* supernatant fractions, where the cells were inoculated for 0.5 h and for 5 h after adding IPTG (Manuscript in preparation). These results suggest that avian MTs were efficiently inducible under the current experimental condition. Each avian MT protein in *E. coli* was quantified after 5 h inoculation. The result indicated a similar production efficiency of recombinant proteins for the two MT isoforms both in cormorant and mallard.

Figure 4 shows a growth rate of *E. coli* after administration of Cd, Ag, Zn, and Cu. Up to 100 μM of Cd concentrations had no apparent effect on the overall growth rate of the *E. coli* harboring control and each avian MT expression vector.

However, at 500 and 1000 μM of Cd, *E. coli* transfected with each MT showed a significantly higher growth rate than the control vector, but no isoform- and species-dependent growth rate was observed among transformed MTs. It is generally accepted that basic function of MT is to protect cells from the toxic effects of heavy metals. The heterologous expression of mouse MT1 endowed resistance in *E. coli* up to 830 μM of Cd (Hou *et al*., 1988). Other investigations showed that in *E. coli* (JM105) expressing human MT2 after Cd exposure (893 μM), about 30% of Cd was present in the form of Cd-binding MT, indicating a greater tolerance of *E. coli* transformed with human MT2 than that with control vector (Odawara *et al*., 1995). Expression of MT1 and MT2 from cormorant and mallard also endowed *E. coli* with resistance to high levels of Cd (500 and 1000 μM), suggesting that avian MTs could be involved in detoxification of intracellular Cd.

In contrast, no significant difference in growth rates between the cells transformed with control and each avian MT expression vector was observed by treatment with Ag (0–500 μM), Zn (0–2000 μM), and Cu (0–2000 μM) (Fig. 4). In previous studies, *E. coli* expressing human MT2 that were incubated in the medium containing Zn (0–4600 μM) and Cu (0–4600 μM) showed no resistance (Odawara *et al*., 1995), whereas the expression of mouse MT1 in *E. coli* showed a detoxification potential to those heavy metals (Hou *et al*., 1988). The difference in the ability of resistance between mouse MT1 and human MT2 in *E. coli* is unclear. It has been reported that *E. coli* showed resistance to Zn, Cu, or Ag by their intrinsic heavy metal regulatory machinery (Brown *et al*., 1991; Cooksey, 1994; Helmann, 1997; Nies and Brown, 1997). In this study, *E. coli* carrying control vector showed similar resistance to Zn, Cu, or Ag, compared with cells expressing each avian MT isoform. It is possible that a native control system in *E. coli* participated primarily in the regulation of Zn, Cu, or Ag, thus detoxification ability of the avian MT heterologously expressed in *E. coli* might be submerged by their indigenous control system. The physiological role and metal-binding mechanism of each avian MT isoform deserve further investigation.

The present study provides the first direct evidence on the inducibility of avian MT isoforms by specific elements and functional significance of each avian MT isoform to detoxify intracellular heavy metals. Our *in vitro* approaches demonstrated the validity to predict the response of MTs to element exposure in a wild avian population.

Acknowledgments—This study was supported by Grants-in-Aid for "21st Century COE Program" and "Global COE Program" from the Ministry of Education, Culture, Sport, Science and Technology, Japan. The award of the JSPS (Japan Society for the Promotion of Science) Postdoctoral Fellowship for Foreign Researchers in Japan to Dong-Ha Nam (ID No. 06058) is acknowledged.

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