

Original Article

## Effects of organic and inorganic mercury(II) on gene expression via DNA conformational changes

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**ABSTRACT** — The effects of organic and inorganic mercury ions on gene expression were studied by *in vitro* cell-free assays for transcription and translation. While organic mercury (methylmercury chloride, MeHgCl) showed no effects, treatment of template DNA with inorganic mercury (HgCl<sub>2</sub>) inhibited mRNA synthesis in a bacteriophage T7 RNA polymerase transcription system. The inhibited transcription resulted in reduced protein synthesis after the subsequent application of the transcripts to a translation system consisting of Sf21 insect cell lysate. Treatment of mRNA with inorganic mercury also reduced translation, although this inhibitory effect was weaker than the effect produced by DNA exposure. Treatment of DNA and RNA with mercury did not increase oxidative damage such as strand cleavage and base oxidation. Instead, circular dichroism spectrometry demonstrated that mercury ions, not methyl mercury, drastically changed strand conformation of DNA and RNA. Therefore, the gene expression inhibition observed in this study was thought to be caused by crossbridging of DNA bases with mercury ions, which blocked the transcriptional machinery. Taken together with reports on biological conversion of organic mercury to inorganic forms in animals, our results show that transcriptional inhibition via conformational changes in DNA could be a toxic mechanism involved in mercury poisoning.

**Key words:** Mercury toxicity, Gene expression, DNA binding, Demethylation

### INTRODUCTION

Mercury (Hg) is present in the environment in its elemental, inorganic, and organic forms. In humans, while intestinal absorption of elemental and inorganic Hg is very low, organic Hg compounds such as methylmercury (MeHg) are almost completely resorbed via enterohepatic circulation, and thus accumulate at high levels in the body (Naganuma and Imura, 1984). MeHg poisoning causes severe dysfunction of the central nervous system. Catastrophic human mercury exposure has occurred in several areas around the world, including Japan, and many patients currently suffer from the sequelae of such exposure. In Japan, mercury poisoning causes Minamata disease in people who have ingested biologically concentrated MeHg in fish caught in the Shiranui Sea area. The adverse health effects of Hg ingestion through seafood constitute a global concern (UNEP, 2013). The toxicological mechanisms of Minamata disease have been reviewed based on observations of tissue samples from afflicted patients (Eto, 1997); however, the molecular

mechanisms through which MeHg induces central nervous system-specific toxicity are not fully understood.

Thiol groups (R-SH) of protein cysteine residues can be targeted by Hg cations such as Hg<sup>2+</sup> and MeHg<sup>+</sup> due to their high affinity for thiolate anions (R-S<sup>-</sup>). Although the cellular abundance of selenol groups (R-SeH) is lower than that of thiol groups, Hg prefers to bind to selenols because they are about 1,000 times stronger acids than thiols, with a pK<sub>a</sub> of approximately 5.2 vs. 8.3 for thiols (Sugiura *et al.*, 1976). Selenocysteine is translationally incorporated into selenoproteins and forms a catalytic site due to its low pK<sub>a</sub>. Several selenoproteins, such as glutathione peroxidase 1 and thioredoxin reductase, have been reported as targets through which Hg-induced oxidative damage is mediated (Usuki *et al.*, 2011).

Although recent transcriptomic and proteomic analyses revealed a comprehensive set of cellular factors responding to Hg exposure (Hwang *et al.*, 2011; Toyama *et al.*, 2011; Vendrell *et al.*, 2007), it is noteworthy that previous studies have reported the effects of Hg on mRNA and protein syntheses using radiolabeled substrates with brain

tissue from regions including the cerebellum, a specific target of Hg (Sarafian and Verity, 1985; Syversen, 1982; Yoshino *et al.*, 1966). These previous reports suggested the hypothesis, which has not been studied recently, that Hg might exert toxicity via modulation of gene expression itself, in addition to altering the profile of gene expression that occurs as a cellular response to Hg. Chemical interaction between Hg and nucleic acids has been studied in detail (Kuklenyik and Marzilli, 1996; Ono and Miyake, 2003; Onyido *et al.*, 2004). Therefore, we hypothesized that DNA and/or RNA could be targets of Hg through which its toxicity is mediated. In the present study, we investigated the effects of Hg binding to DNA and RNA on their structure and function using circular dichroism (CD) spectrometry and *in vitro* gene expression systems, respectively. Our results suggest that perturbation of gene expression, especially at the transcriptional level, could be involved in the toxicity of Hg.

## MATERIALS AND METHODS

### Materials

Mercury(II) chloride (HgCl<sub>2</sub>) was purchased from Katayama Chemical Industries Co., Ltd. (Osaka, Japan). Methylmercury(II) chloride (MeHgCl) was purchased from Alfa Aesar (Ward Hill, MA, USA). Other chemicals were of the best commercially available grade. Calf thymus DNA, guanosine (G), 2'-deoxyguanosine (dG), and 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 8-Hydroxyguanosine (8-oxoG) was purchased from Alexis Biochemicals (San Diego, CA, USA). Nuclease P1 was purchased from Yamasa Shoyu (Chiba, Japan). Calf intestine alkaline phosphatase and *Hind* III were purchased from Roche Diagnostics (Basel, Switzerland). Chlorophenol red-β-D-galactopyranoside (CPRG), diethylenetriamine-*N,N,N',N''*-pentaacetic acid (DTPA), and ethylenediamine-*N,N,N',N'*-tetraacetic acid disodium salt (EDTA) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

### Plasmid DNA preparation

Plasmid DNA pTD1-Gal was a gift from Dr. Toru Ezure at Shimadzu Corp. (Kyoto, Japan) (Ezure *et al.*, 2010). *Escherichia coli* DE21 (laboratory stock) was transformed with pTD-Gal using a Ca<sup>2+</sup>-containing solution and the transformant was cultured overnight in the presence of 100 µg/mL ampicillin. The plasmid was purified using a kit (Plasmid Mega, Qiagen, Hilden, Germany) and quantified based on relational expression by the following equation: DNA (µg/mL) = A<sub>260</sub> × 50.

### mRNA synthesis by *in vitro* transcription

Plasmid DNA was linearized with *Hind* III before being used as a template for an *in vitro* transcription system using bacteriophage T7 RNA polymerase (Script-MAX, Toyobo, Osaka, Japan). The procedure was performed according to the manufacturer's instructions. The mRNA transcript coding for *lacZ* was purified with a kit (RNeasy mini, Qiagen) and quantified based on relational expression by the following equation: RNA (µg/mL) = A<sub>260</sub> × 40.

### Hg treatment of DNA or RNA

To prepare Hg-treated samples, plasmid DNA or synthesized *lacZ* mRNA (1 mM) was mixed with the indicated concentration of HgCl<sub>2</sub> or MeHgCl in 4 mM phosphate buffer (pH 7.8) at 37°C for 2 hr. The mixture was divided for analyses via HPLC, electrophoresis, and *in vitro* transcription or translation. For HPLC, the DNA or mRNA was purified via ethanol precipitation and subjected to measurement of 8-oxodG or 8-oxoG as described below. For electrophoresis and *in vitro* reactions, Hg-treated mixtures were used without ethanol precipitation to avoid possible detachment of bound Hg. Preincubation assays with the enzyme solutions confirmed that the effects of unbound Hg on gene expression in the reaction system were negligible.

### Measurement of base oxidation

Hg-treated DNA or mRNA (100 µM base) was dissolved in 20 mM sodium acetate buffer (pH 5.0) after ethanol precipitation and subsequently digested to nucleosides with nuclease P1 (3.4 units) at 37°C for 30 min followed by calf intestine alkaline phosphatase (1.3 units) at 37°C for 1 hr in 0.1 M Tris-HCl (pH 7.5). Levels of 8-oxodG and 8-oxoG were analyzed by an HPLC instrument (LC-10 series, Shimadzu) equipped with an electrochemical detector (ECD, Coulochem II, ESA, Chelmsford, MA, USA). The HPLC conditions were as follows: the column was an ODS-80Ts (Tsk-gel, 150 × 4.6 mm i.d., Tosoh, Tokyo, Japan), the column temperature was 25°C, the flow rate was 1 mL/min, and the detection wavelength was 254 nm (for dG or G). The ECD conditions were: guard cell, 400 mV; E, 150 mV; R, 100 µA; filter, 2; output, 1 V (channel 1); E, 300 mV; R, 200 nA; filter, 10; output, 1 V (channel 2).

### DNA cleavage assay

A mixture containing plasmid DNA (100 µM base) was incubated for 2 hr at 37°C with the indicated concentrations of HgCl<sub>2</sub> or MeHgCl in 4 mM sodium phosphate buffer (pH 7.8), and 10 µL of the mixture was subject-

ed to electrophoresis using a 1% agarose gel containing ethidium bromide in 40 mM Tris-acetate/1 mM EDTA (TAE buffer, pH 7.8). The fluorescent band image was recorded by an enclosed camera on a UV illuminator.

### RNA cleavage assay

Synthesized mRNA was mixed with RNA loading buffer AG (BioDynamics Laboratory Inc., Tokyo, Japan) and formaldehyde, heated at 75°C for 3 min, and loaded into non-denaturing 1% agarose gels for electrophoresis in TAE buffer. Migration of the RNA was detected as a fluorescent image.

### *In vitro* translation

The *lacZ* mRNA obtained by *in vitro* transcription was translated into protein by a cell-free system using an extract of insect cell Sf21 according to the manufacturer's instructions (Transdirect Insect Cell, Shimadzu). The reaction was performed for 5 hr at 25°C in a 25- $\mu$ L system containing 4.5  $\mu$ L of the reaction solution from *in vitro* transcription.

### Protein activity assay

The protein obtained by the *in vitro* translation was evaluated for  $\beta$ -galactosidase activity by a colorimetric assay. The assay solution (50  $\mu$ L) containing the translation reaction mixture (10  $\mu$ L) and 0.1 mg/mL CPRG was incubated at 37°C for 30 to 60 min. The activity was measured as a relative value against the control (without Hg) sample.

### CD spectrophotometry

Calf thymus DNA or mRNA (100  $\mu$ M base) was mixed with HgCl<sub>2</sub> or MeHgCl in 2 mL of 4 mM sodium phosphate buffer (pH 7.8) containing DTPA (5  $\mu$ M) at room temperature. CD spectra were recorded on a CD spectrophotometer (J-725, Jasco, Tokyo, Japan) under the following measurement conditions: light path length, 1 cm; range, 230-350 nm for DNA or 200-340 for mRNA; band width, 1.0 nm; sensitivity, 200 mdeg; resolution, 0.5 nm; response, 2 sec; speed, 200 nm/min.

## RESULTS

### Oxidative damage in Hg-treated DNA

We investigated oxidative DNA damage by measuring 8-oxodG formation and strand cleavage in plasmid DNA following treatment with HgCl<sub>2</sub> or MeHgCl. Neither 8-oxodG nor strand cleavage were detected after treatment with either form of Hg at concentrations up to 200  $\mu$ M (Figs. 1A and 1B; Figs. 2A and 2B).

### Transcription of Hg-treated DNA

To investigate gene transcription from Hg-exposed DNA, *in vitro* transcription was performed using a DNA plasmid containing the reporter gene *lacZ*. Transcripts representing rapidly migrating "shorter" mRNAs were detected at concentrations of HgCl<sub>2</sub> greater than 10  $\mu$ M (Fig. 1C). The abundance of the shorter mRNAs increased with increasing concentrations of HgCl<sub>2</sub>, and full-length transcripts were not detected after treatment with 100  $\mu$ M HgCl<sub>2</sub>. In contrast, MeHgCl treatment did not induce any detectable change in transcript length (Fig. 2C). Preincubation of RNA polymerase with HgCl<sub>2</sub> or MeHgCl did not change the activity of the enzyme (data not shown).

### Translation of mRNA prepared from Hg-treated DNA

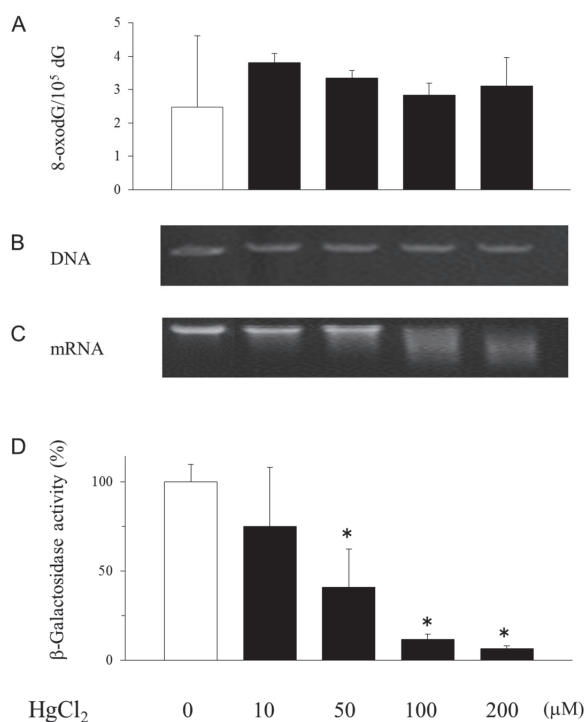
Following the *in vitro* transcription of the Hg-exposed plasmid DNA, *in vitro* translation of the resulting transcript was performed. The  $\beta$ -galactosidase enzyme activity of the protein product decreased with increasing concentrations of HgCl<sub>2</sub> (Fig. 1D). This decrease was consistent with the protein levels detected via western blotting (data not shown). No significant change was observed in the  $\beta$ -galactosidase enzyme activity of the protein product from mRNA prepared from the plasmid DNA exposed to MeHgCl (Fig. 2D).

### Effects of Hg on mRNA oxidative damage and translation

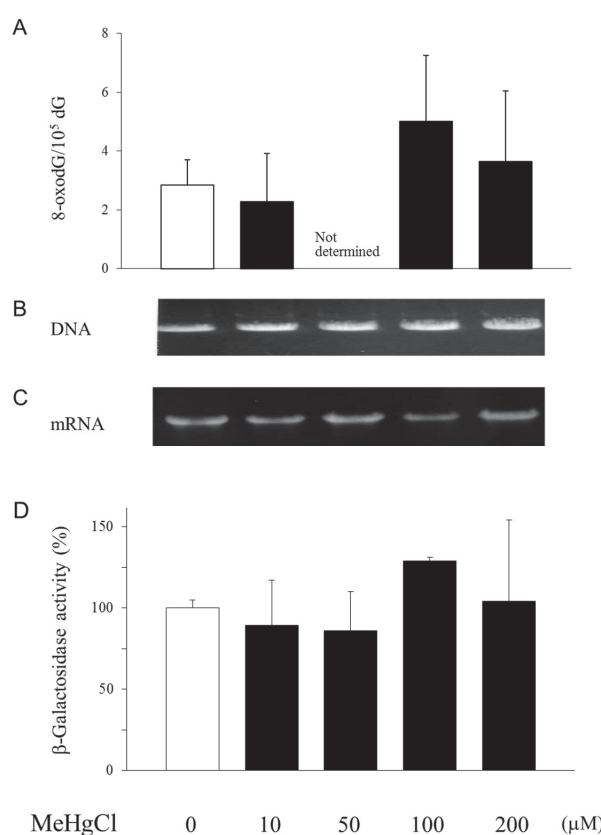
The effects of HgCl and MeHgCl on mRNA were investigated, as with DNA, via assays for oxidative damage and translational inhibition. Intact mRNA prepared by the *in vitro* transcription system was treated with HgCl<sub>2</sub> or MeHgCl and subjected to HPLC, electrophoresis, or *in vitro* translation. Oxidative damage was not detected (Figs. 3A and 3B), but activity of the translational product was decreased by exposure to 500  $\mu$ M HgCl<sub>2</sub> (Figs. 3C). This concentration is approximately 10 times greater than that required to exert an inhibitory effect on transcription following exposure of DNA to HgCl<sub>2</sub>. The exposure of mRNA to MeHgCl produced no significant differences in oxidative damage or translational activity (Fig. 4).

### Conformational changes in DNA and mRNA induced by Hg

Hg-induced DNA conformational changes were assessed based on the alteration of CD spectra. A positive Cotton effect at 280 nm, which is a characteristic of B-form DNA, decreased with increasing concentrations of HgCl<sub>2</sub> and became negative at concentrations greater than 10  $\mu$ M (Fig. 5A). HgCl<sub>2</sub> also changed the CD spectra of



**Fig. 1.** HgCl<sub>2</sub>-induced DNA damage and effects on gene expression: A, 8-oxodG; B, DNA cleavage; C, mRNA; D, enzyme activity. Each column and bar indicates mean  $\pm$  S.D. and the band images are representative of at least 3 independent reactions. Asterisks indicate that the mean value is significantly different from the control value (Hg 0  $\mu$ M) at  $P < 0.01$  with Student's *t*-test.



**Fig. 2.** MeHgCl-induced DNA damage and effects on gene expression: A, 8-oxodG; B, DNA cleavage; C, mRNA; D, enzyme activity. Each column and bar indicates mean  $\pm$  S.D. and the band images are representative of at least 3 independent reactions.

mRNA, but the effective concentrations were almost 10 times greater than those that produced an effect on DNA (Fig. 5B). MeHgCl induced no detectable conformational change in the CD spectra of DNA or mRNA (Figs. 5C and 5D).

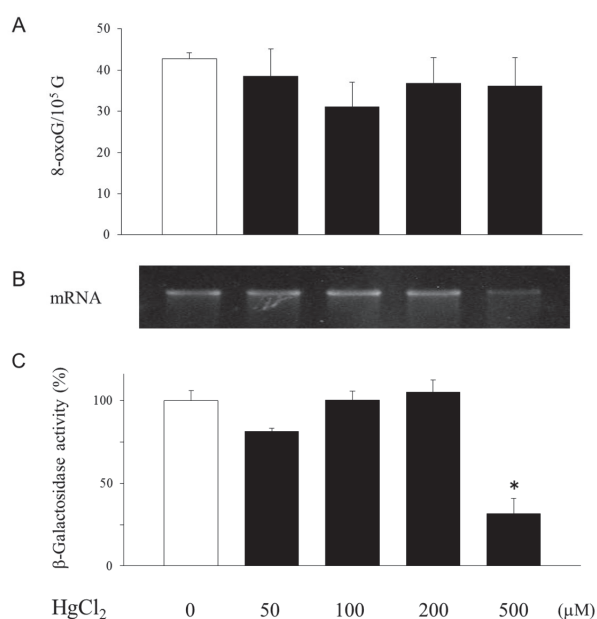
## DISCUSSION

### DNA/RNA strand modification and gene expression inhibition

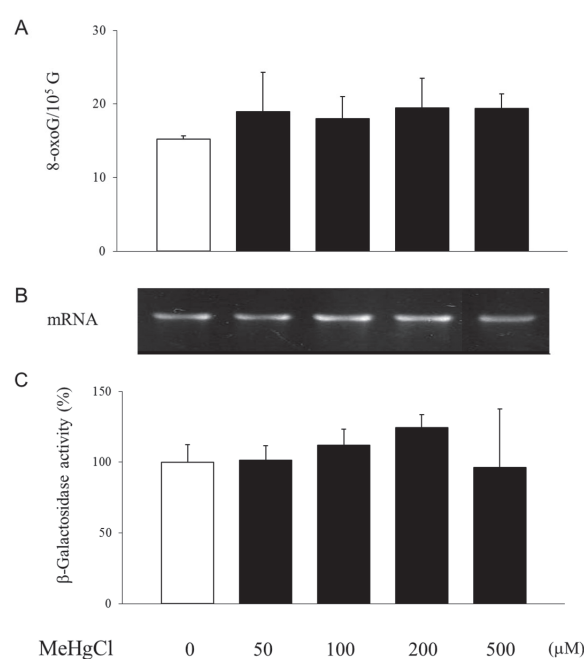
The present study assessed the effects of Hg interaction with DNA/RNA on gene expression using *in vitro* reconstituted transcription and translation systems. Our results revealed that inorganic Hg ions repress gene expression mainly at the transcriptional level, and transcriptional inhibition was accompanied by a conformational change in the template DNA. The CD spectra indicated drastic conformational changes from the normally-ob-

served B-DNA to reverse-wound Z-DNA (Gruenwedel, 1994). Among the metal ions, silver (Ag<sup>+</sup>) and mercury (Hg<sup>2+</sup>) induce CD phase transition at relatively low molar ratios of less than 0.5 in comparison to nucleotides (Rossetto and Nieboer, 1994). Reduced processibility of RNA polymerase has been reported for Z-DNA (Butzow *et al.*, 1984), although the precise influence of this change was not clear in our system. Blocking transcriptional machinery at positions where Hg is bound to the coding regions of reporter genes may result in aberrant short transcripts. The insertion of Hg<sup>2+</sup> into an A/T-rich region was shown to produce a "bulge" structure by NMR study of oligo-DNA duplexes (Froeystein and Sletten, 1994). A DNA base crosslinked with Hg<sup>2+</sup> would prevent RNA polymerases from dividing the sense and antisense DNA strands, which precedes nascent mRNA synthesis. Although methylmercury ion (MeHg<sup>+</sup>) also binds to various nucleotides with comparable affinity

## Structural and functional modification of DNA by mercury ions



**Fig. 3.** HgCl<sub>2</sub>-induced mRNA damage and effects on gene expression. A, 8-oxoG; B, mRNA cleavage; C, enzyme activity. Each column and bar indicates mean  $\pm$  S.D. and the band images are representative of at least 3 independent reactions. Asterisks indicate that the mean value is significantly different from the control value (Hg 0  $\mu$ M) at  $P < 0.01$  with Student's  $t$ -test.



**Fig. 4.** MeHgCl-induced mRNA damage and effects on gene expression: A, 8-oxoG; B, mRNA cleavage; C, enzyme activity. Each column and bar indicates mean  $\pm$  S.D. and the band images are representative of at least 3 independent reactions.

to that of Hg<sup>2+</sup> (Simpson, 1964), MeHg<sup>+</sup> cannot form a crosslink due to the occupation of a binding site with a methyl group. Thus, MeHg<sup>+</sup> is unable to affect DNA conformation and gene expression. Higher concentrations of Hg<sup>2+</sup> were needed to produce translational inhibition than were needed to produce transcriptional inhibition. This difference could be explained by differences in structure; mRNA is usually single-stranded and thus contains fewer positions for Hg-mediated crossbridging than double-stranded DNA. Moreover, ribosomes on the mRNA are capable of resolving Hg-mediated secondary structures that prevent protein translation.

### Transcriptional inhibition and neurotoxicity

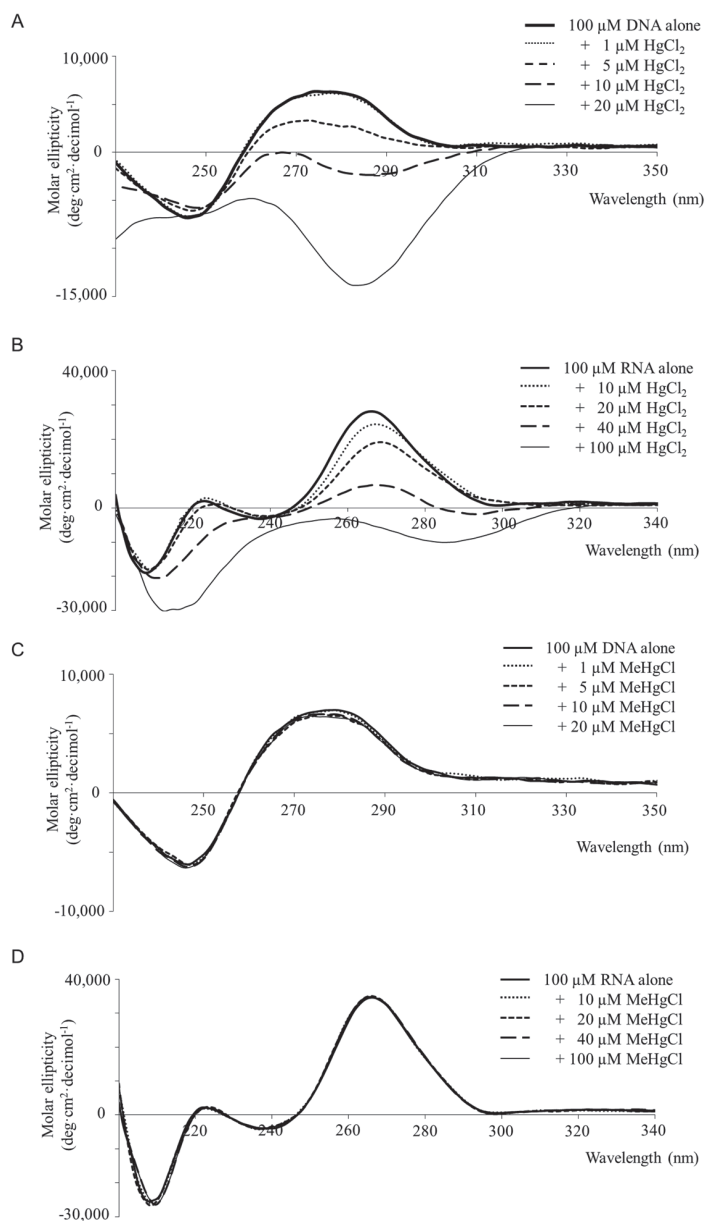
DNA damage is considered a cause of carcinogenesis in proliferative cells. Although neuronal cells are less proliferative in adults, studies have revealed that DNA damage disrupts proper functioning of the nervous system (Hetman *et al.*, 2010). Transcription from template DNA may be influenced by various DNA damage types, including strand breaks, base oxidation, and adduct formation. Transcriptional repression has been reported to

induce atypical neuronal cell death that is distinct from apoptosis, necrosis, or autophagy (Hoshino *et al.*, 2006). In addition to protein expression via mRNA synthesis, transcription contributes gene regulation via non-coding RNAs (Tal and Tanguay, 2012) and genomic stability via transcription-coupled DNA repair (Jaarsma *et al.*, 2011). Together with our present study, these results suggest that aberrant transcription of Hg-bound DNA underlies the neurotoxic mechanisms of Hg.

### Toxicological activity of organic and inorganic Hg

In the present study, HgCl<sub>2</sub> affected the conformation of DNA/RNA, and thus affected translation and transcription processes, whereas MeHgCl did not. However, in general, the chemical forms showing stronger neurotoxicity are organic Hg forms such as MeHgCl, rather than inorganic Hg forms such as HgCl<sub>2</sub>. This inconsistency could be reconciled by assuming that MeHg<sup>+</sup> is converted into Hg<sup>2+</sup> in target organs by demethylation. Although the bond between Hg and methyl group in MeHg<sup>+</sup> is stable, it can be cleaved by reaction with reactive oxygen spe-





**Fig. 5.** Effects of Hg treatment on the conformation of DNA and RNA: A, DNA with HgCl<sub>2</sub>; B, mRNA with HgCl<sub>2</sub>; C, DNA with MeHgCl; D, mRNA with MeHgCl.

cies (Chen *et al.*, 2003; Suda *et al.*, 1991). This demethylation was reported to be possible in biological systems using tissue culture or slices (Shapiro and Chan, 2008; Yasutake and Hirayama, 2001). Accumulation of inorganic Hg in the brain has been observed in primates after long-term exposure to MeHgCl (Vahter *et al.*, 1995). In a 22-year follow-up study of a MeHgCl-exposed family in the United States, the detected mercury was almost completely found in the inorganic form (Davis *et al.*, 1994).

MeHg<sup>+</sup> easily crosses the blood-brain barrier, while Hg<sup>2+</sup> crosses poorly. Therefore, Hg<sup>2+</sup> tends to be trapped inside the brain by the demethylation of MeHg<sup>+</sup>. Although the present results were obtained with *in vitro* cell-free systems that do not simulate pharmacokinetics processes such as metabolism, they suggest that DNA and RNA represent critical targets of Hg toxicity, and the mechanisms underlying this effect will be determined by future studies.

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**Conflict of interest----** The authors declare that there is no conflict of interest.

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