REVERSAL OF HEAVY METAL-DIRECTED INHIBITION OF RNA SYNTHESIS IN ISOLATED MOUSE LIVER NUCLEI

Yukimasa HAYASHI and Ei-ichi MIKAMI
Institute for Developmental Research, Aichi Colony, Kasugai, Aichi 480-03, Japan

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1. Introduction

The molecular mechanism of the heavy metal ion toxicity to living cells has remained uncertain. Inhibitory effects of heavy metal ions on RNA synthesis have been observed in isolated rat liver nuclei [1–4] and in the purified *Escherichia coli* RNA polymerase system [5]. On the other hand, exposure to sublethal doses of heavy metals such as zinc, cadmium, mercury and copper induces synthesis of small metal-binding proteins, metallothioneins, in a wide range of mammals and cultured mammalian cells. It is believed now that thionein is a protein that detoxifies some of the heavy metal ions [6]. Here, we present evidence that apothioneins (metal-free thioneins) are very effective proteins in suppressing the heavy metal-directed inhibition of RNA synthesis and that metallothioneins are not inhibitory to RNA synthesis nor do they suppress heavy metal-directed inhibition. Moreover, it is illustrated that the inhibited activity of RNA synthesis in isolated nuclei regains the original level by the addition of apothionein, implying that heavy metal ions bind to some components of the RNA synthesis machinery in a reversible manner.

2. Materials and methods

2.1. *Nucleus preparation*

Livers of 2–3 month male mice (C57BL/6J) were washed extensively with phosphate-buffered saline and homogenized in a Potter-Elvehjem type homogenizer with 6 vol. 2.2 M sucrose–3.3 mM $CaCl_2$ at 0°C. The homogenate was filtered through 4 layers of gauze and overlaid on 10 ml (1/2 vol. homogenate) of cold 2 M sucrose containing N-buffer which consisted of 10 mM Tris–HCl (pH 8.0), 3 mM $CaCl_2$, 0.5 mM dithiothreitol and 2 units/ml rat liver RNase inhibitor. Nuclei were pelleted by centrifugation at 51 000 × *g* for 60 min at 4°C in a Hitachi RPRS 25 rotor, suspended in 0.3 M sucrose containing N-buffer with the aid of a loosely-fitted and hand-stroked teflon homogenizer. The nucleus suspension was overlaid on an equal volume of solution containing 25% glycerol, 50 mM Hepes–Na(pH 8.0), 5 mM Mg-acetate and 2 units/ml RNase inhibitor, and centrifuged at 1000 × *g* for 7 min at 4°C in a Hitachi RPRS 14 rotor. Pelleted nuclei were suspended again in a small volume of the underlaid solution and stored in small aliquots at −75°C until use. Optical microscope examination of isolated nuclei displayed no intact cell contamination and <2% broken nuclei.

2.2. *Preparation of metallothioneins and apothioneins*

Cadmium-induced metallothionein-I and -II were prepared from livers of C57BL/6J mice by almost the same procedure as in [7]. The modifications were reductions of possible metallothionein dimers by 10 mM dithiothreitol and subsequent dialysis against 10 mM $CdCl_2$ before the second Sephadex G-50 column. The modifications increased Cd^{2+} contents of metallothionein-I and -II from ~4 mol to 6.4 and 6.2 mol, respectively.

Apothionein-I and -II were prepared from cadmium-induced metallothionein-I and -II by releasing metals as in [8].

2.3. *RNA synthesis in isolated nuclei*

The standard reaction mixture of RNA synthesis consisted of 50 mM Hepes–Na (pH 8.0), 5 mM Mg-acetate, 200 mM KCl, 10% glycerol, 2 units/ml rat liver RNase inhibitor, 50 μM [^{3}H]UTP (5 μCi/nmol), 500 μM each of ATP, GTP, CTP and 2 × 10^7 nuclei/

ml. The reaction was started by the addition of nuclei and carried out at 25°C, in 50 μl or 100 μl total vol. The chloride salts of heavy metals and metallothionein were added to the standard reaction mixture. At the end of the reaction, 20 μl aliquots were applied to Whatman GF/C glass filter discs and the discs were plunged into cold 6% trichloroacetic acid for 10 min, washed by shaking slowly in a sectioned stainless-steel screen, 4 times with cold 6% trichloroacetic acid and once with cold 99% ethanol, dried and counted with a toluene-based scintillation cocktail in a Beckman LS-233 counter.

2.4. *Isotopes and chemicals*

The materials used in these experiments were obtained as follows: [5-^{3}H]UTP (10–12 Ci/mmol) and [5-^{3}H]CTP (17-18 Ci/mmol) from The Radiochemical Center; RNase inhibitor from rat liver from Searle Diagnostic; ATP, GTP, UTP, CTP and bovine serum albumin from Sigma; glass filter GF/C from Whatman; chloride salts of heavy metals and other chemicals from Nakarai Chem., Kyoto.

3. Results and discussions

3.1. *Inhibition of RNA synthesis in isolated nuclei by heavy metals*

RNA synthesis in isolated mouse liver nuclei was examined in the presence of heavy metal ions at various concentrations. As shown in fig.1, metal ions, except Mg^{2+}, exhibited concentration-dependent inhibitions. Among them, the inhibitory effects of Pb^{2+} and Cd^{2+} were marked, while Mn^{2+} was inhibitory only at high concentrations. The order of inhibition by these metal ions was similar to that observed in

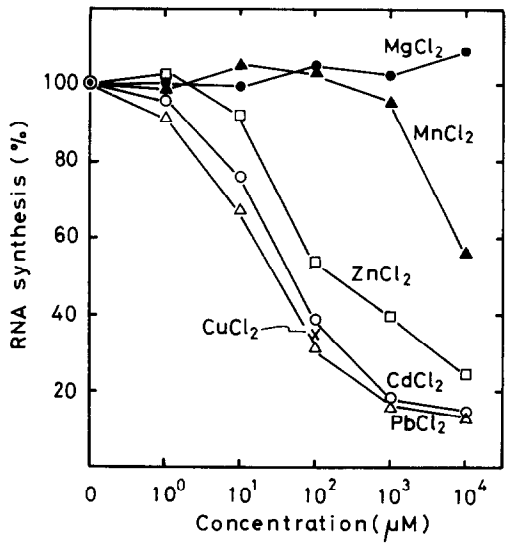

Fig.1. Effects of increasing concentrations of metal salts on RNA synthesis in isolated nuclei. Standard reaction mixture (50 μl) of RNA synthesis in isolated nuclei incorporated on av. 14.6 pmol [^{3}H]UMP in 30 min at 25°C.

Table 1
Suppression of heavy metal-directed inhibition of RNA synthesis

	$PbCl_2$		$CdCl_2$		$ZnCl_2$		$CuCl_2$	
Control (+ no metal)	14.50	100	14.90	100	14.00	100	14.4	100
+ Metal 100 μM	4.62	31.9	6.12	41.1	6.31	45.0	4.63	32.1
+ Metal + MT-I[a] 7.0 μg	4.61	31.8	6.27	42.1	5.98	42.7	5.66	39.3
+ Metal + MT-II[a] 7.0 μg	4.93	34.0	5.97	40.1	5.76	41.2	5.28	36.7
+ Metal + AT-I[a] 5.9 μg	15.0	103.5	16.8	112.7	15.6	111.7	15.7	109.0
+ Metal + AT-II[a] 5.4 μg	13.5	93.2	16.0	107.3	13.1	93.7	15.1	105.1
+ Metal + BSA[b] 3.1 μg	5.15	35.5	5.65	37.9	5.53	39.5	4.18	29.1
+ Metal + BSA[b] 6.2 μg	5.55	38.3	7.73	51.9	6.05	43.2	5.04	35.0
+ Metal + Cysteine 0.1 mM	–		7.08	47.5	–		–	
0.2 mM	–		8.71	58.5	–		–	
0.4 mM	–		10.6	71.2	–		–	
1.0 mM	–		13.7	91.9	–		–	

[a] Concentrations of metallothionein-I, -II, apothionein-I and -II were 20.4, 20.1, 19.2 and 17.9 μM, respectively
[b] Bovine serum albumin

Incorporations of [^{3}H]UMP in 30 min per 50 μl of the standard reaction mixture are indicated (pmol %). Figures are averages of triplicate samples

the system of purified *E. coli* RNA polymerase and DNA [5], although the extents of inhibition at lower concentrations of metal ions were larger in the present system.

Inhibition of RNA synthesis by Pb^{2+}, Cd^{2+}, Zn^{2+} and Cu^{2+} at 100 μM ranged from 50–70%. The result apparently indicates that the toxicity of a heavy metal to the mammalian cell is at least partly due to inhibition of RNA synthesis.

3.2. *Suppression of the heavy metal-directed inhibition*

Metallothioneins are inducible proteins believed to detoxify heavy metal ions [6]. However, it also has been reported that the injection of metallothioneins into animals brings about more profound toxicity than free cations [9,10]. We examined the effects of metallothioneins, apothioneins (metal-free thioneins) and bovine serum albumin (as control) on RNA synthesis and on the heavy metal-directed inhibition of RNA synthesis.

As shown in table 1, metallothioneins and bovine serum albumin had little effect on the heavy metal-directed inhibition of RNA synthesis, while apothioneins strongly suppressed the inhibition. Apothioneins themselves were not stimulators of RNA synthesis but rather weak inhibitors (fig.2A). Comparing the suppressing activity of apothionein with that of cysteine which is assumed to react with Cd^{2+} [4], the former is >50-times that of cysteine on the molar basis (table 1). The results in fig.2 also indicate that the suppressing activity of apothionein possibly resulted from binding of Cd^{2+} since 18 μM apothionein suppressed the inhibition almost completely (if 1 mol apothionein binds 6–7 mol Cd^{2+}, 18 μM is just sufficient to bind 100 μM Cd^{2+}.). On the other hand, 20 μM metallothionein which contained 100 μM or more Cd^{2+}(determined by atomic absorption) showed no inhibitory effect on RNA synthesis (fig.2A) and no suppression of the inhibition (fig.2B).

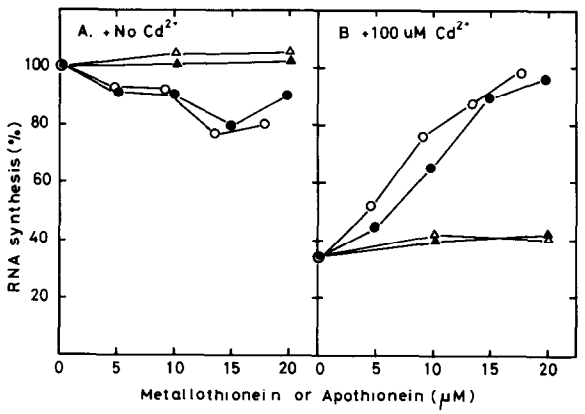

Fig.2. Effects of increasing concentrations of apothionein and Cd-thioneins on RNA synthesis in the presence or absence of $CdCl_2$. Reaction conditions were as in fig.1. Apothionein or Cd-thionein was added as the indicated final concentration just prior to the start of the reaction: (–●–) apothionein-I; (–○–) apothionein-II; (–▲–) Cd-thionein-I; (–△–) Cd-thionein-II.

3.3. *Reversal of Cd^{2+}-directed inhibition by subsequent addition of apothionein*

The inhibition of an enzyme reaction is often irreversible on account of the irreversible inactivation of the enzyme and/or of the irreversible binding of the inhibitor to a component of the enzyme reaction. To examine the reversibility of the heavy metal-directed inhibition of RNA synthesis, cadmium was chosen as a representative of heavy metals. After RNA synthesis had started in the presence of 100 μM Cd^{2+}, an aliquot of the reaction mixture was transferred to pre-warmed tube containing the same volume and concentration of salts, [^{3}H]UTP, ATP, GTP, CTP, $CdCl_2$ and 40 or 36 μM apothionein. As shown in fig.3, the addition

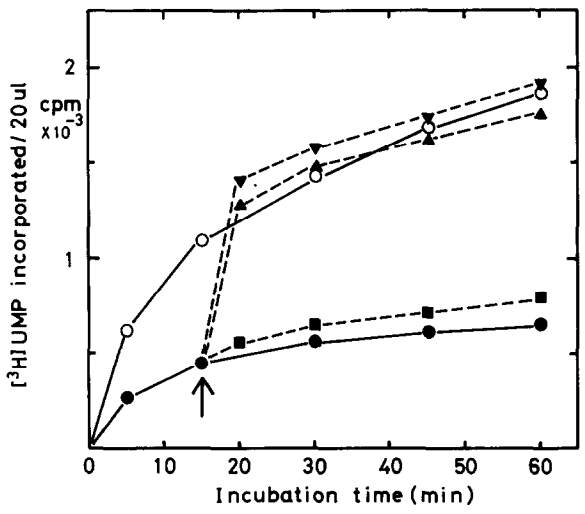

Fig.3. Reversal of Cd^{2+}-directed inhibition of RNA synthesis. Aliquots of Cd-added reaction mixture were transferred at 15 min (→) to tubes containing apothionein or bovine serum albumin and other reaction constituents including $CdCl_2$: (–○–) no $CdCl_2$; (–●–) 100 μM $CdCl_2$; (–▲–) 100 μM $CdCl_2$ + apothionein-I 20 μM; (–▼–) 100 μM $CdCl_2$ + apothionein-II 18 μM; (–■–) 100 μM $CdCl_2$ + bovine serum albumin 930 μg/ml.

of the apothionein clearly allowed RNA synthesis to resume, while the same amount of bovine serum albumin showed only little such activity. The slight recovery of RNA synthesis by the addition of bovine serum albumin might be caused by the weak activity of serum albumin to bind Cd^{2+} [11] or by the dilution of nuclei on transferring to the new reaction mixture. The result implies that inhibitions of RNA synthesis by heavy metals are reversible processes and that the RNA synthesis machinery in nuclei is fully active after the inhibition is reversed by the addition of apothionein (also see table 1). However, it should be noted that the assay of RNA synthesis in isolated nuclei described here usually measures RNA accumulation which includes effects on many components of the system other than RNA polymerase and DNA. The target of heavy metals in the inhibition of RNA synthesis in isolated nuclei is under study.

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