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# Metallothionein mediates gene expression of 3.1 mRNA (PTZ17) related to epileptic seizure

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**Abstract Genes differentially expressed in association with disruption of the metallothionein gene were screened using two hepatic stellate cell lines isolated and established from the livers of normal 129/Sv (IMS/N cells) and transgenic mice deficient in the genes for metallothionein-I and -II (IMS/MT (−) cells). We found one cDNA (tentatively named NM31) that was expressed only in IMS/N cells. Transfecting IMS/MT (−) cells with the genes for both metallothionein-I and -II resulted in NM31 expression. These results suggest that metallothionein is essential for NM31 gene expression. The nucleotide sequence of NM31 (294 bp) was identical to the 3′ region of 3.1 mRNA (PTZ 17), which is abundant in the embryonic mouse brain and is related to chemically induced seizures. The present study indicates that metallothionein mediates the expression of specific genes. This is a novel explanation for some of the functions of metallothionein.** 

*Key words:* Metallothionein; Gene expression; NM31; 3.1 mRNA; PTZ 17; Stellate cell

## 1. Introduction

Metallothionein(s) is a small protein found in most eukaryotic species. Cysteine residues account for approximately 30% of the total amino acid content of metallothionein [1,2] which has roles in protection against the toxic effects of heavy metals, xenobiotics and γ-irradiation, as well as in drug resistance, homeostasis of essential metals and free radicals scavenging [1,3–5]. The high content and intermolecular localization of cysteine residues in metallothionein are highly conserved among species; these facts are considered to be extremely important to metallothionein function [1,6]. On the other hand, nuclear localization of metallothionein in mammalian cells has been reported by several investigators [7–9], indicating that metallothionein may also be associated with nuclear functions. We speculated that metallothionein is involved in gene expression. We therefore screened the genes that are expressed when the metallothionein gene is disrupted.

## 2. Materials and methods

### 2.1. Isolation and immortalization of stellate cells

According to the method of Kitamura et al. [10], hepatic stellate cells were isolated from the livers of transgenic mice deficient in the genes for metallothionein-I and -II [11] and from normal 129/Sv mice. The cells obtained were immortalized by infection of SV40 virus. To confirm immortalization, expression of SV40 T antigen was determined by RT-PCR (to be described in detail elsewhere).

### 2.2. Differential display screening

Total RNA was extracted from exponentially growing cells or from mouse liver using guanidinium thiocyanate-phenol-chloroform [12]. Differential display proceeded as described by Liang and Pardee [13]. Total RNA (0.1 μg) was converted to single-stranded cDNA using MMLV-reverse transcriptase and primers anchored by one base in a volume of 20 μl. The solution (1 μl) containing cDNA was then added to a PCR reaction mixture (10 μl) containing 2 μM of the anchored and random primers, 2 μM dNTP, 0.5 μCi of [α-$^{32}$P]dCTP and 0.5 units of *Taq* polymerase (Boehringer). PCR proceeded using a thermal cycler (MP; Takara) through 40 cycles of 94°C for 30 s, 40°C for 2 min and 72°C for 30 s followed by 72°C for 5 min. The amplified cDNAs (3.5 μl) were separated on a 6% sequencing gel, blotted on 3M paper, dried and exposed to X-ray film. Bands of interest were eluted from the gel by boiling in water. DNA was precipitated in ethanol, then re-amplified using the same primer set and PCR conditions except the dNTP concentrations were 20 μM and no isotope was added. PCR samples were separated on a 1.5% agarose gel, extracted and used as a probe for Northern hybridization.

### 2.3. Northern blot analysis

Total RNA (20 μg) was resolved by electrophoresis on 0.8% agarose/formaldehyde gels, then transferred to nylon membrane (Hybond-N+, Amersham), and UV cross-linked. The membrane was then hybridized overnight with random-primed [$^{32}$P]dCTP-labelled cDNA fragments obtained as above. The membrane was washed and exposed to X-ray film overnight at −80°C with an intensifying screen. The membrane was stripped and re-probed with $^{32}$P-labelled GAPDH cDNA as an internal control.

### 2.4. Gene transfer

IMS/MT (−) cells were incubated with a calcium phosphate precipitate containing 12 μg of DNA (10 μg of pKH plasmid containing the MT-I and -II genes [14] and 2 μg of pcDEBD containing hygromycin-B phosphotransferase as a selective marker) for 7 h. This mixture was replaced with normal medium and incubated for a further 48 h. Stably transfected cells were selected by culture in the presence of hygromycin-B (300 μg/ml, Boehringer) for about 14 days. Clones resistant to hygromycin-B were selected and expanded.

## 3. Results and discussion

We isolated and established SV40-immortalized stellate cell lines from the livers of transgenic mice deficient in the genes for metallothionein-I and -II [11] and from normal 129/Sv mice (controls). These cells were designated as IMS/MT (−) (immortalized mouse stellate cells from MT-null mouse) and IMS/N (immortalized mouse stellate cells from normal mouse), respectively. We examined total RNA samples from both cell lines by differential display [13], and we found one cDNA (tentatively named NM31) that was expressed only in IMS/N cells (Fig. 1a). Northern hybridization using NM31 as a probe confirmed that this gene was expressed at negligible

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levels in IMS/MT (−) cells (Fig. 1b). These results suggested that metallothionein(s) is involved in NM31 gene expression. To confirm this notion, plasmid pKH containing mouse metallothionein-I and -II genes [14] (a kind gift from Dr. R.D. Palmiter) was introduced into IMS/MT (−) cells. Stable transformants called IMS/MT (−)/pKH, expressed both normal and disrupted mutant metallothionein I and II mRNA as shown in Fig. 2a. Synthesis of metallothionein protein was induced by zinc chloride in IMS/N and IMS/MT (−)/pKH, but not in IMS/MT (−) cells. Moreover, IMS/MT (−)/pKH cells were significantly less sensitive than IMS/MT (−) cells to cadmium, indicating that IMS/MT (−)/pKH cells produce functional metallothionein proteins. We then compared the level of NM31 gene expression in IMS/MT (−) and IMS/MT (−)/pKH cells by Northern hybridization. NM31 was expressed at high levels in IMS/MT (−)/pKH cells (Fig. 2b). This finding suggests that NM31 gene expression is essentially mediated by metallothionein. It is believed that most of the biological functions of metallothionein are due to an abundance of cysteine residues. The present study presents a new concept to explain the biological roles of metallothionein: it may mediate the expression of specific genes.

Next, we examined the effect of induction of synthesis of metallothionein on expression of NM 31. The level of expression of NM31 gene in IMS/N cells determined by Northern blot analysis was not changed by the treatment with zinc chloride whereas the mRNA level of metallothionein-I was significantly increased. This result suggests that the basal level of expression of metallothionein in IMS/N cells might be enough to mediate the expression of NM31 gene.

The nucleotide sequence of NM31 (294 bp) was identical to the 3′ region of 3.1 mRNA [15], which is abundant in the embryonic mouse brain. The gene for 3.1 mRNA is conserved at least in the mouse and in humans, where it is mainly expressed in the cerebellum, hippocampus and olfactory bulb [15]. On the other hand, Kajiwara et al. [16] also isolated the gene for this 3.1 mRNA (PTZ17) as the gene related to chemically induced seizures. The expression of 3.1 mRNA in the mouse brain is significantly depressed by an intraperitoneal administration of pentylenetetrazole, which induces seizures [16]. During the pentylenetetrazole-induced bursting activity, which is characteristic of convulsions, intracellular calcium is released from storage sites and moved to the inner surface of the cell membrane in neurons [17,18]. The injection of 3.1 mRNA into *Xenopus* oocytes potentiates the pentylenetetrazole-induced calcium inward current and an intracellular calcium increase [16]. On the other hand, a brain-specific isoform (MT-III) of metallothionein has been isolated and observed to inhibit survival of cultured rat neurons and to be deficient in the brains of patients with Alzheimer's disease [19,20]. Erickson et al. [21] reported that mice deficient in metallothionein-III are more susceptible to seizures induced by kainic acid. Therefore, metallothionein-III might play a role in the expression of 3.1 mRNA in the brain. We could not observe expression of metallothionein-III mRNA in both IMS/MT (−) and IMS/N cells. We then transfected the metallothionein-III gene into IMS/MT (−) cells to obtain transformants stably expressing metallothionein-III (IMS/MT (−)/MT-III). The levels of NM31 mRNA in IMS/MT (−)/MT-III cells determined by Northern hybridization were almost equivalent to IMS/N cells (data not shown). These results suggest that NM31 gene expression is also mediated by metallothionein-III. It is possible to conclude that metallothioneins might be involved in chemically induced seizures by mediating 3.1 mRNA gene expression.

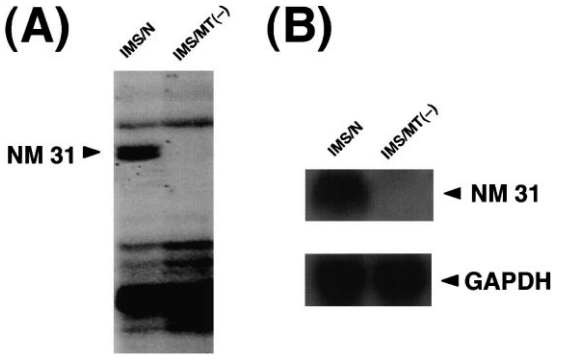


Fig. 1. A: Gene expression in IMS/N and IMS/MT (−) cells. Total RNA extracted from both cell lines was the template for reverse transcription and subsequent PCR amplification in the presence of [$^{32}$P]dCTP. The amplified products were resolved on 6% DNA sequencing gels and visualized by autoradiography. The NM31 arrow indicates an mRNA species amplified in IMS/N, but not in IMS/MT (−) cells. B: Northern hybridization of NM31 mRNA in IMS/N and IMS/MT (−) cells. NM31 cDNA (excised from the gel shown in A) was re-amplified and labelled with [$^{32}$P]dCTP. GAPDH mRNA was determined as a loading control. The results were confirmed by three separate experiments.

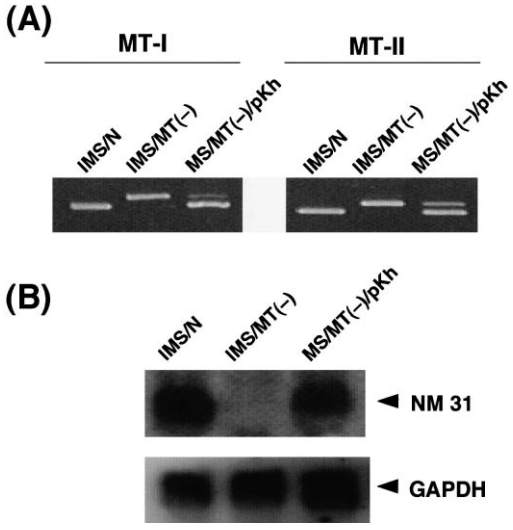


Fig. 2. Effect of introduction of plasmid pKH, carrying the genes for metallothionein-I and II, into IMS/MT (−) cells on expression of metallothionein-I (MT-I) and -II (MT-II) and NM31 mRNA. RNAs from IMS/N, IMS/MT (−) and the transformed cell line (IMS/MT (−)/pKH) were analyzed by RT-PCR using primers specific for metallothionein-I and -II, respectively [11] (A), or by Northern blotting analysis using NM31 cDNA as a probe (B). The results were confirmed by three separate experiments.

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