Original MRE-binding Transcriptional Factor Gene in Normal Humans is ZRF, not MTF-1

Kayoko Kita,[a] Nobuhiko Miura,[a,1] Minoru Yoshida,[b] Mitsunobu Matsubara,[c] Yutaka Imai,[d] and Akira Naganuma*[,a]

[a]*Laboratory of Molecular and Biochemical Toxicology, Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai 980–8578, Japan,* [b]*Department of Chemistry, St. Marianna University School of Medicine, Kawasaki 216–8511, Japan,* [c]*Second Department of Internal Medicine, Graduate School of Medicine, Tohoku University, Sendai 980–8574, Japan, and* [d]*Laboratory of Clinical Pharmacology and Therapeutics, Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai 980–8578, Japan*

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Metallothionein gene is transcriptionally regulated by heavy metals through *cis*-acting metal responsive elements (MREs). Two proteins, metal-regulatory transcription factor-1 (MTF-1) and zinc regulatory factor (ZRF), have been isolated and cloned from human cells as MRE-binding transcriptional factor (MREBT). These proteins are almost identical to each other, except for only one base substitution at codon 185 that causes an amino acid change from histidine to tyrosine. This single amino acid difference has been reported to influence zinc-responsive transcriptional activities. In this study, we determined the nucleotide sequence of the region containing codon 185 in DNA samples obtained from normal Japanese (n = 30) and three human-derived cultured cell lines. The findings indicate that all subjects have the same sequence identical to ZRF, suggesting that ZRF is the original MREBT gene in normal humans, and MTF-1 is its minor variant.

Key words —— ZRF, MRE-binding transcriptional factor, MTF-1, human, metallothionein

INTRODUCTION

Cadmium contamination of foods is of particular concern because it accumulates in the human body with an extremely long biological half-life of 15–20 years.[1,2] Overaccumulation of cadmium in human tissues has been shown to cause a variety of adverse health effects such as kidney dysfunction,[3–5] hypertension,[6,7] diabetes,[8,9] disturbed calcium metabolism[10] and osteoporosis.[11,12] Metallothionein (MT), a low-molecular weight cysteine-rich protein, has been shown to have a protective effect against cadmium toxicity.[1,13] Transcription of mammalian MT genes is regulated by metal-regulatory transcription factor-1 (MTF-1),[14–17] which binds to the *cis*-acting regulatory sequences of the MT promoter termed metal-responsive elements (MREs).[18,19] Zinc regulatory factor (ZRF) was also isolated from HeLa cell nuclear extract as an MRE-binding transcriptional factor (MREBT) by Otsuka *et al*.[20] The amino acid sequence of ZRF is almost identical to human MTF-1 (hMTF-1) with only one difference at amino acid 185 in the second zinc finger domain [histidine (CAC) in hMTF-1 and tyrosine (TAC) in ZRF].[21] Koizumi *et al*.[22] reported that a reporter gene expression, which was driven by MREs, was induced by zinc in the hMTF-1 overexpressing cells, but not in the ZRF overexpressing cells, although basal levels of reporter gene expression were significantly elevated by overexpression of each in both variants. The single amino acid difference between these two MREBT variants may influence MT gene expression and the extent of manifestation of cadmium toxicity in humans. Therefore, we determined the genetic variation of codon 185 in the MREBT gene in Japanese individuals and several human cell lines.

MATERIALS AND METHODS

DNA Isolation —— DNA was obtained from blood samples of 30 unrelated Japanese individuals, and stored at –20°C until analysis. Written informed consent, approved by the Institutional Review Board of Tohoku University School of Medicine, was obtained from all individuals.

Polymerase Chain Reaction —— A 239 bp fragment (corresponding to codons 137 to 216) containing the zinc finger-I, -II and half of -III region of hMTF-1 (Fig. 1A) was amplified using 100 ng of each DNA sample. PCR was performed in a PCR buffer solution containing 50 pmol of each primer

[1]Present address: Division of Health Effects Research, National Institute of Industrial Health, Kawasaki, Kanagawa 214–8585, Japan.

*To whom correspondence should be addressed: Laboratory of Molecular and Biochemical Toxicology, Graduate School of Pharmaceutical Sciences, Tohoku University, Aoba-ku, Sendai 980–8578, Japan. Tel.: +81-22-217-6870; Fax: +81-22-217-6869; E-mail: naganuma@mail.pharm.tohoku.ac.jp

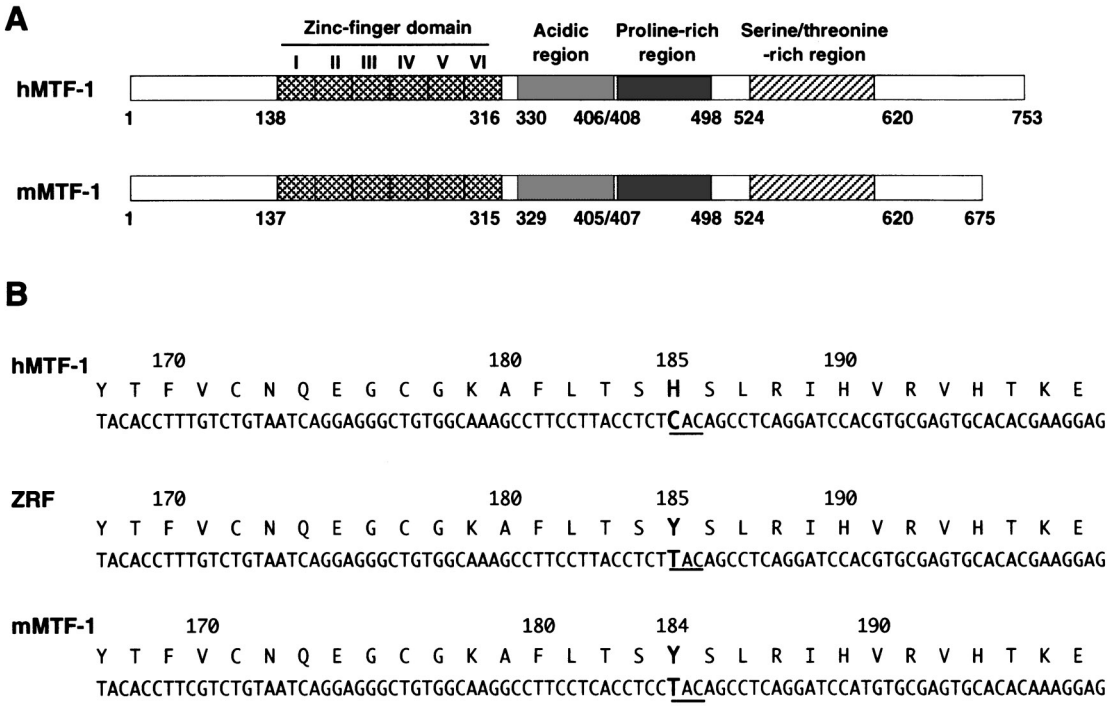


Fig. 1. Structural Features of Human and Mouse MRETBs[14,23,21]

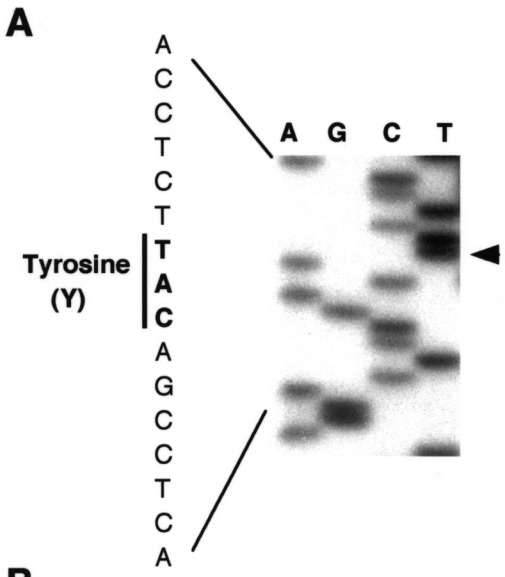
(A) Schematic representation of hMTF-1 and mMTF-1. (B) Nucleotides and amino acids sequences of the second zinc finger region of hMTF-1, ZRF and mMTF-1. Amino acids are indicated in the one letter code above the nucleotide sequences.

(forward, 5′-GTAAAGCGGTACCAATGTAC-3′; reverse, 5′-CTGTACAGTGTGTTGAATGC-3′), 1.5 mM of $MgCl_2$, 0.001% (w/v) gelatin, 200 μM dNTP mix, and 1.25 units of Taq polymerase in a total volume of 50 μl. Samples were heated at 95°C for 10 min, then subjected to 35 cycles at 96°C for 30 sec, 57°C for 30 sec and 72°C for 30 sec. PCR products were purified using the High Pure PCR Product Purification Kit (Boehringer Mannheim GmbH, Mannheim, Germany) according to the manufacturer's instructions. The samples were stored at –20°C until analysis.

Sequence Analysis —— Amplified PCR products were subjected to sequence analysis using a Thermo Sequenase Cycle Sequencing Kit (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) according to the manufacturer's instructions. The 5′-ends of forward or reverse primers (as above, 10 pmol) were labeled with [γ-32P]ATP by T4 polynucleotide kinase as sequencing primers. Samples were mixed with loading buffer, heat denatured, and resolved on 6% sequencing polyacrylamide gel. Electrophoresis was performed at 80 W in 89 mM Tris-borate buffer (pH 8.0) containing 2 mM EDTA. Gel was dried on Whatmann 3MM paper and visualized by autoradiography.

RESULTS AND DISCUSSION

Two variants of human MREBT, hMTF-1[23] and ZRF,[20] have been independently isolated from different human-derived cell lines. A difference in the amino acid sequence of these two variants was only observed at codon 185 located in the second zinc finger domain (Figs. 1A and 1B).[21,23] To examine the genetic variation in this domain of MREBT, we amplified the zinc finger fragment including the first, second and half of the third zinc finger domains (Fig. 1A), and determined the nucleotide sequence of this region in 30 normal Japanese individuals. As shown in Fig. 2, the nucleotide sequence of codon 185 in all Japanese individuals examined in this study (n = 30) was found to be TAC, which is identical to ZRF, and no subject with CAC was observed. Moreover, the nucleotide sequence of the whole region examined (239 bp) was also completely identical to ZRF in these 30 Japanese individuals. These findings suggest that the major gene of MREBT is ZRF in normal Japanese. We also examined the partial nucleotide sequence of the MREBT gene in human-derived cultured cells, HeLa-S3, HepG2 and HEK293, using the same protocol as for the Japanese individuals. All these human cells also showed that codon 185 and other regions of the MREBT gene



B

No. of samples	TAC	CAC
30	30	0

Fig. 2. Direct Sequence Analysis of the 239 bp Fragment of Second Zinc Finger Region in MREBT

(A) A representative result of the sequence analysis for a normal Japanese subject.(B) Nucleotide sequence of codon 185 in the MREBT gene of normal Japanese subjects (n = 30). All thirty Japanese subjects and three human cultured cell lines (HeLa-S3, HepG2 and 293 cells) showed the same sequence (TAC).

examined (239 bp) were completely identical to ZRF. These findings suggest that tyrosine (TAC)-185 in the second zinc finger region may be the original amino acid at that position in human MREBT. Therefore, we conclude that ZRF is likely the original gene of MREBT, and hMTF-1 is a minor variant with a single amino acid substitution.

The hMTF-1[23] was cloned as a human homologue of mouse MTF-1 (mMTF-1),[14] and the overall structure of hMTF-1 is very similar to that of mMTF-1 except for an additional 78 amino acids in hMTF-1 (see Fig. 1A).[23,24] The six zinc-finger domains of MTF-1 proteins were more highly conserved between mouse and human than the other regions, and only two amino acid differences were noted (amino acids 185 and 234 for hMTF-1).[23,24] ZRF, in contrast to hMTF-1, has the same residue (tyrosine) at amino acid 185 as mMTF-1 (Fig. 1B), and there is only one difference between ZRF and mMTF-1 in the six zinc-finger domains at amino acid 234 (glutamate in ZRF and glutamine in mMTF-1).[14,21] Brugenera *et al.*[23] and Muller *et al.*[24] reported that metal responses were partially restored by transfection of mMTF-1 into an MTF-1 deficient mouse cell line, but hMTF-1 exhibited a more pronounced metal response. Radtke *et al.*[25] fused the *N*-terminal part containing all the zinc finger regions to a constitutive heterologous activation domain of the viral activation protein (VP16), and demonstrated that the zinc responses of the zinc finger region of hMTF-1 were greater than those of mMTF-1. Koizumi *et al.*[22] indicated that overexpression of either hMTF-1 or ZRF constitutively activated the MRE-driven reporter gene, but further induction by exogenous zinc was only observed in the cells overexpressing hMTF-1, even though their amino acid sequences were identical except for one amino acid. The single amino acid difference in the second zinc finger region between hMTF-1 and ZRF or mMTF-1 (Fig. 1B) may reflect their functions that are activated in response to metals, such as zinc and cadmium. Although the common human MREBT gene was found to be ZRF in this study, the inducibility of metallothionein and the cadmium sensitivity of people who have its variant, such as MTF-1, may be different from people having the original gene.

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