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Cloning, chromosomal mapping and characterization of the human metal-regulatory transcription factor MTF-1

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ABSTRACT

Metallothioneins (MTs) are small cysteine-rich proteins that bind heavy metal ions such as zinc, cadmium and copper with high affinity, and have been functionally implicated in heavy metal detoxification and radical scavenging. Transcription of metallothioneins genes is induced by exposure of cells to heavy metals. This induction is mediated by metal-responsive promoter elements (MREs). We have previously cloned the cDNA of an MRE-binding transcription factor (MTF-1) from the mouse. Here we present the human cDNA equivalent of this metal-regulatory factor. Human MTF-1 is a protein of 753 amino acids with 93% amino acid sequence identity to mouse MTF-1 and has an extension of 78 amino acids at the C-terminus without counterpart in the mouse. The factors of both species have the same overall structure including six zinc fingers in the DNA binding domain. We have physically mapped the human MTF-1 gene to human chromosome 1 where it localizes to the short arm in the region 1p32–34, most likely 1p33. Both human and mouse MTF-1 when produced in transfected mammalian cells strongly bind to a consensus MRE of metallothionein promoters. However, human MTF-1 is more effective than the mouse MTF-1 clone in mediating zinc-induced transcription.

INTRODUCTION

Metallothioneins (MTs) are small ubiquitous cysteine-rich proteins that selectively bind heavy metal ions such as zinc (Zn), cadmium (Cd), copper (Cu) and others. In mammals, MT protein concentrations are elevated upon exposure to metal, various hormones, interleukin 1, interferon injection or viral infection (for review, see refs. 1–4). There are at least four metallothionein genes, two of which (I and II) are metal-inducible and another two (III and IV) are constitutively expressed, however with a restricted tissue specificity (ref. 5 and R.D.Palmiter, personal communication). Elimination of metallothionein I and II genes by targeted gene disruption renders null mutant mice highly sensitive to cadmium intoxification (6, 7). The ability of metallothionein genes to be induced by heavy metals is controlled by metal regulatory elements (MREs) present in MT gene promoters in multiple, non-identical copies. Previously our group has identified and cloned a mouse transcription factor, designated MTF-1, that binds to these metal-responsive promoter elements (8, 9).

Mouse MTF-1 is a zinc finger factor that specifically binds to metallothionein gene promoters and activates them in transfected cells (9). However, activation was found to be mostly constitutive, and some uncertainty remained about the role of MTF-1 in mediating heavy metal-responsive transcription. Here we report the cloning and functional characterization of the human counterpart (hMTF-1) of mouse MTF-1. Its primary structure is very similar to that of the mouse factor, however in transfected cells human MTF-1 mediates a more pronounced metal response of a reporter gene than does the mouse factor, indicating that it is critically involved in zinc induction of metallothionein genes.

MATERIALS AND METHODS

Screening of a human cDNA library

The human B-cell cDNA library was constructed by Nicholas J.MacDonald and maintained by Andreas Sailer (20). The library was made from the induced Namalwa line NM864 (treated 4 hours with Newcastle Disease Virus (NDV) in the presence of cyclohexmide) and NM878 uninduced cell cDNA. The cDNAs were randomly primed, ligated to *Eco*RI linkers, size fractionated over an agarose gel (cut off at 800 bp) and cloned into λgt11 (Amersham).

The mouse MTF-1 probe (*Sna*BI, *Nco*I), prepared according to standard methods (21), was used to screen approximately 1.2×10^6 phages of the Namalwa cDNA library. The protocol for reduced stringency hybridization from (22) was slightly

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modified. Both prehybridization and hybridization were done for 13 hours at 47°C in 28% formamide, 2% SDS, 10×Denhardt's solution, 5×SSCP, 0.06 mg/ml yeast RNA and 0.3 mg/ml heparin, except that in the hybridization solution salmon sperm DNA was replaced by mouse MTF-1 probe (1.5×10^6 dpm/ml). The filters were washed twice at room temperature in 2×SSC and 0.2% SDS for 5 min, followed by an additional 15 min at 53°C with 0.1×SSC and 0.1% SDS.

After plaque purification, the cDNA was subcloned into the pBluescript II SK⁻ vector (Stratagene) for sequence determination by the Sanger dideoxynucleotide technique (23) using the double stranded Nested Deletion Kit (Pharmacia).

Three λgt11-clones were found: (i) A λ-clone which probably represented an incorrectly processed transcript and contained the segments 17I (−83 to +85 relative to ATG and +429 to +1133) and B3 (+1134 to +1629), (ii) λ-clone13 (+231 to +1332), (iii) λ-clone J3 (+1761 to +2259).

Gap filling by PCR and sequencing

The λ-clone 13 sequence data and the Namalwa library, as a source of cDNA, were used to PCR-clone the fragment +85 to +231. The anti-sense oligonucleotide, MTFPCR1, 5′-GGACATTCCGACTGCAGAGT-3′ spanning the region +389 to +370 of hMTF-1 and two standard λ-gt11 primers located near the cloning site, *Eco*RI, 105.1 5′-GACTCCTGGAGCCCCGTCAGTATC-3′ or 105.2 5′-ACTGGTAATGGTAGCGACCGGC-3′ were used. All PCR experiments were performed with 0.5 U Super-Taq enzyme and standard Super-Taq buffer from P.H.Stehelin and Cie AG (Basel) under standard conditions: The PCR was hot started and allowed to proceed for 30 cycles under the following conditions: denaturation at 95°C for 50 sec, annealing at 60°C for 1 min and extension at 72°C for 3 min. The PCR reaction produced DNA fragments of about 450−500 basepairs. These DNAs were purified on an agarose gel and subcloned into pBluescript II SK⁻ for sequencing. Three independent clones were sequenced and found to be identical. Clone 4_1 was used for further subcloning in the expression vector pcDNA I (*Invitro*gen corporation).

Since our efforts to fill the remaining gap with the B-cell cDNA library were unsuccessful, we prepared total RNA from tissue of a hepatocellular carcinoma of a 55-year old man according to ref. (24). A sample of 10 μg of total RNA was used as a template for first strand cDNA synthesis in 50 mM Tris−HCl (pH 8.3), 75 mM KCl, 10 mM DTT, 2.5 mM dNTPs and 10 μg/ml oligo dT (17 mer). The strand synthesis was carried out for one hour at 37°C in the presence of 200 U M-MLV (Moloney Murine Leukemia Virus) reverse transcriptase and the product was directly used for PCR. The sense primer corresponding to the nucleotide sequence of human MTF cDNA +1495 to +1514, was 5′-CTTTCTGTTGTTGCTGGGGC-3′ and the anti-sense primer +1894 to +1872 was 5′-ATGCCTCTTCTTGTTTGATGATG-3′. Five independent clones were sequenced. A consensus clone designated 8_3 was used for further subcloning in the expression vector pcDNA I.

The sequence information for human MTF-1 was used to scrutinize the previously obtained mouse cDNA and led to some changes at the following nucleotide positions in the mouse sequence (numbering according to the human sequence): Insertions at 181 (GAA), 1899 (G), 1907 (G) and 1959 (TTCTT); deletions at 293 (CAA), 1981 (G) and 2168 (C) and substitutions at 850 (A to G), 851 (A to G), 1807 (A to G), 1862 (A to T) and 1863 (A to T). These changes in the mouse sequence have been updated in the EMBL database.

Construction of DNA templates and expression vectors

The expression vector pc DNAI-hMTF corresponding to the entire ORF was cloned by ligating six fragments previously cloned into pBluescript II SK⁻ vector (Stratagene). pc DNA I (*Invitro*gen corporation) was cut with *Bam*HI and *Xho*I and the fragments 4_1 *Bam*HI/*Ssp*I, 13 *Ssp*I/*Eco*RI, B3 *Eco*RI/*Nco*I, 8_3 *Nco*I/*Sac*I and J3 *Sac*I/*Xho*I were ligated to the vector.

The reporter and reference plasmids used in the transfection assays are based on the OVEC-1 reporter gene (10). The 4× MREd-reporter used has been previously described from (9). The human MT-IIA-reporter was constructed by removing the human MT-IIA upstream region by *Hin*dIII, *Bam*HI digestion out of pHS1 (a gift of M.Karin) at positions −780 to +70. The fragment was blunt-ended and ligated into the OVEC-1 reporter previously digested with *Sac*I/*Pst*I and rendered blunt-ended.

Cell culture and DNA transformation

HeLa cells were grown in DMEM (GIBCO) supplemented with 2.5% fetal calf serum (GIBCO), 2.5% newborn calf serum (GIBCO), 100 U/ml penicillin and 100 U/ml streptomycin and 2 mM L-glutamine. The cells were transfected by the calcium phosphate co-precipitation method (25, 26). For zinc induction, cells were treated with 100μM $ZnSO_4$ for 4 h.

Bandshift assay

Preparation of nuclear extracts from HeLa cells was performed as described from (27). In order to overexpress hMTF-1, we transfected the eukaryotic expression vector pcDNAI-hMTF and the SV40 T-antigen expression clone (CMV-Tag;) (28) into HeLa cells. In the bandshift assay (29, 30), binding reactions were carried out by incubating 7.5 fmol double stranded end-labeled DNA, MREd (9), with 5 μg of nuclear protein and 5 μg poly(dI-dC) in a buffer containing 12 mM HEPES pH 7.9, 12% glycerol, 5mM NaCl, 50 mM KCl, 5mM $MgCl_2$, 0.6 mM DTT and 100 μM $ZnSO_4$. After 15 min incubation at room temperature, the reaction mixture was loaded on a 4% polyacrylamide gel in 0.25×TBE buffer and electrophoresed at 10 V/cm at room temperature.

For competition experiments, 2 pmoles of unlabeled, double stranded oligonucleotide containing either an MRE site (MREd, MRE-s) or an Sp1 site from HSV-1 (9) was added prior to the addition of the extract. Signal intensities of the protein−DNA complexes were quantified using a PhosphorImager.

MREd:
5′-CGAGGGAGCTCTGCACTCCGCCCGAAAAGTG-3′
3′-TCGAGCTCCCTCGAGACGTGAGGCGGGCTTTTCACAGCT-5′
MRE-s:
5′-CGAGGGAGCTCTGCACACGGCCCGAAAAGTG-3′
3′-TCGAGCTCCCTCGAGACGTGTGCCGGGCTTTTCACAGCT-5′
Sp1 (HSV1):
5′-CGAGGCCCCGCCCAG-3′
3′-TCGAGCTCCGGGGCGGGTCAGCT-5′

Transcription analysis

RNA isolation and S1 nuclease mapping were performed as previously described (10). 20 μg of reporter plasmid, 1 μg of OVEC -ref-1 (with an SV40 enhancer upstream of the TATA box) and the indicated amount of pcDNAI-hMTF were used per 100 mm plate. Salmon sperm DNA was added to equalize the

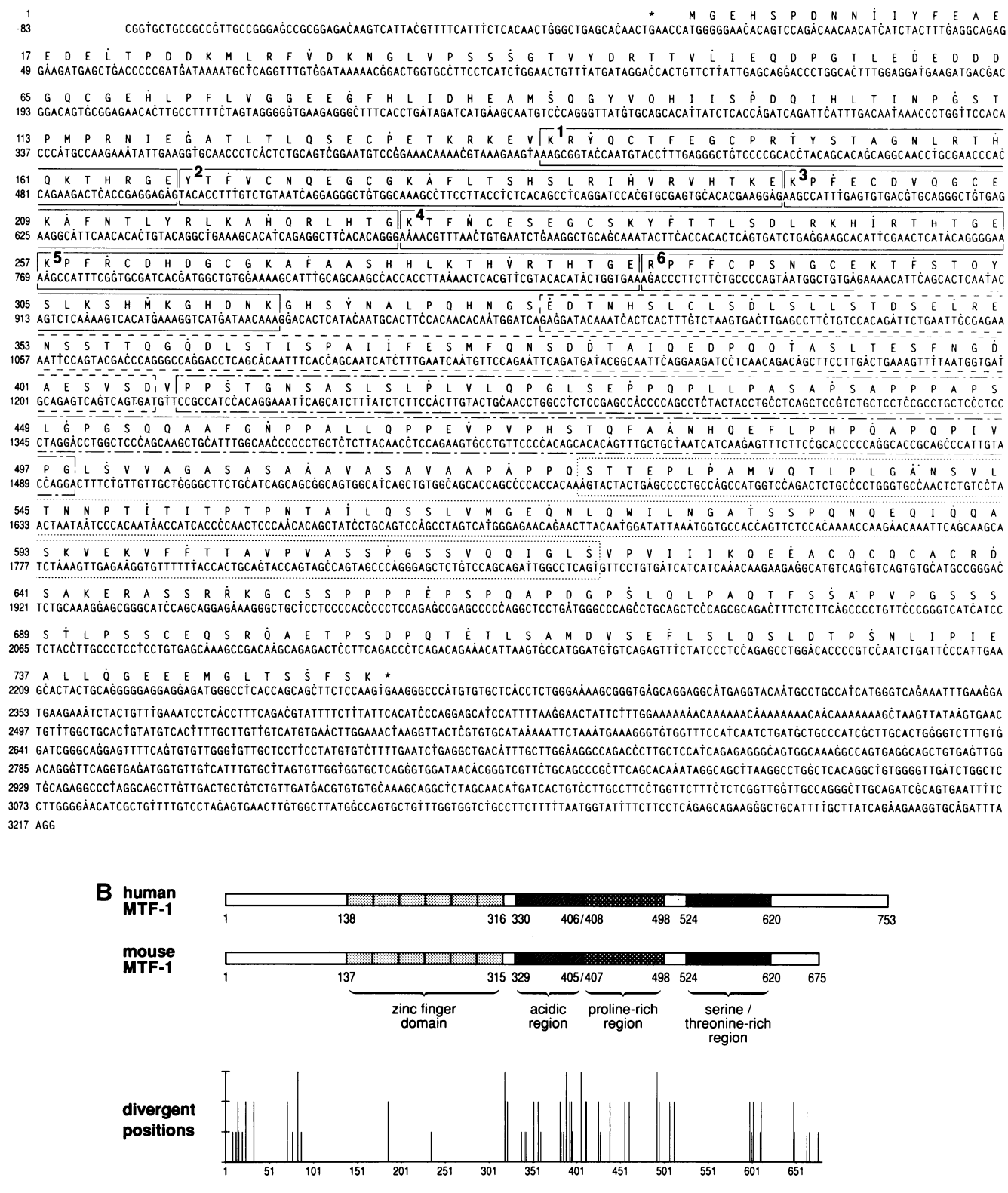

Figure 1. Structural features of human MTF-1. (**A**) sequence of human MTF-1. (EMBL/GenBank/DDBJ accession number X78710 HSMTFMR). Amino acids are indicated in the one letter code above the human MTF-1 nucleotide sequence. Stop codons TGA and TAG are indicated by asterisks. Zinc fingers are boxed with a solid line. Acidic, proline-rich and serine/threonine-rich activation domains are boxed with short lines (__), broken lines (__.__) and dots (.), respectively (32). (**B**) schematic representation of human and mouse MTF-1. A map of human and mouse amino acid sequence differences is depicted (short and medium size vertical bars indicate positions of highly or less conservative single amino acid exchange, respectively; long bars denote insertion/deletion or non-conservative exchange of single amino acids). The overall structures of human and mouse MTF-1 (9) are very similar, with a unique N terminus, six zinc fingers of the Cys2 His2 (TFIIIA) type and three putative activation-type domains, however the human factor extends 78 amino acids beyond the mouse factor.

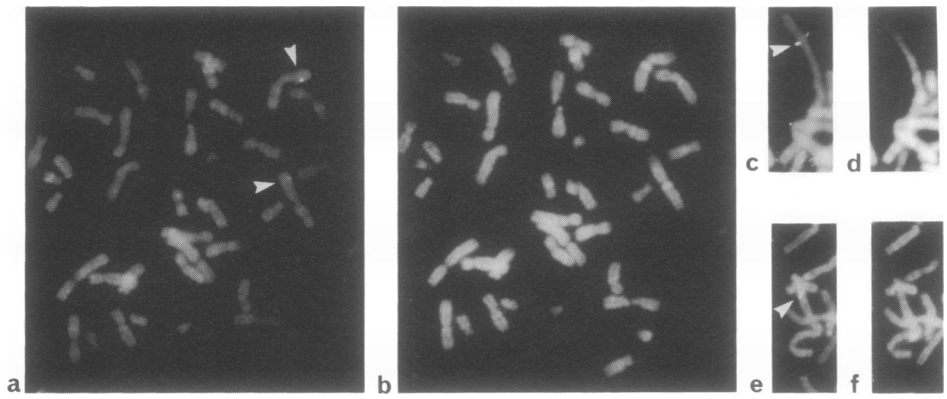

Figure 2. Chromosomal mapping of human MTF-1. Shown are whole and partial metaphases with signals from *in situ* hybridization with the biotinylated hMTF probe. (a,c,e) Normal male chromosomes counter-stained with propidium iodide. Hybridization sites on chromosome 1 (1p33) are indicated by arrows. (b,d,f) DAPI staining of the same metaphases for chromosome identification.

total amount of transfected DNA per plate. For heavy metal induction, $ZnSO_4$ was added to the medium to a final concentration of 100 μM at 4 hours before cell harvesting. S1 nuclease mappings were quantified using a PhophorImager and the data were normalized using OVEC-ref signals.

Because the complete human MT-IIA promoter, including its TATA box and leader, was fused to the OVEC reporter gene, MT-IIA-driven transcripts also hybridize to the OVEC upstream DNA sequence and thus migrate more slowly than the usual OVEC transcripts.

Gene mapping by *in situ* hybridization

The hMTF-1 cDNA probe used for chromosome mapping extended from −83 to +372 and was *PstI* ligated to the sequence +704 to +1133 in order to delete the most generally conserved Zn-fingers 1−3 and also part of the Zn-finger 4.

The probe was nick-translated with biotin-14-dATP and hybridized *in situ* at a final concentration of 20 ng/μl to metaphases from two normal males. The fluorescence *in situ* hybridization (FISH) method was modified from that previously described (31) in that no preassociation was necessary and chromosomes were stained before analysis with both propidium iodide (as counterstain) and DAPI (for chromosome identification).

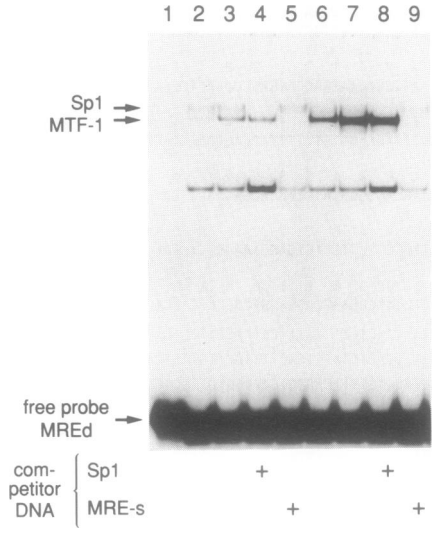

Figure 3. Bandshift analysis of human MTF-1. Nuclear extracts from HeLa cells transfected with either SV40 T-antigen expression plasmid alone (lane 2−5; negative control) or cotransfected with SV40 T-antigen and hMTF-1 expression clone (6−9) were analyzed for hMTF-1 by electrophoretic mobility shift assay. The oligonucleotide corresponding to the strong binding site MREd binds both MTF-1 and Sp1 but can be distinguished by the use of different competitors.

RESULTS

Structure

Using a cDNA probe from mouse MTF-1, we have screened a lambda phage cDNA library from Namalwa cells, a human Burkitt's lymphoma B type line. Three positive phage clones were analyzed and found to contain non-overlapping MTF-1 segments. Two small remaining gaps were closed with PCR-amplified cDNA segments. The cDNA assembled from the five overlapping DNA segments was 3302 nucleotides long and contained an open reading frame for a protein of 753 amino acids (Figure 1A). The overall structure of the predicted human MTF-1 protein (hMTF-1) is the same as that of the mouse: a unique N terminal region is followed by six zinc fingers of the TFIIIA type (Cys2 His2) and three distinct putative activation domains with characteristic clusters of acidic residues, prolines, and serines/threonines. Human and mouse MTF-1 proteins are very similar with 93% amino acid identity, and are even more strictly conserved in the DNA-binding zinc fingers (Figure.1B). Curiously, the human protein is 78 amino acids longer at the C terminus, which explains its slower migration in bandshift assays and denaturing polyacrylamide gel electrophoresis 'Southwestern blot' (data not shown). The human cDNA also includes 83 and 957 nucleotides of leader and trailer sequence, respectively, and thus extends in both 5′ and 3′ direction beyond the mouse cDNA. We note that not only the protein-coding sequence, but also leader and trailer are highly conserved between human and mouse. In both species the leader sequence contains a potential reading frame that terminates with a TGA just 3 nucleotides upstream of the MTF-1 initiator AUG (see also

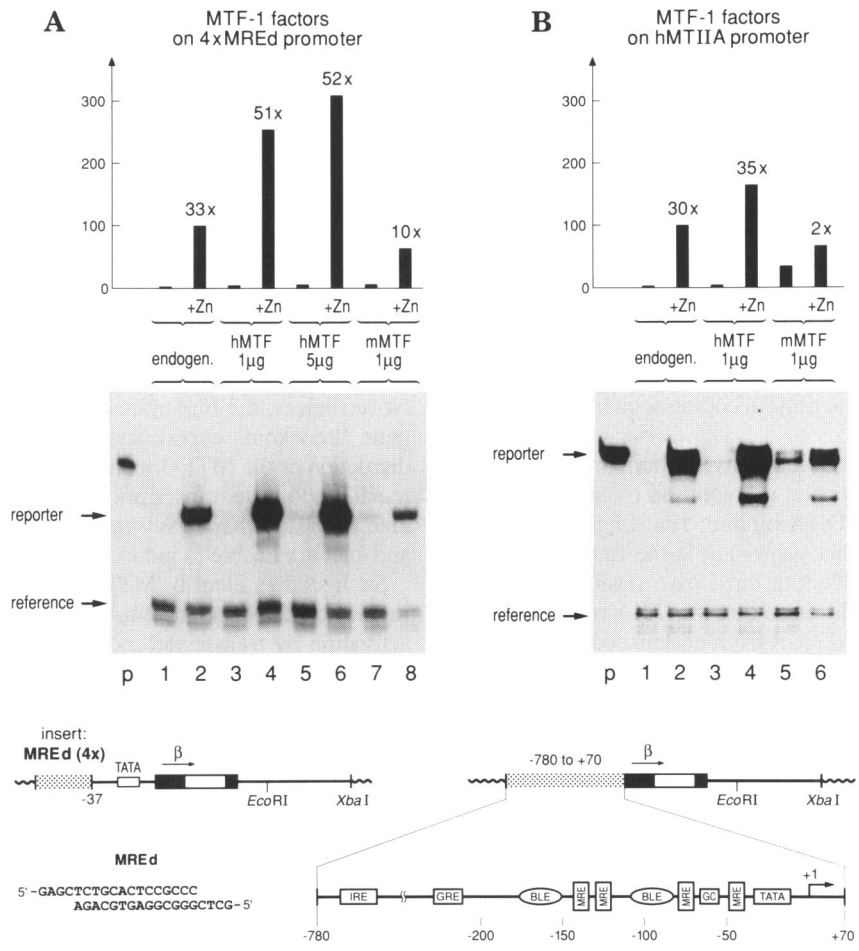

Figure 4. Effect of MTF-1 on metal-induced transcription. (**A**) Expression studies with 4×MREd promoter (schematically shown at the bottom left). Zinc induction without transfected MTF-1 was 33 fold above the uninduced transcription level. Expression of hMTF-1 at 1 µg and 5 µg amounts of expression plasmid raised induction values to 51 and 52 fold, respectively. Transfection of mouse MTF-1 raised basal level transcription and yielded only a 10 fold zinc induction. The autoradiogram of S1 nuclease mapping of transcripts is shown underneath the bar diagram. (**B**) Expression studies with human MTIIA promoter (schematically shown at bottom right, adapted from ref. 11. MRE, metal-responsive element; GC, GC-box/Sp1 binding site; BLE, basal level element; GRE, glucocortiocoid responsive element; IRE, interferon-responsive element). The ratio of metal-induced over uninduced transcription increases from 30 fold without extra MTF-1 to 35 fold after transfection of HeLa cells with 1 µg human MTF-1 expression clone, while mouse MTF-1 reduces the induction ratio to factor 2.

Discussion). In addition, both trailers have an A-rich stretch, which in the mouse demarcates the 3′ end of the cDNA and presumably has served as a fortuitous primer binding site during cDNA synthesis. The human cDNA, that was obtained by random priming, is much longer in the 3′ end but lacks, like the mouse cDNA, the characteristic poly A site AATAAA. Thus it is likely that the mRNA sequence extends at the 5′ and/or the 3′ end beyond the known cDNA.

Mapping

For chromosomal mapping of human MTF-1, a biotinylated probe was prepared from the cDNA and used to stain human metaphase chromosomes. Twenty-four metaphases from a normal male were examined for fluorescent signals. Twenty-two of these metaphases showed a signal on one or both chromatids of chromosome 1 in the region 1p32−1p34.2; 67% of this signal was at 1p33 (Figure 2). There was a total of 5 non-specific background dots observed in these 24 metaphases. A similar result was obtained from hybridization of the probe to 20 metaphases from a second normal male (data not shown).

Expression and activity

We expressed the hMTF-1 cDNA clone in mammalian cells to see whether an active protein of the correct size was produced. cDNA sequences were inserted into an expression vector under the control of the strong enhancer/promoter of cytomegalovirus (CMV). The vector also contained an SV40 origin of replication for efficient amplification in the presence of viral T-antigen. HeLa cells were cotransfected with expression plasmids for MTF-1 and SV40 T-antigen. When nuclear extracts from such transfected HeLa cells were analyzed by bandshift, a very strong band of a size indistinguishable from human MTF-1 was observed (Figure 3). Competition experiments with unspecific and MRE-specific oligonucleotides confirmed the identity of the band as being MTF-1.

Transcriptional activity of hMTF-1 was analyzed by transfecting the cDNA expression clone into HeLa cells, together with the OVEC reporter gene (10). The reporters had either a promoter with 4 tandem copies of a strong metal-responsive element (MREd) or one copy of the complete human

metallothionein IIA enhancer/promoter (11, 12). Without transfected MTF-1, the metal-responsive promoters 4×MREd and hMTIIA stimulated transcription 33 fold and 30 fold upon zinc treatment, respectively (Figure 4). Expression of additional human MTF-1 in these cells raised both basal and induced transcription, thus boosting induced transcript levels to 51 and 35 fold, respectively. By contrast, mouse MTF-1, as shown earlier (9) had a tendency to increase uninduced transcription, and in this particular experiment even reduced the inducible expression of the reporter gene.

DISCUSSION

We have cloned the complete protein coding sequence of human MTF-1.

This factor is highly conserved between human and mouse, two mammals that are separated in evolution by some 70 Mio yr. Not unexpectedly, the DNA-binding zinc fingers are most strictly conserved. Somewhat surprising is the finding that the entire cDNA sequence, including third base positions, is also highly conserved. This could mean that the DNA or the mRNA serves yet another function(s) apart from protein coding (see e.g. ref. 13).

In contrast to the generally high sequence conservation between the two species, human MTF-1 is extended by 78 amino acids at the C terminus as compared to mouse MTF-1. Does this mean that the human factor has recently grown through the loss of a stop codon, or that the mouse factor has become truncated? The latter possibility is more probable, because the factors of man, monkey, bovine, deer (Tragulus javanicus) and rabbit migrate identically, while only mouse and rat contain the fast-migrating form (E.Brugnera, F.Radtke and W.Schaffner, unpublished data).

It remains to be seen whether the seemingly greater ability of the cloned human factor to confer metal inducibility as compared to the mouse factor (Figure 4) is the result of this longer C terminus. Experiments with chimeric human/mouse proteins should clarify this point. It may also be interesting to see whether the shortened mouse MTF-1, unlike human MTF-1, is deficient in responding to any of the numerous stress conditions that can induce metallothionein gene transcription (14).

Interestingly, in both species the cDNA appears to contain a conserved open reading frame in the leader upstream of the MTF-1 coding sequence, with a TGA stop codon just 3 bp upstream of the initiator ATG. The role of this putative reading frame, if any, remains to be seen. In this context it is interesting to note that TGA is the stop codon that can, in special cases, be translated into an amino acid and thus would allow for readthrough translation (15). Alternatively, leader and trailer sequences may be conserved because they are utilized for translation of alternative MTF-1 splice forms. However, there is no evidence so far for alternative splicing, and incidentally also no evidence for a gene family of MTF-1 related factors (R.Heuchel, F.Radtke, O.Georgiev and W.Schaffner, unpublished). Both of these findings, if substantiated, would make MTF-1 rather unique among mammalian transcription factors. Another peculiarity is represented by the highly conserved A-rich stretch in the 3′ trailer sequence. We note that also other transcription factors, including Octamer factors 1 and 2 have similar A stretches in the trailer. Their function, if any, is not known.

We physically mapped the hMTF-1 gene to human chromosome 1 where it localizes to the short arm in the region 1p32−34, most likely 1p33. This region harbors several disease loci that do not bear any obvious relation to a deficiency in metal homeostasis, except perhaps Mendes da Costa disease (erythrokeratodermia variabilis) (16) whose symptoms are relieved by retinoic acids or retinoid analog treatments. Since zinc is needed for mobilization of vitamin A, one may envisage an indirect involvement of MTF-1 in this disease. Another skin disease, acrodermatitis enteropathica (17), can be virtually cured by simple zinc treatment, but this locus has not been mapped as yet.

Previously, we cloned MTF-1 from mouse. When the protein was expressed from transfected cDNA it could activate metallothionein promoters, but activation was largely constitutive. Nevertheless, the importance of MTF-1 is confirmed by recent gene 'knockout' experiments in cultured mouse cells, where disruption of the MTF-1 locus results in a complete loss of heavy metal-responsive transcription. In these knockout cells, not only zinc but also other heavy metals like cadmium, copper, nickel and lead are unable to induce metallothionein transcription (18).

Such studies identify MTF-1 as an essential factor for metal regulated transcription, but they do not explain the constitutive activation by transfected mouse MTF-1. One possibility could be that, since all mammalian cells analyzed so far contain high levels of MTF-1, any assay system with additional MTF-1 expression is hampered by the background from the endogenous metal-responsive system and effects due to the extra MTF-1 may be seen only at very high factor concentration. It is also possible that MTF-1 is a positively acting factor whose availability is regulated by a negative factor, similar to the NFκB-IκB system, and that this inhibitor is titrated by excess of transfected MTF-1 (9, 19). Despite these possible constraints, our study shows that the human MTF-1 protein, which is more inducible than the mouse factor, activates transfected natural and synthetic metal-inducible promoters in response to zinc treatment, thus underlining the importance of this factor for heavy metal-regulated transcription.

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