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Analysis of the Heavy Metal-Responsive Transcription Factor MTF-1 from Human and Mouse

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Abstract—*Heavy metal-induced transcription in mammalian cells is conferred by the metal-responsive 70 kDa transcription factor MTF-1 which contains six zinc fingers and at least three activation domains. In previous cell transfection experiments we have shown that the zinc finger region confers an about 3 fold metal inducibility of transcription, due to its differential zinc binding. However, we also noted that human MTF-1 was more metal-responsive than the mouse factor (about 10 fold versus 3 fold, respectively). Here we analyze this difference in more detail by using chimeric human-mouse factors and narrow the critical region to a 64 amino acid stretch immediately downstream of the zinc fingers, overlapping with the acidic activation domain. A short human segment of this region (aa 313–377) confers efficient metal induction to the mouse MTF-1 when replacing the corresponding mouse region. However, high metal inducibility requires an unaltered MTF-1 and is lost when human MTF-1 is fused to the general activation domain of herpesvirus VP16. Wild type and truncation mutants of MTF-1 fused to VP16 yield chimeras of high transcriptional activity, some exceeding the wildtype regulator, but only limited (about 3 fold) heavy metal inducibility.*

INTRODUCTION

Heavy metals, notably zinc and cadmium, induce the production of small cysteine-rich proteins, so called metallothioneins (reviewed in 1–3), which bind heavy metals and help to detoxify the cell (4, 5). Metallothionein production is induced at the transcriptional level via short conserved DNA sequence motifs, so-called metal responsive elements (MREs) (6–8). We have previously identified a key factor for heavy metal-induced transcription in mammalian cells termed MTF-1 (9–11, for review see also 12). In transfection experiments an expression vector with MTF-1 cDNA can induce transcription from synthetic or natural promoters containing MREs. In such transient expression assays the human MTF-1 factor is able to confer a higher metal inducibility than the mouse factor (11). In a preliminary characterization of the different domains of MTF-1, we have found that both human and mouse MTF-1 contain six zinc fingers and three activation domains for transcriptional activation (13). Here we have further characterized the metal transcription factors from both human and mouse. In particular we have narrowed down the region responsible for high metal inducibility to a region over-

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lapping the acidic activation domain of human MTF-1.

MATERIALS AND METHODS

Plasmids Constructs. The 4xMREd-reporter plasmid, as well as the reference plasmid, used in the transient transfection assay, have been described by Westin et al. (9, 14). The mouse MTF-1 expression plasmid was described before (10). Human MTF-1 was cloned from pcDNAI-hMTF (11) into pCT-mMTF-1 (10) by replacing the mouse MTF-1 portion, resulting in pCT-hMTF-1.

The chimera constructions were done by swapping parts of the expression plasmids from human MTF-1 sequences to the mouse MTF-1, or vice versa, using the indicated restriction enzymes (Figure 1 and Figure 2A). Briefly, hBR/m, amino acids 1–312 from mouse MTF-1 were fused to the short intervening piece of human MTF-1, aa 314–377, and the clone was completed by addition of mouse MTF-1 aa 377–675; hRA/m consists of the mouse sequences from aa 1–376 and 449–675, and aa 378–449 of human MTF-1; hRS/m contains aa 1–376 and 524–675 from the mouse and an insert of human MTF-1, aa 378–523; mRS/h contains the intervening protein sequence, aa 377–524 from the mouse embedded in human MTF-1, aa 1–377 and 525–753.

In the POU chimera, human MTF-1 zinc fingers were replaced by the POU domain (15, 16) of Oct-2A (17) which was cloned in pBluescript SK (Stratagene). The POU domain was inserted into the expression plasmid pCT-hMTF-1 as follows: pCT-hMTF-1 was cut with *Asp* 718, *Bsp*H I and *Cla* I. The *Cla* I/*Asp* 718 and the *Bsp*H I/*Cla* I sequences were ligated via bridging oligonucleotides (AAHMP1, 5′-GTACCAACCATCCCACC-3′; AAHMP2, 5′-TCGGGGTGGGATG-GTTG-3′; PBHMP1, 5′-GTGCGGCC-3′; BPHMP2, 5′-CATGGGCCGCACTGCA-3′) to the sequences cut out by *Ava* I/*Pst* I of the Oct-2A-pBluescript-derivative (17).

The hMTF-1/VP16 fusions were constructed by blunt end ligation, fusing the VP16 C-terminal 116 aa that include the 80 aa activation domain (18) to the indicated restriction sites of human MTF-1 in Figure 3A. For technical reasons, the *Sca* I/VP16 fusion contains only the very 80 aa activation domain of VP16. However, we found in independent Ga14 fusion experiments that both the 116 aa as well as the 80 aa segment were equally active (K. Seipel, O. Georgiev and W. Schaffner, unpublished data).

Cell Culture. HeLa and 3T3 cells were cultured in DMEM medium (Gibco) supplemented with 2.5% fetal calf serum, 2.5% newborn serum, 100 U/ml penicillin, 50 μg/ml streptomycin and 2 mM L-glutamine. The Ras or T-antigen expressing MTF-$1^{+/-}$ and MTF-$1^{-/-}$ cells (13) were cultured in the same medium described above except that there was 10% fetal calf serum and no newborn calf serum added.

DNA Transfection and Cadmium Induction. The DNA transfection experiments were performed by the calcium co-precipitation method (19, 20), RNA isolation and S1 nuclease mapping were described before (14). Per 100 mm cell culture petridish 10 μg of reporter plasmid, 1 μg of OVEC-REF (9) and 2 μg of the different expression plasmids were used. Salmon sperm DNA was added in order to equalise the amount of transfected DNA per plate. For metal induction experiments 50 mM cadmium chloride was added to a final concentration of 50 μM 4 hours before harvesting the cells.

RESULTS

The Acidic Domain of Human MTF-1 Contributes to High Metal Inducibility

In our previous studies on MTF-1, we have shown that the zinc fingers themselves confer a several fold metal inducibility, but that the particularly high inducibility of the human factor can be localised to a region downstream of the zinc fingers (13).

In order to delineate the region of high inducibility in the human MTF-1, we have constructed a series of new chimeric molecules from the factors of the two species (see diagrams in Figure 2A). These include not only bipartite chimeras but also fusion molecules where a small MTF-1 segment from one species is inserted into the background of the other species.

These chimera were transfected into a cell line which is null mutant for MTF-1, i.e. does not produce any MTF-1 of its own. This MTF-$1^{-/-}$ mutant cell line had been generated by gene replacement via homologous recombination of mouse embryonic stem (ES) cells followed by differentiation and immortalization (13, 21). As is shown in Figure 1 and Figure 2, the critical region for high metal response is located between amino acid 313 and 377, since all the chimeras that contain this region from human MTF-1 show the high inducibility typical for the human factor. This segment is largely overlapping with the acidic activation domain. Some minor contribution may still come from regions outside, because a patchwork chimera which contains only this small segment from the human factor in a background of mouse MTF-1 is inducible but not quite as highly as the simple chimeras (Figure 2B).

Fusion of the VP16 Activation Domain to MTF-1 Results in Very Strong Activators of a Limited Inducibility

In a previous study, we have shown that the human MTF-1 is quite sensitive towards alterations, notably C-terminal deletions which can render the factor essentially nonfunctional (13). In order to possibly improve the transcriptional activity of such truncation mutants, and also to see whether a better metal regulatory factor could be artificially created by adding an extra activation domain, we fused the very strong 80 amino acid activation domain of the Herpesviral activator protein VP16 (= vmw65, αTIF) (18) to various deletion mutants of MTF-1, as shown schematically in Figure 3A. Indeed, the addition of the VP16 domain yielded extremely strong activators, whereby the fusion to the complete MTF-1 was far more potent than wild type MTF-1 in our transfection experiments (38 fold and 3.5 fold in absence and presence of cadmium, respectively) (Figure 3B). As expected, the presence of the VP16 domain also restored a significant activity to the C-terminal truncation mutants whose activities otherwise were on the borderline of detection (13). All the mutants fused to VP16 yielded an about three fold metal inducibility which is typically associated with the zinc finger region of both human and mouse MTF-1 (13, 21). Therefore, we conclude that addition of the VP16 domain cannot rescue the high inducibility exerted by wild type human MTF-1. These results confirm and extend a previous result with a single type of MTF-1 zinc finger/VP16-fusion protein (13). The difficulty of dissecting the heavy metal response is also underlined by another experiment, where the six zinc fingers of MTF-1 were replaced by the DNA binding "POU" domain of Octamer transcription factor. This chimera was constructed such that the spacing between the flanking domains was kept as in human MTF-1 (described in Materials and Methods). Although the chimera induced transcription from "octamer" site promoters, it was refractory to metal induction (data not shown).

Trivial Reasons are Unlikely to Explain the Differences between Mouse and Human MTF-1

We performed several further experiments to elucidate the difference in metal response between human and mouse MTF-1.

Already earlier on we had excluded a simple species specificity: firstly, the metal responsive elements used for our studies were either a general consensus one (MRE-s) or a specific one (MREd), derived from the mouse MT-Ia promoter. With both these

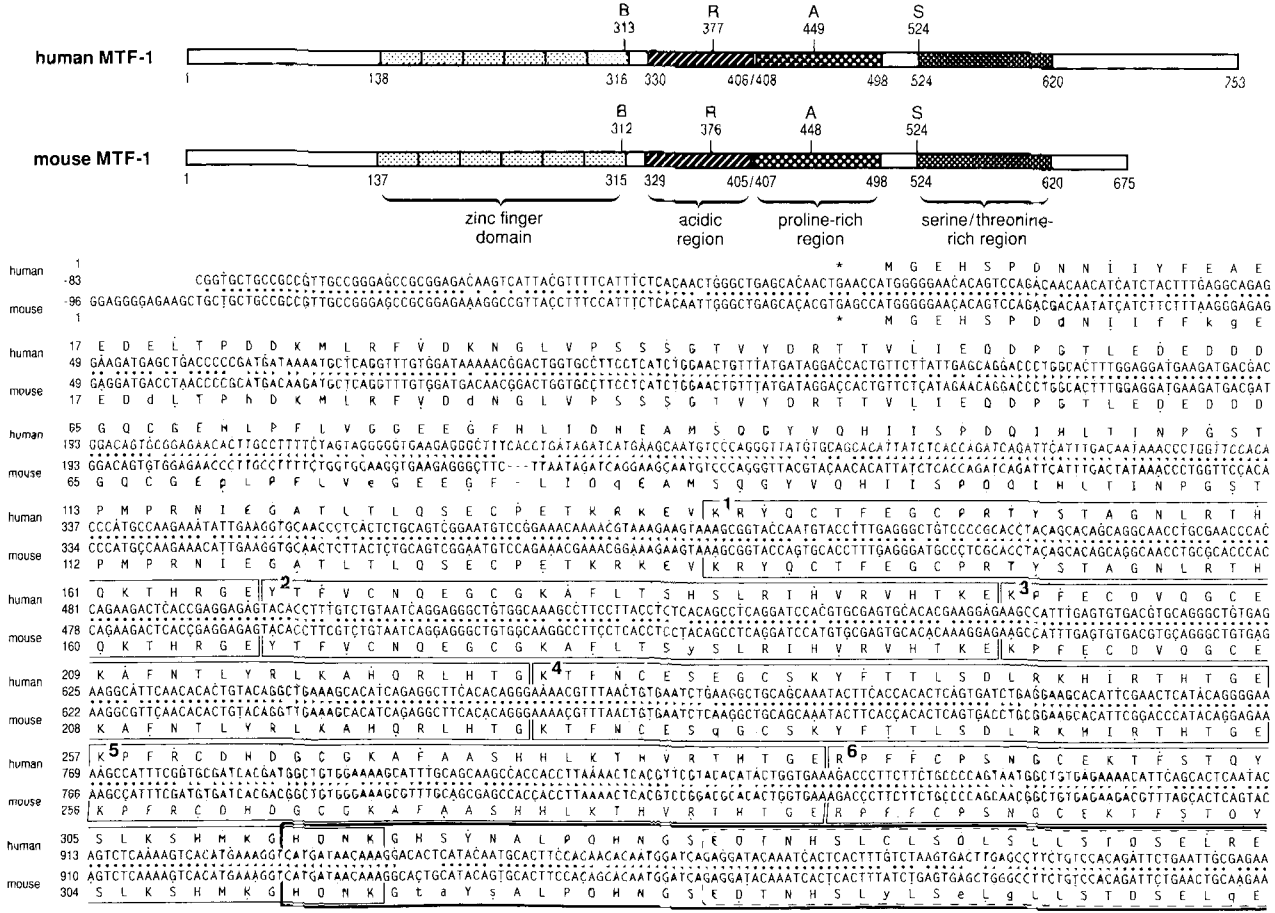


Fig. 1. Structural comparison of human and mouse MTF-1. Schematic representation of the two proteins with their different domains, as characterized earlier (10, 11, 13) is shown on top. Underneath is a comparison of the human and mouse cDNA clones (10, 11). Identical nucleotides are indicated by a dot. Zinc fingers are in thin frame and numbered; acidic, proline-rich and serine/threonine-rich domains are indicated by (_), (___) and (. . . . .), respectively. The critical region conferring high metal inducibility to human MTF-1, overlapping with the acidic region, is framed in bold. Amino acids of mouse MTF-1 differing from the human protein are indicated by lower case letters.

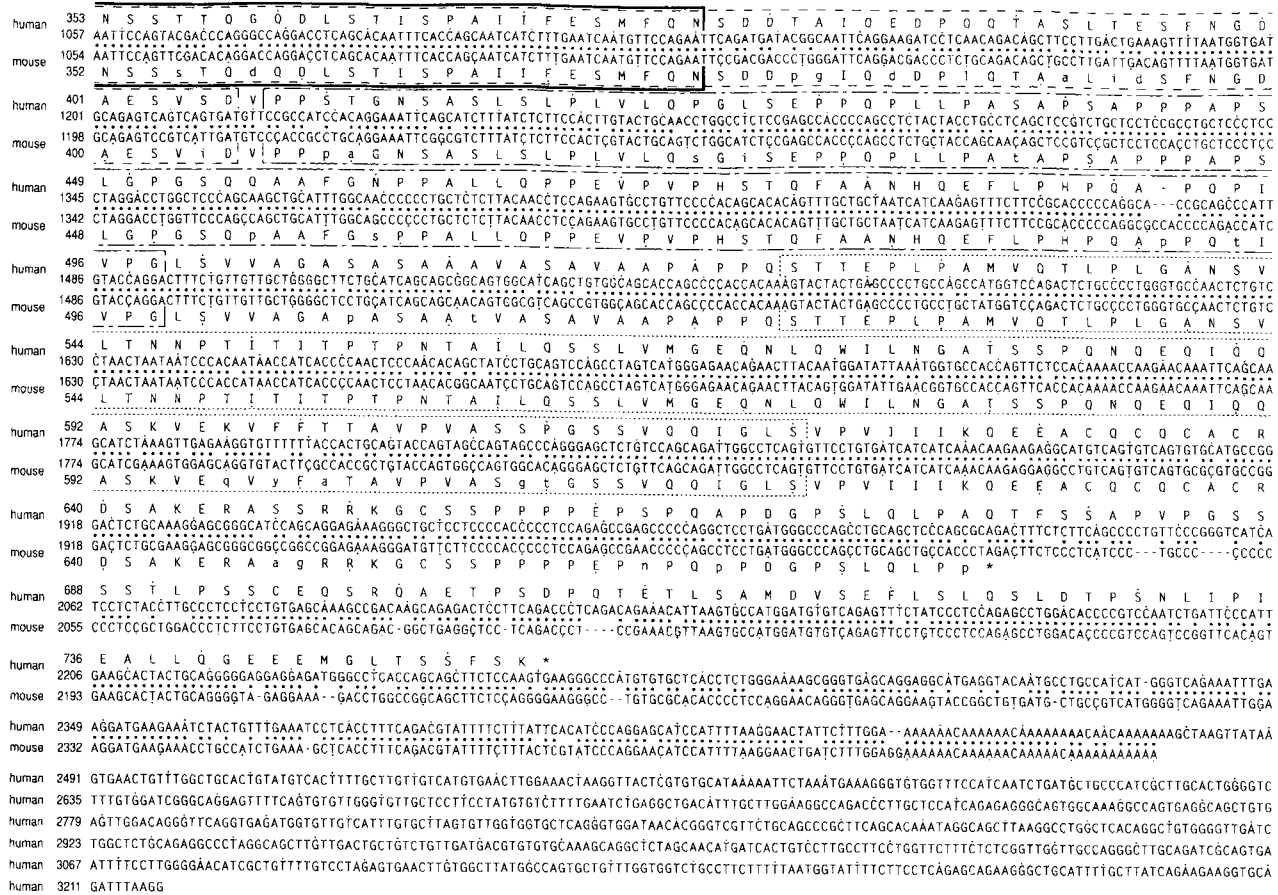


Fig. 1. (Continued)

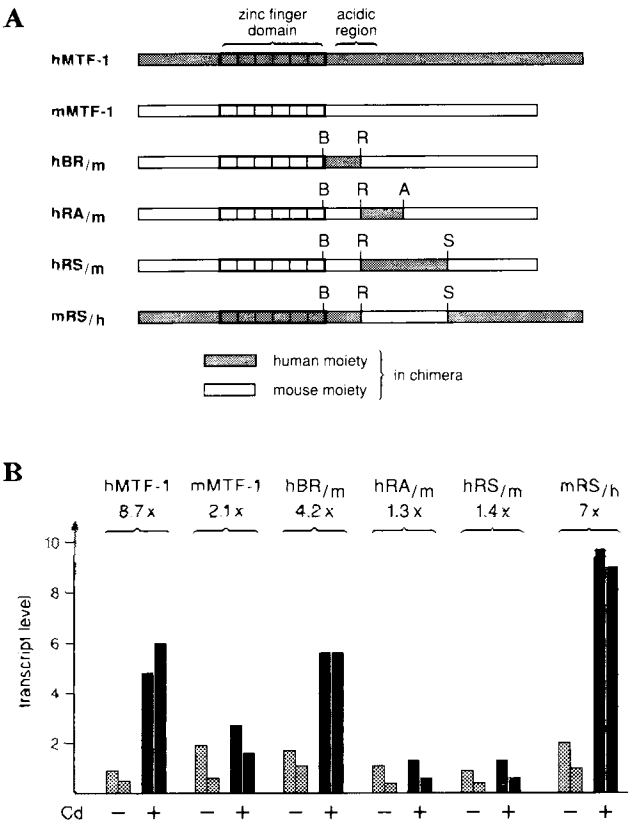
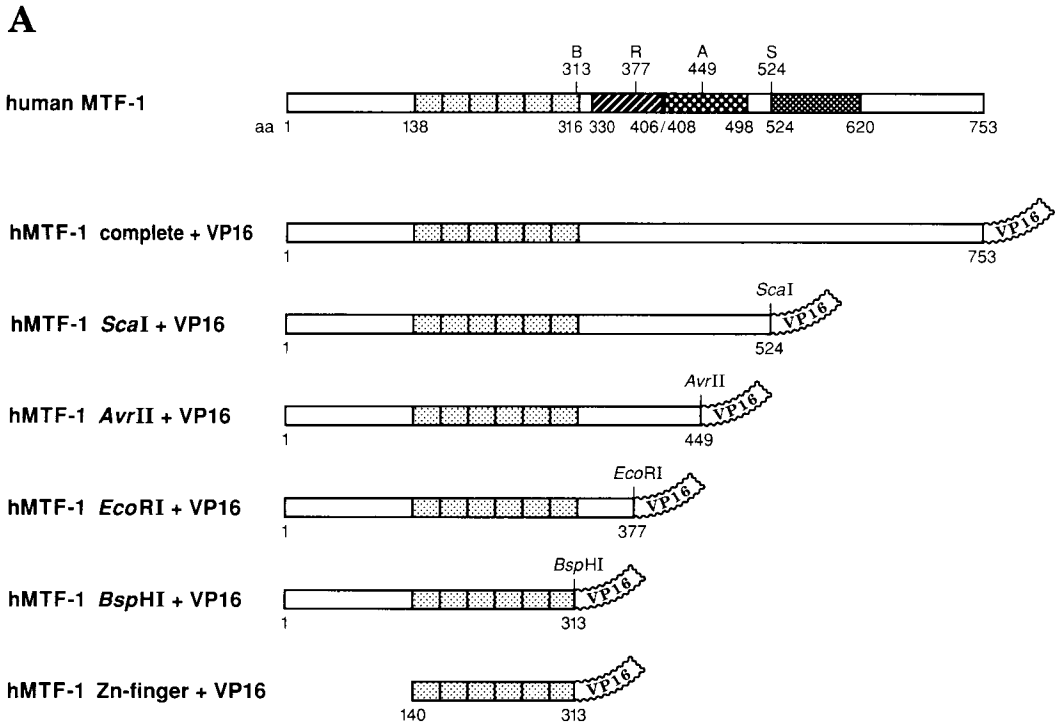
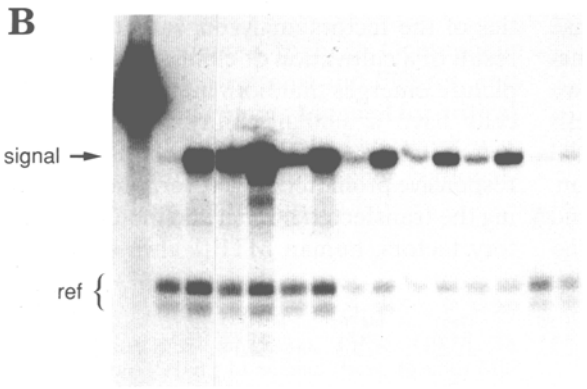


Fig. 2. Metal induction conferred by mouse-human chimeric MTF-1 proteins. (A) Schematic representation of the chimera between human and mouse MTF-1. B, BspH I; R, EcoR I; A, Avr II; S, Sca I; (B) metal induced transcription with different MTF-1 proteins. Each activator was tested with and without cadmium exposure of transfected MTF-1$^{-/-}$ null mutant mouse cells. The induction value (8.7× for hMTF-1, for example) is the average induction from two independent experiments (indicated by the double bars). All expression values were normalized to the transcript level of a cotransfected reference gene (OVEC-ref) which is not heavy metal inducible (9, 14).

promoter elements, the relative inducibility remained characteristic for human versus mouse MTF-1 irrespective of whether they were transfected into mouse or human cells; i.e. even in mouse cells and on a mouse-derived MRE the human factor performed better than the one from the mouse. However, the mouse MTF-1 cDNA had originally been cloned from a cultured cell line, thus we could not exclude the possibility that the cDNA clone studied by us contained a critical mutation, either as a result of the long period of cell culture, or perhaps from the cloning process. Since the region between the *Bsp*H I and *Eco*R I site was the one which conferred the typical mouse or human properties to the chimeric factors, we re-isolated this region from mouse liver poly A$^+$ RNA, using a combination of reverse transcriptase and polymerase chain reaction (RT-PCR). The sequence obtained was indistinguishable from our mouse cDNA clone analysed before (framed in bold in Figure 1; data not shown). We also had to consider another point. As we had noted in the transfection experiments, mouse MTF-1 was always expressed about four fold higher than the human factor, even though both were cloned in the same expression vector background. Therefore, it seemed possible that





	P	1	2	3	4	5	6	7	8	9	10	11	12	13	14
hMTF-1		+		–		–		–		–		–		–	
hMTF-1-VP16		–		compl.		*Sca*I		*Avr*II		*Eco*RI		*Bsp*HI		Zn-f.	
Cd		–	+	–	+	–	+	–	+	–	+	–	+	–	+
fold induction		27		2.5		2.9		3.5		3.5		3.2		2.6	

Fig. 3. Strong activity and low inducibility of MTF-1/VP16 chimeras. (A) Schematic representation of human MTF-1 (top) and chimeric factors; (B) transcript mapping with S1-nuclease. P, free probe DNA; signal, specific reporter transcript; ref, transcript from the reference gene (9, 14) as a transfection standard. Each activator was tested with and without cadmium exposure of MTF-1$^{-/-}$ recipient cells. Lanes 1 and 2, transfection with wild type human MTF-1. Lanes 3–14, transfection with hMTF-1-VP16 chimeras. The extent of transcriptional induction by cadmium treatment is indicated at the bottom. To calculate for each MTF-1 variant the ratio of heavy metal-induced versus uninduced expression ("fold induction" underneath the autoradiogram), expression values ("signal") were normalized to reference gene ("ref") expression.

the mouse factor, unlike the human one, was present in saturating amounts in transfected cells, thus causing a higher basal transcription and hence a lower extent of inducibility. However, bandshift analyses of the various chimeras unambiguously identified the zinc finger region, rather than the acidic domain, to be responsible for the different level of protein expression: whenever a chimera contained the mouse zinc finger region it showed the typical four fold higher expression characteristic for mouse MTF-1, thus higher protein expression was not linked to lower metal inducibility (data not shown). Taken together, these data imply that the higher inducibility of the human factor is based on a genuine difference between species, rather than an artifact.

In Spite of Weak Metal-Induction by Recombinant Mouse MTF-1, Mouse Cells Show Good Metal Response: Evidence for a Cofactor?

After having established that the mouse factor had an intrinsically lower metal inducibility as compared to the human MTF-1, we wondered whether perhaps mouse cells would have an intrinsically lower potential for metal-induced reporter gene activation. For this, we analysed mouse 3T3 cells and two variants of differentiated ES-cells (Figure 4), heterozygous for MTF-1, one immortalised by the Ras oncogene and the other by SV40 T-antigen. While the two heterozygous MTF-$1^{+/-}$ cell lines indeed showed a modest inducibility of 5 and 6 fold, respectively, mouse 3T3 cells showed a similar metal inducibility (11×) as the human Hela cells (14×). Although this analysis is by no means exhaustive, it nevertheless implies that mouse cells show an intrinsic metal inducibility similar to the one of primate cells. The relatively lower inducibility of the Ras and T-antigen immortalised MTF-$1^{+/-}$ cells is best explained by the reduced MTF-1 expression level, due to elimination of one allele by homologous recombination.

DISCUSSION

In conclusion, our data strongly suggest that the different expression levels and different metal responses of human and mouse MTF-1 proteins are genuine properties of the factors analyzed, rather than the result of a cultivation or cloning artifact. The picture emerges that both human and mouse cells have a similar ability to respond to heavy metal stress by activating metal-responsive promoters. However, when analyzing the transfected human and mouse regulatory factors, human MTF-1 always exerts a

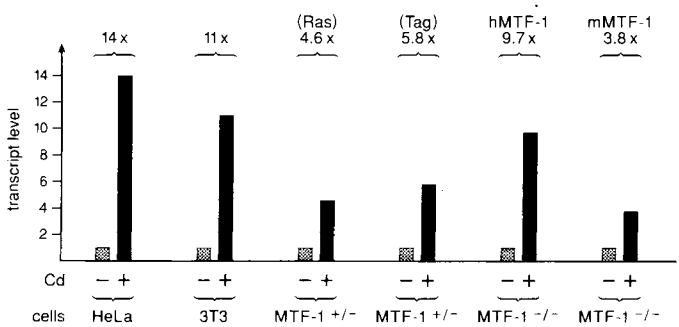


Fig. 4. Comparison of heavy metal response in different cells. Human Hela and mouse 3T3 cells were transfected with a reporter gene without exogenous MTF-1. Also, no exogenous MTF-1 was added to MTF-$1^{+/-}$ heterozygous ES-derived immortalized cells. For comparison, the last two panels on the right hand side show null mutant ES cells transfected with human MTF-1 and mouse MTF-1. Transcript levels with and without cadmium were normalized to a cotransfected reference gene (9, 14).

higher metal inducibility than the mouse factor (Figure 2B and Figure 4). This raises the interesting question whether or not MTF-1 is the only factor controlling metal inducibility. Previous experiments with null mutant cells have shown that MTF-1 is not only essential for the induction of transcription by zinc, but also by cadmium, nickel, copper and lead (21). However, even though the essential role of MTF-1 for metal induction is well established, this does not exclude any requirement for a specific cofactor(s) which is also metal-responsive. R. Palmiter and his group have recently provided indirect evidence for such a cofactor (22). Our data are also compatible with a substantial contribution by a limiting cofactor, which would contribute to metal-induced transcription in concert with MTF-1. The mouse may depend more on such a cofactor than human.

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