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Participation of upstream stimulator factor (USF) in cadmium-induction of the mouse metallothionein-I gene

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ABSTRACT

The roles of the bHLH-Zip protein, upstream stimulatory factor (USF), in mouse metallothionein-I (MT-I) gene expression were examined. The promoter contains a putative USF binding site which overlaps an antioxidant response element (ARE) located at –101 bp relative to the transcription start point. The USF/ARE composite element increases basal expression of the mouse MT-I gene, and partly mediates response to oxidative stress. However, other functions of this composite element and the *in vivo* roles for USF in MT-I promoter functions have not been examined. We report studies which indicate that USF participates via the USF/ARE element in cadmium responsiveness of the mouse MT-I promoter. During the course of these studies a second, higher affinity USF binding site at –223 bp was identified. Stable and transient transfection assays in mouse hepatoma cells, using the USF/ARE in the context of a minimal promoter and site-directed and truncation mutants of the MT-I promoter, revealed that the USF and the ARE sites contribute to cadmium (2–30 μM) but not zinc responsiveness, and to basal promoter activity. Overexpression of dominant–negative (dn)USF in co-transfection assays significantly attenuated cadmium induction of the USF/ARE in the context of a minimal promoter, and attenuated cadmium, but not zinc, induction of the intact MT-I promoter. A consensus E-box (CACATG) at –223 bp in the MT-I promoter was also found to bind USF *in vitro*, and to be constitutively footprinted *in vivo*. The interaction of USF with E-box1 was apparently 10-fold stronger than that with the USF/ARE. However, in contrast, E-box1 was not a strong basal promoter element nor was it metal ions responsive in mouse Hepa cells. In conclusion, these studies demonstrate a role for USF in cadmium-specific induction of the mouse MT-I gene, but bring into question an obligate role for USF in regulating basal activity of this gene. The data further suggest that USF interacts with ARE-binding proteins to influence MT-I gene expression.

INTRODUCTION

Metallothioneins (MT) are cysteine-rich heavy metal binding proteins (1). In the mouse, MT-I and MT-II participate in detoxification of transition metals (2,3), zinc homeostasis (4) and protection against oxidative stress (5). MT-I gene transcription is induced by the heavy metals zinc and cadmium (6). Five metal response elements (MRE) in the proximal promoter participate in this induction (7) and in mediating transcriptional response of MT genes to oxidative stress (8,9). The Zn-finger transcription factor MTF-1 (MRE-binding transcription factor-1) (10,11) plays an essential role in metal (12,13) and oxidative stress-induced MT-I gene expression (9).

Another oxidative stress-responsive element in the MT-I promoter has been mapped to the –101 bp region (8). This region contains an antioxidant response element (ARE) consensus sequence (TGACnnnGC) (14). The ARE (also called electrophile response element) mediates induction of glutathione-*S*-transferase Ya subunit and the quinone reductase (Qr) genes in response to redox cycling xenobiotics and H_2O_2 (14,15). Many MT promoters contain a single perfectly matched consensus ARE sequence (8). The ARE may be negatively regulated by the bZip proteins Fos and Fra-1, and positively regulated by Nrf2-small Maf heterodimers in response to electrophilic agents (16,17). In the mouse MT-I promoter (and the hamster MT-I promoter), the ARE overlaps a previously identified USF binding site (CRCGTGRY) (18).

USF is a member of the bHLH-Zip protein superfamily which includes Myc, Max, Mad and TFE3 (19,20). These proteins can each recognize the core E-box sequence CACGTG. Three isoforms of USF (USF1, USF2a and USF2b) have been described (21–23). The 43 kDa USF1 and the 44 kDa USF2 polypeptides are encoded by separate genes (21,22) while alternate splicing gives rise to the USF2a and USF2b isoforms (23). USFs form DNA-binding homo- and heterodimers (22–24), but can also interact with several different bZip transcription factors (25–28). USF is ubiquitously expressed (21), and has been shown to positively or negatively influence the expression of a myriad of genes (23,29–33).

USF binding *in vitro* was localized to the USF/ARE region of the mouse MT-I promoter, while deletion of the USF/ARE, in the context of the MT-I promoter, reduced basal expression in *in vitro*

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transcription reactions (18) and *in vivo* in transiently transfected cells (7). This suggested that USF activated the mouse MT-I promoter. However, the existence of the ARE was unknown at that time, and our knowledge of the functions and structures of the bHLH-Zip proteins has advanced considerably. Our finding that the USF/ARE in the mouse MT-I promoter is responsive to oxidative stress led us to further investigate the roles of USF in MT-I promoter function. In this study we identified a second, higher affinity USF binding site, an E-box, located at –223 bp in the mouse MT-I promoter and obtained evidence which suggests that USF participates in the cadmium induction of this gene via the USF/ARE composite element.

MATERIALS AND METHODS

Reporter plasmids

The proximal mouse MT-I promoter fragments –250 to +66 and –200 to +66 (numbers relative to the transcription start point in the MT-I gene) were amplified by PCR using –720CAT (8) as template. These MT-I promoter regions, and –150 to +62 promoter, –150ΔUSF/ARE to +62 promoter (deletion of –100 to –89) and the minimal –42 to +62 promoter, also described previously (8), were subcloned into a luciferase reporter (Luc) vector, pGL-2 basic (Promega Biotech, Madison, WI). Four tandem copies of the USF/ARE (USF/ARE$_4$) with the MT-I minimal promoter (–42 to +62) (8) were also subcloned into pGL-2 basic. The TATA box and transcription start point were provided by the MT promoter in these fusion genes. The following MT-I promoter elements were cloned as a single forward oriented copy into the *Bgl*II site which precedes the adenovirus major late minimal promoter (34,35) in pTi-Luc (provided by Dr William Fahl, University of Wisconsin, Madison, WI):

E-box1: GATCTGTTCCACAC**GT*CA*CA*TG***GGTCGTCCTATC
USF/ARE: GATCCGCGGGG**CGCGTGAC**TAT**GC**GTGGGCTGGA
mutUSF/ARE: GATCCGCGGGGGCC**GTGAC**TAT**GC**GTGGGCTGGA
USF/mutARE: GATCCGCGGGG**CGCGTGAC**TATAAGTGGGCTGGA
mutUSF/mutARE: GATCCGCGGGGGCC**GTGAC**TATAAGTGGGCTGGA

In these constructs the TATA box and transcription start point were provided by the adenovirus major late minimal promoter. A dominant–negative human USF1 expression vector, pCX-DN-USF1, was kindly provided by T. Kadesch (32). Oligonucleotides were synthesized by the Biotechnology Support Center (University of Kansas Medical Center, KS).

Transient transfection assay

Hepa cells were cultured in complete medium (DMEM-high glucose supplemented with 2% fetal bovine serum) and transfected using the calcium phosphate precipitation method with modifications (36). Hepa cells were seeded at a density of ~80 000 cells/well in 24-well plates, and co-transfected 24 h later with the reporter plasmids (250–400 ng) and SV-βGal (Promega Biotech) transfection control plasmid (300 ng). The total DNA transfected (600 or 700 ng) was normalized by the addition of pBluescript KS DNA (Stratagene, San Diego, CA). Cells were washed with Dulbecco's phosphate-buffered saline (PBS) 18–20 h post-transfection, and cultured for 20–24 h in complete medium. Where indicated, cells were treated during this period by the direct addition of $ZnSO_4$ and $CdCl_2$ to the culture medium. In experiments involving co-transfection of a dominant–negative expression plasmid pCX-dnUSF1, the control transfections and the titration samples included the expression vector with no insert (CMV-EX) (9) to bring the total DNA transfected to 850 ng/well. Cells were lysed and assayed for Luc and β-galactosidase (βGal) activities using the luminescence assays as described (36). Luc activity was normalized to βGal activity to correct for transfection efficiency. Statistical significance was determined using analysis of variance and student *t*-test. Differences were considered significant when the *P*-value was <0.001. Values are given as mean ± standard deviation (SD) or standard error of the mean (SEM), as indicated. In some experiments, immunoblotting, as described below, was used to monitor expression of dnUSF in the transfected cells.

Immunoblotting

Whole cell extracts were prepared by solubilizing transiently transfected cells in 2× concentrated SDS–sample buffer (200 µl per well). Equal amounts of extract (per cell volume) were resolved on 10% SDS–polyacrylamide gels (acrylamide:bis-acrylamide = 30:0.8) with the discontinuous buffer formulation of Laemmli (37) and then transferred to Nitrocellulose membranes (Midwest Scientific, St Louis, MO), using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA). After blocking, membranes were probed overnight at 4°C with anti-USF1 antibody (Santa Cruz Biochemicals, Santa Cruz, CA), diluted 1:10 000 in TBST (15 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.05% Tween-20), then subsequently incubated with goat anti-rabbit peroxidase-conjugated antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 90 min at room temperature. Specific protein complexes were visualized with the enhanced chemiluminescence (ECL) system (Amersham Life Sciences, Arlington Heights, IL).

In vivo genomic footprinting

In vivo genomic footprinting was performed as described in detail by Dalton *et al.* (9). In brief, Hepa cells were exposed to 0.1% dimethyl sulfate (DMS) and genomic DNA was purified and subjected to piperidine cleavage at positions of methylated guanines. The cleaved DNA was then amplified by ligation-mediated PCR (LM-PCR), using mouse MT-I promoter specific primers (9,38,39). Reaction conditions (38,39) were modified as described (9).

Electrophoretic mobility shift assay (EMSA)

EMSA was performed using nuclear and whole cell extracts as described previously (9,40,41). Proteins from nuclear extracts (5–10 µg in 2–3 µl) or whole cell extracts (20 µg in 1 µl) were incubated in buffer containing 12 mM HEPES, pH 7.9, 60 mM KCl, 0.5 mM DTT, 12% glycerol, 5 mM $MgCl_2$, 0.2 µg poly dI/dC/µg protein and 8 fmol end-labeled double-stranded oligonucleotide (5000 c.p.m./fmol), in a total volume of 20 µl for 20 min on ice (9,40). In antibody supershift experiments, 1 µg of USF1- or USF2-specific antibody was pre-incubated with nuclear extracts for 2 h on ice before addition of the labeled binding site. EMSA was performed using the E-box1, USF/ARE, mutUSF/ARE and USF/mutARE oligonucleotides described above, and an Sp1 binding site oligonucleotide (42) described previously (9). Protein–DNA complexes were separated electrophoretically at 4°C in a 4% polyacrylamide gel (acrylamide:bisacrylamide = 80:1) at 15 V/cm. The gel was polymerized and run in buffer consisting of 0.19 M glycine–25 mM Tris, pH 8.5, 0.5 mM EDTA. After electrophoresis,

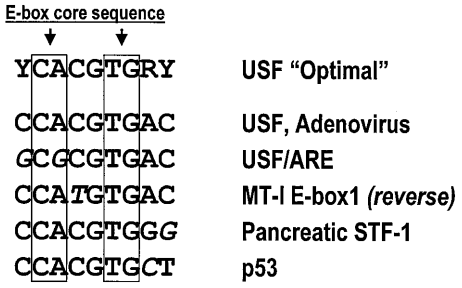

Figure 1. Alignment of the E-box1 and USF/ARE sequences in the proximal promoter of the mouse MT-I gene with consensus USF binding sites. USF-binding sites in the proximal MT-I and other promoters are aligned. The optimal USF binding site was defined by capture and reamplification of random sequence oligonucleotides bound by recombinant USF (20). The adenovirus USF binding site (major late transcription factor) is a high affinity USF binding site (76). The USF/ARE binding site was identified by *in vitro* methylation protection and mobility shift assays (8,18). The MT-I E-box1 USF binding site was identified by homology, as reported herein. The pancreatic STF-1 gene is expressed in the islets due, in part, to this USF binding site (56). USF transactivates the p53 promoter via this E-box (29). The bolded bases are those that differ from the USF 'optimal' binding site motif. Underlined are the core E-box bases.

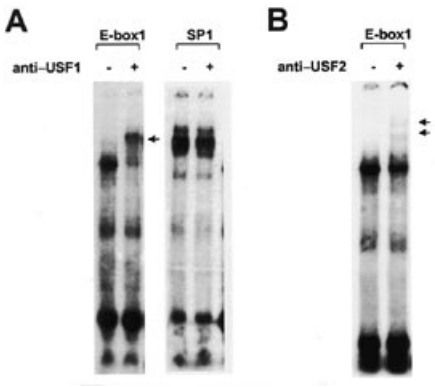

Figure 2. USF1 and USF2 are components of the E-box1 binding complex. Nuclear extracts were prepared from Hepa cells and analyzed by EMSA as described in Materials and Methods. Antisera against USF1 (**A**) or USF2 (**B**) were added to the EMSA binding reaction, allowed to react with USF, and then ^{32}P-labeled E-box1 or Sp1 oligonucleotides were added and protein–DNA complexes were allowed to form. Protein–DNA complexes were separated by PAGE and detected by autoradiography. The arrows point to the supershifted E-box1 binding complexes. The Sp1 oligonucleotide served as a control for specificity of the supershift.

the gel was dried and labeled complexes were detected by autoradiography.

RESULTS

In the mouse MT-I promoter, E-box1 is found at –223 bp relative to the transcription start point. E-box1 and USF/ARE in the MT-I promoter and the high affinity USF binding site in the adenovirus major late promoter are identical in the 3′ half site (GTGAC), but differ by 1 (E-box1) or 2 (USF/ARE) bases in the 5′ half site (Fig. 1). The core E-box sequence CAnnTG is not conserved in the USF/ARE (C**G**nnTG). Therefore, it was of interest to examine USF binding to these mouse MT-I promoter sequences. EMSA was performed using nuclear extracts prepared from mouse Hepa cells. A single binding complex was detected, and the mobility of this complex was retarded by preincubation of the extract with antibodies against USF1 or USF2 (Fig. 2). The USF1 antisera had no effect on Sp1 binding complexes in the same nuclear cell extracts (Fig. 2), nor did the USF2 antisera (data not shown).

The base specificity of E-box1–USF interactions was examined by competition EMSA, in which labeled E-box1 was incubated with nuclear extracts in the presence of increasing molar excess of unlabeled competitors. The amount of radioactivity in the specific E-box1-binding complex was quantitated by radioimaging the dried gel, and the molar excess of competitor required to achieve 50% inhibition was calculated (Fig. 3). Competitors with mutations in the E-box core bases (**CA**nn**TG**), and those with mutations in the bases immediately flanking the core dinucleotides CA or TG were ineffective competitors. In contrast, mutation of three bases 5′ to this core sequence had no effect on the ability to efficiently compete for E-box1-complex formation. These data suggest that the eight bases (T**CA**CA**TG**G) of E-box1 play a role in specific interactions with USF. Remarkably, competition EMSA using the previously identified USF/ARE in the MT-I promoter indicates that USF binds to E-box1 with an apparent 10-fold higher affinity than it does to the USF/ARE, under these binding conditions (Fig. 3).

		Molar Excess of Oligos for 50% Competition of E-box1 binding
*GATC*TGTTCCACACGT**CA**CA**TG**GGTCGTCCTATC	E-BOX1 (S)-223	5
*GATC*TGTTCCACACGT**AT**CATGGGTCGTCCTATC	mut1E-BOX1 (S)	400
*GATC*TGTTCCA**TA**G**A**TCACATGGGTCGTCCTATC	mut2E-BOX1 (S)	5
*GATC*TGTTCCACACGTCACA**AT**GGTCGTCCTATC	mut3E-BOX1 (S)	300
*GATC*TGTTCCACACG**G**CA**G**ATGGGTCGTCCTATC	mut4E-Box1 (S)	> 800
*GATC*TGTTCCACACGTCAC**T**TG**T**CGTCCTATC	mut5E-BOX1 (S)	> 800
*GATC*CGCGGGG<u>CG**CGTGAC**</u>TATG<u>C</u>GTGGGCTGGA	USF/ARE (S)	50

Figure 3. E-box1 core bases and flanking bases are critical for USF binding, and E-box1 binds USF with 10-fold higher apparent affinity than does the USF/ARE. Nucleotide specificity of the E-box1 binding complex in Hepa cell nuclear proteins was determined by competition EMSA. An excess (0–800-fold molar excess) of the indicated unlabeled oligonucleotides was titrated into the EMSA binding reaction containing labeled E-box1 oligonucleotide and nuclear proteins. The amount of radioactivity in the USF-binding complex after electrophoresis was quantitated by radioanalytic analysis of the dried gel, and the approximate molar excess of each competitor required to achieve 50% inhibition of complex formation is shown.

Whether E-box1 interacts with proteins *in vivo* was examined using genomic footprinting, although this method does not reveal the identity of proteins which may bind to this site. Genomic footprinting was accomplished by LM-PCR of bases –250 to –30 in the MT-I promoter, as previously reported (9). Guanine residues involved in protein–DNA interactions were visualized as either less intense (protected) or more intense (hypersensitive) compared with invariant G residues in the promoter, and by comparison with DNA from untreated control cells. Treatment of Hepa cells with H_2O_2, *t*BHQ or zinc caused genomic footprints to be rapidly induced over five of the MREs in the MT-I promoter (–42 to –150 bp) and to be changed over the USF/ARE (–101 bp) (9). Here we report analysis of the upstream region of E-box1 which begins with core base –223 (**C**A) and ends with core base

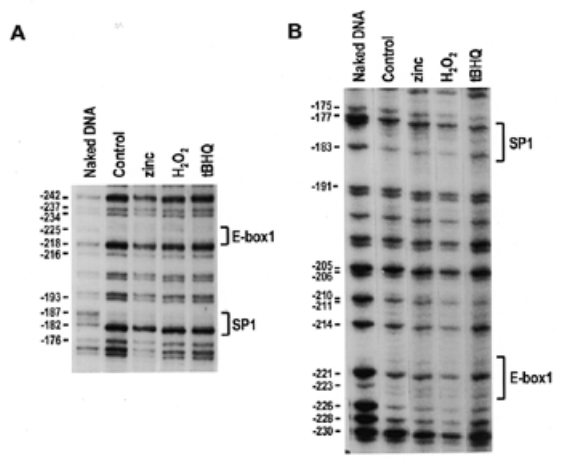

Figure 4. *In vivo* genomic footprinting reveals constitutive occupancy of E-box1 in mouse Hepa cells. Hepa cells were incubated for 1 h in medium containing 100 µM zinc, 2.5 mM H_2O_2, or 200 µM tBHQ. These agents induce expression of the mouse MT-I gene in Hepa cells (9). Cells were treated briefly with DMS to methylate guanine residues and genomic DNA was purified. To generate a G-ladder (naked DNA), purified genomic DNA was methylated with DMS *in vitro*. Methylated DNA was cleaved with piperidine, and the MT-I promoter fragments of the sense strand (**A**) and antisense strand (**B**) were specifically amplified using LM–PCR as described previously (9). LM–PCR products were separated on a 6% sequencing gel and detected by autoradiography. Locations of protected and/or hypersensitive G residues in the Sp1 binding site and E-box1, are indicated. Constitutive footprints were detected in untreated as well as treated control cells over E-box1 and the Sp1 site. In the more proximal region of the MT-I promoter (–42 to –150) these treatments induced footprints over the MREs and altered the footprint over the USF/ARE (9).

–218 (T**G**). *In vivo* footprinting revealed a strong constitutive footprint covering an 18 bp region (Fig. 4) centered over E-box1. This footprint was essentially unchanged after induction of the gene. A strong constitutive footprint on the guanine-rich sense-strand (Fig. 4A) was noted over the Sp1 binding site (–187 to –179), as reported previously (9). In addition, protection of guanine –225 was apparent, as was protection of guanine –216 which immediately flanks an E-box1 core base (Fig. 4A). The *in vivo* footprint over E-box1 was striking when examined on antisense-strand (Fig. 4B). The E-box1 footprint extended ~5 bp upstream (–228 bp) and 8 bp (–210 bp) downstream of the core bases. In contrast, the Sp1 footprint is weak on this strand. This large footprint over and around E-box1 suggests that several proteins may interact with this site in Hepa cells.

In initial experiments designed to examine the functions of the USF/ARE, Hepa cells were stably transfected with a fusion gene consisting of a concatenate of the USF/ARE (USF/ARE_4) promoting expression of βGeo. In agreement with previous studies, this gene was responsive to H_2O_2 (8), but not *t*BHQ (9). Remarkably, we also noted that this gene was responsive to cadmium, but not to zinc (data not shown).

To further explore these findings, cadmium-responsiveness of the USF/ARE also examined in transient transfection assays using mouse Hepa cells. A single copy of USF/ARE is present in the MT-I promoter (8). Therefore, we examined the ability of a single copy of the USF/ARE to direct response to cadmium when placed in front of a minimal promoter in a Luc reporter vector (Fig. 5A). Our previous study showed that at least two factors

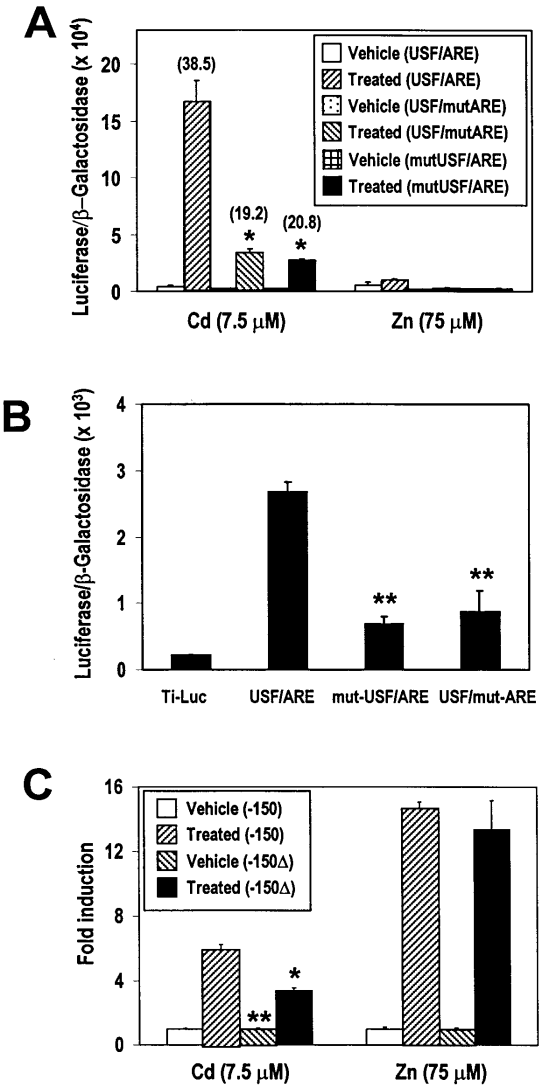

Figure 5. A single copy of USF/ARE directs response to cadmium and deletion of the USF/ARE from the MT-I promoter reduces response to cadmium in transient transfection assays. Hepa cells were transiently co-transfected with the indicated reporter genes and SV-βGal as an internal control for transfection efficiency. Transfected cells were treated overnight with the indicated concentrations of cadmium or zinc and then assayed for Luc and βGal activities. Data represent the mean ± SEM of 12 determinations. (**A**) The USF/ARE, mutUSF/ARE and USF/mutARE oligonucleotides, described in Materials and Methods, were cloned as a single forward-oriented copy in front of the adenovirus major late minimal promoter in pTi-Luc. Data are expressed as the mean ± SEM of the Luc/βGal activity ratio for each sample. The fold-induction relative to the untreated sample is given in parentheses. * indicates *P* values of <0.001 between treated USF/ARE and the mutUSF/ARE or the USF/mutARE. (**B**) Basal activity of the USF/ARE and mutUSF/ARE and USF/mutARE reporter constructs are shown. ** indicates *P* values of <0.001 between USF/ARE and mutUSF/ARE or USF/mutARE. (**C**) Hepa cells were transiently transfected with a Luc reporter gene driven by the first 150 bp of the MT-I promoter or this promoter region in which the USF/ARE was deleted by PCR (8). For each fusion gene transfection, the data are expressed as the fold induction of the Luc/βGal ratio in metal treated cells relative to that in untreated cells. * indicates *P* values of <0.001 between treated –150ΔUSF/ARE and treated –150.

bind to the composite USF/ARE (8). USF binds to the site (CGCGTGAC) and another protein(s) binds to the ARE (TGACTATGC). Core bases are underlined and mutation of these bases abolishes protein binding *in vitro* (8). The functional contribution

of individual binding sites was examined (Fig. 5A and B). In addition, we determined whether deletion of this element from the proximal –150 bp of the MT-I promoter effected cadmium induction (Fig. 5C). The single copy USF/ARE was dramatically induced by cadmium, but not by zinc, in the transiently transfected cells, and the USF/ARE promoted high basal level expression of the reporter gene. Mutation of the USF (mutUSF/ARE) or of the ARE (USF/mutARE) attenuated the relative cadmium induction by ~50% (Fig. 5A). In contrast, these mutations reduced basal activity 80–87 or 68–76%, respectively (Fig. 5B). Mutation of both the USF and the ARE (mutUSF/mutARE) reduced basal promoter activity to near that of the minimal promoter alone and abolished Cd responsiveness (data not shown). An MRE-Luc vector (MRE-d_5-Luc; 8) transfected in a parallel experiment was responsive to cadmium and to zinc in these Hepa cells (data not shown).

Site-directed deletion of the USF/ARE from the 150 bp MT-I promoter (–150ΔUSF/ARE) caused an 81.3% reduction or 5-fold decrease in basal transcriptional activity ($P < 0.001$), as reported previously (7,8,18) (data not shown). This deletion also reduced cadmium-responsiveness to a level of 55% of control in transient transfection assays (Fig. 5C). In contrast, induction by zinc was not effected by deletion of the USF/ARE from the proximal MT-I promoter (Fig. 5C). Induction by zinc, and partly by cadmium, is mediated by multiple MREs in the proximal MT-I promoter.

The potential role of USF in cadmium induction of the USF/ARE was examined by co-transfection of a dnUSF expression vector in which the basic DNA-binding domain was deleted (43). The USF/ARE-Luc vector was co-transfected with increasing amounts of the dnUSF vector, as well as with the SV-βGal transfection control plasmid. Cadmium induction was quantitated (Fig. 6A) and expression of the dnUSF monitored by western blotting (Fig. 6B). Over-expression of dnUSF clearly attenuated cadmium induction of the USF/ARE in a dose–response manner. However, dnUSF had no effect on expression of the transfection control (SV-βGal) plasmid or on the basal expression of the USF/ARE. A similar experiment was performed using the MT-I promoter (Fig. 7). Hepa cells were transiently transfected with Luc fusion genes containing –250 or –150 bp of the MT-I promoter or with a single copy of E-box1 in the minimal promoter construct (Fig. 7). The 100 bp region between –250 and –150 bp contains E-box1, an Sp1 binding site, and potentially other promoter elements. However, deletion of this region had no effect on cadmium inducibility of the MT-I promoter (data not shown). In sharp contrast, over-expression of dnUSF attenuated cadmium induction of the 250 bp MT-I promoter to about one third of the control level, but had no effect on zinc induction (Fig. 7A). We further noted that dnUSF has no effect on Cd- or Zn-induction of an MREd-Luc reporter gene (data not shown) which further suggests that this effect of dnUSF is specific to Cd. Deletion of the region between –250 and –150 bp slightly reduced basal expression of the reporter gene, and co-transfection of dnUSF had little effect on the basal activity of either promoter construct (Fig. 7B). In addition dnUSF had no effect on the expression of the internal transfection control plasmid (SV-βGal). We also examined the ability of a single copy of E-box1 to direct response to cadmium when placed in front of a minimal promoter in a pTi-Luc reporter vector. E-box1 did not confer significant cadmium responsiveness, nor did it exert significant effects on the basal activity of this minimal promoter (Fig. 7C). Furthermore,

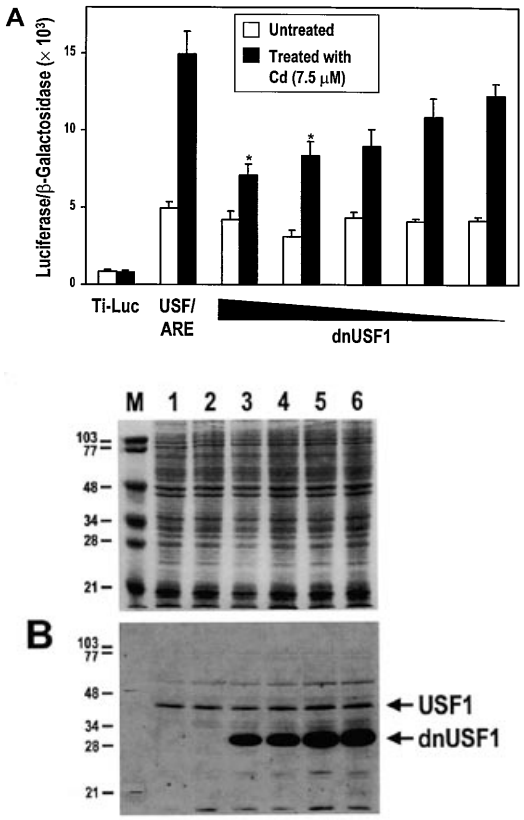

Figure 6. Over-expression of dominant–negative USF interferes with cadmium induction of the USF/ARE in transient transfection assays. (**A**) Hepa cells were transiently transfected with the indicated reporter gene and the indicated transfections also contained an expression vector for dnUSF (25–150 ng/well). The empty CMV expression vector was added to bring the total DNA transfected to the same level in each well. The dnUSF construct lacks the basic domain of human USF1 and cannot bind to DNA (32). Transfected cells were treated overnight with the indicated concentration of cadmium and then assayed for Luc and βGal activities. Data represent the mean Luc/βGal ratio ± SEM of 12 determinations. * indicates P values of <0.001 between the treated USF/ARE, and the treated sample co-transfected with the dnUSF. (**B**) Immunoblot detection of USF and dnUSF in whole cell extracts from Hepa cells transiently co-transfected with dnUSF. Blots were probed with USF1 antiserum. M, relative molecular weight markers; lanes 1 and 2, no dnUSF; lanes 3 and 4 (150 ng dnUSF), Lanes 5 and 6 (300 ng dnUSF).

over-expression of dominant–negative USF did not effect E-box1 basal activity or cadmium induction (Fig. 7C).

These data suggest that USF participates in activation of the MT-I gene in response to cadmium by interacting with ARE-binding factors through the USF/ARE composite element. USF and ARE binding activities were examined in whole cell extracts prepared from Hepa cells during treatment with cadmium. EMSA was performed using the mutUSF/ARE and USF/mutARE oligonucleotides to differentiate between USF binding and ARE-binding activities, as described (8). Supershift and competition EMSA demonstrated that USF is a major component of the USF/mutARE–protein complex (8). The identity of proteins in the ARE binding complex is unknown. Both USF and ARE-binding activity were detected in control cells and USF activity remained unchanged during cadmium treatment (Fig. 8). In contrast,

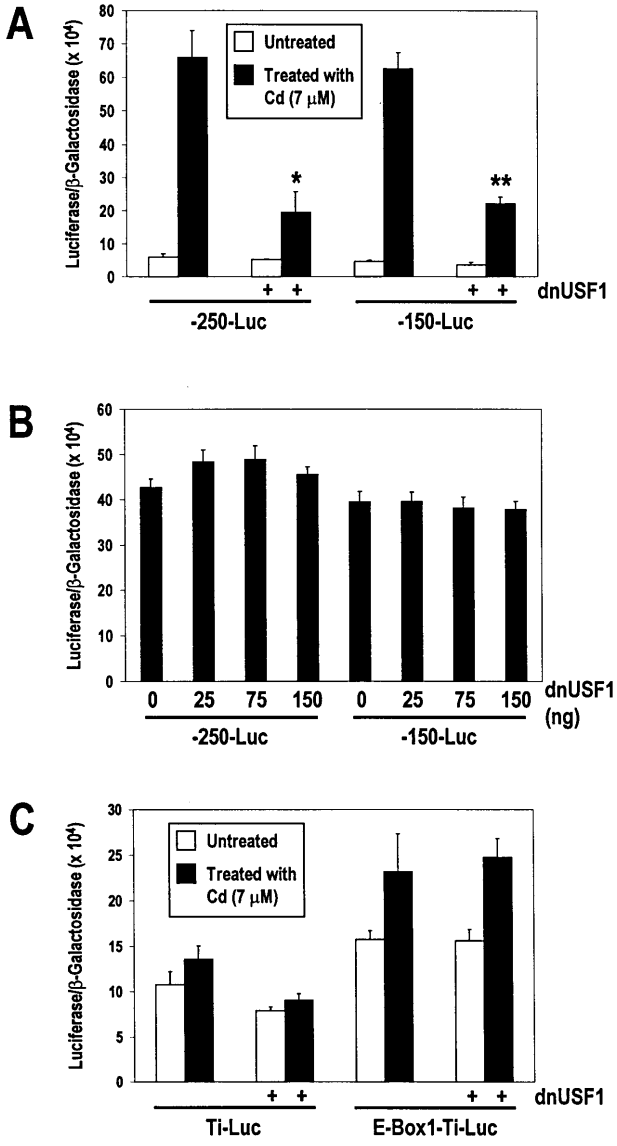

Figure 7. Over-expression of dominant-negative USF interferes with cadmium induction, but not zinc induction or basal activity of the MT-I proximal promoter, and E-box1 has little effect on basal expression or cadmium induction in transient transfection assays. (**A**) Hepa cells were transiently co-transfected as described in the legends to Figures 5 and 6. Luc expression was driven by the 250 bp MT-I promoter which was co-transfected with the indicated amount of the dnUSF expression vector. All transfections contained the empty CMV expression vector such that the same DNA concentration was transfected in each well. Transfected cells were treated with cadmium (7.5 μM) or zinc (75 μM) for 24 h and assayed for Luc and βGal activities. Data represent the mean ± SEM of six determinations of the Luc/βGal ratio. * indicates *P* values of <0.001 between Cd-treated –250-Luc and that cotransfected with dnUSF and treated. (**B**) The effects of over-expression of dnUSF on the basal activity of the –250-Luc and –150-Luc promoters is shown. The –250-Luc and –150-Luc reporters were co-transfected with the indicated amount of dnUSF expression vector plus empty CMV expression vector to equalize DNA concentration in each transfection, and basal Luc expression was measured relative to βGal. Values represent the mean ± SD and were not significantly different at $P < 0.001$. (**C**) The E-box1 oligonucleotide was cloned as a single forward-oriented copy in front of the adenovirus major late minimal promoter in pTi-Luc. Hepa cells were transfected with the indicated reporters plus (+) or minus the dnUSF (150 ng) expression vector plus empty CMV expression vector. Transfected cells were treated with cadmium and assayed for Luc and βGal, and data represent the mean ± SEM of twelve determinations.

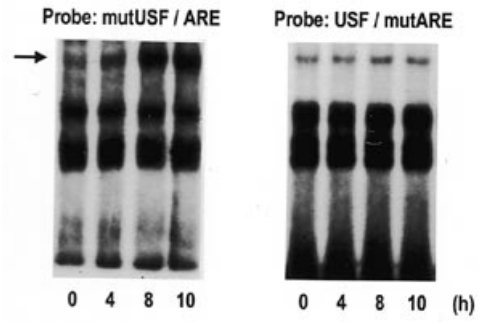

Figure 8. ARE binding activity is increased in whole cell extracts from cadmium treated Hepa cells. The stably transfected Hepa cells used in Figure 5 were incubated in medium containing 10 μM cadmium for the indicated times. Whole cell extracts were prepared (9,40,41) and analyzed by EMSA using the mutant USF/ARE and USF/mutant ARE oligonucleotides (8), as described in the legends to Figures 2 and 3. Supershift and competition EMSA demonstrated that USF is a major component of the USF/mutant ARE–protein complex (8). The identity of proteins in the ARE binding complex is unknown.

ARE-binding activity increased significantly during cadmium treatment. Increased ARE-binding activity was noted by 4 h and was dramatic by 10 h (Fig. 8).

DISCUSSION

These studies examined the roles of the bHLH-Zip protein USF in regulating the basal and induced expression of the mouse MT-I gene. Since members of the bHLH-Zip transcription factor family recognize the E-box sequence CACGTG, putative USF binding sites in the MT-I promoter could also represent binding sites for other family members, including Myc–Max or Max–Max (19) or TFE3 dimers (32). At present, we have no definitive information on the *in vivo* functional roles of USF in regulating MT gene expression. Previous studies demonstrated *in vitro* binding for USF within the MT-I promoter (18), and it was subsequently noted that this site (–101 bp) was a composite element consisting of an overlapping ARE (8). The putative USF binding site in the composite USF/ARE has a base substitution in the 5′-E-box core dinucleotide (CGCGTG) and a flanking base which makes it a relatively low affinity binding site (18). Nonetheless, deletion of the USF/ARE reduces basal expression of the MT-I promoter in transfected cells (7,8,18) and USF can stimulate the *in vitro* transcription of the mouse MT-I promoter (18). We recognized a second putative USF binding site, an E-box located at –223 bp in the mouse MT-I promoter (44). This led us to re-examine the roles of USF in MT-I promoter function. Three experimental approaches were taken. We examined protein interactions with these elements *in vitro* and *in vivo*, the structure and function of these elements in stably and transiently transfected cells, and the effect of over-expression of dnUSF on the transcriptional activity of these elements.

The results of this study demonstrate that the two putative USF binding sites in the MT-I promoter have different functions and interact differently with USF *in vitro*, and perhaps *in vivo*. E-box1 binds USF *in vitro*, and is nearly identical (8/9 bases) with an 'optimal' extended USF binding site (20). *In vivo* footprinting in Hepa cells suggested that E-box1 is bound by protein constitutively. Western blot analysis of Hepa cell extracts, and EMSA using antisera against other members of the bHLH-Zip protein

superfamily (Myc, Max) (data not shown) suggests that USF1 is the most abundant bHLH-Zip family member in these cells. Thus, although these data do not formally exclude the possibility that other proteins actually bind to E-box1 *in vivo*, these studies suggest that USF does. E-box1 had little effect on basal expression or metal-induction of the MT-I promoter and dnUSF did not significantly affect basal activity of E-box1 or the MT-I promoter. These data suggest that USF binding to E-box1 does not lead to obligatory transcriptional activation or repression of transcription, at least in mouse Hepa cells. Binding of USF to the IgH enhancer does not activate transcription (32) and activation of *Xenopus* MyoD transcription is inhibited by USF (33). It is conceivable that E-box1 participates in cell-specific expression of the mouse MT-I gene (6,44) which occurs in developing hepatocytes (45,46), endoderm cells of visceral yolk sac (47), placental spongiotrophoblasts (6,48) and maternal deciduum (44). E-boxes are important in regulating muscle- (49,50), neuronal- (51,52), pancreas- (53) and hematopoietic-specific (19,54) genes. USF has been suggested to play a critical role in cell-specific gene expression in pancreatic islets (55,56), ovarian granulosa (57), muscle (58) and red blood cells (59).

The composite USF/ARE was previously identified as a binding site for USF *in vitro* (8,18). USF interacts weakly with this site relative to the USF site in the adenovirus major late promoter (18), and relative to E-box1 in the mouse MT-I gene. *In vivo* footprinting previously revealed a constitutive footprint over the USF/ARE (9,60) and changes in this footprint were noted after induction of MT-I gene expression by oxidative stress or metals. Protein interactions within, and immediately around, the USF site and with the ARE site were changed during induction (9,60). We previously found, using extracts from control cells and cells treated acutely with oxidative stress, that USF and ARE-binding protein(s) independently interact with the USF/ARE *in vitro* (8). The identity of this ARE binding activity is unknown, but it did not contain c-Jun (8). We have yet to examine the potential interactions of Nrf or Maf family members with the USF/ARE.

The USF/ARE has strong basal promoter activity (8,18,61), and this activity reflects the synergistic interactions of the USF site and the ARE site. In apparent conflict with the previous findings that purified USF can augment the *in vitro* transcription of the MT-I promoter (18), we noted that over-expression of dnUSF had little effect on the *in vivo* basal activity of the USF/ARE or the intact MT-I promoter. This suggests the likelihood that proteins other than USF are important for basal activity of the USF/ARE *in vivo*. ARE sequences have been suggested to regulate basal activity of several antioxidant genes (15,62–66). MTF-1 regulates all basal expression of the endogenous and transfected MT-I promoter in embryonic stem cells (12) which suggests that the USF/ARE does not participate in basal level expression in those cells. The basal promoting activity of the USF/ARE may, therefore, be cell-specific.

Previous studies showed that the USF/ARE was partly responsible for induction of MT-I gene expression in response to H_2O_2 (8), but not to redox cycling quinones (9). Remarkably, the USF site and the ARE site were also found to increase transcription of the MT-I promoter in response to cadmium. A recent study also suggested a role for USF in cadmium induction of the rat heme oxygenase-I gene (67). We noted that dnUSF significantly antagonized cadmium induction of the USF/ARE, as well as that of the intact MT-I promoter. Previous studies did not report a significant effect of deletion of the USF/ARE on metal induction of the MT-I promoter (7), and manipulation of MTF-1 expression by targeted deletion of both genes in embryonic stem cells (12) or by expression of antisense MTF-1 in BHK cells (13) eliminated metal responsiveness of the MT-I gene. Thus, MTF-1 plays a key role in regulating MT gene expression in response to metal ions. The DNA-binding activity of MTF-1 is reversibly regulated by zinc interactions with the Zn-finger domain (40). Interestingly, cadmium does not cause a rapid increase in MTF-1 binding activity (41). This suggests that zinc and cadmium may activate MT-I promoter function by overlapping, yet distinct signal transduction pathways. Cadmium can cause oxidative stress and the depletion of glutathione (68), as well as effect the activity of signal transduction molecules (69–72) and evoke superoxide anion production by macrophages (73). Data presented here indicate that in mouse Hepa cells, USF also plays an *in vivo* role in cadmium induction of the mouse MT-I gene. In addition to MTF-1, the USF/ARE could serve to specifically augment or prolong the responsiveness of the MT-I gene to cadmium relative to zinc and relative to the other mouse MT genes. USF could help maintain an open chromatin structure to facilitate interactions of the MT-I promoter with other factors. The cell-specificity of this role of USF warrants investigation.

Cadmium induction of the USF/ARE involves both the ARE site and USF. Mobility shift assays demonstrated *in vitro* binding of protein(s) with the ARE in control cells (8), and as shown here increased ARE-binding activity was detected after cadmium induction. These results suggest that in the mouse MT-I promoter USF may physically interact with ARE-binding proteins. Very recent studies have demonstrated that USF can, in fact, interact with several different bZip transcription factors (25,26), including the ARE binding proteins Fra1 (27) and c-Maf (28). USF activity can involve other proteins and adjacent promoter elements. A composite CCAAT-binding protein-USF binding site mediates TGF-β1 induction of the human type 1 plasminogen activator inhibitor gene (74), and a three-protein complex containing USF occurs at the immunoglobulin μ heavy chain gene enhancer in B cells (75). The proteins which interact with the ARE in the MT-I promoter are unknown, but our data suggest that they play an important role in cadmium induction of this gene.

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REFERENCES

1 Kagi,J.H.R. (1991) *Methods Enzymol.*, **205**, 613–626.
2 Masters,B.A., Kelly,E.J., Quaife,C.J., Brinster,R.L. and Palmiter,R.D. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 584–588.
3 Michalska,A.E. and Choo,K.H.A. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 8088–8092.
4 Dalton,T.P., Fu,K., Palmiter,R.D. and Andrews,G.K. (1996) *J. Nutr.*, **126**, 825–833.
5 Lazo,J.S., Kondo,Y., Dellapiazza,D., Michalska,A.E., Choo,K.H.A. and Pitt,B.R. (1995) *J. Biol. Chem.*, **270**, 5506–5510.
6 Andrews,G.K. (1990) *Prog. Food Nutr. Sci.*, **14**, 193–258.

7 Stuart,G.W., Searle,P.F., Chen,H.Y., Brinster,R.L. and Palmiter,R.D. (1984) *Proc. Natl Acad. Sci. USA*, **81**, 7318–7322.
8 Dalton,T.P., Palmiter,R.D. and Andrews,G.K. (1994) *Nucleic Acids Res.*, **22**, 5016–5023.
9 Dalton,T.P., Li,Q.W., Bittel,D., Liang,L.C. and Andrews,G.K. (1996) *J. Biol. Chem.*, **271**, 26233–26241.
10 Radtke,F., Heuchel,R., Georgiev,O., Hergersberg,M., Gariglio,M., Dembic,Z. and Schaffner,W. (1993) *EMBO J.*, **12**, 1355–1362.
11 Brugnera,E., Georgiev,O., Radtke,F., Heuchel,R., Baker,E., Sutherland,G.R. and Schaffner,W. (1994) *Nucleic Acids Res.*, **22**, 3167–3173.
12 Heuchel,R., Radtke,F., Georgiev,O., Stark,G., Aguet,M. and Schaffner,W. (1994) *EMBO J.*, **13**, 2870–2875.
13 Palmiter,R.D. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 1219–1223.
14 Favreau,L.V. and Pickett,C.B. (1993) *J. Biol. Chem.*, **268**, 19875–19881.
15 Jaiswal,A.K. (1994) *Biochem. Pharmacol.*, **48**, 439–444.
16 Venugopal,R. and Jaiswal,A.K. (1996) *Proc. Natl Acad. Sci. USA*, **93**, 14960–14965.
17 Itoh,K., Chiba,T., Takahashi,S., Ishii,T., Igarashi,K., Katoh,Y., Oyake,T., Hayashi,N., Satoh,K., Hatayama,I., Yamamoto,M. and Nabeshima,Y. (1997) *Biochem. Biophys. Res. Commun.*, **236**, 313–322.
18 Carthew,R.W., Chodosh,L.A. and Sharp,P.A. (1987) *Genes Dev.*, **1**, 973–980.
19 Beckmann,H., Su,L.K. and Kadesch,T. (1990) *Genes Dev.*, **4**, 167–179.
20 Bendall,A.J. and Molloy,P.L. (1994) *Nucleic Acids. Res.*, **22**, 2801–2810.
21 Sirito,M., Lin,Q., Maity,T. and Sawadogo,M. (1994) *Nucleic Acids. Res.*, **22**, 427–433.
22 Gregor,P.D., Sawadogo,M. and Roeder,R.G. (1990) *Genes Dev.*, **4**, 1730–1740.
23 Viollet,B., Lefrancois Martinez,A.M., Henrion,A., Kahn,A., Raymondjean,M. and Martinez,A. (1996) *J. Biol. Chem.*, **271**, 1405–1415.
24 Sirito,M., Walker,S., Lin,Q., Kozlowski,M.T., Klein,W.H. and Sawadogo,M. (1992) *Gene Expr.*, **2**, 231–240.
25 Blanar,M.A. and Rutter,W.J. (1992) *Science*, **256**, 1014–1018.
26 Meier,J.L., Luo,X., Sawadogo,M. and Straus,S.E. (1994) *Mol. Cell. Biol.*, **14**, 6896–6906.
27 Pognonec,P., Boulukos,K.E., Aperlo,C., Fujimoto,M., Ariga,H., Nomoto,A. and Kato,H. (1997) *Oncogene*, **14**, 2091–2098.
28 Kurschner,C. and Morgan,J.I. (1997) *Biochem. Biophys. Res. Commun.*, **231**, 333–339.
29 Reisman,D. and Rotter,V. (1993) *Nucleic Acids Res.*, **21**, 345–350.
30 Gao,E., Wang,Y., Alcorn,J.L. and Mendelson,C.R. (1997) *J. Biol. Chem.*, **272**, 23398–23406.
31 Kirschbaum,B.J., Pognonec,P. and Roeder,R.G. (1992) *Mol. Cell. Biol.*, **12**, 5094–5101.
32 Carter,R.S., Ordentlich,P. and Kadesch,T. (1997) *Mol. Cell. Biol.*, **17**, 18–23.
33 Lun,Y., Sawadogo,M. and Perry,M. (1997) *Cell Growth Differ.*, **8**, 275–282.
34 Smale,S.T. and Baltimore,D. (1989) *Cell*, **57**, 103–113.
35 Means,A.L., Slansky,J.E., McMahon,S.L., Knuth,M.W. and Farnham,P.J. (1992) *Mol. Cell. Biol.*, **12**, 1054–1063.
36 Johnson,J.A. and Nathanson,N.M. (1994) *J. Biol. Chem.*, **269**, 18856–18863.
37 Laemmli,U.K. (1970) *Nature*, **227**, 680–685.
38 Mueller,P.R. and Wold,B. (1989) *Science*, **246**, 780–786.
39 Garrity,P.A. and Wold,B.J. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 1021–1025.
40 Dalton,T.D., Bittel,D. and Andrews,G.K. (1997) *Mol. Cell. Biol.*, **17**, 2781–2789.
41 Bittel,D., Dalton,T., Samson,S., Gedamu,L. and Andrews,G.K. (1998) *J. Biol. Chem.*, **273**, 7127–7133.
42 Briggs,M.R., Kadonaga,J.T., Bell,S.P. and Tjian,R. (1986) *Science*, **234**, 47–52.
43 Kadesch,T. (1993) *Cell Growth Differ.*, **4**, 49–55.
44 Liang,L., Fu,K., Lee,D.K., Sobieski,R.J., Dalton,T.P. and Andrews,G.K. (1996) *Mol. Reprod. Dev.*, **43**, 25–37.
45 Andrews,G.K., McMaster,M.T., De,S.K., Paria,B.C. and Dey,S.K. (1993) In Suzuki,K.T., Imura,N. and Kimura,M. (eds), *Metallothionein III: Biological Roles and Medical Implications*. Birkhauser Verlag, Basel, Switzerland, pp. 351–362.
46 De,S.K., Dey,S.K. and Andrews,G.K. (1990) *Toxicology*, **64**, 89–104.
47 Andrews,G.K., Adamson,E.D. and Gedamu,L. (1984) *Dev. Biol.*, **103**, 294–303.
48 De,S.K., McMaster,M.T., Dey,S.K. and Andrews,G.K. (1989) *Development*, **107**, 611–621.
49 Weintraub,H. (1993) *Cell*, **75**, 1241–1244.
50 Weintraub,H., Davis,R., Tapscott,S., Thayer,M., Krause,M., Benezra,R., Blackwell,T.K., Turner,D., Rupp,R., Hollenberg,S. *et al* (1991) *Science*, **251**, 761–766.
51 Kageyama,R., Sasai,Y., Akazawa,C., Ishibashi,M., Takebayashi,K., Shimizu,C., Tomita,K. and Nakanishi,S. (1995) *Crit. Rev. Neurobiol.*, **9**, 177–188.
52 Vaessin,H., Caudy,M., Bier,E., Jan,L.Y. and Jan,Y.N. (1990) *Cold Spring Harb. Symp. Quant. Biol.*, **55**, 239–245.
53 Walker,M.D., Park,C.W., Rosen,A. and Aronheim,A. (1990) *Nucleic Acids Res.*, **18**, 1159–1166.
54 Baer,R. (1993) *Semin. Cancer Biol.*, **4**, 341–347.
55 Read,M.L., Clark,A.R. and Docherty,K. (1993) *Biochem. J.*, **295**, 233–237.
56 Sharma,S., Leonard,J., Lee,S., Chapman,H.D., Leiter,E.H. and Montminy,M.R. (1996) *J. Biol. Chem.*, **271**, 2294–2299.
57 Morris,J.K. and Richards,J.S. (1996) *J. Biol. Chem.*, **271**, 16633–16643.
58 Ojamaa,K., Samarel,A.M. and Klein,I. (1995) *J. Biol. Chem.*, **270**, 31276–31281.
59 Bresnick,E.H. and Felsenfeld,G. (1993) *J. Biol. Chem.*, **268**, 18824–18834.
60 Mueller,P.R., Salser,S.J. and Wold,B. (1988) *Genes Dev.*, **2**, 412–427.
61 Chaudhuri,G., Cuevas,J., Buga,G.M. and Ignarro,L.J. (1993) *Am. J. Physiol., Heart Circ. Physiol.*, **265**, H2036–H2043.
62 Li,Y. and Jaiswal,A.K. (1992) *J. Biol. Chem.*, **267**, 15097–15104.
63 Xie,T., Belinsky,M., Xu,Y. and Jaiswal,A.K. (1995) *J. Biol. Chem.*, **270**, 6894–6900.
64 Rushmore,T.H. and Pickett,C.B. (1990) *J. Biol. Chem.*, **265**, 14648–14653.
65 Angel,P. and Karin,M. (1991) *Biochim. Biophys. Acta*, **1072**, 129–157.
66 Daniel,V. (1993) *Crit. Rev. Biochem. Mol. Biol.*, **28**, 173–207.
67 Maeshima,H., Sato,M., Ishikawa,K., Katagata,Y. and Yoshida,T. (1996) *Nucleic Acids Res.*, **24**, 2959–2965.
68 Stohs,S.J. and Bagchi,D. (1995) *Free Radic. Biol. Med.*, **18**, 321–336.
69 Tang,N. and Enger,M.D. (1993) *Toxicology*, **81**, 155–164.
70 Chao,S.H., Bu,C.H. and Cheung,W.Y. (1995) *Arch. Toxicol.*, **69**, 197–203.
71 Vig,P.J.S. and Nath,R. (1991) *Biochem. Int.*, **23**, 927–934.
72 Smith,J.B., Dwyer,S.D. and Smith,L. (1989) *J. Biol. Chem.*, **264**, 7115–7118.
73 Amoruso,M.A., Witz,G. and Goldstein,B.D. (1982) *Toxicol. Lett.*, **10**, 133–138.
74 Riccio,A., Pedone,P.V., Lund,L.R., Olesen,T., Olsen,H.S. and Andreasen,P.A. (1992) *Mol. Cell. Biol.*, **12**, 1846–1855.
75 Rao,E., Dang,W., Tian,G. and Sen,R. (1997) *J. Biol. Chem.*, **272**, 6722–6732.
76 Sawadogo,M. and Roeder,R.G. (1985) *Cell*, **43**, 165–175.