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Activation of the Metallothionein-I Gene Promoter in Response to Cadmium and USF *in Vitro*

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To elucidate the molecular mechanism of metallothionein (MT) gene activation in response to various inducers, we constructed a G-less mouse MT-I promoter and transcribed in HeLa nuclear extract. The MT-I gene was transcribed efficiently in this extract and initiation of transcription occurred at the correct site (+1). Transcription of the MT-I gene was stimulated three- to fivefold in the nuclear extract from the cadmium-treated cells relative to the extract from the untreated cells. The MT-I promoter was also activated three- to fourfold by recombinant USF1, a helix-loop-helix-leucine zipper DNA binding transcription factor that recognizes the major late transcription factor (MLTF) binding site on the MT-I promoter. To our knowledge, this is the first report of the activation of MT-I promoter *in vitro* by a toxic metal and by the transcription factor USF.

Metallothioneins (MT) are cysteine-rich low molecular weight proteins that are expressed in most vertebrates and invertebrate species (1-3). The two major isoforms of this protein present in the mammals, MT-I and MT-II, are coordinately regulated in mouse (4). The MT-I and MT-II genes are inducible by a variety of factors that include group IIB heavy metals, glucocorticoid, phorbol esters, free radicals such as reactive oxygen species, exposure to UV and other DNA-damaging agents such as some DNA-intercalating anticancer drugs and stress (1-3,5,6). The biological functions of MTs have not been completely elucidated. They have been implicated in the detoxification of heavy metals, maintenance of metal homeostasis (2,3) and protection against oxidative damage of the cells (7). We have demonstrated the interaction of a 33 kDa protein with a 26 bp cis element of the mouse MT-I gene, designated MRE-c′, and its role in the basal expression of MT-I gene *in vivo* (8). A metal regulated transcription factor (MTF1) has been cloned and the expression of the cDNA ectopically enhances MT-I gene transcription even under normal levels of the metal (9,10).

The development of the G-less cassette by Sawadogo and Roeder (11) has revolutionized the *in vitro* transcription system, as it facilitates the detection of properly initiated transcripts and distinguishes them from randomly or spuriously initiated RNAs. The reproducibility and accuracy of transcription achieved with such construct prompted us to take advantage of the system to study regulation of MT-I expression. Numerous studies have demonstrated the induction of MT gene *in vivo* after cadmium treatment (see reviews 1-3). To date, there has been no study that demonstrates the induction of MT-I gene in a cell free system after cadmium treatment. In this paper, we report for the first time the induction of MT-I gene transcription by cadmium in cell free extracts and also demonstrate that recombinant USF1 (upstream stimulatory factor 1) can activate MT-I promoter *in vitro.*

MATERIALS AND METHODS

Cell culture. HeLa cells were grown in Spinner flasks in DMEM supplemented with 5% FBS and antibiotics at 37°C in humidified atmosphere. For cadmium treatment, cells were grown under similar conditions and treated with 15 μM $CdCl_2$ (final concentration) for 4 hr. Cells were grown to a density of 5×10^5 cells/ml and harvested.

Preparation of HeLa nuclear extract. HeLa cells were harvested by low speed centrifugation, nuclear extracts were prepared according to the procedure of Shapiro et al (12) and were stored in small aliquots at −70°C.

Construction of a MT-I promoter G-less template. The presence of guanosine residues at position +1 and +9 handicaps cloning of the MT-I promoter directly into the G-free cassette plasmid (pC_2AT) constructed by Sawadogo and Roeder (11). The strategy involved cloning the amplified MT-I promoter containing 324 nucleotides upstream of the +1 site and substituted G-residues by polymerase chain reaction (PCR) upstream of a 377 bp synthetic G-free cassette. A

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Abbreviations used: MT, metallothionein; MLTF, major late transcription factor; USF, upstream stimulatory factor.

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mutant oligonucleotide termed E5 (5′-CTCAGCACGTGGAAGTGG-TGGTGAGGGTTA-3′) complementary to the upper strand was synthesized (Ransom Hill Bioscience, CA). This oligonucleotide conserved most of the sequence from the +1 to +17 nucleotide with the exception that the G residues at +1 and +9 were substituted by C residues and an A residue at position +15 was substituted by a C residue to facilitate the generation of a restriction enzyme site Pml I. The reaction was performed using 100 ng of pMT24/CAT DNA (8), 1 μM each of M13 reverse primer as the 5′ end primer and the mutant oligonucleotide E5 as the 3′ end primer, 200 μM of each dNTPs and 5 units of Hot Tub DNA polymerase (Amersham). After 25 cycles of amplification using a Perkin Elmer Cetus thermal cycler, the amplified DNA was purified using Wizard PCR preps DNA purification system (Promega) and digested with restriction enzymes Pst I and Pml I. The resultant fragment was cloned in the Pst I - Eco RV site of pRVC$_2$AT (13) (kindly provided by Dr. Aubrey Thompson, University of Texas Medical Branch, Galveston, TX). The resultant recombinant contains a 394 bp G-free fragment (+1 to +17 bp of the MT-I gene and 377 bp of the synthetic G-free cassette) downstream of the MT-I promoter and is designated pMTC$_2$AT. The plasmid DNA was purified by routine CsCl gradient centrifugation and used as circular templates in transcription assays.

In vitro transcription assay. In general, the transcription reactions (25 μl) contained 1 μg of pMTC$_2$AT template DNA, 2 μg of sonicated herring sperm DNA (non-specific DNA), 24, 36, 48 or 72 μg of extract, 0.4 mM dATP, CTP and 0.1 mM 3′-O-methyl-GTP (Pharmacia Biotech), 10 μCi of [α-^{32}P] UTP, 6 mM $MgCl_2$, 5 mM creatine phosphate, 10% glycerol, 50-60 mM KCl and 15 units of RNase T1. The DNA template and extract were incubated at room temperature for 10 min. Transcription was then initiated by addition of the nucleotides and incubated at 30°C for 45 min. Reactions were terminated by adding stop buffer (12). RNA was purified by phenol/chloroform extraction and ethanol precipitation, separated on 7M Urea-5% polyacrylamide gel and visualized by autoradiography.

In vitro translation of USF1 and analysis of the translation products. The TNT T7-coupled Reticulocyte lysate system (Promega), a coupled transcription-translation system, was used to translate the USF1 protein. Briefly, 1 μg of pSVUSF1 (14) (a cDNA encoding USF1, kindly provided by Dr. Michele Sawadogo, M. D. Anderson Cancer Center, Houston) was transcribed and translated according to the manufacturer's protocol in 50 μl reaction volume. As a control the Luciferase gene was also transcribed and translated. Reactions were carried out in presence of ^{35}S-Methionine (Amersham) for obtaining the labeled proteins or in presence of cold amino acid mixtures. Five microlitre aliquots of the ^{35}S-Methionine labeled reactions were mixed with 20 μl of SDS sample buffer and heated at 100°C for 3 min. The labeled proteins were separated by SDS-10% polyacrylamide gel electrophoresis and visualized by fluorography using Amersham's Amplify reagent, dried under vacuum and exposed to X-Omat AR film (Kodak) at −70°C.

Electrophoretic mobility shift analysis (EMSA). Electrophoretic mobility shift analysis using labeled mouse MT-I promoter-specific MLTF oligonucleotide was performed according to the procedure described by Datta and Jacob (8) with *in vitro* translated USF1, luciferase and HeLa nuclear extracts. In competition experiments, the nuclear extract was incubated with 100 × excess of cold oligonucleotides SP1 and MLTF for 10 min prior to addition of the radiolabeled probe. The DNA protein complexes were separated by non-denaturing gel electrophoresis and visualized by autoradiography.

Transfection and CAT assay. HeLa cells were grown in Dulbecco's minimal essential medium and 10 % FBS (Atlanta Biologicals) and were split 1:15 the day before transfection. Cells were transfected by Calcium phosphate procedure (15) and harvested 48 h post transfection. In some samples, cells were treated with 5 μM $CdCl_2$ 16 h prior to harvest. Cells were collected by scraping, resuspended in 100-150 μl of 250 mM Tris-HCl (pH 8.0) and cell lysate was prepared by three cycles of repeated freeze-thaw. Following normalization for protein concentrations, the cell lysates were assessed for CAT activity by the method of Gorman et al. (16) and for β Gal activity by the method of Rosenthal (17).

RESULTS AND DISCUSSION

Optimal conditions required for in vitro transcription of the mouse metallothionein I gene. Prior to evaluation of the MT-I promoter activity in nuclear extracts prepared from cadmium-treated HeLa cells, the optimal conditions for *in vitro* transcription of the G-less MT-I promoter construct were established. First, transcription of a fixed amount of the template was analyzed by varying the amounts (24 to 72 μg) of the nuclear extracts (Fig. 1A). The optimal amount of DNA required for efficient transcription was also determined using increasing concentrations of pMT(C$_2$AT) construct and 72 μg of HeLa nuclear protein (Fig. 1B). Maximal transcription was obtained at 800 ng to 1 μg of template DNA (Fig. 1B, lanes 1 to 5). The optimal $MgCl_2$ concentration required to obtain efficient transcription was also determined (Fig. 1C). The amount of MT transcripts increased when the $MgCl_2$ concentration was raised from 0 to 6 mM whereas it decreased at 8 mM $MgCl_2$ (Fig. 1C, lanes 1-5). The optimal activities of both basal and induced transcription occurred at 6 mM $MgCl_2$. Since specific *in vitro* initiation can normally occur in the absence of the substrate GTP (18), the substitution of G residues in the pMT(C$_2$AT) construct did not affect transcription. Under all the conditions, transcription of the G-less cassette yielded the anticipated 394 nucleotide transcript (see Fig. 1A-C). However, transcripts smaller than the anticipated 394 nucleotide-long product were also observed. These products probably represent spuriously initiated or non-specific transcripts that are not completely digested by RNase T1. Similar short length transcripts were also observed in studies utilizing G-less constructs for the immunoglobulin heavy chain gene (IgH) core promoter (19) and the rat growth hormone (rGH) gene promoter (20).

Transcriptional induction by cadmium. Earlier studies have demonstrated that the MT-I gene promoter can be induced by a variety of heavy metals which include cadmium (see reviews, 1-3). To determine whether the G-less MT-I promoter can be activated *in vitro* in nuclear extracts prepared from Cd-treated HeLa cells, the MT-I construct was transcribed in untreated cell extract and in nuclear extract prepared from Cd-treated cells (Fig. 2A). Both the extracts transcribed the gene efficiently. However, a 3-5 fold increase in the amount of the transcript was observed when the extracts from the Cd-treated cells were used for transcription (Fig. 2A, compare lane 2 with lane 1). To determine the relative efficiency of the *in vitro* sys-

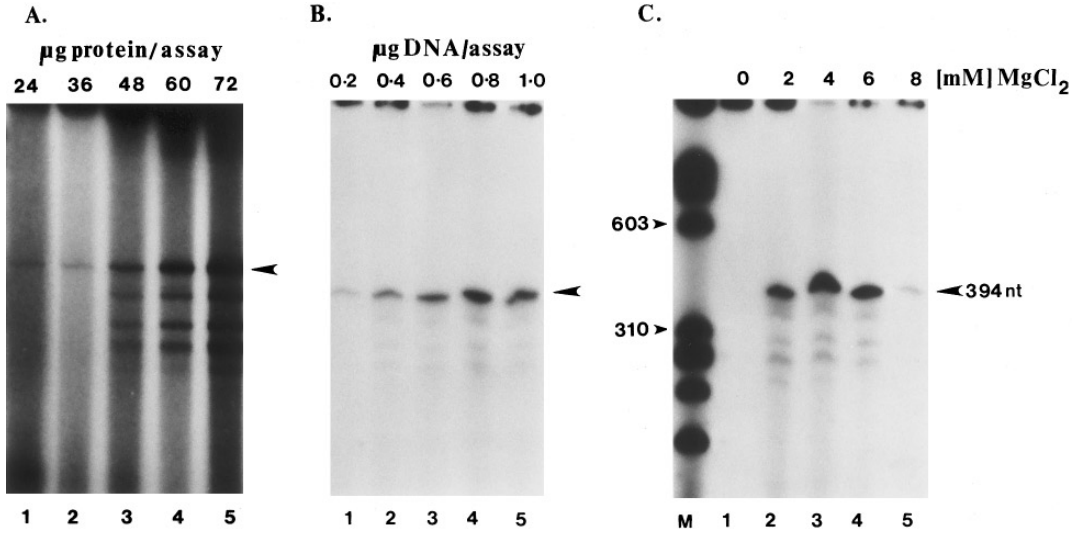

FIG. 1. Optimization of ideal transcription conditions. *In vitro* transcription assays were performed as described under Materials and Methods. (A) Transcription in presence of 1 μg of pMT(C_2AT), 2 μg of sonicated herring sperm DNA and increasing amounts of HeLa nuclear extract (24-72 μg; lanes 1 to 5 respectively). (B) Transcription in presence of 72 μg HeLa nuclear extract and increasing amounts of pMT(C_2AT) template DNA (200 ng - 1 μg; lanes 1 to 5). The total DNA in the reaction mixture was adjusted to 3 μg using sonicated herring sperm DNA. (C) Transcription assay performed with varying amounts (0-8 mM) of $MgCl_2$, 72 μg of HeLa nuclear extract and 1 μg of pMT(C_2AT) DNA. Lane 1, 0 mM; lane 2, 2 mM; lane 3, 4 mM; lane 4, 6 mM and lane 5, 8 mM $MgCl_2$. M, ϕX174 Hae III digested marker DNA. Arrowheads indicate the specific 394 nt long transcript.

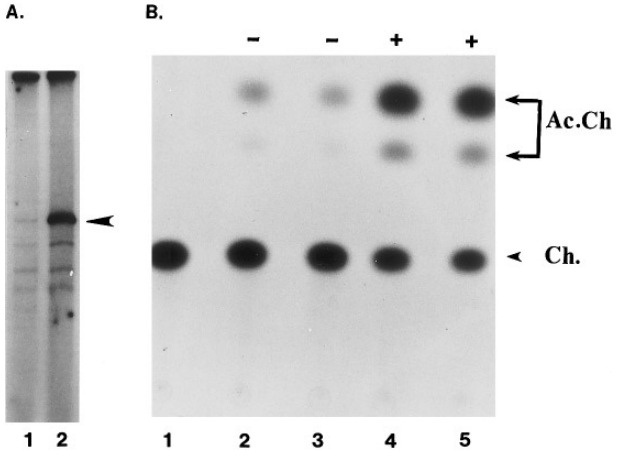

FIG. 2. Activation of MT-I gene in HeLa cells by cadmium. (A) Transcription of pMT(C_2AT) in HeLa nuclear extracts. Lane 1, *in vitro* transcription was performed using 1 μg of pMT(C_2AT) template in presence of 36 μg of HeLa nuclear extract (untreated); lane 2, similar reaction condition in the presence of 36 μg of nuclear extract prepared from cells treated with 15 μM $CdCl_2$. Arrowhead represents the specific 394 nucleotide-long transcript. (B) Representative autoradiogram showing the CAT activity of MT promoter-CAT fusion constructs. HeLa cells were transiently co-transfected by the calcium phosphate precipitation method [24] with 10 μg of pMT24 CAT construct and 3 μg of pSVβgal. Lane 1, mock transfection. Cells were cultured for 16 h in absence (−) (lanes 2 and 3) or presence (+) (lanes 4 and 5) of 5 μM $CdCl_2$ and harvested, extracts were prepared and assayed as described under Materials and Methods. Ch., Chloramphenicol; Ac.Ch, Acetylated Chloramphenicol.

tem with respect to activation of MT-I gene expression, HeLa cells were transfected with MT24/CAT (8) containing the MT-1 gene promoter linked to the bacterial chloramphenicol acetyl transferase (CAT) gene. The result of such an analysis (Fig. 2B) showed that the MT-I promoter can indeed be activated in cells treated with $CdCl_2$. A 4-6 fold increase in the CAT activity was observed in cells treated with $CdCl_2$, which is consistent with that observed *in vitro.*

Transcriptional activation of the MT-I promoter by recombinant USF1. Before we studied the activation of MT-I promoter by USF *in vitro,* we examined whether recombinant USF1 interacts with the MLTF (Major late transcription factor) binding site on the MT-I promoter. USF1 was synthesized from its cDNA by coupled transcription-translation reaction. As anticipated, a 43 kDa product was obtained by this reaction whereas the luciferase cDNA yielded a 61 kDa polypeptide that was used as a control (Fig. 3A). Electrophoretic mobility shift assay with USF1 and MLTF oligonucleotide yielded a major complex (Fig. 3B, lane 2) whereas the *in vitro* translated luciferase cDNA did not produce a complex with the MLTF oligonucleotide (Fig. 3B, lane 1). Electrophoretic mobility shift assay with labeled MLTF oligonucleotide and HeLa nuclear extract yielded two typical complexes (Fig. 3C), as observed with the Adenovirus MLP USF binding site (21). The two complexes C1 and C2 represent specific interaction, as it was efficiently competed by cold MLTF

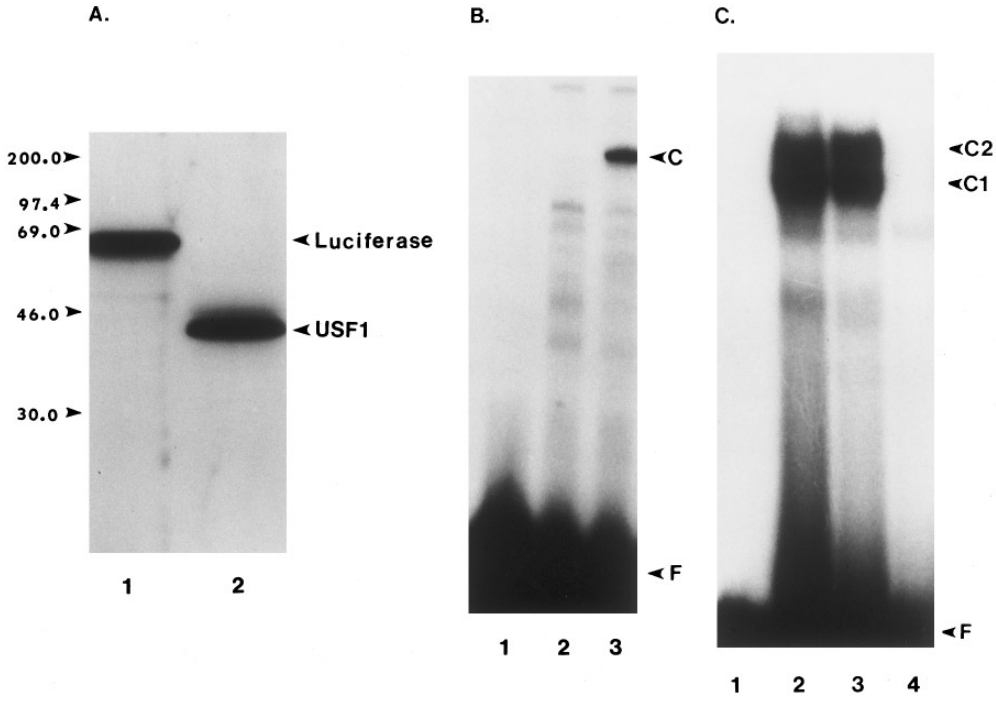

FIG. 3. (A) A Coupled transcription-translation of USF1 and Luciferase cDNAs in TNT T7 reticulocyte lysate system. USF1 and Luciferase were produced by coupled transcription-translation of their cDNAs in presence of ^{35}S-Methionine (see Materials and Methods for details). Five microlitre aliquot of each reaction was subjected to electrophoresis on 10% SDS-10% polyacrylamide gel and processed for autoradiography, as described in Materials and Methods. Lane 1, Translated product of Luciferase cDNA; lane 2, translated product of USF1 cDNA. Prestained protein standards (Amersham) are indicated in kDa. (B and C) Electrophoretic mobility shift assay (EMSA) using recombinant USF1, HeLa nuclear extract and labeled MLTF oligonucleotide. (B) EMSA was performed as described under Materials and Methods using the *in vitro* translated USF1 and ^{32}P-labeled MLTF oligonucleotide. Lane 1, free probe; lane 2, MLTF probe incubated with 5 μl of control reticulocyte lysate; and lane 3, MLTF oligonucleotide incubated with 5 μl of the lysate containing the USF1. (C) EMSA was performed using HeLa nuclear extract (12 μg) and ^{32}P-labeled MLTF oligonucleotide. Lane 1, free probe; lane 2, MLTF oligonucleotide incubated with nuclear extract; lanes 3 and 4, MLTF oligonucleotide incubated with nuclear extract in presence of 100 $\times$ excess of cold SP1 oligonucleotide and cold MLTF oligonucleotide, respectively. C, C1, and C2 represent the DNA-protein complexes, and F indicates the free MLTF probe.

oligonucleotide (Fig. 3C, lane 4) and not by the non-specific competitor SP1 (Fig. 3C, lane 3). The single complex detected in the EMSA with recombinant USF1 is identical to that obtained with endogenous USF. Since the monomer of USF does not bind DNA (22), the single complex detected with recombinant USF1 must be present in the reaction as a homodimer.

We then investigated whether the recombinant USF1 can activate the MT-I promoter in the *in vitro* system. For this purpose, transcription assay was performed with 1 μg of the G-less MT-I promoter construct and 36 μg of HeLa nuclear extract. Since fractionation is known to result in the loss of an essential component(s) required for USF function (18), unfractionated HeLa nuclear extract was used in this experiment. The strategy of endogenous USF depletion using oligonucleotide affinity method was also not attempted, as it is known to affect the basal level of transcription (23). Since the purpose of our experiment was to determine activation of the G-less MT-I promoter by the addition of exogenous transcription factor, USF1 synthesized *in vitro* was added directly to the transcription reaction in increasing amounts. The amount of the 394 nucleotides-long transcript increased with increasing amounts of the translation product (Fig. 4, lanes 2-7). A 3-4 fold increase in the amount of transcript was consistently observed in response to the exogenously added USF1 relative to recombinant luciferase added as the control protein (Fig. 4, lane 1). It has been demonstrated earlier that the MT-I gene transcription *in vitro* can be dramatically reduced following the deletion of the MLTF-binding site on the MT-I promoter to which the transcription factor USF binds (24). These experiments showed for the first time that recombinant USF1 recognizes the MLTF-binding site on the MT-I promoter and activates transcription of the MT-I promoter *in vitro*.

Our laboratory has in the past developed tissue-specific cell extracts that transcribe ribosomal RNA gene by RNA polymerase I and MT-I gene by RNA polymer-

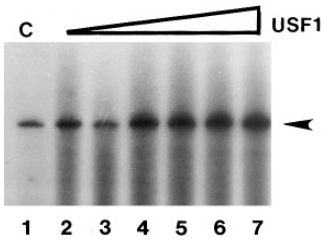

FIG. 4. Activation of the G-less MT-I promoter construct by in vitro-translated USF1. The template was transcribed in presence of 36 μg of HeLa nuclear extract. Lane 1, Basal level of transcription in presence of 3 μl of reticulocyte lysate containing luciferase produced by coupled transcription-translation of its cDNA (C, Control); lanes 2–7: transcription in presence of increasing amounts (0.5 μl to 3 μl) of reticulocyte lysate containing USF1. The specific transcript is shown by an arrowhead. Transcription levels were quantitated by densitometry of the autoradiogram.

ase II (25-27). Although this tissue-derived extract was capable of transcribing pol II-driven genes (26,27) the efficiency and reproducibility of this system were not satisfactory. With the development of a G-less cassette of the MT-I promoter in this study, we now have a highly reproducible and efficient system to study MT gene expression *in vitro.*

In conclusion, we have demonstrated that HeLa nuclear extract can transcribe a G-less cassette containing MT-I promoter efficiently, nuclear extracts from cadmium treated cells activate the promoter to the same extent as observed *in vivo* and the promoter can be activated by recombinant USF1. To our knowledge, this is the first report where an efficient and highly reproducible cell-free system has been used to study the activation of MT-I promoter by the environmental toxicant, cadmium and to demonstrate that USF1 can activate MT-I promoter *in vitro.* It is anticipated that this system can be used to study MT-I expression under a variety of environmental stress conditions, particularly to elucidate the molecular mechanism(s) of the promoter activation.

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