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CELLULAR ADAPTATIONS TO GENE AMPLIFICATION

by

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Sociology of 0340
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   General 0679
   Ancient 0289
   Linguistics 0290
   Modern 0291
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   General 0401
   Classical 0294
   Comparative 0295
   Medieval 0297
   Modern 0298
   African 0316
   American 0591
   Asian 0305
   Canadian (English) 0352
   Canadian (French) 0355
   English 0593
   Germanic 0311
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   Middle Eastern 0315
   Romance 0313
   Slavic and East European 0314

PHILOSOPHY, RELIGION AND THEOLOGY
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   General 0318
   Biblical Studies 0321
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   Philosophy of 0322
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Anthropology
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   Physical 0327
Business Administration
   General 0310
   Accounting 0272
   Banking 0770
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Canadian Studies 0385
Economics
   General 0501
   Agricultural 0503
   Commerce-Business 0505
   Finance 0508
   History 0509
   Labor 0510
   Theory 0511
Folklore 0358
Geography 0366
Gerontology 0351
History
   General 0578

Ancient 0579
Medieval 0581
Modern 0582
Black 0328
African 0331
Asia, Australia and Oceania 0332
Canadian 0334
European 0335
Latin American 0336
Middle Eastern 0333
United States 0337
History of Science 0585
Law 0398
Political Science
   General 0615
   International Law and
      Relations 0616
   Public Administration 0617
Recreation 0814
Social Work 0452
Sociology
   General 0626
   Criminology and Penology ... 0627
   Demography 0938
   Ethnic and Racial Studies 0631
   Individual and Family
      Studies 0628
   Industrial and Labor
      Relations 0629
   Public and Social Welfare 0630
   Social Structure and
      Development 0700
   Theory and Methods 0344
Transportation 0709
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Women's Studies 0453

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   Plant Pathology 0480
   Plant Physiology 0817
   Range Management 0777
   Wood Technology 0746
Biology
   General 0306
   Anatomy 0287
   Biostatistics 0308
   Botany 0309
   Cell 0379
   Ecology 0329
   Entomology 0353
   Genetics 0369
   Limnology 0793
   Microbiology 0410
   Molecular 0307
   Neuroscience 0317
   Oceanography 0416
   Physiology 0433
   Radiation 0821
   Veterinary Science 0778
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Biophysics
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Biogeochemistry 0425
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Geodesy 0370
Geology 0372
Geophysics 0373
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Paleoecology 0426
Paleontology 0418
Paleozoology 0985
Palynology 0427
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Physical Oceanography 0415

HEALTH AND ENVIRONMENTAL SCIENCES
Environmental Sciences 0768
Health Sciences
   General 0566
   Audiology 0300
   Chemotherapy 0992
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   Education 0350
   Hospital Management 0769
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   Immunology 0982
   Medicine and Surgery 0564
   Mental Health 0347
   Nursing 0569
   Nutrition 0570
   Obstetrics and Gynecology . 0380
   Occupational Health and
      Therapy 0354
   Ophthalmology 0381
   Pathology 0571
   Pharmacology 0419
   Pharmacy 0572
   Physical Therapy 0382
   Public Health 0573
   Radiology 0574
   Recreation 0575

Speech Pathology 0460
Toxicology 0383
Home Economics 0386

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Pure Sciences
Chemistry
   General 0485
   Agricultural 0749
   Analytical 0486
   Biochemistry 0487
   Inorganic 0488
   Nuclear 0738
   Organic 0490
   Pharmaceutical 0491
   Physical 0494
   Polymer 0495
   Radiation 0754
Mathematics 0405
Physics
   General 0605
   Acoustics 0986
   Astronomy and
      Astrophysics 0606
   Atmospheric Science 0608
   Atomic 0748
   Electronics and Electricity 0607
   Elementary Particles and
      High Energy 0798
   Fluid and Plasma 0759
   Molecular 0609
   Nuclear 0610
   Optics 0752
   Radiation 0756
   Solid State 0611
Statistics 0463

Applied Sciences
Applied Mechanics 0346
Computer Science 0984

Engineering
   General 0537
   Aerospace 0538
   Agricultural 0539
   Automotive 0540
   Biomedical 0541
   Chemical 0542
   Civil 0543
   Electronics and Electrical 0544
   Heat and Thermodynamics ... 0348
   Hydraulic 0545
   Industrial 0546
   Marine 0547
   Materials Science 0794
   Mechanical 0548
   Metallurgy 0743
   Mining 0551
   Nuclear 0552
   Packaging 0549
   Petroleum 0765
   Sanitary and Municipal 0554
   System Science 0790
Geotechnology 0428
Operations Research 0796
Plastics Technology 0795
Textile Technology 0994
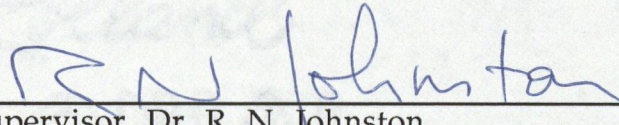
PSYCHOLOGY
General 0621
Behavioral 0384
Clinical 0622
Developmental 0620
Experimental 0623
Industrial 0624
Personality 0625
Physiological 0989
Psychobiology 0349
Psychometrics 0632
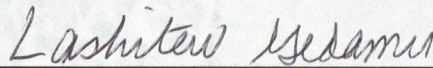Social 0451

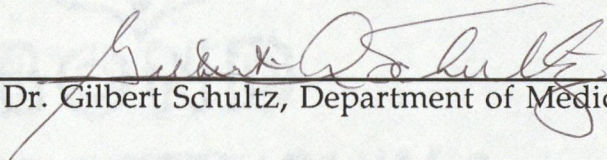THE UNIVERSITY OF CALGARY

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Cellular Adaptations to Gene Amplification" submitted by Lauri Lintott in partial fulfillment of the requirements for the degree of Master of Science.
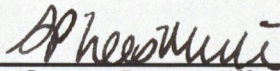
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ii

ABSTRACT

The mechanisms by which the activities and abundance of transcription factors are regulated are not well understood. Binding of a metal regulatory factor (MRF) to the metal regulatory element (MRE) of the metallothionein (MT) promoter is thought to confer metal inducibility to the MT gene. The MRF has not been clearly identified, and we do not know the precise mechanism by which it regulates MT transcription or how its abundance and activity are regulated. I have used the novel technique of directed amplification to increase the number of chromosomally located MT promoters in CHO cells 125 to 1500 fold. This concomitant increase in MREs apparently had no effect on the sensitivity of CHO cells to Cd or on the inducibility of endogenous MT gene expression. However, gel shift assays indicate that cells with increased numbers of MREs may have higher levels of MRF(s).

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Dedicated to

my husband Eric Zimmerman, without whose stalwart love and support I may never have completed this project, and to our daughter Nicole who has brought incredible joy and meaning into my life.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

BLE   basal level enhancer

bp   base pair

BSA   bovine serum albumin

CAT   chloramphenicol acetyltransferase

CHO   Chinese hamster ovary

CIP   calf intestinal alkaline phosphatase

CsTFA   Cesium Trifluoroacetate

dhfr   dihydrofolate reductase

DNA   deoxyribonucleic acid

DTT   dithiothreitol

EC_{50} the effective concentration of Cd required to reduce PE by 50%.

GCBF   GC binding factor

GRE   glucocorticoid regulatory element

GTH   glycine, thymidine and hypoxanthine

HEPES   N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]

hMT-I   human metallothionein-I

hMT-II$_A$ human metallothionein-II$_A$

HPLC   High-pressure liquid chromatography

kbp   kilo base pair

kD   kilo Dalton

LB   Luria and Burrows

MCBF   MRE binding factor

MLTF   Major Late Transcription Factor

mMT-I   mouse metallothionein-I

MOPS	3-(N-Morpholino) Propane-Sulfonic Acid
MPA	metallothionein amplified promoter
MRE	metal regulatory element
MRF	metal regulatory factor
mRNA	messenger RNA
MT	metallothionein
MTX	methotrexate
OLB	oligonucleotide labeling buffer
PE	plating efficiency
PIC	preinitiation complex
pol II	RNA polymerase II
RNA	ribonucleic acid

I. INTRODUCTION

1. Regulation of eukaryotic gene expression

A central goal in molecular biology is the elucidation of how gene expression, a multistep process, is regulated. For Class II genes (genes that encode a protein product) the main steps in this process are transcription of a precursor RNA from a DNA template, processing the precursor into a mature messenger RNA (mRNA), transport of the mRNA into the cytoplasm and translation of the mRNA to a polypeptide. All of these processes involve several discrete steps, each a potential target for regulatory action.

One of the earliest events in gene expression that is tightly regulated is initiation of transcription. Instead of transcribing all genes at all times, a cell produces transcripts whose products are required at a particular point in its life cycle. At the same time the cell is able to respond to extracellular signals by changing the repertoire of transcripts produced. Regulation of transcript initiation is known to require several components, two of which are promoters, cis-acting DNA sequences topologically contiguous with the gene they regulate, and transcription factors, trans-acting proteins that bind to these sequences and effect transcription.

Class II genes are transcribed by the enzyme RNA polymerase II (pol II). The first step in transcription is the formation of a stable preinitiation complex (PIC) that is made up of pol II and several accessory proteins (Roeder, 1991; Zawel and Reinberg, 1992). Assembly of the PIC at the transcription start site requires the presence of either of two cis-elements, the TATA box or the initiator (Inr) element. The TATA box, located about 30 bp upstream of the transcription start site, binds the general transcription factor TFIID, the first step in an ordered process in which pol II and the other accessory proteins

assemble to form the PIC. The Inr element encompasses the transcription start site and binds an unidentified protein. Assembly of the PIC at the Inr element requires the same accessory factors as those involved at the TATA box, but how a stable PIC is formed is not yet understood (Zawel and Reinberg, 1992). Following stable PIC formation, hydrolysis of ATP by the PIC results in initiation of transcription (Roeder, 1991).

The TATA box or the Inr promoter element along with pol II and the PIC accessory proteins are necessary and sufficient for specific initiation of transcription of all Class II genes and so are referred to as core promoter elements and general transcription factors respectively. A core promoter alone directs very low levels of transcription *in vitro*. Additional cis-acting elements that can modulate transcription levels are usually found in Class II promoters. For example, the GC box found upstream of the TATA box in many promoters binds the ubiquitous transcription factor Sp1 which induces higher levels of transcription (Kadonga *et al.*, 1986). Other elements such as the metal regulatory elements of metallothionein or the heat shock elements of Hsp70 can alter transcription in response to specific intracellular conditions (Maniatis *et al.*, 1987; Mitchell and Tjian, 1989; Dynan and Tjian, 1985).

Most of the gene-specific elements and factors appear to regulate transcript initiation by either promoting or repressing formation of the PIC. They do this by altering either the rate of formation of a stable PIC (Roeder, 1991; Zawel and Reinberg, 1992), or the availability of the TATA box or Inr element (Roeder, 1991).

Promoter elements are often viewed as passive tethering sites for transcription factors and the transcription factors as the active effectors of transcription. However, this model is oversimplified as the availability of a

promoter element can be altered at the DNA level. For example, methylation of CpG islands can interfere with transcription factor binding (Cedar, 1988). Chromatin topology, heterochromatinization, or nucleosome assembly on DNA can also make promoter elements inaccessible to transcription factors (Wolffe, 1992).

Even if a promoter element is available, the transcription factor that interacts with it may not always be present. Several transcription factors are known to be developmentally regulated. For example, the MyoD family of transcription factors which regulate a cascade of muscle specific genes, are found only in myoblasts and skeletal muscle tissue of vertebrates (Olson 1990, Olson 1992). Other factors may be found in many different cell types but only under certain conditions. For example, Fos protein, which is part of the transcription factor AP1, is transiently induced by a wide variety of agents (Angel and Karin, 1991). In general, neither the role of transcription factor abundance in transcriptional regulation, nor the mechanisms by which this abundance is regulated, are understood.

The activity of a transcription factor that is present may be regulated by post-translational modifications or by protein-protein interactions. Post-translational events that modulate factor activity include phosphorylation–dephosphorylation and glycosylation (Maniatis *et al.*, 1987; Mitchell and Tjian, 1989; Ptashne, 1989). Protein-protein interactions such as dimerization can activate or repress transcription factor activity, or even change the factor's specificity or its function (Shaw, 1990; Karin, 1990; Lamb and McKnight, 1991).

In summary, the regulation of transcription initiation of Class II genes is an intricate process. The promoters of Class II genes can contain combinations of several cis-elements each of which can interact with one or more

transcription factors. Factors that recognize these elements may or may not be present in a given cell at any particular time. Even if a transcription factor is present its function can be altered by post-translational modifications or interactions with other factors. In addition, the availability of a given promoter will vary from one cell type to another. These multiple levels of control make understanding transcriptional regulation in eukaryotes particularly difficult.

Over the past few years intense research has resulted in the identification of numerous cis-acting elements. However, much less is known about the proteins that interact with these sites, mainly because isolation of transcription factors is often difficult as they are usually present in relatively low abundance. The modes of action of the factors that have been isolated are not yet well understood and for most, two central question remain unanswered: 1) by what mechanical and biochemical mechanisms do these factors, once bound to their target promoters, activate transcription, and 2) how are levels and activities of transcription factors themselves regulated?

2. The study of transcriptional regulation.

In order to better understand transcriptional regulation of gene expression we need to understand how transcription factors that interact with cis-acting elements are able to effect transcription. An obvious way to study these questions is to isolate and clone transcription factors and then to study their regulation and how they interact with other components of the transcriptional machinery. However, isolation of transcription factors has proven to be difficult and has been successfully accomplished for only a few so far. This has resulted in a need for indirect methods of studying

transcription factors. Such methods include gel shift, DNA footprinting and competition assays.

DNA footprinting assays allow visualization of the direct contacts made between a protein and its specific recognition site. While footprinting analyses are commonly performed with purified factor, they can also be performed with crude nuclear extracts or even *in vivo*. In gel shift assays, protein, either purified or from crude extracts, is incubated in a binding reaction mix with a DNA probe and complexes that form with the probe are visualized by electrophoresis of the mixture through a non denaturing gel. DNA footprinting and gel shift assays can be used to determine the specificity of a protein-DNA interaction, and what cellular conditions might effect such an interaction.

The competition assay usually involves transient transfection of cells with a plasmid bearing a promoter region linked to a reporter gene, along with varying amounts of competitor DNA that consists of the same region on a separate plasmid. If factors that act on these sequences are limited in abundance, then increasing amounts of competitor DNA should result in a decrease or increase in activity from the reporter gene, depending on whether it is a positive or negative regulatory factor respectively (Scholer *et al.*, 1986). There are two main difficulties with this method. First, it is not clear that transiently introduced genes will necessarily be regulated in the same manner as the endogenous genes. Secondly, this approach does not allow for competition with the endogenous chromosomal genes.

A new method using directed gene coamplification provides a way to achieve stable competition between chromosomally integrated genes or gene fragments and endogenous genes. This method takes advantage of the ability

of cells to survive methotrexate (MTX) selection by amplifying the gene for dihydrofolate reductase (*dhfr*). Dhfr is an essential enzyme involved in the conversion of dihydrofolate to tetrahydrofolate, an important step in the synthesis of purines and thymidylate, precursors in the synthesis of DNA. MTX, a folic acid analog, inhibits the action of the dhfr enzyme by binding irreversibly to its active site. Cells can survive MTX exposure, usually by overproducing dhfr, and this is achieved by amplification of its gene (Johnston and Kucey, 1988; Schimke, 1984b; Schimke, 1984a; Schimke, 1988). During gene amplification, DNA sequences within several kilobases of *dhfr* will be passively coamplified (Johnston and Kucey 1988).

Briefly, the gene coamplification technique involves cloning of the gene or gene fragments of interest into a plasmid bearing the *dhfr* gene. The linearized plasmid is transfected into tissue culture cells where it integrates randomly into the chromosomal DNA. Transformed cells are selected with increasing concentrations of MTX, resulting in amplification of the *dhfr* gene and accompanying sequences.

This technique was used to amplify the promoter of the heat shock gene *hsp70* to 10,000 copies (Johnston and Kucey, 1988). Upon exposure to heat shock, these cells were unable to synthesize hsp70 and had much lower survival rates than controls. This suggested that amplified *hsp70* regulatory regions were able to compete with endogenous genes for the trans-acting heat shock regulatory factor.

Gene coamplification could be particularly useful for studying the regulation of transcription factors that have not yet been isolated since it allows one to test what the effect of manipulation of transcription factor levels *in vivo* may be. At high levels of amplification, the additional cis-

elements would bind the regulatory factors and prevent them from interacting with the endogenous promoter, resulting in a reduction or an increase in the endogenous gene expression. Amplification and subsequent counter-selection could also be used to increase the levels of endogenous transcription factor. Increasing factor levels in this manner could make them easier to isolate.

I am interested in using the gene amplification technique to study the transcriptional regulation of the MT gene in response to metal. MT is a good candidate for this method because, although the cis-acting regulatory regions for metal regulation are well defined, the trans-acting factors that bind these regions are not. Indirect evidence (reviewed below) indicates that a factor that interacts with a cis-acting control region positively affects MT transcription in response to metal. The two questions I want to address are, 1) what effect will amplification of the MT promoter have on the expression of endogenous MT genes and 2) can cell lines with amplified MT promoter be secondarily selected for an increased level of regulatory factor?

3. Metallothionein

a) Background

MT is a relatively low molecular weight protein (6000 – 7000) that has a high heavy metal content. It has an unusual amino acid content that includes a high percentage of cysteine residues (up to 30%), all of which are present in reduced form and are involved in the coordination of heavy metal ions through mercaptide bonds (Fowler *et al.*, 1987). MTs are found throughout the animal kingdom, in some plants, yeast and prokaryotes. In mammals they are most abundant in the liver, kidney, pancreas and

intestines but are also found in many other tissues (Hamer, 1986). Usually MT is localized to the cytoplasm, however, it has also been observed to accumulate in lysosomes (Johnson *et al.*, 1981) and occasionally, during development, in the nucleus (Wlostowski, 1992).

Based on sequence homology, MTs have been assigned to three classes (Fowler *et al.*, 1987). Class I includes mammalian MT and those from other phyla with related primary sequence. Class II MTs have no, or very distant homology to mammalian MT (or to each other). Class III MTs, more commonly referred to as γ–glutamylcysteinyl peptides or phytochelatins, are atypical polypeptides that are not directly encoded by structural genes (Mehra and Winge, 1991; Rauser, 1990).

Among Class I MTs there is extensive sequence homology and a high correspondence of Cys residues. There are two major Class I MT isoforms referred to as MT-I and MT-II that differ by a single negative charge at neutral pH (Hamer, 1986). In many organisms there are several sub forms of MT-I separable by HPLC and these are specified by subscripts as in MT-I$_A$ and MT-I$_B$. So far all organisms studied have no more than a single MT-II gene.

Until recently it was thought that Class I and II MTs were exclusively found in animals and Class III in plants, with yeast having either type. However, MTs of Class II have subsequently been found in the plants *Mimulus guttatus*, and *Maize* (Miranda *et al.*, 1990; de Framond, 1991) and the yeast *C. glabrata* is reported to have both Class I and III MTs (Miranda *et al.*, 1990). Perhaps it is only a matter of time before Class III MTs are found in animals.

The fact that MT binds to and its production is stimulated by heavy metals such as Zn, Cu, Cd and Ag, has long suggested that it is involved in

detoxification and/or homeostasis of heavy metals. A homeostatic function is supported by the ability of MT to exchange Zn with other proteins (Li *et al.*, 1980; Zeng *et al.*, 1991) and to reactivate Zn or Cu requiring apoenzymes *in vitro* (Udom and Brady, 1980; Brouwer and Brouwer-Hoexum, 1989). In addition MT levels increase dramatically during processes requiring high levels of Zn such as development and injury healing (Andrews *et al.*, 1991; De *et al.*, 1991; Webb, 1987; Wlostowski, 1992). A role for MT in protection from heavy metal toxicity is supported by the correlation between resistance to metals such as Cd in cell culture and in whole animals, and high levels of MT and amplification of the MT gene (Cismowski *et al.*, 1989; Roch *et al.*, 1982; Huang *et al.*, 1987; Grady *et al.*, 1987).

b) Transcriptional regulation

MT expression is regulated primarily at the transcriptional level, although evidence for some post-transcriptional regulation has been found (Sadhu and Gedamu, 1989; McCormick *et al.*, 1991). Transcriptional regulation of MT was first demonstrated for heavy metal and glucocorticoid stimulation in HeLa cells (Karin *et al.*, 1980; Karin and Herschman, 1981) and in human skin fibroblasts (Richards *et al.*, 1984). Since these initial studies, regulation of MT gene expression has been extensively studied in mammalian cell culture and in yeast.

All mammals have a single MT-II and at least one MT-I gene. While humans have several functional MT-I genes, rodents appear to have only one. In most cells MT is found to be expressed at low levels constitutively and to increase dramatically in response to a wide range of agents (reviewed in Kägi and Schäffer, 1988). The ability of different agents to induce high levels of MT expression varies from cell type to cell type. The inducibility of

different MTs, especially of the large family of human MT-Is, also varies depending on cell type and the inducing agent (Sadhu and Gedamu, 1988; Varshney *et al.*, 1986). The functional significance of differential expression in this large family of human MT-Is is not yet understood.

The transcriptional regulation of MT has been studied mainly for the mouse MT-I (mMT-I) and the human MT-II$_A$ (hMT-II$_A$) genes. A cartoon of the regulatory region of these two gene is presented in Figure 1. The functional significance of each of the cis-elements and the factors that interact with them will be discussed below.

Basal level expression

MT is expressed at low basal levels in cells that have not been exposed to heavy metals (Karin *et al.*, 1980). Basal level expression of both mMT-I and hMT-II$_A$ is directed by a TATA box and one or more GC boxes (Lee *et al.*, 1987; Karin *et al.*, 1987). In addition to these elements, hMT-II$_A$ has two basal level enhancer elements (BLE) that are responsible for its high level of basal expression compared to other hMT genes (Richards *et al.*, 1984; Scholer *et al.*, 1986; Haslinger and Karin, 1985). Full basal level expression of mMT-I requires the binding site for major late transcription factor (MLTF) (Carthew *et al.*, 1987).
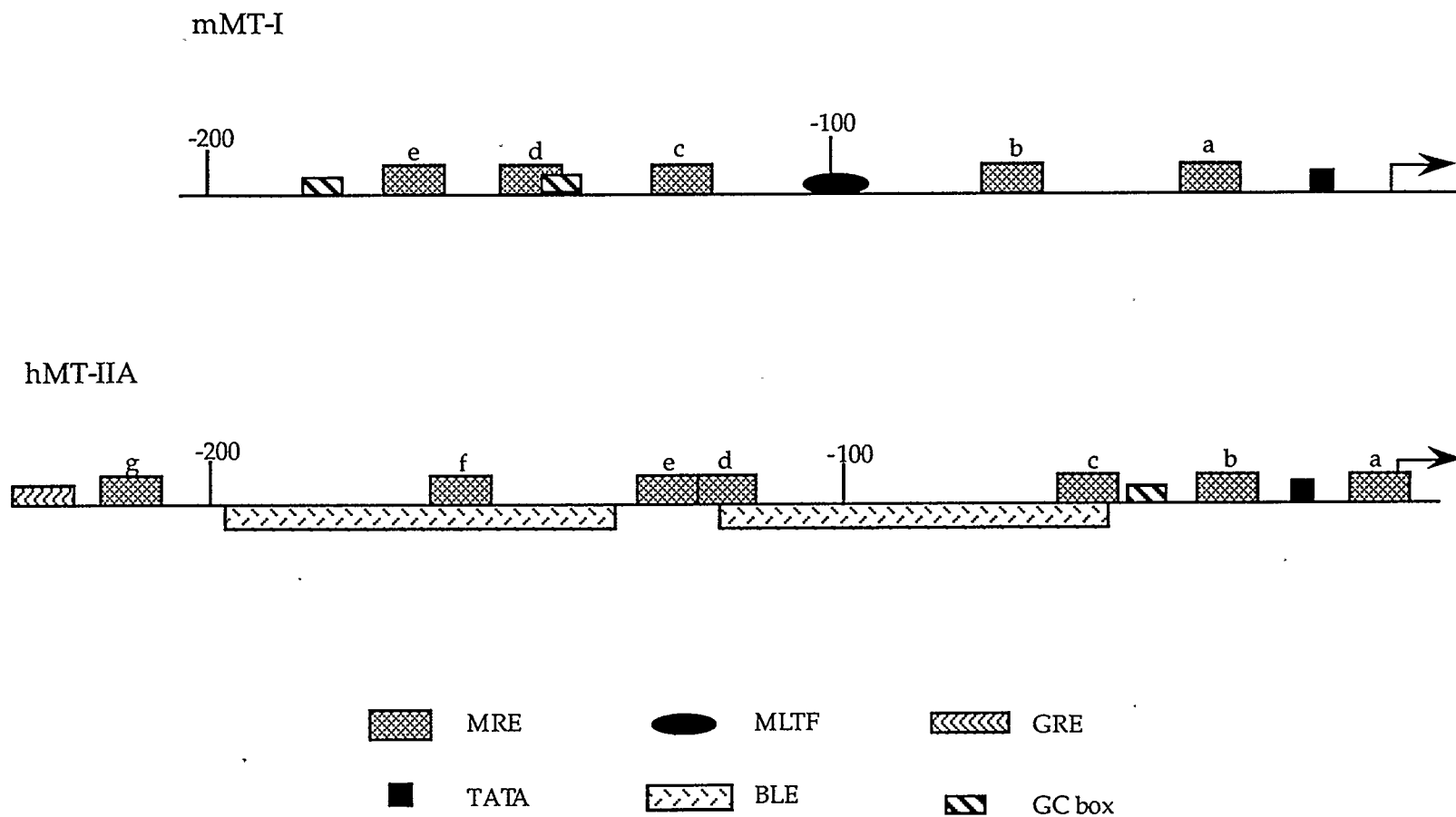
mMT-I

hMT-IIA

MRE   MLTF   GRE

TATA   BLE   GC box

Figure 1. Cartoon of the promoter regions of the mMT-I and hMT-II$_A$ genes.

Promoter elements are as indicated in the legend.

These upstream promoter sequences are bound by trans-acting factors that positively effect transcription of the MT genes. The TATA box is bound by the general transcription factor TFIID that is required for assembly of the RNA polymerase complex (see above). The GC box is bound by the ubiquitous transcription factor Sp1 (Lee *et al.*, 1987). The BLE of hMT-IIA is bound by several factors including, AP1, AP2 and AP4 as well as others that have not yet been identified (Lee *et al.*, 1987; Imagawa *et al.*, 1987; Mitchell *et al.*, 1987).

Metal Induction

The unifying biological characteristic of MTs is the rapid increase in their mRNA and protein levels when cells or organisms are exposed to heavy metals (Hamer, 1986). Evidence indicates that this induction is primarily at the transcriptional level. In nuclear run on assays, the bulk of mMT-I mRNA accumulation in liver and kidney, within an hour of injecting a mouse with Cd, Zn, Cu or Hg, could be accounted for by the increased transcription of mMT-I (Durnam and Palmiter, 1981). Similar results were obtained for hMT-IIA when a human cell line was pulse labeled for 15 to 30 minutes, although the overall induction level was much lower than that observed for mMT-I (Karin *et al.*, 1984a). In addition, MT promoter regions that do not include any transcribed sequences are able to render heterologous genes metal responsive [hMT-IIA; (Karin *et al.*, 1984b; Serfling *et al.*, 1985): mMT-I (Stuart *et al.*, 1984; Serfling *et al.*, 1985; Carter *et al.*, 1984; Stuart *et al.*, 1985; Searle *et al.*, 1985)].

It has been well established that the region of the MT gene responsible for the transcriptional response to metals consists of several short *cis* acting sequences called metal responsive elements (MREs). Deletion analysis and

linker scanning mutagenesis were used initially to define the MRE as a 12 bp motif in the mMT-I promoter (Karin *et al.*, 1984a; Karin *et al.*, 1984b; Stuart *et al.*, 1985; Carter *et al.*, 1984). Serfling *et al.* (1985) found that a region of the hMT-II$_A$ or mMT-I promoter including several copies of this 12 bp motif could confer metal responsiveness to a SV40 promoter. Insertion of at least 2 copies of the 12 bp motif upstream of a heterologous gene, not normally metal inducible, renders it metal responsive (Stuart *et al.*, 1984; Stuart *et al.*, 1985; Searle *et al.*, 1985; Searle *et al.*, 1987).

All mammalian MT promoters contain MREs with homology to a consensus sequence of 15 nucleotides, CTNTGCRCNCGGCCC (where N= any nucleotide, R= purine) first defined by aligning MREs from mMT-I with MREs from several other MT genes (Searle *et al.*, 1985; Imbert *et al.*, 1990). Within this sequence is a more tightly conserved core consensus of seven nucleotides (TGCRCNC) (Searle *et al.*, 1985). The importance of this core sequence in heavy metal induction is supported by a study using point mutations of the mMT-I MREd (within a promoter MREs are specified by lower case letter with the one closest to the initiation site being MREa). Point mutations within the heptanucleotide core reduce metal inducibility more severely than those within the less conserved flanking regions (Culotta and Hamer, 1989).

For some time it was thought that Sp1 played a role in metal induction because MREs in mammalian promoters often overlap with GC rich regions that have homology to the Sp1 binding site. However, Culotta and Hamer (Culotta and Hamer, 1989) found that this was not the case since point mutations in the GC region outside of the core MRE had little or no effect on metal inducibility. As well, Westin and Schaffner (Westin and Schaffner,

1988) showed that a promoter with a mutated GC box that can no longer bind Sp1 is still capable of responding to heavy metals.

Experimental evidence suggests that binding of a factor, the metal responsive factor (MRF), to the MRE induces transcription of MT genes in response to heavy metal. The first evidence for a positively acting MRF came from competition experiments in which Séguin *et al.* (1984) found that excess mMT-I promoter sequences resulted in the loss of metal inducibility of a mMT-I promoter linked to a CAT reporter gene. Gel shift and *in vitro* footprinting assays demonstrated that a protein did indeed bind specifically to the MRE (Séguin and Hamer, 1987; Séguin and Prévost, 1988; Séguin, 1991; Westin and Schaffner, 1988; Foster and Gedamu, 1991; Koizumi *et al.*, 1992a; Koizumi *et al.*, 1992b; Radtke *et al.*, 1993).

There is a strong correlation between the binding of MRF to MRE and metal induction of MT. In general this is shown by demonstrating that MRE sequences that bind a factor in gel shift or footprint assays are also capable of conferring metal responsiveness to a reporter gene, but mutant MREs, that cannot bind MRF, no longer confer metal induciblity (Westin and Schaffner, 1988; Foster *et al.*, 1988; Imbert *et al.*, 1989).

Since the binding of a factor to MRE correlates with metal induction of MT, it might be expected that this interaction would be regulated by metal induction. *De novo* footprints do form on MREs in cells treated with Cd (Andersen *et al.*, 1987; Mueller *et al.*, 1988) and Zn (Mueller *et al.*, 1988). However, footprints formed on MREs *in vitro* are apparently unaffected by heavy metals (Séguin and Hamer, 1987; Séguin and Prévost, 1988) and there is variability in the effect of metals on the binding of proteins to MREs in gel shift assays.

Several groups have been able to demonstrate a Zn (but not Cd or Cu) induced complex in gel shift assays, either when Zn is added to the binding reaction (Westin and Schaffner, 1988; Koizumi *et al.*, 1992b) or when cells are exposed to Zn before nuclear protein is extracted (Radtke *et al.*, 1993; Czupryn *et al.*, 1992). Other groups have not been able to demonstrate an effect of metals on gel shift complexes (Koizumi *et al.* 1991; Foster and Gedamu, 1991). One group has even reported a gel shift complex that is inhibited by low concentrations of Zn or Cd, although this could be a general response rather than a specific one (Koizumi *et al.* 1992a).

Only two groups have reported detecting a gel shift complex that was induced by metals other than Zn. Czupryn *et al.* (Czupryn *et al.*, 1992) detected a complex that appeared only after cells were induced with Cd, Cu, or Zn, but the specificity of this complex for MRE sequence is questionable as it was competed by both non specific (poly dIdC) as well as specific competitor, and competitions with mutant MRE sequences were not done. Anderson *et al.* (1990) also reported a Cd dependent complex in extracts from rat cells. This complex appeared only when the DNA probe had two functional MREs and was competed for by an oligo containing wild type MREs, but not by one with mutant MREs.

These conflicting results may be attributable to the use of different MREs, different nuclear extracts or different gel shift conditions, by different groups. For example, only Anderson *et al.* (Andersen *et al.*, 1990) used a DNA probe with more than one MRE and this may be why only they saw a Cd inducible shift.

Identification and isolation of the MRF has proven to be a difficult task due in part to the fact that most MREs are GC rich and can weakly bind Sp1.

Despite this, several proteins (listed in Table 1) have been identified that specifically bind to sequences containing an MRE. Imbert *et al.* (1989) isolated a mouse nuclear factor of 74 kD (MBF-1) that binds specifically to trout MT-B MREa. Koizumi *et al.* (1991) partly purified a 112 kD protein, MREBP, that binds to hMT-II$_A$ MREa in band shift assays. Labbé *et al.* (1993) isolated a protein of 108 kD (MEP-1) that can bind to several different MREs. Radtke *et al.* (1993) have cloned a 72.5 kD factor called MTF-1 that binds to MRE only if extracted from cells treated with Zn. Although some of these proteins exhibit metal specific binding to MRE, their involvement in metal regulation of MT is questionable since with one exception there is no evidence that they can enhance transcription in a metal-dependent manner.

The one exception to this is MBF-1 (Imbert *et al.*, 1989) that was tested for its ability to stimulate transcription of a CAT reporter driven by a trout MT promoter, in an *in vitro* transcription assay. Addition of purified MBF-1 and Zn stimulated CAT transcription. Zn alone also induced transcription but to a lower level than Zn + MBF-1, perhaps by activating endogenous MBF-1. MBF-1 alone had no effect on transcription. However, it is not clear whether or not this factor is truly an MRE binding factor since it would only footprint on one mouse MRE, MREe, even though it was isolated from mouse cells.

Table 1. Factors that bind specifically to MREs.

Name	Size kD	Purified	Effect of heavy metals on binding.
MTF-I	72.5	yes	Only binds in nuclear extract from cells that are Zn induced.[a]
MREBP	112	partial	Zn or Cd added to extract reduces binding.[b]
MBF-I	74	yes	No effect on footprint.[c]
MEP-I	108	yes	Zn can reconstitute ExoIII footprint following 1,10-phenanthroline treatment.[d]
MRE-BF-1	86	no	Metal treatment of cells before extraction inhibits binding.[e]
MRE-BF2	2 x 28	no	Binds only in extracts from cells induced with Cd, Cu, or Zn.[e]
ZRF	116	yes[f]	Addition of Zn to extract induces binding.[g]
p39	39	no	Addition of Cd to extract or induction of cells with Cd induces a new shift.[h]

[a] (Radtke et al., 1993)
[b] (Koizumi et al., 1991; Koizumi et al., 1992a)
[c] (Imbert et al., 1989)
[d] (Labbé et al., 1993)
[e] (Czupryn et al., 1992)
[f] (Otsuka et al., 1992)
[g] (Koizumi et al., 1992b)
[h] (Andersen et al., 1990)

In the yeast, *Saccharomyces cerevisiae,* a transcription factor called ACE1 (or CUP2) has been isolated and evidence suggests that it is responsible for metal regulation of the yeast MT gene CUP1 (Fürst *et al.,* 1988; Welch *et al.,* 1989). CUP1 in yeast lacking the ACE1 gene is not Cu inducible and introduction of the ACE1 gene on an expression plasmid results in restoration of Cu inducible expression (Thiele, 1988; Welch *et al.,* 1989). In gel shift assays, *in vitro* synthesized ACE1 protein and extracts from yeast overproducing ACE1 form the same discrete complex with an oligonucleotide corresponding to the Cu regulator element (UASc) (Fürst *et al.,* 1988). Formation of the protein-UASc complex in both cases was stimulated by addition of Cu. A yeast strain overproducing ACE1 has a higher level of the CUP1 transcript than the wild type strain both in non-inducing and inducing conditions, suggesting a role for ACE1 in both basal and Cu induced transcription (Fürst *et al.* 1988).

The ACE1 protein is a 24 kD protein that is localized to the nucleus. The amino-terminal domain has several cysteine residues arranged in Cys-X-Cys sequences similar to CUP1 and it most likely binds metals in a manner similar to MT. This domain also has a net positive charge that could give it DNA-binding activity. It appears that binding of Cu activates ACE1 DNA binding by altering its tertiary structure (Fürst *et al.,* 1988; Fürst and Hamer, 1989).

Other inducers

Besides heavy metals, expression of MT in mammals is known to be induced by a vast array of agents including mitogens, cytotoxic agents, carcinogens and some steroid hormones. Glucocorticoids, serum factors, and TPA all induce hMT-II$_A$ expression at the transcriptional level (Angel *et al.,*

1986; Imbra and Karin, 1987). mMT-I is also induced by glucocorticoids (Hager and Palmiter, 1981). Glucocorticoids induce expression through the glucocorticoid receptor complex that binds to the glucocorticoid responsive elements (GRE)s found in the promoters of all MT-II genes (Plisov *et al.,* 1991). Serum factor and TPA induction of MT-II genes is mediated though AP1 (Angel *et al.,* 1987; Lee *et al.,* 1987) and AP2 (Mitchell *et al.,* 1987; Imagawa *et al.,* 1987) binding sites in the BLE.

Cytotoxic agents such as UV light and mitomycin C also stimulate transcription of MT but the mechanism of their action is unknown (Angel *et al.,* 1986). Similarly α-interferon stimulates MT transcription through an unknown pathway (Friedman and Strak, 1985). Bacterial endotoxin induces transcription of mMT-I through a pathway independent of metals and glucocorticoid hormones and this activation appears to involve a unique region of the MT promoter (Durnam and Palmiter, 1984).

4. Experimental strategy

The questions I wanted to address is this thesis were, whether multiple chromosomally located copies of the MT promoter could alter the responsiveness of endogenous MT genes to metal and if so could this be exploited to create a cell line that overproduces the MRF. To engineer cell lines with multiple copies of the MT promoter I used a modified amplification technique.

Although gene co-amplification has been successfully employed in our laboratory to amplify whole genes or promoter regions, difficulties have been encountered. The main problem was that during co-amplification deletions and rearrangements take place that can result in loss of, or mutation of the co-amplified sequence. This resulted in the unequal amplification of *dhfr* and human MT-I$_G$ genes in a previous experiment (Dutler, 1989).

In order to circumvent the problem of loss or mutation of co-amplified sequences I used a modified technique that I call directed amplification. In this technique I used the MT promoter to drive the expression of the *dhfr* gene, thereby forcing the cells to amplify the promoter intact.

The MT promoter was a good candidate for a study of directed amplification because in cells not exposed to metals its transcriptional activity should not involve MRF. Therefore, amplification of the MT promoter region should not have affected the MRF pathway or viability of the cells. Once the desired level of amplification was reached cells could be exposed to heavy metals to see what effect the high copy number of chromosomally located MT promoters (and MREs) would have on the cells. Since previous experiments suggested that the MRF was present in limited abundance (Séguin *et al.*, 1984) I expected that at high amplification levels MRF would be titrated out by the additional MREs. Such cells would presumably not be able to induce high levels of MT expression and were predicted to be hypersensitive to heavy metals.

Metal hypersensitive cells could be used for a second round of selection with heavy metal. CHO cells can become resistant to heavy metals by amplification of the MT gene (Crawford *et al.*, 1985; Gick and McCarty, 1982). However, hypersensitive cells produced by titrating out MRF would not be expected to be able to use this route. My prediction was that these cells would become more metal resistant by increasing the level of MRF instead.

Briefly, my experimental strategy involved transfection of a *dhfr* cDNA, under the regulation of a MT promoter, into the Chinese hamster ovary (CHO) cell line DG44. These CHO cells have no functional endogenous *dhfr* gene and thus require supplements of thymidylate, glycine and

hypoxanthine in order to survive. Transfected cells were selected for stable integration of MT:*dhfr* by removal of supplements, and then exposed to stepwise increases in MTX concentration. Once high levels of amplification were achieved cells were tested for metal sensitivity by exposing them to Cd (chosen because it is especially cytotoxic). Any hypersensitive cells would be secondarily selected for Cd resistance.

The hMT-I$_G$ promoter was selected for these experiments because it has a GC and a TATA box (which together can direct low basal levels of transcription) and several MREs, but has no other consensus cis-elements (Foster *et al.*, 1988). The hMT-I$_G$ promoter has also been shown to be metal regulated (Foster *et al.*, 1988). Under non-inducing conditions the transcription of *dhfr* in the MT:*dhfr* construct would be driven by the TATA and GC boxes. Once the construct was amplified cells could be stimulated with metal to determine what effect the additional MREs would have on endogenous MT expression.

II. MATERIALS AND METHODS

1. Composition of buffers used.

Table 2. Buffers used.

Name	Composition
DNA loading buffer[a]	0.25% bromophenol blue 40% (w/v) sucrose
RNA loading buffer[a]	50 % glycerol 1 mM EDTA 0.4 % bromophenol blue
LB medium[a]	10 % Tryptone 5 % yeast extract 10 % NaCl pH 7.0
TE[a]	10 mM Tris·Cl 1 mM EDTA pH as indicated for procedure
STE buffer[b]	0.1 M NaCl 1 mM EDTA 50 mM Tris-HCl pH 7.5
PEG ligation buffer	200 mM Tris·Cl 50 mM $MgCl_2$ 50 mM DTT 500 µg/ml BSA 15 % Polyethylene glycol (PEG 8000)
100 X Denhardts[a]	1 % Ficoll 1 % polyvinylpyrrolidone 1 % BSA
20X SSC[a]	3 M NaCl 0.3 M sodium citrate pH 7.0

Name	Composition
OLB (5 X oligonucleotide labeling buffer)[a]	250 mM Tris 25 mM $MgCl_2$ 5 mM β-mercaptoethanol 2 mM each dATP, dGTP, dTTP 1 M HEPES (pH 6.6) 1 mg/ml oligonucleotides
PBS (phosphate buffered saline)[a]	140 mM NaCl 2.7 mM KCl 10 mM Na_2HPO_4 2 mM KH_2PO_4 pH 7.4
TAE[a]	0.04 M Tris-acetate 0.001 M EDTA
10X MOPS[a]	0.2 M MOPS (pH 7.0) 0.05 M NaAc (pH 7.0) 1.0 mM EDTA (pH 8.0)
1 X TBE[c]	89 mM Tris base 89 mM boric acid 2.5 mM EDTA pH 8.3
1 X TB	89 mM Tris base 89 mM boric acid pH 8.3
10 X CIP buffer[b]	500 mM Tris-HCl (pH 9.0) 10 mM $MgCl_2$ 1 mM $ZnCl_2$ 10 mM spermidine

a (Sambrook *et al.*, 1989)

b (Berger and Kimmel, 1987)

c (Sealey and Southern, 1990)

2. Nucleic acid isolation

a) Bacterial Cultures

All liquid bacterial cultures were grown in standard LB broth at 37 °C with constant shaking. For selection of single colonies, bacteria were plated on LB agar and grown at 37 °C. Where required ampicillin was added to a final concentration of 10 µg/ml or tetracycline was added to a final concentration of 15 µg/ml. For long term storage, bacteria were grown in LB broth to stationary phase and frozen at – 80 °C in 15% glycerol.

b) Plasmid DNA

Large scale isolation and purification of plasmid DNA from bacterial cultures of 100 to 500 ml was carried out using a QIAGEN column and the procedure specified by the manufacturer (QIAGEN Inc.).

Small scale isolation of plasmid DNA from bacterial cultures of 5 to 10 ml was carried out using the product Gene Clean as outlined by the manufacturer (BIO101).

c) Chromosomal DNA

Two 10 cm plates of confluent tissue culture cells were used for each extraction. Medium was removed and the cells were rinsed in cold PBS. The cells were lysed with a 5 ml solution of 1% SDS in 200 mM Tris, 20 mM EDTA (pH 8.0) and transfered to a 12.5 ml polypropylene tube. DNA was sheared with a large bore pipette, treated with RNase A (final concentration 100 µg/ml) for 4 hr at 37 °C followed by Proteinase K (final concentration 100 µg/ml) at 37 °C overnight. The lysate was then extracted once with an equal volume of phenol:chloroform:isoamyl alcohol (24:24:1) and the DNA precipitated with Na acetate (final concentration 0.3 M) and 2.5 volumes of

95% ethanol for 5 min at room temp. Precipitated DNA was pelleted by centrifugation at 22 000 g for 30 min, air dried and dissolved in TE (pH 8.0).

d) Total RNA

Growth media was removed from a 150 cm^2 flask of confluent cells and the cells lysed with 2.5 ml RNA extraction buffer containing: 50% guanidine isothiocyanate (w/v), 0.2 M Na-citrate (pH 7), 0.02% sarcosyl (w/v) and 0.0072% 2-mercaptoethanol (v/v). The lysed cells were then layered onto 1 ml of CsTFA (density 1.51 g/ml) in a sterile 4 ml Beckman quick seal conical tube and centrifuged in a SW41 rotor for 8 to 15 hours at 30 000 rpm. The top of the conical tube was cut off with a razor blade and the CsTFA and RNA extraction buffer carefully pipetted off the RNA pellet. After allowing the tube to drain upside down for a few minutes the RNA was dissolved in 50 μl of sterile TE (pH 7.8), transferred to a sterile tube and heated for 10 min at 65 °C. RNA was stored at − 80 °C until needed.

3. Manipulation of DNA

a) Restriction endonuclease digestion

Digestion of DNA with restriction endonucleases was carried out according to the manufacturer's instructions.

b) Agarose Gel Electrophoresis

Electrophoresis of DNA

Agarose gels were used to separate, identify and purify DNA restriction fragments. Agarose gel electrophoresis of DNA was carried out as described in Sambrook *et al.* (1989).

Electrophoresis of RNA

RNA was electrophoresed in a denaturing gel of 1.5% agarose (w/v) 16% formaldehyde (v/v) in 1X MOPS buffer. A 6 µl sample of RNA was prepared by adding 2.5 µl 10 X MOPS, 4 µl formaldehyde and 12.5 µl deionized formamide, heating to 65 °C for 10 min, and cooling on ice. Just before loading the sample 2 µl of RNA loading buffer and 1 µl of 10 mg/ml ethidium bromide was added. Electrophoresis was carried out at 50 – 60 milliamps in 1X MOPS buffer for 2 to 4 hr.

c) Purification of DNA fragment from agarose gel

Plasmid DNA fragments of specific sizes were isolated by first digesting the plasmid with the appropriate restriction enzyme(s). The digested plasmid was electrophoresed through an agarose gel and the correctly sized fragment cut out with a razor blade. The DNA fragment was then isolated from the agarose using the product Gene Clean according the manufacturer's instructions.

d) Cloning

Dephosphorylation

To prevent self ligation of vector in non-directional cloning (vector digested with one enzyme) the vector was dephosphorylated (5' phosphates removed). Following enzyme digestion and purification of a vector, 5' phosphates were removed by incubating 2 µg of DNA in a total volume of 50 µl containing 1X CIP buffer and 0.25 units calf intestinal alkaline phosphatase (CIP), for 2 hr at 56 °C. An additional 0.25 units alkaline phosphatase was added and incubation at 56 °C continued for another 2 hr. The reaction was stopped by adding 50 µl STE buffer, 5 µl 10% SDS and incubating at 65 °C for

15 min. The DNA was extracted 2·X with an equal volume of phenol:chloroform:isoamyl alcohol (24:24:1) pH 8.0, precipitated with 2.5 volumes 95% ethanol and dissolved in 20 µl TE (pH 8.0).

The vector was checked for self ligation by ligating a small amount for 2 hr and then running it on an agarose gel next to a lane containing the unligated vector. If any self ligation was apparent dephosphorylation was repeated.

When cloning was directional (vector digested with two enzymes) the vector was not dephosphorylated.

Ligation

Ligations were carried out in a 20 µl total volume containing 0.1 to 0.2 µg of DNA (a molar ration of vector:insert of 1:4 to 1:5), 1X PEG ligation buffer and 1 unit T4 ligase. This mixture was incubated at room temperature overnight. 10 µl of the ligation mixture was used to transform competent *E. coli* as described below.

Transformation

Competent *E. coli* were prepared as follows. Bacteria were grown to log phase in 50 ml of LB broth (OD_{600} 0.6), and pelleted by centrifugation at 4000 rpm for 10 min. The pellet was dissolved in 5 ml ice cold 0.1 M $CaCl_2$ and left on ice 15 min. The cells were pelleted again by centrifugation and dissolved in 2 ml of ice cold 15 % glycerol in 0.1 M $CaCl_2$. 100 µl aliquots were frozen in liquid nitrogen and stored at − 80 °C.

For each transformation 100 ng of plasmid DNA was added to 100 µl of competent bacteria (pre-thawed on ice). After sitting on ice 30 min the bacteria were heated at 42 °C for 90 s and cooled on ice for 2 min before adding

800 µl of SOC medium. The entire volume was spread on a LB plate containing the appropriate antibiotic.

4. Cell Culture Maintenance

Tissue culture cells were routinely grown in a 37 °C incubator under 5% CO_2. All manipulations of cells were carried out in a sterile laminar flow hood. Unless otherwise indicated the medium used was α-MEM containing 10% dialyzed fetal calf serum and streptomycin, penicillin, and fungizone. Where required glycine, thymidine, and hypoxanthine (GTH) were added to a final concentration of 30 µM each. For routine maintenance, and when selecting cells with MTX, media were changed every 3 – 4 days and cells were subcultured when they reached confluence. Cells that were MTX resistant were maintained in media which contained the appropriate concentration of MTX. For subculturing, cells were treated with trypsin–EDTA to release them from the culture flask substratum and a portion of the cells transferred to a new flask containing fresh media. For storage, trypsinized cells were frozen above liquid nitrogen in 10% DMSO, and then submerged in liquid nitrogen.

5. Transfection

Transfection of DG44 cells was carried out using the CaPO$_4$–DNA precipitation method (Parker and Stark, 1979).

DG44 cells transfected with *dhfr* containing plasmids were selected for stable incorporation of this DNA by removal of the GTH supplements. Under this condition only cells with a functional *dhfr* gene will survive.

6. Amplification

Selection of cells with MTX was carried out in a stepwise fashion beginning with 2 nM MTX. Cells were subcultured at low density in media

containing the appropriate concentration of MTX. Cells were kept at this level of MTX until survivors were growing well, about 3 – 4 weeks. Cells were then subcultured at the next level of MTX. The levels of MTX used for selection were 2 nM, 8 nM, 50 nM, 250 nM, 1 μM, 5 μM, 20 μM.

7. Southern Transfer

Chromosomal or plasmid DNA was isolated, digested with the appropriate enzyme, applied to an agarose gel and electrophoresed to separate fragments. After photographing the gel, fragmentation, denaturation, neutralization and transfer of DNA to Hybond N+ nylon membrane was carried out according to the specifications of the membrane manufacturer, Amersham. After allowing for complete transfer, the DNA was fixed to the membrane by laying the membrane on a pad of filter paper soaked in 0.4 M NaOH for 30 min. The membrane was briefly washed in 5 X SSC, sealed in a hybridization bag and stored at – 20 °C until hybridization could be performed.

8. Northern Transfer

RNA was isolated and run on an agarose gel as described above. The RNA was then transfered to Hybond N+ nylon membrane in the same manner as DNA except the gel was not pre-treated. After allowing 24 – 48 hr for complete transfer the RNA was fixed to the membrane by laying the membrane on a pad of filter paper soaked in 0.05 M NaOH for 5 min. The membrane was briefly washed in 5 X SSC, sealed in a hybridization bag and stored at – 20 °C until hybridization could be carried out.

9. Probe labeling

a) Random priming

DNA probes used for detecting RNA and DNA on nylon membranes were labeled by the random priming method with minor modifications (Feinberg and Vogelstein, 1983). The DNA template was prepared by digesting the appropriate vector and isolating the fragment to be used as probe template from an agarose gel as described above. MT promoter probe template was obtained from pLL4 (*Sac* I – *Pst* I), *dhfr* probe template was obtained from pLL3 (*Pst* I – *Pst* I). The probe used for detecting endogenous MT RNA was the CHMT-1 cDNA (pLL2, *Bgl* II – *Sac* I). For plasmid maps see Results. Labeling reactions consisted of 0.2 – 1 µg of linear DNA, 1 X OLB, 0.8 µg/µl BSA (bovine serum albumin), 50 µCi of $\alpha^{32}P$ dCTP and 1 unit of Klenow. After incubating at room temperature overnight, the reaction mix was heated to 65 °C for 15 min with 2.5 µl tRNA (10 mg/ml), 30 µl of 7.5 M ammonium acetate and 2.5 µl of 0.25 M EDTA. 300 µl of 95 % ethanol was added and the DNA precipitated for 30 min at – 80 °C, pelleted in a micro centrifuge, dried and dissolved in 1 X TE (pH 8.0). Probes were stored at –80 °C and denatured just prior to adding to hybridization solutions, either by alkaline treatment for 2 min (0.2 N NaOH) or by boiling for 2 min and snap cooling on ice.

b) End Labeling

Synthetic oligonucleotides used in band shifts were end labeled on the 5' OH with T4 polynucleotide kinase as described in Sambrook *et al.* (1989). Unincorporated nucleotides were removed using G-50 sephadex Spun-Column Chromatography (Sambrook *et al.*, 1989).

10. Hybridization

Hybridization of Southern Bolts were carried out according to Amersham's specifications (included with Hybond N+ membrane) with minor modifications. Pre-hybridization was carried out for 1 hr at 65 °C in a solution of 5 X SSC, 5 X Denhardt's solution, 0.5 % SDS and 20 µg/ml salmon sperm DNA. Denatured probe was added directly to the pre-hybridization solution (final probe concentration 1.0×10^6 cpm/ml) and allowed to hybridize for 12 to 24 hr at 65 °C. To remove non-specifically bound probe the membrane was washed for 1 hr at 65 °C in 1 X SSC and 0.5 % SDS and an additional 1 hr at 65 °C in 0.1 X SSC and 0.5 % SDS. The membrane was then sealed in a hybridization bag and exposed to X-ray film at –80 °C.

Hybridization of Northern Blots were carried out at 55 °C using either the above procedure or more recently using Amersham's Rapid Hybridization Buffer according the manufacture's specification. Washes were the same as above except at 55 °C.

For second and subsequent probing of the same membrane, probe was striped by immersing the membrane in boiling 0.5% SDS and shaking overnight.

11. Densitometer scanning

Scanning of X-ray films to determine relative levels of signal was carried out with a LKB Ultra Scan XL laser densitometer, according to the manufacture's instructions. Care was taken to make sure the signal being scanned was in the linear range of the X-ray film. Integration was used to calculate relative signal intensities.

12. Cd sensitivity testing

Sensitivity of control and transfected cell lines to Cd toxicity was tested by determining the plating efficiency (PE) of each cell line in media containing Cd. Trypsinized cells were plated at 1000 cells per 10 cm plate. After allowing 2 days for cells to recover from trypsin treatment, the cells were fed fresh media containing $CdCl_2$ (final concentration 0, 0.5 μM, 1.0 μM, 2.0 μM, or 3.0 μM). Each concentration of Cd was tested in triplicate. Cells were fed fresh Cd containing media twice more, 6 days and 9 days following initial plating. Twelve days after the initial plating colonies were fixed to the plates using 95% ethanol and stained with Coomassie Brilliant Blue (Sambrook *et al.* 1989). The colonies on each plate were counted manually and the average number of colonies on three plates at each Cd concentration calculated. Typical plating efficiency with no Cd treatment was 20% to 40% and experiments where efficiency was under 10% were rejected. The PE of cells at each Cd concentration was calculated using the PE without addition of Cd as 100 %.

13. Nuclear Protein Isolation

For each extraction three 150 cm^2 flasks of cells were grown to confluence in media (no MTX added). Nuclear extracts were prepared from these cells as described by Abmayer and Workman (1992). Nuclear protein was quantified using the BioRad Protein Microassay (BSA as standard).

14. Gel Shift

For each gel shift about 20 000 cpm of end labeled probe and 5 – 10 μg of protein was used. Unless indicated otherwise, probe and nuclear protein extract were incubated together for 30 min at room temperature in a binding

buffer containing: 12 mM HEPES (pH 7.9), 5 mM NaCl, 50 mM KCl, 5 mM MgCl$_2$, 4 μg poly(dIdC)·poly(dIdC), 60 μg BSA and 12 % glycerol. Where indicated DTT, Zn (ZnCl$_2$) or Cd (CdCl$_2$) were added to the binding reactions. When competitor DNA was used, the protein and competitor were pre-incubated for 10 min in the binding buffer, then probe was added and the incubation continued for 20 min. Following incubation the mixture was loaded directly onto a vertical polyacrylamide gel that had been pre-run at 30 mAmps for 30 min (4 °C in 0.25 X TB). The gel consisted of: 7% or 5% polyacrylamide (30:1 acrylamide:bisacrylamide), 3 % glycerol, 0.25 X TB, 0.07% ammonium persulphate, and 0.06% TEMED. The samples were electro-phoresed through the gel at 35 mAmps for 2 – 4 hrs under the same conditions as the pre-run. The gel was dried for 1 hr at 80 °C under vacuum and autoradiographed at – 80 °C.

III. RESULTS

1. Plasmid constructs

pLL1. The strategy for constructing pLL1, in which the expression of *dhfr* was driven by the hMT-I$_G$ promoter, is outlined in Figure 2. The 1.5 kb *dhfr* cDNA came from the plasmid D11 (Gasser *et al.*, 1982). The plasmid pMEV1R, containing the hMT-I$_G$ promoter (from - 174 to + 66), into which *dhfr* was cloned, was a gift from Dr. Lashitew Gedamu, Department of Biological Sciences, University of Calgary (Shworak, 1990). The *cat* gene was removed from the *Pst* 1 site in pMEV1R and the vector dephosphorylated. The *dhfr* cDNA from D11 was ligated into the *Pst* 1 site and bacteria were transfected with this construct and selected on Ampicillin plates. Plasmid DNA was purified from Ampicillin resistant colonies using the Gene Clean plasmid preparation and the orientation of *dhfr* was determined using the *Sac* I restriction site. A plasmid containing *dhfr* in the correct orientation was named pLL1. The MT driven *dhfr* construct in this plasmid is referred to as MT:*dhfr*. The pertinent details of pLL1 are shown in the schematic diagram presented in Figure 3.

pLL2. Probing for endogenous MT mRNA production required an easily accessible source of CHO MT for probe synthesis. A CHO MT-I cDNA (CHMT1) previously cloned in pBR322 (Griffith *et all.*, 1983) was sub-cloned into the pSP73 vector (obtained from Promega) using *Bgl* II and *Sac* I restriction sites. A schematic diagram of the pLL2 construct is presented in Figure 4.

pLL3. For making *dhfr* probes for both Northerns and Southerns, *dhfr* was sub-cloned from pLL1 into the *Pst* 1 site of the pSP70 vector (obtained from Promega). A diagram of the new plasmid, named pLL3, is shown in Figure 4.

pLL4. To have a convenient source of hMT-I$_G$ promoter DNA for making probe, *dhfr* was removed from the *Pst* 1 site in pLL1. In Figure 4 is a diagram of the final construct, named pLL4.

2. Transfection and Amplification

The plasmid pLL1 was linearized at the *Pvu* I site and transfected into DG44 cells as described in Materials and Methods. Twelve colonies that showed stable integration of *dhfr* (i.e., were viable without GTH supplements) were selected and cultured separately. Each of these clones was exposed to stepwise increases in MTX concentration as described in Materials and Methods.

Selection by MTX took much longer than was expected from experience in our lab. Cells needed at least 4 weeks to adjust to new levels of MTX. Massive cell death often occurred when cells were exposed to a higher concentration of MTX, making it necessary to culture them at the previous level of MTX for 1 to 2 weeks to allow recovery. Of the original 12 colonies selected, 10 achieved resistance to 20 µM MTX. These clones were designated MPA1-10 (MT Promoter Amplified).
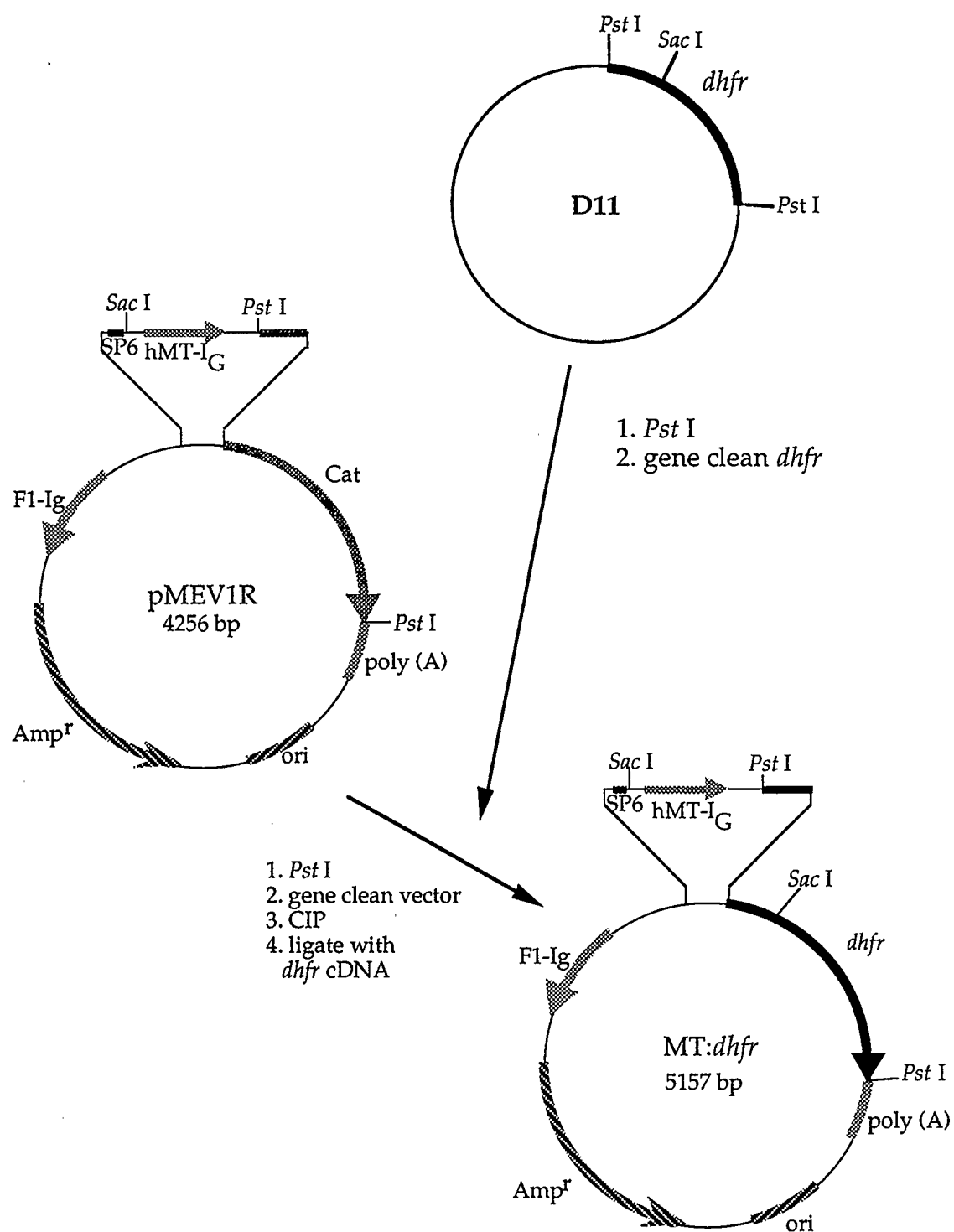
Figure 2. Strategy for the construction of the hMT-I$_G$ promoter driven *dhfr* cDNA.
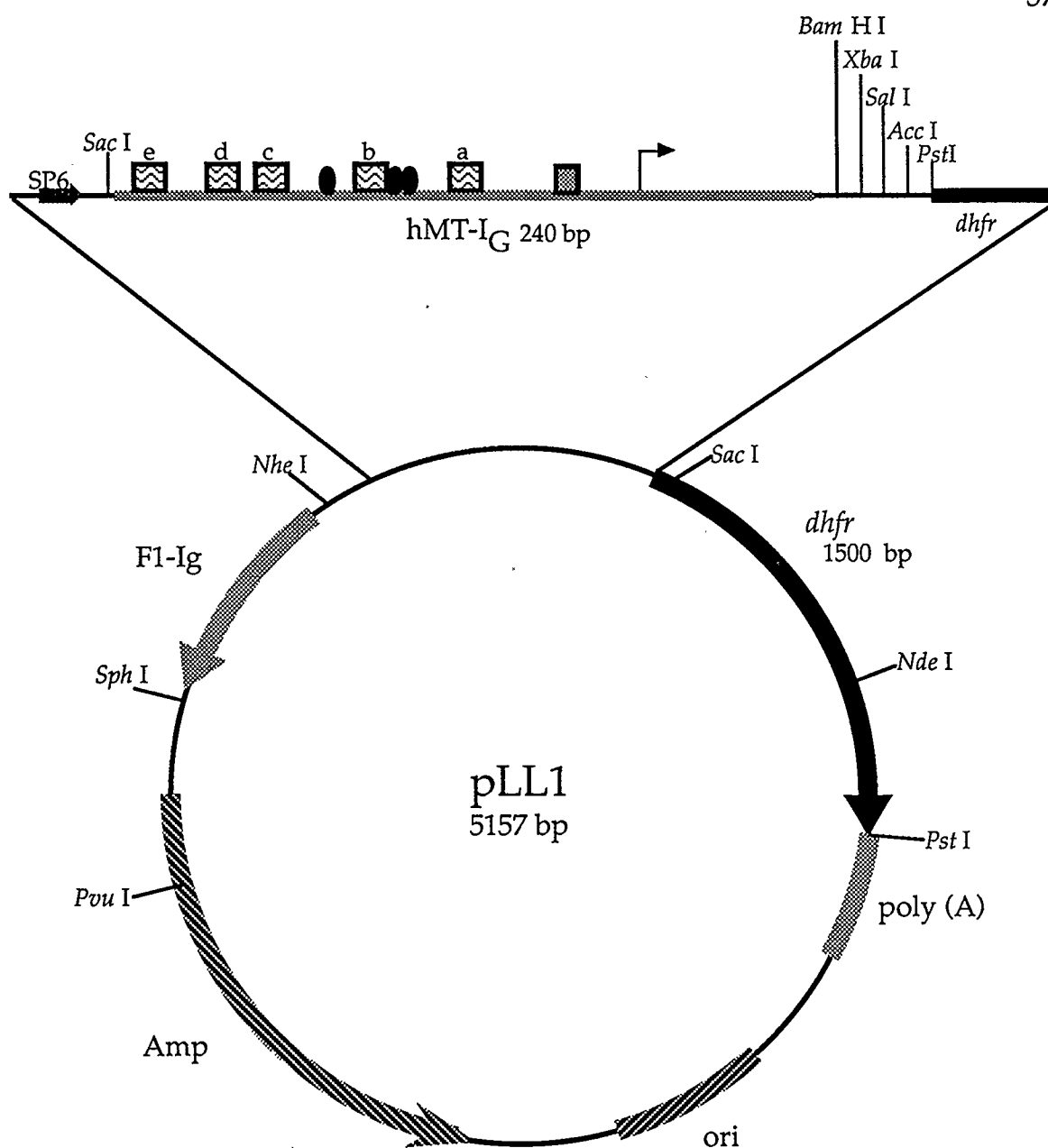
Figure 3. Cartoon of the pLL1 construct. Construction of pLL1 is described in detail in the Results section. Components of the hMT-I$_G$ promoter region are diagrammed as follows; ⊠ indicates a MRE, ▨ is the TATA box, ● indicates a GC box, and ↑ indicates the start site of transcription.
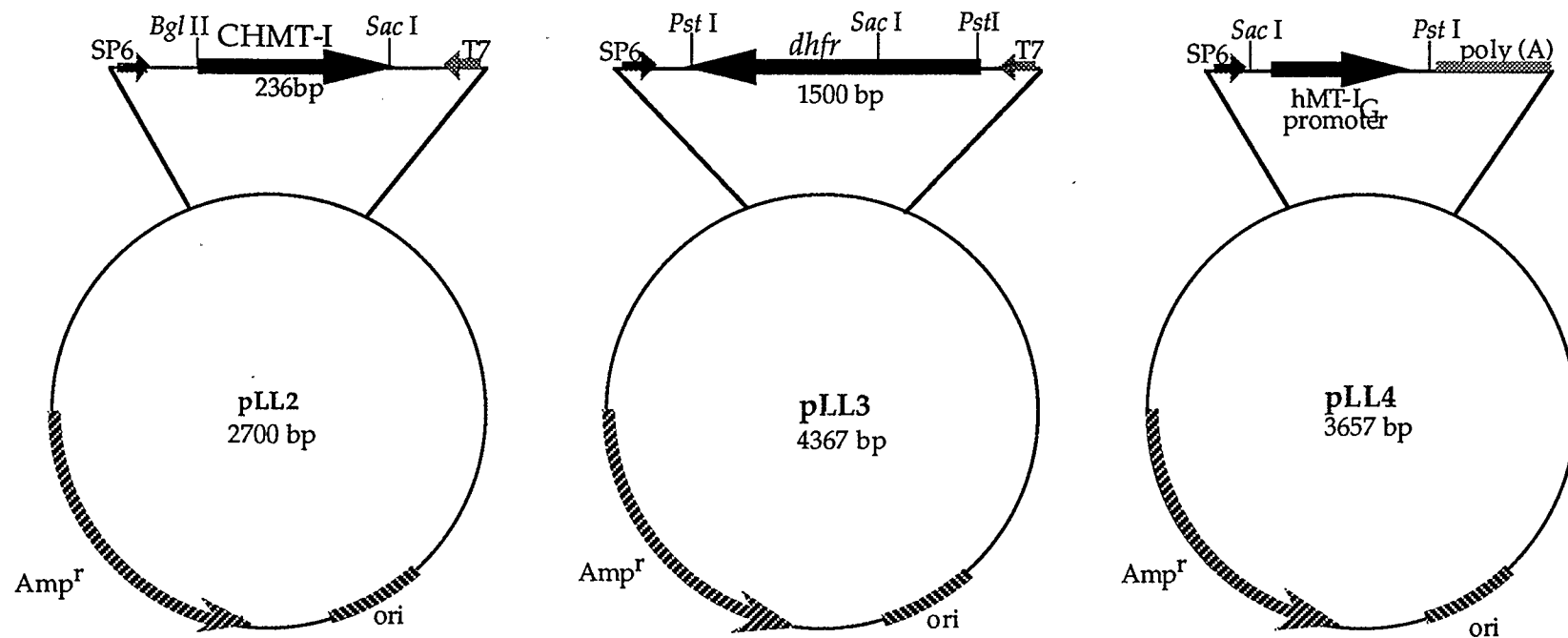
Figure 4. Cartoons of plasmids pLL2, pLL3, and pLL4. Plasmids were constructed as described in text. Features important for this work are indicated.

3. MTX resistant clones have amplified MT:*dhfr* DNA.

MTX resistant clones were screened to determine if they contained intact and amplified MT:*dhfr* sequences. Amplification by MTX selection often results in chromosomal deletions or rearrangements, especially in the amplified region. The enzymes *Nhe* I and *Nde* I were used to digest DNA from MT:*dhfr* transfected cells because they recognize sites on either side of the MT:*dhfr* construct (see the diagram of pLL1, Figure 3). If no rearrangements had occurred this digestion should yield a fragment of 1.5 kb that would hybridize to both the MT promoter probe and the *dhfr* probe.
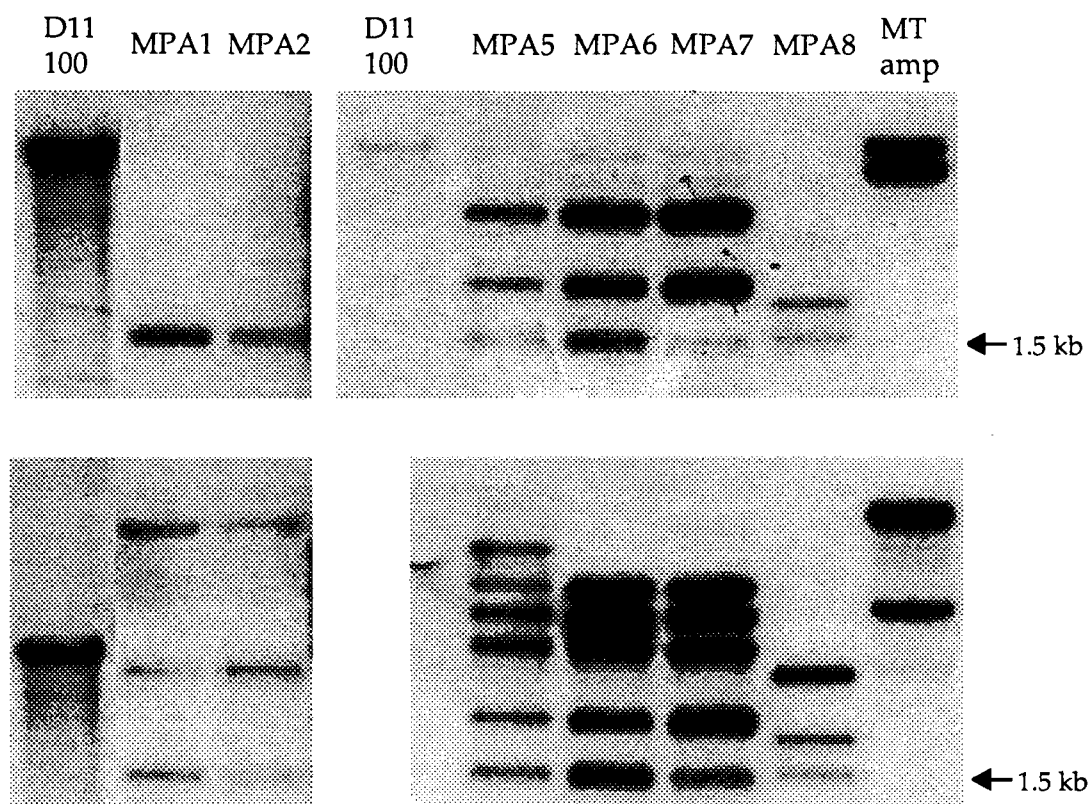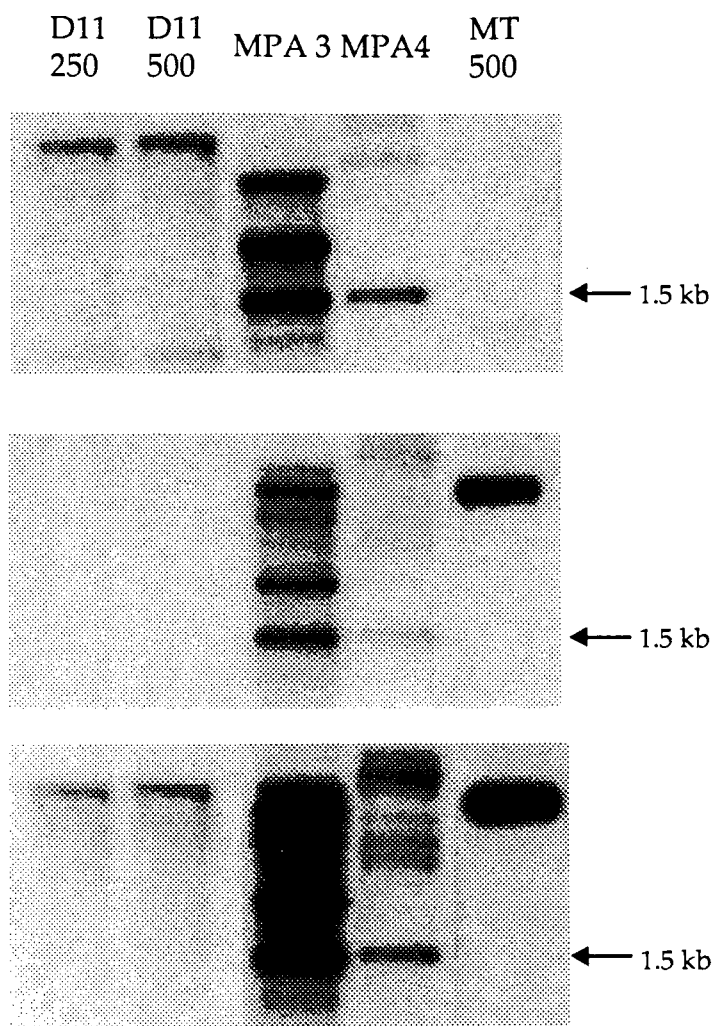
In Figures 5 and 6 are autoradiographs of Southern blots of 8 different MPA cell lines selected for MTX resistance. All the MPA cell lines in these blots were resistant to 20 μM MTX, except for MPA4 which was resistant to 5 μM MTX. Blots were hybridized with random primed *dhfr* cDNA and MT promoter probes, with the probe used first stripped off the membrane before the second probing. The upper panels in both figures show the results of hybridization with a *dhfr* probe. Several different bands are apparent in most clones, with the lowest band corresponding to the expected size of 1.5 kb. The detection of larger bands suggests that there were rearrangements of the MT:*dhfr* gene. In the lower panels of Figures 5 and 6 are autoradiographs of the same membranes seen in the upper panel, stripped of the *dhfr* probe and re-probed with an MT promoter probe. All the bands that hybridized with the *dhfr* probe appeared to hybridize with the MT promoter probe as well, suggesting that the MT:*dhfr* construct was usually amplified intact. However, several bands were detected that hybridized with MT promoter probe but not with *dhfr* probe (compare lower and upper panels of Figures 5 and 6). This suggests that there were some chromosomal rearrangements that led to

Figure 5. Autoradiographs of Southern analysis of MT:*dhfr* amplified clones. Membranes in the upper panel were probed with *dhfr* cDNA. In the lower panel, the same membranes as seen in the upper panels were stripped and reprobed with hMT-I_G promoter probe. Lane D11 100 has 100 copies of *dhfr* cDNA per genomic copy of CHO K1. MT amp is a control cell line in which the entire hMT-I_G gene was co-amplified with *dhfr*. DNA in lanes MPA1, MPA2, MPA5, MPA6, MPA7, and MPA8 was digested with *Nde* I and *Nhe* I. MT amp DNA was digested with *Eco* RI (MT promoter copy number in this cell line previously determined to be 620). The position of the expected 1.5 kb band is marked on the right of each panel.

Figure 6. Autoradiographs of Southern analysis of MT:*dhfr* amplified cell lines. In the top panel the membrane was probed with random primed *dhfr* cDNA. In the middle panel the same membrane as in the top panel was stripped and reprobed with MT promoter probe. The lower panel is a longer exposure of that seen in the middle panel to more easily visualise the bands in MPA4. Lanes D11 250 and D11 500 contain 250 and 500 copies of the *dhfr* cDNA per genomic copy of CHO K1 DNA, respectively. In lanes MPA3 and MPA4, chromosomal DNA was digested with *Nhe* I and *Nde* I. Lane MT 500, 500 copies of the MT promoter per genomic copy of CHO K1 DNA.

separate amplification of the MT promoter and the *dhfr* cDNA. Digested DNA from the cell lines MPA5, MPA6 and MPA7 had similar band sizes, which suggests that they may arise from the same clone. The same may be true for cell lines MPA1 and MPA2, which also exhibited similar banding patterns.

To quantitate the copy number of *dhfr* cDNA and MT promoter in the MPA clones, standards of known *dhfr* or MT promoter copy number were made. *Dhfr* standards were made by digesting purified pLL3 plasmid DNA with *Eco* RI and diluting it into CHO K1 chromosomal DNA to give 100, 250 or 500 copies of the plasmid per single genome copy of CHO K1 DNA (Figure 5: lane D11 100 and Figure 6: lanes D11 250 and D11 500). Similarly for the MT promoter, pLL4 plasmid DNA was diluted to give 500 copies of the plasmid per single genome copy of CHO K1 DNA (lane MT 500 in Figure 5). By comparing the relative signal intensities (measured by laser densitometer scanning) of the D11 or MT promoter standards to the signals obtained for the amplified cell lines, the copy number of both *dhfr* and MT promoter was estimated for MPA clones (see Table 3).

In both Figures 5 and 6 cross hybridization between the MT promoter probe and the D11 standards is apparent. This cross hybridization probably occurred because the MT promoter probe included some plasmid sequence that could hybridize to the plasmid sequence of pLL3 in the D11 standards. Since the *dhfr* probe did not include any plasmid sequence, it did not cross react with the MT promoter standard. Hybridization of the MT probe to the D11 standard was much weaker than its hybridization to the MT promoter

Table 3. Estimated copy number of amplified *dhfr* cDNA and MT promoter.

Clone	*dhfr* copy #	MT promoter copy #
MPA1	20	9
MPA2	30	5
MPA3	3000	1000
MPA4	300	250
MPA5	550	360
MPA6	2500	1400
MPA7	3000	1400
MPA8	460	180
MT-amp	2300	620

The copy numbers of the *dhfr* cDNA and the MT promoter in MPA3 and MPA4 were estimated by comparison to the standards D11 250 and MT 500, as described in the text. For MPA1, MPA2, MPA5, MPA6, MPA7, and MPA8, copy numbers of *dhfr* cDNA were calculated by comparison to D11 100, and copy numbers of the MT promoter were calculated by comparison to MT-amp, whose promoter copy number had previously been estimated to be 620 (by comparison to the MT promoter 500 standard, data not shown).

standard, however this may have resulted in overestimation of the MT promoter copy number in the MPA cell lines.

It is interesting that in several MPA cell lines the MT promoter copy number appeared to be much lower than the *dhfr* copy number, suggesting that some *dhfr* cDNA was amplified without the MT promoter. Without an

MT promoter, these *dhfr* genes may not be transcribed. Alternatively, transcription of the extra *dhfr* genes may be directed by another promoter located near the insertion site of the MT:*dhfr* gene.

4. Cd Sensitivity

I initially hypothesized that high levels of MT promoter would result in titration of MRF by the excess MREs, and that this titration would result in cells losing their ability to respond to Cd exposure with increased MT production. I proposed that this would lead to increased sensitivity of such cells to Cd toxicity.

To determine whether amplification of the MT promoter had affected the sensitivity of cells to Cd toxicity, I determined the plating efficiency (PE) of control and MPA cell lines exposed to various Cd concentrations. In Tables 4 and 5 the PE of each cell line, at a given Cd concentration, is expressed as a percentage of the PE of the same cell line in the absence of Cd. In all experiments PE at each Cd concentration was tested in triplicate.

Table 4 includes a summary of the results of several PE experiments with the control cell lines *dhfr*-amp (DG44 cells transfected with a *dhfr* gene and selected to 5 μM MTX resistance) and DG44 . While there is some variability from experiment to experiment, the effective concentration of Cd required to reduce the DG44 PE to 50% (EC_{50}) was approximately 0.2 μM. For *dhfr*-amp cells, the EC_{50} was between 0.05 and 0.1 μM Cd. This suggests that there may be some inherent variability in sensitivity to Cd among different cell lines even when MT promoter copy number is unaffected.

Table 4. PE of control cells exposed to increasing concentrations of Cd.

	PE at indicated Cd concentration (μM)[a].			
Cell Line	0.05	0.1	0.2	0.3
DG44	ND[b]	78 ±2.0	65±1.4	3.8±1.3
DG44	ND	89±2.0	72±1.5	32±2.0
DG44	ND	76±3.5	46±1.2	25±0.53
dhfr-amp	67 ± 1.7	21 ± 2.7	1.2 ± 0.17	0
dhfr-amp	96 ± 3.7	8.6 ± 0.5	2.2 ± 0.29	0

[a] PE is the number of colonies counted at each [Cd] expressed as % of the number of colonies when no Cd was added. Each [Cd] was tested in triplicate and the average PE of the 3 trials ± the SD is given.

[b] ND indicates not done.

The PEs of 8 MPA cell lines are listed in Table 5. When the PEs of the MPA cell lines were compared to the average PEs of the control cell lines, DG44 and *dhfr*-amp (calculated from Table 3), the EC_{50}'s of the MPA cells all fall within the range of the controls. For MPA2, MPA3, MPA6 and MPA7 the EC_{50} was in the 0.1 to 0.2 μM range, the same range as seen for the parental DG44 cells. The cell lines MPA1, MPA4, and MPA5 had EC_{50}'s between 0.05 and 0.1 μM which was in the range seen for *dhfr*-amp cells. These results suggested that there was no real increase in sensitivity of any MPA cell lines to Cd, contrary to the initial hypothesis.

Table 5. PE of MPA and control cells when exposed to Cd.

|	PE at the indicated Cd concentration (μM).[a]			
	0.05	0.1	0.2	0.3
DG44	ND[b]	81 ± 7.0	62 ± 13	20 ± 15
dhfr-amp	82 ± 21	15 ± 8.8	1.7 ± 0.71	0
MPA1	101 ± 2.9	20 ± 0.5	1.5 ± 0.51	0
MPA2	89 ± 2.7	53± 9.1	15 ± 3.1	0.2 ± 0.13
MPA3	ND	71 ± 2.1	64 ± 3.0	7.8 ±1.0
MPA4	87 ± 2.7	39.2 ± 4.6	16.6 ±1.8	0.12 ± 0.12
MPA5	77 ± 3.3	22 ± 1.8	8.4 ± 1.3	0.5 ± 0.12
MPA6	78 ± 2.6	59 ± 7.0	9 ± 0.45	0.8 ± 0.2
MPA7	104 ± 1.7	70 ± 6.3	3.1 ± 0.16	0.58 ± 0.2

[a] see Table 4.

[b] see Table 4.

Since none of the MPA cell lines showed a significant decrease in EC_{50}, only two were used for further analysis, MPA3 and MPA4. These clones were chosen because they had different levels of amplification and showed distinct banding patterns in the Southern analysis (see Figure 6).

5. The concentration of Cd that induced maximal levels of CHO MT RNA was variable.

There are two plausible explanations for why MPA cell lines were not more sensitive to Cd than were DG44 cells. Competition for MRF may have occurred causing a reduced level of MT, but the cells might have used another strategy to maintain Cd resistance. Alternatively, there may have been no competition for MRF so that MPA cells had the same level of MT as the parental DG44 cells.

Since MT is known to be primarily regulated at the transcriptional level, I used Northern Blot analysis to determine if transcription of endogenous MT was metal inducible in MPA3 and MPA4. Northern Blots were hybridized to random primed CHMT-I cDNA and GAPDH probes. Since MT-I and MT-II in Chinese Hamster are 80% homologous at the nucleotide level, CHMT-I should hybridize to both transcripts (Griffith et al., 1983). GAPDH RNA levels were used to control for RNA loading.

Previous studies with CHO cell lines indicated that 9 hr of exposure to 0.9 μM Cd gave maximal MT levels (Dutler, 1989). I did several time course experiments with different levels of Cd and confirmed that maximal MT RNA levels were always seen 8 to 10 hr after exposure to Cd (data not shown), so all subsequent Cd treatments were carried out for 9 hr. Inducibility of endogenous MT in DG44 cells, at different concentrations of Cd, was tested by Northern blot analysis several times (4 examples are presented in Figure 7) and the level of Cd required to obtain peak MT levels were found to vary from 2 to 4 μM Cd. This was somewhat higher than the level of Cd (0.9 μM) that was previously shown to give peak MT induction in DG44 cells.
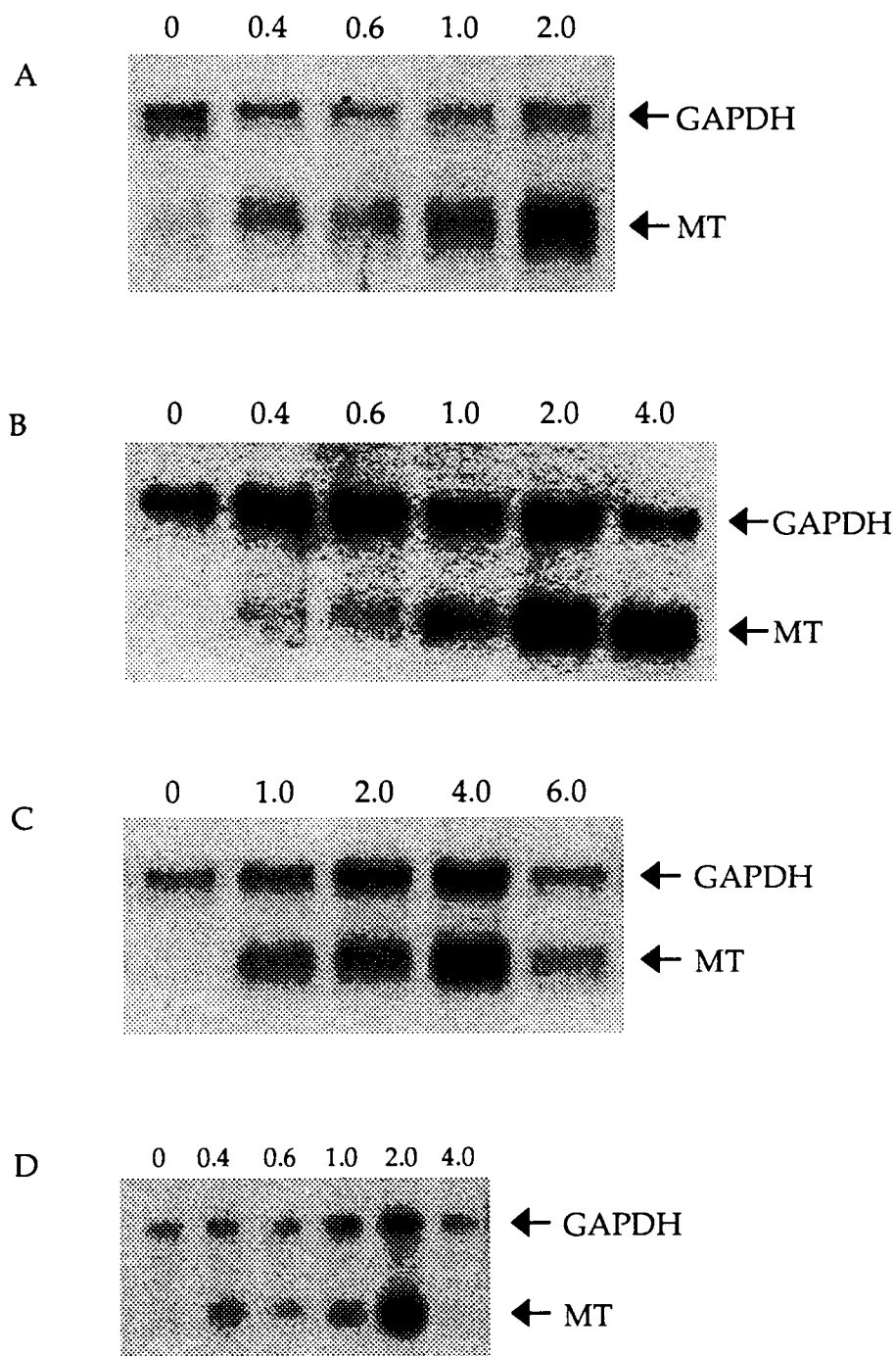
Figure 7. Northern blot analysis of DG44 cells treated with various levels of Cd. Cells were treated with the concentration of Cd (μM) indicated above each lane for 9 hours before total RNA was extracted. All blots were hybridized with CHMT-I and GAPDH probes at the same time and the position of each RNA is indicated on the right hand side of each panel.

A.

B.
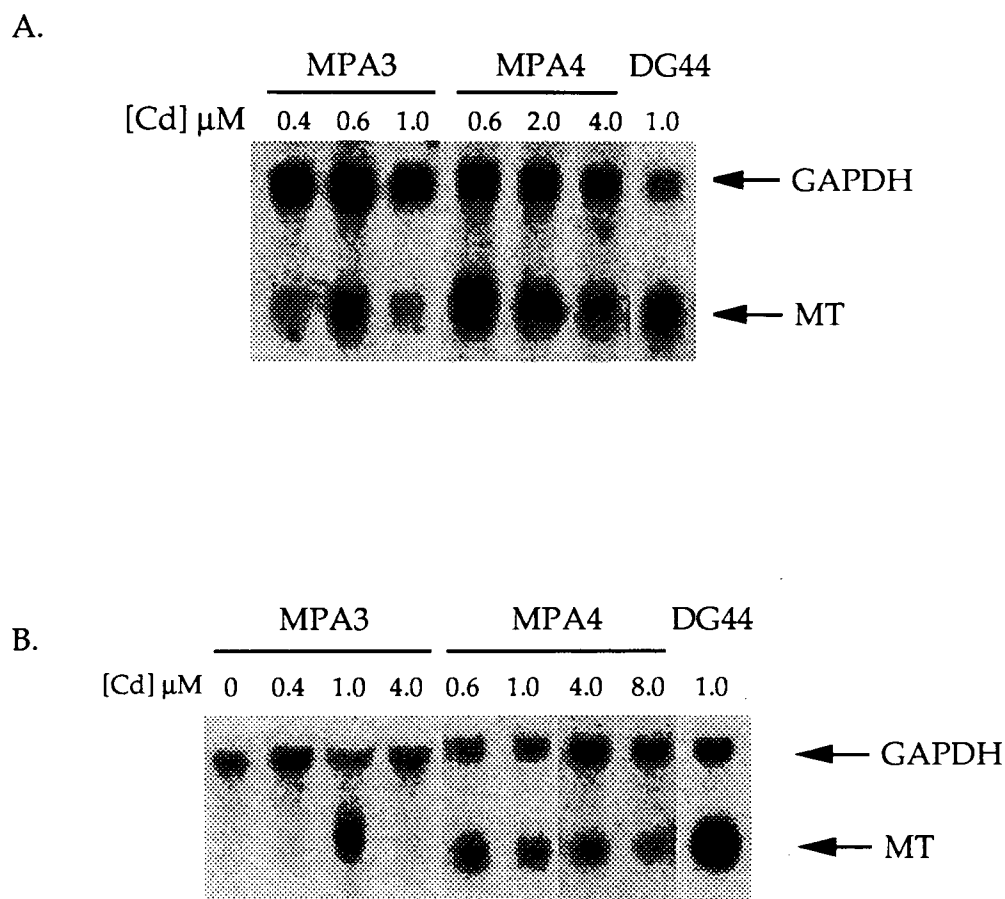
Figure 8. Northern analysis of inducibility of endogenous MT RNA in MPA3 and MPA4 cells. Cells were induced with the Cd concentration indicated above each lane (μM) as described in the text. The RNA extracts used in Northern A are all different from those in B except for Lane DG44 1.0 μM, which is the same in both panels. Membranes were probed as described in the legend for Figure 7. A and B each contain several different exposure times of the same membrane to X-ray film, selected so that signal intensities for hybridization of GAPDH are similar.

6. Endogenous MT mRNA still increases in response to Cd .

Northern blot analysis of MPA3 and MPA4 cells was carried out as described above for the DG44 analysis. Figure 8 shows the autoradiographs of two different sets of MPA3 and MPA4 Northerns. As can be seen in both Figures 7 and 8, there is usually no detectable MT signal when cells are not Cd treated. This prevented the accurate calculation of the degree of MT inducibility. Instead, for each autoradiograph I compared the absolute level of the peak MT RNA signals (measured by laser densitometry and normalized by comparison to GAPDH) in MPA3 and MPA4 relative to that in DG44.

Table 6 contains a summary of the relative levels of MT RNA signals measured in four different Northerns. For each experiment the peak MT signal in MPA3 and MPA4 was expressed as a percentage of the DG44 signal in the same experiment. The peak MT signal in MPA3 and MPA4 relative to the peak in DG44 is variable among different experiments. The variability in the concentration of Cd required to induce peak MT RNA levels, and the variability in the absolute level of peak MT RNA probably reflected variability in the ability of cells to take up and/or respond to Cd.

The peak MT RNA level in MPA4 was found to be 41% to 80% (mean of 65% ± 24) of the peak MT level in DG44 This relatively small reduction in peak levels of MT does not seem significant when the large variability is taken into account. MPA3 had a somewhat lower peak MT signal than MPA4, from 9% to 72% (mean 31% ± 29) of the peak MT level DG44. Again, considering the variability, the reduction in inducible MT RNA levels in MPA3 is relatively small, perhaps no more than 2 fold.

Table 6. Peak MT RNA levels in MPA3 and MPA4 cells relative to peak MT RNA levels in DG44 cells.

	% MT RNA	
Trial	MPA3	MPA 4
1	9	49
2	72	41
3	13	88
4	30	83
$\bar{x}\pm$SD	31±29	65±24

Signals on X-ray films were laser densitometer scanned and the MT signal normalized to the GAPDH signal. For each trial the peak MT signal in MPA3 and MPA4 cells was expressed as a percentage of the peak MT signal in DG44 cells. Trials 1 and 2 correspond to Northerns A and B in Figure 8. The auto-radiographs of trials 3 and 4 are not shown.

For both MPA3 and MPA4, the reduction in MT inducibility observed was not nearly as great as I had expected, as Johnston and Kucey (1988) found that amplification of the *hsp70* control region reduced the inducibility of the endogenous gene to almost undetectable levels. However, it may be significant that in both MPA3 and MPA4 the peak MT signal was always less than the peak signal in DG44.

7. MT promoter driven *dhfr* is Cd inducible.

A possible reason that transcription of endogenous MT in MPA3 and MPA4 cells was still inducible by Cd was that perhaps the amplified MT:*dhfr* had lost some of its functional MREs thereby preventing MRF from binding to the MT:*dhfr* but the basal function of the promoter was retained, allowing *dhfr* synthesis and cell survival. If functional MREs were not present in the MT:*dhfr* gene then Cd treatment should not induce higher *dhfr* expression in MPA3 and MPA4 cells. To determine if this was the case, the level of *dhfr* RNA in Cd treated MPA3 and MPA4 cells was examined using Northern hybridization. Autoradiographs of Northerns probed with random primed *dhfr* cDNA are shown in Figure 9. From these Northerns it was clear that *dhfr* was Cd induced in both MPA3 and MPA4, suggesting that at least some, but not necessarily all, of the MT:*dhfr* amplified sequences had functional MREs.

8. Gel shift assays detected two specific complexes in MPA4, one of which was Zn-dependent

The reduction in Cd inducibility of endogenous MT mRNA levels in the MT promoter amplified cell lines was less than I had originally expected, but did not appear to be due to a loss of functional MREs in the MT:*dhfr* gene. One way to explain these observations is that the cells with amplified MT promoters avoided depletion of the MRF by increasing the level of active MRF. Since the MRF has never been satisfactorily isolated, this possibility was investigated using gel shift assays. This assay allows the detection of nuclear proteins that can bind to the MRE *in vitro*.
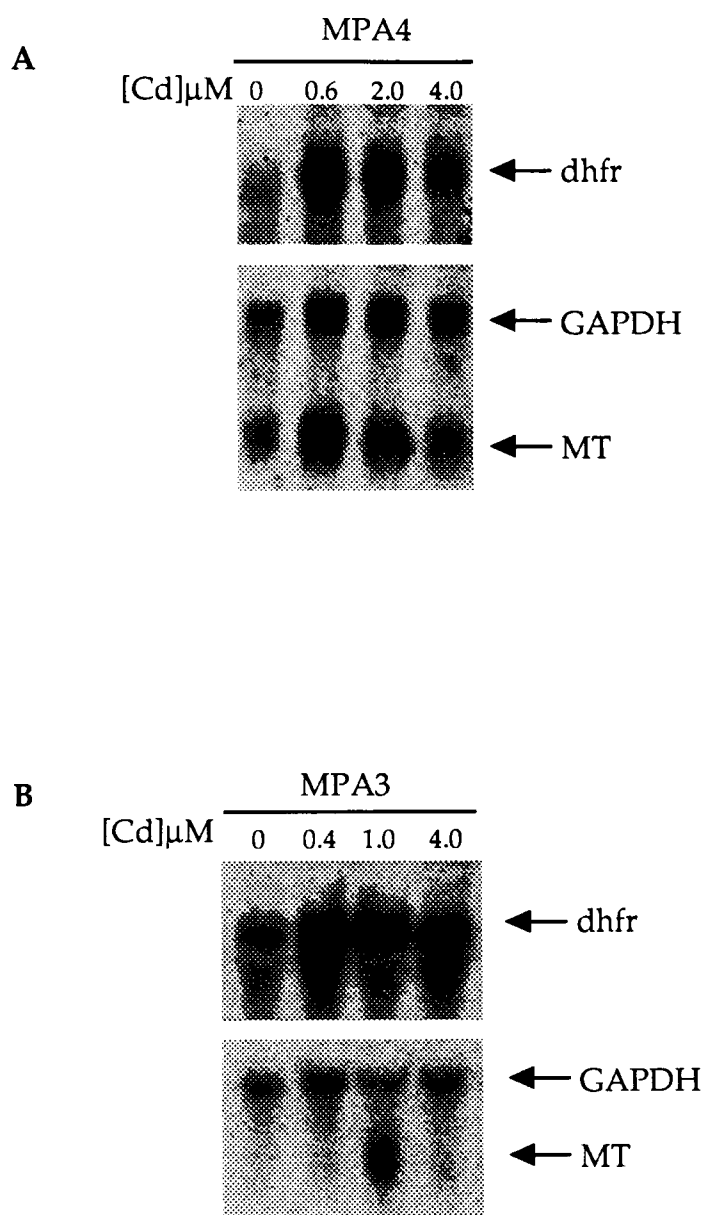
Figure 9. Northern analysis of Cd inducibility of *dhfr* in MPA3 and MPA4 cells. The upper panels of both A and B were probed with random primed *dhfr* cDNA. The lower panels were probed with random primed GAPDH and CHMT-I. The positions of the respective signals are indicated on the right hand side of the panels. The lower panel in A is the same membrane as in Figure 8 A. The lower panel in B is the same membrane as in Figure 8 B.

MT oligo

GACCT`TGCGCCCGGCCC`TTCTGC`TGCACACAGCCC`ACCC

MT' oligo

GACCT**ACACTTTA**GCCC`TTCTGC`**CTAAGTG**AGCCC`ACCC

Sp1 oligo

ATTCGATCGGGGCGGGGCGGAG

Figure 10. Oligonucleotides used in the gel shift assays. Only the top strands of the oligos are shown, the lower strand was a complementary copy. The putative MREs of MT and MT'-oligos are enclosed in boxes. The core MREs that were mutated in MT' are in bold type.

The MRE containing oligonucleotide used in the following experiments was a copy of the hMT-I$_F$ promoter from -175 to -147, and is referred to as MT-oligo (for sequence see Figure 10). This region included two MREs, and had been previously shown to confer Cd inducibility to a CAT reporter gene and to be capable of binding protein in a gel shift (Foster and Gedamu 1991). The hMT-I$_F$ sequence was chosen rather than the hMT-I$_G$ sequence because it conferred higher Cd inducibility to CAT and it was a better competitor in CAT assays and gel shifts (Foster and Gedamu 1991).

In gel shift assays with end labeled MT-oligo two major complexes (called MT-a and MT-b) and several minor complexes were detected in 5% gels (see Figure 11). The minor complexes were difficult to visualize, inconsistent in intensity and did not appear to be specific, and were not investigated further.

The first step in this gel shift assay was to determine what shifts might represent a specific interaction between a MRF and a MT-oligo. A complex involving a MRF should be able to fulfill two criteria: 1) it should be specific for the MT-oligo and 2) its binding to MRE should be regulated by heavy metal. For determining specificity of complexes and the effect of metal on the complexes, MPA4 nuclear protein was used, as this routinely gave better MT-a and MT-b complexes than other cell extracts (see next section).

In gel shifts where the standard binding reaction mix was used (see Materials and Methods) complex MT-a was always visible (see Figure 13A: lane 1). When 50 to 400 μM Zn was added directly to the binding reaction a second complex, MT-b, was also detectable (see Figure 11: lanes 2 – 5). 50 μM Zn gave the best induction of complex MT-b. The formation of complex MT-b not only required the presence of Zn but also relatively high
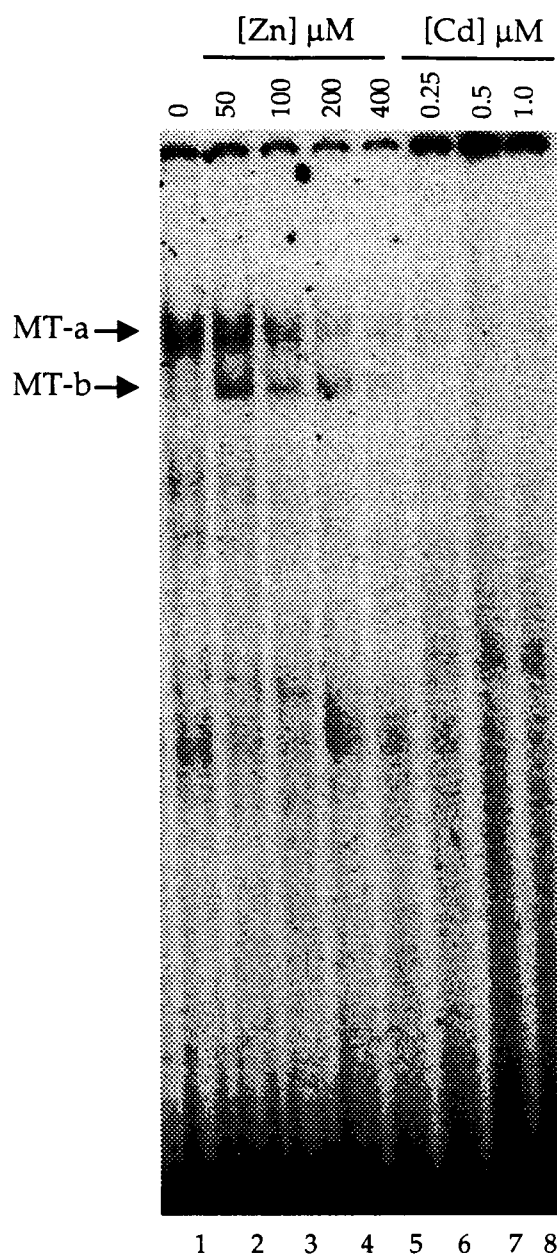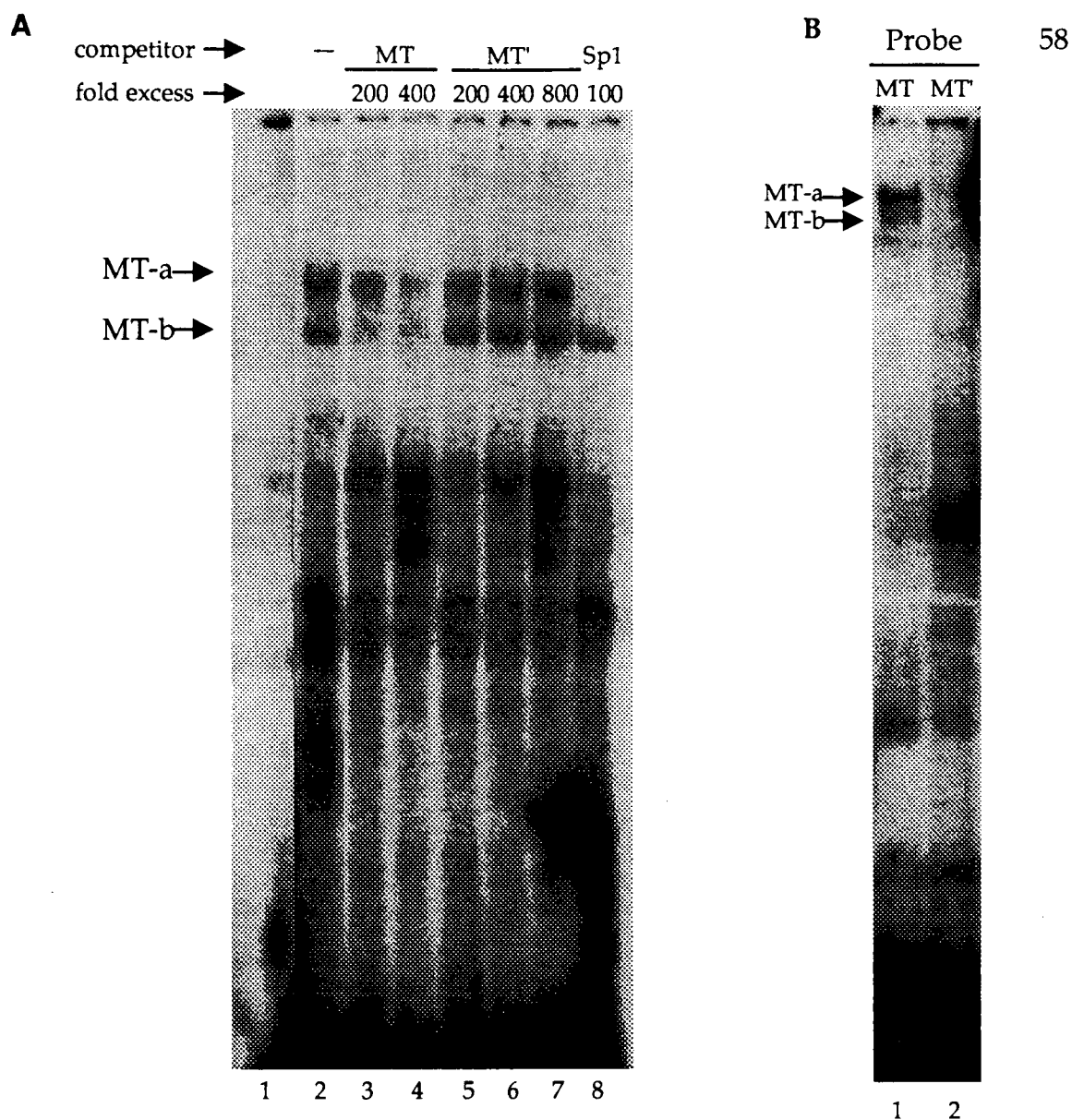
Figure 11. Autoradiographs of gel shift assays showing metal-dependent shifts with the MT-oligo. For each lane the following were added to the standard binding reaction, 5μg MPA4 protein extract (no EDTA in any of the extraction buffers), MT-oligo probe, 10 mM DTT, and the concentration of Zn or Cd indicated above the lane.

concentrations (5 – 10 mM) of the reducing agent DTT. The appearance of MT-b cannot be explained as a general increase in protein-DNA interactions, as 50 μM Zn had no apparent effect on complex MT-a (Figure 11: Lane 2), nor on complexes formed with Sp1 oligo (see below and Figure 15). Higher concentrations of Zn (100 to 400 μM, see figure 11: lane 3 to 5) and all concentrations of Cd tested (0.25, 0.5 and 1.0 μM in Figure 11: lanes 6 to 8) inhibited the formation of complex MT-a. Complexes formed with Sp1-oligo responded in a similar manner to both Zn and Cd (Figure 14 and data not shown), suggesting that the reduction in complex formation may represent a general reduction in DNA-protein interactions. Nuclear proteins for the gel shift presented in Figure 11 were obtained using extraction buffers that did not contain any EDTA. However, the use of standard extraction buffers that included EDTA (see Materials and Methods) had no effect on any of the complexes formed with MT-oligo.

To determine the specificity of complexes MT-a and MT-b, competition assays were performed. In this assay, excess unlabeled oligo is added to the binding reaction and allowed to interact with the nuclear protein for 10 min before labeled oligo is added. If the complexes are specific for MT-oligo then they should not be visible when excess unlabeled MT-oligo is present in the binding reaction. If formation of the complexes requires the presence of core MRE, then mutant MT-oligo (MT'-oligo) should not compete for complex formation with MT-oligo. MT'-oligo is the same sequence as MT-oligo except that the core sequence of the MREs was mutated (sequence in Figure 10).

Addition of 200 fold excess (relative to the amount of probe used), unlabeled MT-oligo completely abolished the Zn-dependent complex (complex MT-b in Figure 12A: lane 3). The same amount of MT'-oligo did

A

competitor → — MT MT' Sp1

fold excess → 200 400 200 400 800 100

MT-a→

MT-b→

1 2 3 4 5 6 7 8

B Probe 58

MT MT'

MT-a→
MT-b→

1 2

Figure 12. Autoradiographs of gel shift assays showing the specificity of interactions with the MT-oligo. In both A and B, all binding reactions contained 5 µg of MPA4 nuclear protein and 50 µM Zn. Complexes are indicated to the left of each panel. A. Competition assays were performed using excess competitor, as indicated at the top of each lane. Samples were run on a 5% polyacrylamide gel. Lane 1, no protein added. B. The probe used is indicated at the top of each lane. The gel contained 7% polyacrylamide.

not compete for this complex (Figure 12A: lane 5). This suggested that formation of complex MT-b required the presence of wild type MREs. A higher concentration of the MT-oligo (400 fold excess) but not the MT'-oligo competed for formation of complex MT-a (Figure 12A: compare lane 4 to lanes 6 and 7). This indicated that while formation of complex MT-a required the presence of wild type MREs, it may have been more abundant than complex MT-b, making complex MT-a harder to compete for. Alternatively, complex MT-a may have a faster on rate and slower off rate than complex MT-b, which would result in MT-b being easier to compete for than MT-a. The specificity of complex MT-a and MT-b for MT-oligo is further supported by the fact that neither complex will form with end-labeled MT'-oligo (Figure 12B).

Because the MT-oligo has GC rich regions that may be capable of binding Sp1 and because Sp1-oligo specific complexes run in the same region of the gel as complex MT-a, I also did competitions with Sp1-oligo. A 100 fold excess of unlabeled Sp1-oligo (sequence in Figure 10) had no effect on complex MT-b, but was found to completely abolish complex MT-a (Figure 11 B, lane 4), suggesting that this complex may involve a Sp1 protein interaction with MT-oligo.

9. MPA3 and MPA4 cells appeared to have more of the metal dependent complex than did DG44 cells

To determine if there was any change in the activity or the level of complexes that form with the MT-oligo, gel shifts were performed in parallel using end labeled MT-oligo and equal amounts of nuclear protein from MPA3, MPA4, DG44 and B11 cells. B11 is a CHO cell line that has been selected for resistance to 50 µM MTX resistance and has approximately 500 copies of the endogenous *dhfr* gene. The results of two such experiments are

shown in Figure 13. In these experiments complex MT-a was more abundant in MPA3 and MPA4 than in DG44s (compare lanes 2 and 3 to lane 1 in Figure 13A; and lane 4 and 5 to lane 2 in Figure 13B). In Figure 13B, complex MT-b was more intense in MPA3 and MPA4 than in DG44 or B11 (compare lanes 4 and 5 to lanes 2 and 3). Complex MT-b was not detected in the gel shift presented in Figure 13A because Zn was not added to the binding reaction. In Table 7 the results of several gel shifts with MT-oligo are summarized. In this table the signal intensity (as measured using laser densitometry) of complexes MT-a and MT-b in MPA3, MPA4 and B11 relative to the signal intensity of the same bands in DG44, is presented.

On average, MT-a and MT-b were 1.3 fold and 2.0 fold more intense, respectively, in MPA3 than in DG44. The same bands were 4.0 fold and 4.6 fold more intense in MPA4 than in DG44. These increases were not simply a result of amplification of *dhfr* as nuclear protein from B11 cells, in which the *dhfr* gene is amplified to 500 copies, showed levels of complexes MT-a and MT-b very similar to those seen in DG44 cells (see Table 7: Trial 4). The specificity of complexes MT-a and MT-b in MPA3, DG44 and B11 cells were found to be the same as those described in the previous section for MPA4 (data not shown).
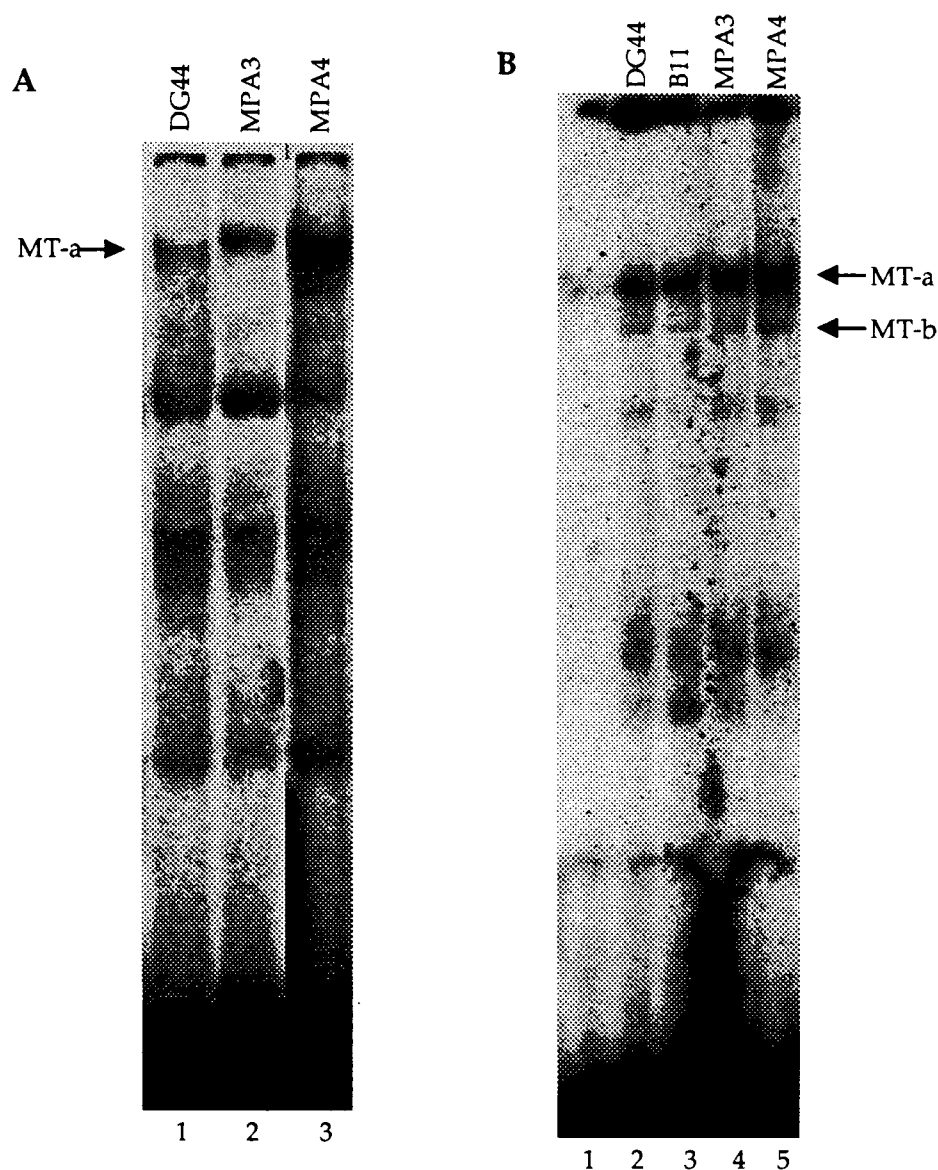
Figure 13. Autoradiographs of gel shifts with MT-oligo probe and nuclear protein from MPA3, MPA4 and control cells. For each lane MT-oligo probe and 5 µg of nuclear protein from the cell line indicated at the top of the lane were added to the standard binding reaction. **A.** This gel shift corresponds to Trial 1 in Table 7. Nuclear protein extractions were obtained using standard buffers. In addition to the above, the binding reaction contained 0.5 mM DTT and no additional Zn, and the gel was 7% polyacrylamide. **B.** This gel shift corresponds to Trial 4 in Table 7. Nuclear protein extractions were obtained using buffers not containing EDTA, the binding reactions contained 10 mM DTT and 50 µM Zn, and the gel was 5% polyacrylamide. Lane 1, no protein added.

Table 7. Intensity of complexes MT-a and MT-b in MPA3, MPA 4 and B11 relative to the intensity of the same bands in DG44.

	Band MT-a			Band MT-b		
Trial	B11	MPA3	MPA4	B11	MPA3	MPA4
1	ND	1.0	6.9	ND	2.4	8.1
2	ND	1.2	4.0	ND	nd	nd
3	ND	1.3	3.1	ND	1.7	3.5
4	0.88	1.5	2.0	1.0	1.8	2.3
$\bar{x} \pm SD$	0.88	1.3±0.21	4.0±2.1	1.0	2.0±0.38	4.6±3.1

Within a single trial, equal amounts of nuclear protein from each cell line, along with equal amounts of MT-oligo probe, were added to the binding reactions. The intensities of the MT bands in MPA3, MPA4, and B11 relative to the intensity of the same bands in DG44 are presented. The intensities of the bands were measured by laser densitometry as described in Materials and Methods. For Trials 1, 2 and 3 nuclear protein was extracted using standard buffers as described in Materials and Methods. For Trial 4, buffers used in nuclear extraction contained no EDTA. In trials 1 and 2, the binding reaction contained 0.5 mM DTT and no additional Zn. In trials 3 and 4, the binding reaction contained 50 µM Zn and 10 mM DTT. ND indicates not done, and nd indicates not detected.

10. Nuclear protein from MPA3 and MPA4 cells did not appear to form more Sp1-oligo specific complexes than did DG44 nuclear protein.

Band MT-a was competed more efficiently with an Sp1-oligo than with MT-oligo (see Figure 11), suggesting that this band may have an Sp1 component. To determine if this was the case, gel shifts were carried out using Sp1-oligo as the probe. Using MPA4 protein, two specific complexes with Sp1-oligo were identified (Figure 14: complex Sp1-a and Sp1-b). A less intense band (Sp1-c in Figures 15 and 16) was frequently observed when gels were exposed to X-ray film long enough. Both complex Sp1-a and Sp1-b were competed with 100 fold excess of Sp1-oligo (Figure 14: Lanes 2 and 5) but not with 400 fold excess of MT-oligo (Figure 14: Lanes 3 and 6). Complex Sp1-c was not competed for by either Sp1 or MT-oligo (data not shown).

In Figure 15, the effect of Zn on the formation of complexes with Sp1-oligo is shown. Neither Zn nor Cd induced the formation of new complexes with Sp1-oligo. Similarly to what was observed for complex MT-a, 50 μM Zn had no effect on any of the Sp1 complexes and higher concentrations of Zn inhibited formation of these complexes.
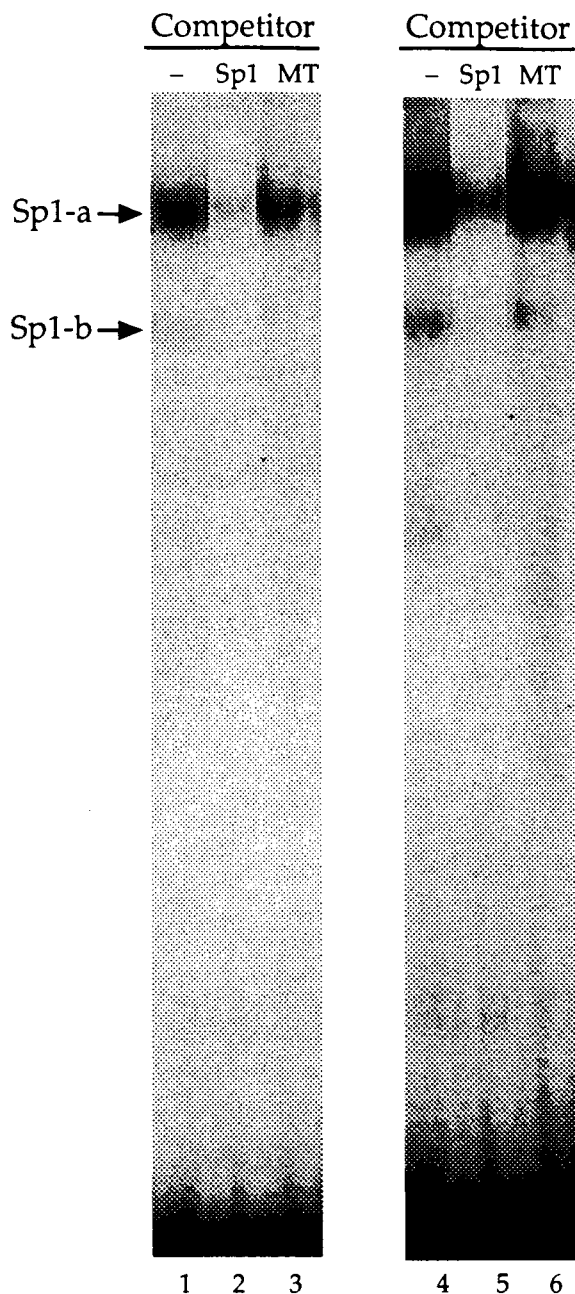
Figure 14. **Autoradiographs of gel shift assays showing specificity of complexes formed with Sp1-oligo.** For each lane the following was added to the standard binding reaction: 5 µg MPA4 nuclear protein, 2.5 mM DTT, the competitor indicated at the top of each lane (Sp1 at 100 fold excess and MT at 400 fold excess), and Sp1-oligo probe. Complexes are indicated to the left of the panels. All lanes were run on the same 7% gel and spliced together for this figure. The right hand panel is a longer exposure of the gel on the left.
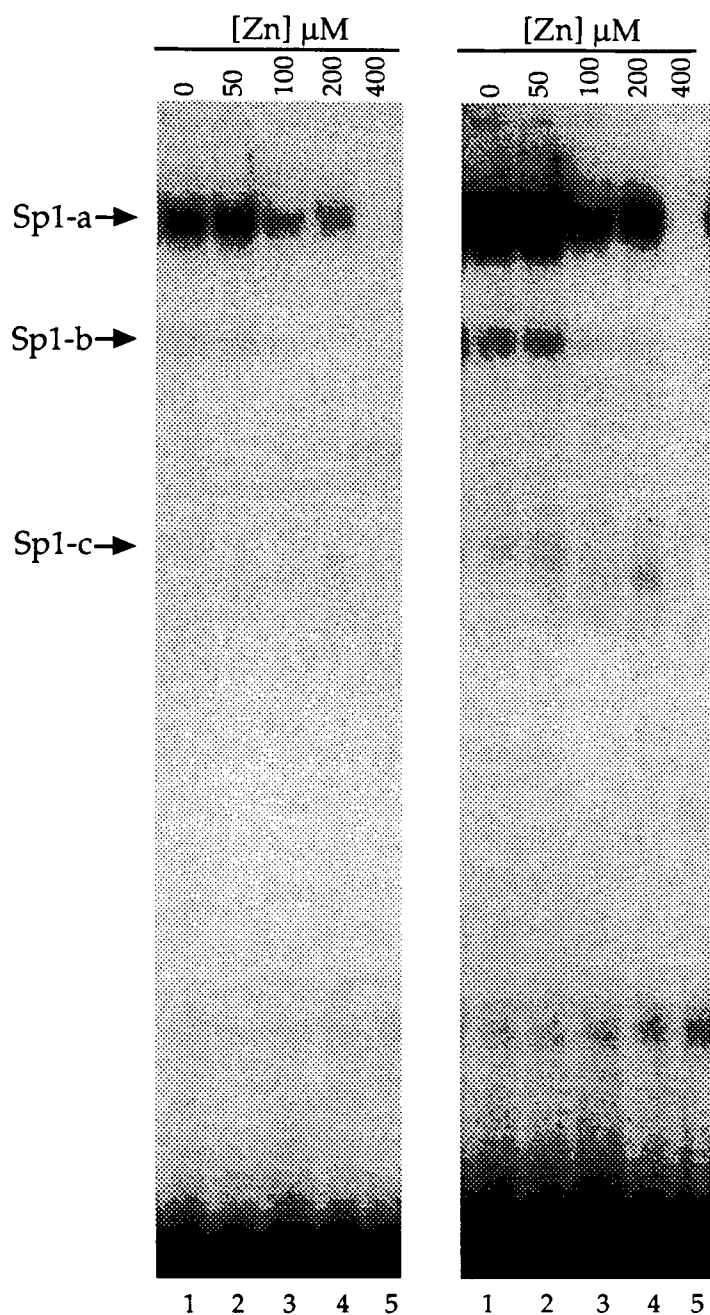
Figure 15. **Autoradiographs** **of gel shifts showing the effect of Zn on complexes formed with Sp1-oligo.** For each lane the following were added to the standard binding reaction: 5 µg MPA4 nuclear protein, 2.5 mM DTT and the concentration of Zn indicated at the top of the lane. The right hand panel is a longer exposure of the gel in the left hand panel. Names of complexes are indicated to the left of the panels. Gels contained 5% polyacrylamide.
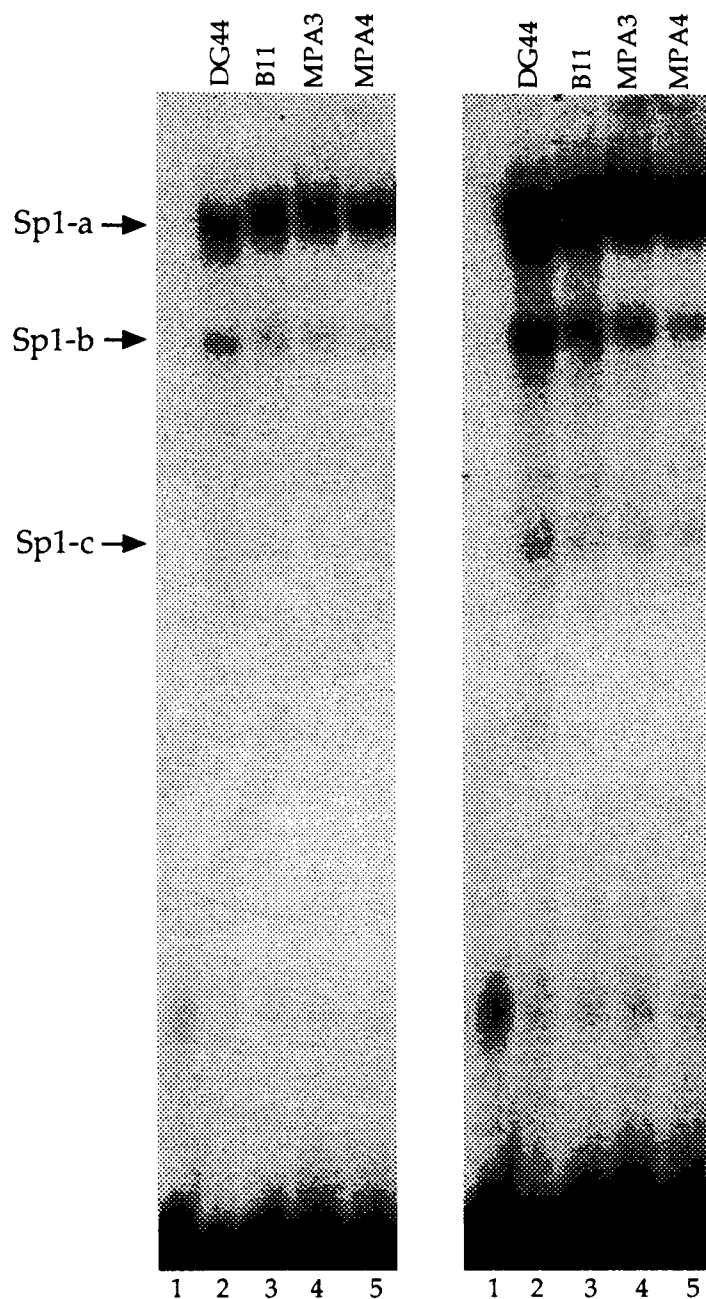
Figure 16. Autoradiographs of complexes formed with Sp1-oligo and nuclear protein from MPA3, MPA4 and control cells. For each lane the following were added to the standard binding reaction: 2.5 mM DTT, 5 µg of nuclear protein from the cell line indicated at the top of the lane, and Sp1-oligo probe. The right hand panel is a longer exposure of the gel in the left hand panel. Names of complexes are indicated on the left hand side. Lane 1, no nuclear protein. The gel was 7% polyacrylamide.

A comparison of the intensities of Sp1-a and Sp1-b complexes among DG44, B11, MPA3 and MPA4, when equal amounts of each nuclear protein were used, is shown in Figure 16. In this gel shift assay it appeared that the level of Sp1-a complex is the same in all 4 nuclear extracts and the level of Sp1-b is actually higher in DG44 than in MPA3 and MPA4. This observation was made in several other gel shifts (data not shown).

11. Complex MT-a may involve some of the same components as complexes Sp1-a and Sp1-b

Addition of 50 µM Zn or Sp1-oligo competitor to the binding reaction had the same effect on complexes MT-a, Sp1-a and Sp1-b, suggesting that all three complexes may involve the same factors. However, the fact that complex MT-a appears to be more intense in MPA3 and MPA4 than in DG44 or B11, while complexes Sp1-a and Sp1-b appear to be the same or less intense in MPA3 and MPA4, suggests that complex MT-a must have some component that is not present in either Sp1-a or Sp1-b.

IV. DISCUSSION

Contrary to my initial hypothesis, amplification of the MT promoter region by 125 to 1500 fold (relative to the 2 endogenous MT promoters) appeared to have no major effect on the sensitivity of CHO cells to Cd toxicity. In addition, metal inducible transcription of the endogenous MT gene in MPA3 and MPA4 cells was at most, only slightly reduced. This result was unexpected as transient competition studies had previously indicated that a positive acting, metal regulatory factor was limited in abundance (Séguin *et al., 1984*).

Analysis of MT RNA levels using Northern blot hybridization proved to be very difficult and perhaps was not the most advisable method. A major problem was the lack of detectable MT RNA signal in non-induced conditions, which made it impossible to calculate a fold induction and thus comparisons of MT RNA inducibility from experiment to experiment were difficult. Perhaps the CHMT-I cDNA sequence, though 80% homologous to CHMT-II, did not hybridize well to the CHMT-II message. Since MT-II is thought to be the source of most of the basal level MT message in CHO and human cells (Griffith, 1985; Sadhu and Gedamu, 1989), poor hybridization of the CHMT-I probe to CHMT-II message could explain the lack of detectable MT message in cells not treated with Cd. Alternatively, there may simply be little or no MT expression in non-induced CHO cells. Perhaps another, more sensitive method, such as RNase protection, would be able to detect MT RNA in non-induced cells.

There are several possible explanations for the apparent lack of competition between the promoters of the amplified MT:*dhfr* gene and the endogenous CHO MT genes: 1) MRF is compartmentalized near the endo-

genous MT genes so that it in unavailable for titration; 2) Hamster MRF has a lower affinity for hMT-I$_G$ MREs than for the MREs of the hamster MT genes; 3) The amplified hMT-I$_G$ promoter has lost some or all of its MREs; 4) MRF is more abundant than was previously thought, and cannot be functionally depleted by increasing the number of its binding sites; 5) Cells have responded to the increase in MREs by increasing the activity or the amount of MRF. The inducibility of MT:*dhfr* construct in MPA3 and MPA4 suggests that at least some human MREs are present and able to interact with hamster MRF, thus making the first three explanations unlikely. Since there is no good way to test the fourth explanation, I decided to investigate the amount of MRF using gel shift assays. Results of the gel shift assays support the fifth explanation, that cells with amplified MT promoter have more MRF than do control cells.

1. The complex MT-b, which is apparently more abundant in MPA3 and MPA4 cells than in control cells, could contain a MRF.

When equal amounts of nuclear protein are allowed to complex with equal amounts of MT-oligo probe, two complexes (MT-a and MT-b) appear to be more abundant in the MT:*dhfr* amplified cell lines MPA3 and MPA4, than in the DG44 cell line. Of these two complexes, MT-b is more likely to involve a MRF since formation of this complex requires the addition of 50 to 200 µM Zn to the binding reaction and the presence of wild type MREs in the probe. In addition, formation of this complex is competed for only by an oligo with wild type MREs, not by an Sp1-oligo nor by an MT-oligo with mutated MREs.

The increase in detectable MT-b complexes in MPA3 and MPA4 was not very large (2 fold and 4.6 fold respectively), but if these complexes do involve an MRF(s) this increase may be biologically significant as the MRF is

thought to normally be low in abundance. It could be argued that the apparent increase in complex MT-b is really only a result of unequal protein activity in the gel shift analysis. However, the fact that this complex is consistently more abundant in MPA3 and MPA4 cells than in DG44 cells, even though the abundance of Sp1-oligo complexes is consistently no different or less in MPA3 and MPA4 than in DG44 cells, argues against this interpretation.

2. Complex MT-a may involve both an MRE binding factor and an Sp1 factor.

Complex MT-a may also have an MRF component, as it bound to an oligo containing wild type MREs and its formation was competed for by the wild type MT-oligo but not by the MT-oligo in which the core MREs are mutated (MT'-oligo) (see Figure 12). However, complex MT-a was competed more efficiently by Sp1-oligo (100 fold excess) than by MT-oligo (400 fold excess) (see Figure 12). The fact that the same amount of Sp1-oligo competitor does not compete for the binding of complex MT-b to MT-oligo indicates that this competition is specific to MT-a. A role for Sp1 in the formation of complex MT-a is further supported by the similar response of complexes formed with Sp1-oligo and complex MT-a, to metal (compare Figures 11 and 15). These results suggest that complex MT-a could be formed by the binding of Sp1 to the GC rich region of the MT-oligo. However, Sp1 protein cannot be the only component in this complex because complexes formed on the Sp1-oligo did not appear to be more abundant in MPA4 than in DG44s but MT-a did appear to be more abundant in MPA4 cells.

One model that could explain these results suggests that the complex MT-a is made up of two or more factors which bind co-dependently to the MT-oligo. One of these factors is limited in abundance and binds specifically

to the core MRE (MRE core binding factor, MCBF). The other factor binds only in conjunction with MCBF to the GC rich region close to the MRE (GC binding factor GCBF), but can bind by itself with high affinity to the Sp1-oligo If MCBF is more limited in abundance than GCBF, then an increase in the level of MCBF would result in more detectable MT-a complex. Since MCBF can bind to the MT-oligo only in conjunction with GCBF, competition with Sp1-oligo, to which GCBF can bind alone, results in loss of detectable MT-a complex. To determine if formation of complex MT-a involves the Sp1 transcription factor, super shifts with Sp1 antibody could be carried out.

Formation of complex MT-a was not induced by addition of Zn to the binding reaction. In fact, concentrations of Zn greater than 50 μM, and all concentrations of Cd tested (0.25 to 8 μM) inhibited the formation of this complex (see Figure 11). Perhaps complex MT-a involves a factor which represses transcription of MT when there are low intracellular levels of metal and this factor is released from the MRE when high levels of metal are present. Alternatively, since this repression of complex formation was also observed for the complexes formed with Sp1-oligo (see Figure 15), it may represent a general inhibition of protein-DNA interactions by Zn and Cd.

3. Relationship of complexes MT-a and MT-b to complexes observed by other researchers.

Of the previously detected Zn-induced complexes that form with MRE containing oligos, the complex ZRF, observed by Koizumi *et al.*, (1992), is most similar to the MT-b complex. The formation of ZRF and MT-b are induced by the addition of Zn to the binding reaction only if high concentrations of DTT (5 to 10 mM) are included. Neither of these complexes were induced by the addition of Cd to the binding reactions. Finally,

Koizumi's ZRF is detected near the top of a 5% polyacrylamide gel, which is similar to the location of the complex, MT-b. However, unlike myself, Koizumi et al., (1992) did not detect a MRE-specific complex above the ZRF.

Westin and Schaffner (1988) detected a complex which appeared when Zn was included in the binding reaction. However, formation of this complex did not seem to require high concentrations of DTT. These researchers also detected a complex, just above the Zn-dependent complex, which was competed with Sp1 consensus oligo. This shift may be related to the MT-a complex that I observed.

The dependence of the Zn induced shift on relatively high DTT concentrations suggests that one or more cysteines in the Zn regulated factor are important for DNA binding. Dependence of protein-DNA interactions on a strong reducing environment in vitro has been observed for several other transcription factors. Such a requirement was observed recently for the Fos–Jun DNA binding activity (Abate et al., 1990; Xanthoudakis et al., 1992). Whether or not reduction/oxidation is used as a mechanism for regulating transcription factor activity in vivo remains to be established. It is possible that the factor that binds the MT-oligo in a Zn dependent manner is present in a reduced form in vivo, and that the nuclear protein extraction procedure results in oxidation of this factor and thus strong reducing conditions are requiredto reconstitute DNA binding in vitro.

Unfortunately, like most other researchers, I was unable to induce the MRE specific shift with the metal Cd. In fact addition of Cd to the binding reaction seemed to inhibit the formation of complex MT-a. If the MRE specific gel shift was really due to the interaction with the MRF then it should be induced by other metals as well. It is possible that the factor which induces

MT transcription in response to Cd is distinct from the factor which responds to Zn and the induction of this factor by Cd *in vitro* is not possible. Alternatively, the MT-b complex seen in these experiments may not be involved in the metal induction of MT.

4. MPA4 cells appear to have more MRF factor than MPA3 cells

From the level of MT promoter amplification (1000 copies in MPA3 and 250 copies in MPA4) I would predict that if the factor interacting with MT-oligo was a positive-acting, metal-inducible transcription factor, it would be more abundant in MPA3 than in MPA4. However, complex MT-a is consistently more abundant in MPA4 cells than in MPA3 cells (see Table 7). In fact, the abundance of this complex is almost the same in MPA3 and DG44 cells. In addition complex MT-b is more abundant, by 2.4 fold, in MPA4 cells than in MPA3 cells (see Table 7). There are several possible explanations for these results. First, it is possible that these two cell lines have used different mechanisms to adapt to the increase in MT promoter copy number. Alternatively, even though MPA3 has a higher MT promoter copy number than MPA4 the amplified promoter in MPA3 may not have as many functional MREs as MPA4. Loss of MREs due to mutation or deletion would not be detected in Southern blots since the probe I used for detecting MT promoter levels was made by random priming the whole MT promoter DNA sequence and therefore would be able to hybridize to partial hMT-I$_G$ promoter sequence.

The lower abundance of MT-a and MT-b in MPA3 could be the reason why the metal induction of endogenous MT RNA in these cells is apparently lower than in MPA4 and DG44 cells. However, MPA3 cells were slightly more resistant to Cd than MPA4 cells, suggesting that they may be using an

alternative mechanism for protecting themselves against the toxic effects of Cd. In CHO cells selected for resistance to Cd the level of MT does not always correlate with the level of Cd resistance, suggesting that other proteins besides MT may be involved in detoxification of heavy metals (Crawford *et al.*, 1985).

5. Implications of the increase in complexes MT-a and MT-b.

The possibility that cells not exposed to heavy metal stress could increase the level of MRF in response to an increase in their binding site is somewhat surprising and has several implications. First, if MRF is a positive-acting transcription factor, it suggests that the cells have some way of counting the number of MREs present, even though experimental evidence to date indicates that *in vivo* MRF is not bound until cells are stimulated with heavy metal (Mueller *et al.*, 1988; Andersen *et al.*, 1987). Alternatively, the MRF may be bound at all times, but was not detected in *in vivo* footprinting assays because it binds with low affinity in cells not exposed to metal. This could allow the cell to keep track of the number of MRF it needs. Another possibility is that the factor visualized in these gel shifts is not a positive-acting factor but a negative one, and that its level has been increased in MPA3 and MPA4 because relief of inhibition of MT expression when cells are not exposed to high levels of metal, is detrimental to cells.

Increased levels of a MRE binding factor has been reported for a Cd resistant mouse cell line (Labbé *et al.*, 1993). However, the relationship of this factor to MT-b or MT-a cannot be determined since these researchers did not show any gel shift assays of this factor.

Adaptation of MT:*dhfr* transformed DG44 cells to each increase in MTX took longer than was expected, based on previous work in our lab. Perhaps this is a reflection of the need for these cells to not only amplify MT:*dhfr*

sequences, but to concurrently increase the level or activity of one or more factors that interact with the hMT-I$_G$ promoter region. Alternatively, slow acclimatization to MTX may result from the deregulation of *dhfr* transcription by replacement of native *dhfr* promoter with an MT promoter, or loss of post-transcriptional regulation of *dhfr* because *dhfr* cDNA does not have its normal introns or untranslated 5' and 3' sequences.

6. Future Research

The experiments presented in this thesis give a solid background upon which many interesting projects could be based. First, I would recommend that further work to better characterize, and perhaps identify, the factors which are more abundant in MPA3 and MPA4, be carried out. South-Western analysis, UV-crosslinking, or cutting out bands MT-a and MT-b and running them on a SDS-page gel, could all be used to determine the size(s) of the factor(s) which interact with MT oligo. This would allow one to determine if one or more of these factors was similar in size to an already isolated MRE specific factor. If any of the factors appear to be unique then an attempt to purify them may be in order. To this end the over-expression of these factors in MPA3 and MPA4 may be an asset.

Once the factors of complexes MT-a and MT-b have been isolated they could be tested in a functional assay. An *in vitro* transcription assay for MT promoters has recently been established (Susan Samson, personal communication) and could be used for determining the role of these factors in metal induction of the MT gene.

The gel shift assays appear to be a promising method for further characterization of the MRF. One question that still needs to be answered is why Zn is usually the only metal that can induce a metal specific shift on

MRE-oligo. Since MT expression in mammals is known to be transcription-aly induced by several other metals, it is not unreasonable to expect that these other metals could also induce MRE specific shifts. I believe that a Cd-inducible shift exists but that the right conditions for producing it *in vitro* have not yet been established. For example, it may be that the MRF which responds to Cd is destroyed during the nuclear protein extraction procedure, and perhaps adjustments to this procedure would allow it to be extracted intact. Consequently, I would recommend that further work be carried out in an attempt to define these conditions.

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