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**EXPRESSION OF MAMMALIAN METALLOTHIONEIN GENES IN *ESCHERICHIA COLI* AND IN *SACCHAROMYCES CEREVISIAE***

Y.-M. Hou
(Ph.D. Thesis)

August 1986

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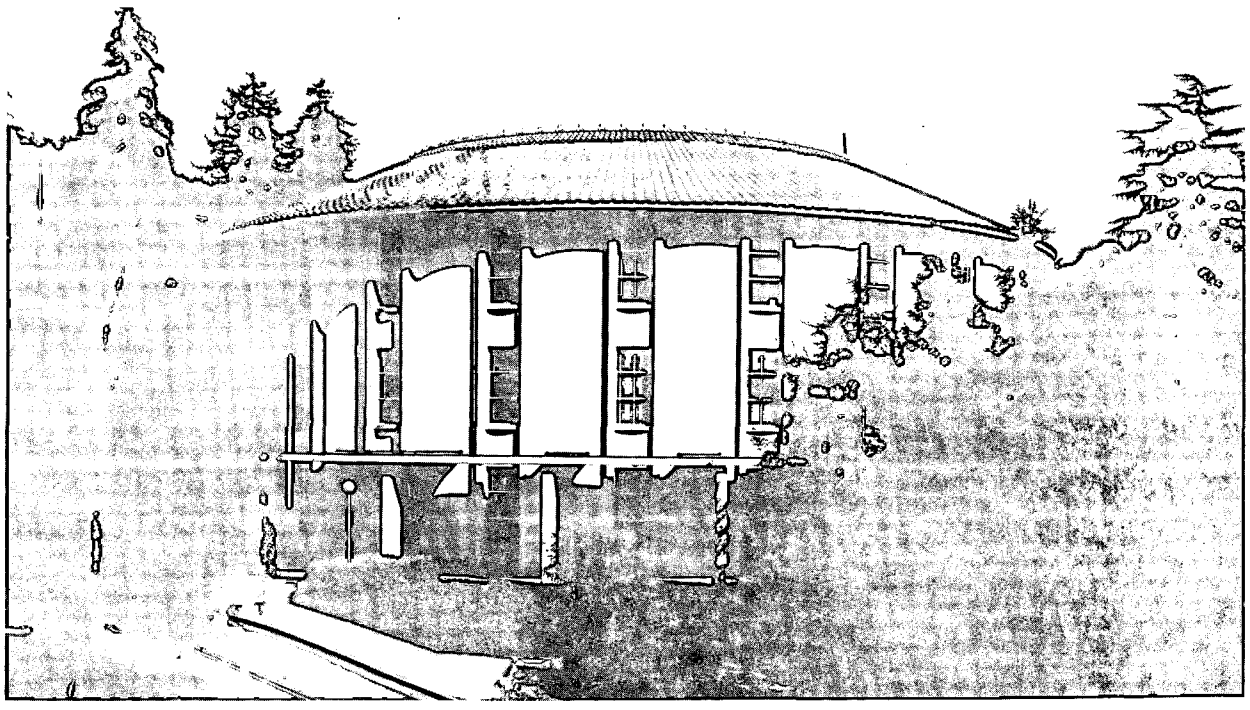

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# Expression of Mammalian Metallothionein Genes in *Escherichia coli* and in *Saccharomyces cerevisiae*

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Ph.D. Thesis

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August 1986

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# Abstract

Expression of Mammalian Metallothionein Genes

in *Escherichia coli* and in *Saccharomyes cerevisiae*

by

Ya-Ming Hou

The methallothionein genes of mouse and human were expressed in *Escherichia coli* and in *Saccharomyces cerevisiae*, respectively. The mammalian metallothionein genes code for a group of proteins that are low molecular weight, rich in cysteine and capable of binding heavy metal ions such as cadminum ($Cd^{+2}$) and zinc ($Zn^{+2}$). Expression of a metallothionein gene renders bacteria and yeast more resistant to heavy metal ions under a variety of growth conditions. This effect is specific for metallothionein because if gene expression is inactivated, bacteria and yeast become sensitive to metals. In addition, bacteria show a preferential incorporation of $Cd^{+2}$ ions upon gene expression and contain a protein species that is readily labelled by [$^{35}$S]cysteine. Yeast also contain a protein of a similar molecular weight as the mammalian species but show characteristics that indicate the cells are under stress. Expression of metallothionein has a toxic effect on the microorganism, especially for yeast.

The amounts of metallothionein detectable in both bacteria and yeast lysates are low. Several experiments suggest that metallothionein is quickly degraded in the presence of cell lysate. The low level of gene expression and/or the degradation of the gene product make it difficult to detect the expressed metallothionein in the bacterial or yeast lysate.

This dissertation is dedicated to my parents, Ting-Hsih and Shu-Ching, who gave me my start in science and supported me in every way possible.

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# Abbreviations

| | |
|---|---|
| $amp^r$ | resistant to ampicillin |
| BRL | Bethesda Research Laboratories |
| bp | base pair |
| BSA | bovine serum albumin |
| Ci | Curie |
| cpm | counts per minute |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| DNase | deoxyribonuclease |
| EDTA | ethylenedinitrilotetraacetic acid |
| hr | hour |
| Kb | kilobase pair |
| min | minute |
| Mr | molecular weight |
| MT | metallothionein |
| NMR | nuclear magnetic resonance |
| OAc | $CH_3COO^-$ |
| OD | optical density |
| RNA | ribonucleic acid |
| RNase | ribonuclease |
| rpm | revolutions per minute |
| sec | second |
| SDS | sodium dodecyl sulfate |
| SV40 | simian virus 40 |
| $tet^r$ | resistant to tetracycline |
| Tris | tris(hydroxymethyl)aminomethane |
| tRNA | transfer ribonucleic acid |
| uv | ultraviolet |

# CHAPTER 1

## Introduction

### 1.1. General Characteristics

Metallothioneins (MTs) are a class of low molecular weight (Mr 6000-7000), cysteine rich proteins which bind heavy metal ions (Kägi and Nordberg, 1979; Vasak and Kägi, 1983; Dalgarno and Armitage, 1984; Neilson *et al.*,1985). The protein was first isolated from equine renal cortex by Margoshes and Vallee in 1957 (Margoshes and Vallee, 1957). Since then, similar proteins have been isolated from kidney, liver, and intestines of a variety of animal species (Käge and Nordberg, 1979, Tzunoo *et al.*, 1978; Kojima *et al.*, 1976; Weser *et al.*, 1973; Buhler and Kägi, 1974), and from invertebrates (Olafson *et al.*, 1979), yeast (Prinz and Weser, 1975), fungi (Lerch, 1980), and recently bacteria (Higham and Sadler, 1984). The best characterized mammalian forms to date contain a single polypeptide chain of 61 amino acids, of which 20 or 21 are cysteine. The chain has seven binding sites for the heavy metal ions cadmium (Cd) and/or zinc (Zn) ions (Kojima *et al.*, 1976). The other MTs have been isolated with bound mercury (Hg) and copper (Cu) ions as well. Under nonphysiological conditions *in vitro*, the protein can bind metal ions such as gold (Au), silver (Ag), lead (Pb), nickel (Ni) and cobalt (Co) (Winge elvit et al., 1975; Winge *et al.*, 1981).

There are two major subgroups of MTs, referred to as MT-I and MT-II, based on their

electrophoretic properties (for example, see ref. 6 for mouse liver MTs). Other methods further resolve the MT-I group into several distinct molecular forms known as MT-IA, MT-IB, etc. The various isoforms exhibit small differences in their binding affinity for metal ions, but otherwise, have similar biochemical properties. The multiplicity of MTs has been confirmed by gene cloning experiments. In most vertebrate species examined, the MTs are encoded by a multigene family. The functional significance of multiple forms is not fully understood, but from the standpoint of protein biosynthesis as discussed in the next section, this divergence may play a role in regulating MT synthesis *in vivo*.

Amino acid sequences of mammalian MT isoforms are strongly conseved and the positions of all 20 cysteine residues are invariant (Kägi and Nordberg, 1979; Lerch *et al.*, 1982). This is demonstrated in Figure 1-1, where the number of residues that appear at a given sequence position along the polypeptide chain is presented (Winge *et al.*, 1984). At most, four different amino acids are found at a particular residue position. Two invariant stretches, at the amino terminus (residues 1-7) and in the center of the molecule (residues 29-38), are observed in addition to the cysteine positions.

Figure 1-1 also shows the two metal-binding domains of mammalian MTs, $\alpha$ and $\beta$. These two domains are generated by a subtilisin reaction after selective removal of Zn ions from the protein with EDTA (Winge and Miklossy, 1982). It should be noted that MTs exist in a random conformation in the absence of metal ions (Vasak *et al.*, 1981) and that binding of metals converts them to a stable, compact conformation which is resistant to many proteases (Feldman *et al.*, 1978). The cleavage catalyzed by subtilisin, for example, is not easily obtained by other proteases (Winge and Miklossy, 1982). The $\alpha$ domain comprises residues 30 through 61 and the $\beta$ domain comprises the amino terminus to residue 29. The junction between $\alpha$ and $\beta$ occurs at one of the invariant stretches. The gene sequence of mouse MT-I has exons corresponding to residues 1 to 9, 10 to 31, and 32 to 61 (Glanville *et al.*, 1981). The $\alpha$ domain correlates with the third exon. The assignment of amino acid residues in the $\alpha$ and $\beta$ domains is consistent with that of X-ray crystal data (Furey *et al.*, 1986). The $\alpha$ domain carries the type A cluster (4-metal cluster) and the $\beta$ domain carries the type B cluster (3-metal cluster) (see section 1.4.). The isolated $\alpha$ and $\beta$ domains of rat MT-II have been shown to be capable of binding metals with the same

stoichiometry as the intact molecule (Eklund *et al.*, 1976).

### 1.2. Induction by Heavy Metal Ions and Hormones

MTs are inducible in various tissues and cells by treatment with metal ions (Kägi and Nordberg, 1979; Kägi *et al.*, 1980) or glucocorticoids (Karin and Herschman, 1980). The induction is manifested up as an enhanced production of MT mRNA (Durnam and Palmiter, 1981; Beach and Palmiter, 1981). Beach and Palmiter had further shown that in cadmium-resistant mouse cells, the MT gene was also amplified (1981). There are two DNA sequences, lying close to the promoter for the MT gene, which separately mediate the induction of the gene by heavy metal ions and by glucocorticoid hormones (Karin *et al.*, 1984). Recent studies by Richards *et al.* (1984) have shown that the affinity of cis-acting elements for inducers is unique for each individual isoform of in the MT gene family. For example, the gene for human MT-II appears to be regulated more efficiently by glucocorticoids than does the gene for isoform I. This may be a mechanism by which animals control the biosynthesis of MTs via more than one independent pathway.

The regulatory region responsible for induction by heavy metal ions contains a sequence, identified as the heavy metal ion responsive element (MRE) (Karin *et al.*, 1984; Carter *et al.*, 1984; Stuart *et al.*, 1984; Stuart *et al.*, 1985). This sequence is shared by the DNA sequences of many other metal-binding proteins (Berg, 1986). It has been suggested that many proteins with the ability to form metal-binding domains are involved in nucleic acid binding or gene regulation (Miller *et al.*, 1985; Berg, 1986). The presence of MRE in the regulatory region of MT would suggest that a metal-dependent transcription factor recognizes and binds to the MRE sequence. This signal is then transmitted over distance and stablizes the formation of an open promoter complex with RNA polymerase (Davison *et al.*, 1983). Since MT itself contains many metal-binding domains, it is possible that MT may act as a trans-acting factor for turning on and off its own gene expression. Such an autoregulation function has been demonstrated for the yeast copperthionein (Hamer *et al.*, 1985), which binds to and is induced by copper (Fogel and Welch, 1982; Butt *et al.*, 1984). However, the autoregulation function of MT has not been vigorously tested.

In addition to glucocorticoid and heavy metal ions, Friedman and Stark (1985) recently

showed that transcription of human MT-IIA gene is induced by $\alpha$-interferon. A similar effect by interferon was observed on the transcription of the histocompatibility locus antigen (HLA) gene. A consensus sequence 5′ to the MT-II and the HLA genes was subsequently localized. This consensus sequence shares no homology with the MT regulatory sequences respective to glucocorticoid and to metal ions (Karen *et al.*, 1984), and thus is a new area for future investigation.

Another upstream region of the human MT-IIA gene has sequence homology to the enhancer element of SV40 (Scholer *et al.*, 1986). Because a competition effect was observed between the expression from the MT promoter and from the SV40 promoter *in vivo*, it is suggested that the expression of MTs is also regulated by at least one cellular factor (the trans-acting factor) which is involved in the viral activity of SV40 (Scholer *et al.*, 1986). In fact, the regulatory region of human MT-IIA contains various sequences found in the SV40 enhancer and in the adenovirous enhancer (Haslinger and Karin, 1985; Hearing and Shenk, 1983), implying that the competition for regulatory proteins may be a common mechanism by which mammalian cells control gene expression.

### 1.3. Biological Functions of MTs

Since the biosynthesis of MT can be induced in response to exposure of an organism to Zn or Cd salts, MTs may play important roles in zinc metabolism and in the detoxification of cadmium and perhaps other heavy metals (Kägi and Nordber, 1979; Kojima and Kägi, 1978). Without induction, the MTs that are naturally present in an animal's liver and kidney may serve as the major storage form for the essential trace elements, Zn and Cu. Once either ion reaches a certain threshold level, the transcription rate of MT genes increases, leading to synthesis of more protein to bind the excess metal. On the other hand, the level of intracellular Zn regulates the turnover rate of MTs; when Zn is in short supply, MTs are rapidly degraded (Karin *et al.*, 1981). Conceivably, MTs could potentially modulate many important processes that involve Zn-requiring enzymes, e.g. DNA polymerase, RNA polymerase, alcohol dehydrogenase, and aspartate carbamoyltransferase, and Cu-requiring enzymes, e.g. amine oxidase, cytochrome c oxidase, tyrosinase, and dopamine-$\beta$-hydroxylase. The way that MTs carry Zn or Cu to different intracellular compartments and interact with different classes of enzymes is thought to be analogous to that of calmodulin in calcium metabolism.

The metal-binding role of MTs has recently been extended to the control of cell differentiation based on several cytological observations. Two examples that support this idea are as follows. First, the hepatic concentration of MT in neonatal rats is 20 times that of adults, but decreases rapidly and reaches the adult level at approximately 28 days (Wong and Kluassen, 1979). Second, the concentration of hepatic MT is maximal during gestation in the mouse embryo and decreases abruptly after day 15 of gestation (Andrews *et al.*, 1984). The predominant form of fetal MT is a soluble Zn-protein, but an insoluble Cu-form is also present. Since over 50% of the total fetal Zn is in the liver, and most of this is associated with MT, the protein may be a major reserve of metal ions during development.

Furthermore, since MTs are synthesized in response to UV radiation (Karin, 1985), the proteins may act as free radical scavengers as part of a protective system. Such a function has been demonstrated *in vitro* (Thornalley and Vasak, 1985). Alternatively, the protein may serve as the sources of Zn ions for DNA repair enzymes that are activated after irradiation. It has been shown that several Zn-requiring enzymes can be reactivated by the transfer of Zn from MTs to the apoenzymes (Udom and Brady, 1980).

### 1.4. Structures of the Metal-Binding Domains

The metal-binding capacity of MT is related to its high sulfhydryl content. With 30% of the amino acid residues being cysteine in the mammalian protein, there are no disulfide bridges or free sulfhydryl groups as shown by various spectroscopic measurements (Kägi and Vallee, 1961; Weser *et al.*, 1973; Rupp and Weser, 1978; Vasak *et al.*, 1981). Kägi *et al.* proposed that all of the cysteinyl sulfurs are directly involved in metal complexation (Kägi *et al.*, 1974). This has been confirmed by cadmium-113 nuclear magnetic resonance studies with crab and rabbit MTs. These experiments showed that each metal ion is bound to four thiolates, with some of the sulfurs forming bridges between adjacent metal atoms, and that three or four metal atoms are organized into individual clusters (Otvos and Armitage, 1979; Otvos and Armitage, 1980a; Otvos *et al.*, 1982; Boulanger et al., 1983). In the case of rabbit MT containing 7 moles of metal/mole of protein, one cluster contains four metals and the other contains three; they are designated cluster A and cluster B, respectively (Boulanger *et al.*, 1983). The postulated structures of these two clusters account for the known participation of all 20 of the cysteine residues in metal ligation,

11 in cluster A and 9 in cluster B. In the case of crab MT containing 6 moles of metal/mole of protein, the two separate metal-thiolate clusters are identical with the mammalian type B cluster in that three metal ions are found in each cluster (Otvos *et al.*, 1982). The polynuclear-cluster structure of MT was recently confirmed by Furey *et al.* in a crystal structure study of rat MT-II, containing 5 $Cd^{+2}$ and 2 $Zn^{+2}$ ions (1986). The cysteine sulfur coordination of the metal clusters A and B, as resolved in a 2.3 angstrom resolution electron density map, is depicted in Figure 1-2. Both the terminal and bridging thiolates present in the two metal clusters participate in a tetrahedral coordination for every metal ion involved.

The primary sequence of MTs contain redundant cysteine sequences, e.g. CXC occurs seven times (C, cysteine residue, X, variable residues), CXXC occurs six times, CXXXC occurs four times, and CC occurs 2 times. X-ray crystal data reveal that most of the cysteines at juxtapositions (15 out of 19) are coordinated to the same metal. As a result, numerous short loops arise from the constraints due to the metal coordination.

Evidence exists that the distribution of metal ions in the two clusters is not random. Crystal data show that both Zn sites are located in cluster B, each coordinated with a CXXC sequence. This is similar to the Zn site in liver alcohol dehydrogenase (Nielson and Winge, 1985). Other evidence suggests that Cu(I) is preferentially bound to the $\beta$ domain as shown by an independent cadmium-113 NMR study with calf liver MTs (Briggs and Armitage, 1982). The functional significance of the metal-binding specificity and preference in the $\beta$ domain is not clear at present.

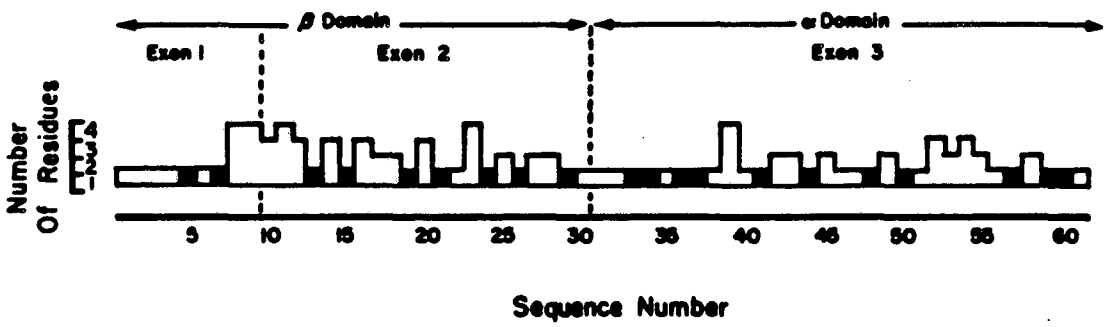
Both the $\alpha$ and $\beta$ domains also bind Cu(I) with a stoichiometry of six metals per domain (Nielson et al, 1985). This would require that all nine cysteines in the $\beta$ domain be bridging thiolates and that the terminal and bridging thiolates in the $\alpha$ domain be rearranged to accommodate six metals. The implication is that the protein is likely to change its conformation depending on type of the metals it binds. Another cadmium-113 NMR study with metal-reconstituted rabbit liver MT-II has indicated that multiple conformers exist in low ionic strength solution thus providing evidence for the structural flexibility of MTs in general (Vasak *et al.*, 1985). Indeed, the identity of the bound metal ions in mammalian MTs is known to vary, subject to such factors as the tissue origin, stage of development, and exposure to heavy metals both *in vivo* and *in vitro* (Kägi and Nordberg, 1979). As discussed earlier, a wide range of heavy metal ions, including

Au(I), Ag(I), Cu(I), Hg(II), Cd(II), Zn(II), Ni(II), and Co(II), have been found associated with MTs. Each of these metal ions has a unique coordination geometry, distance and van der Waals radius. The conformational changes within the protein and the degree of the changes in order to accommodate different metals are not understood.

### 1.5. Purpose of Study

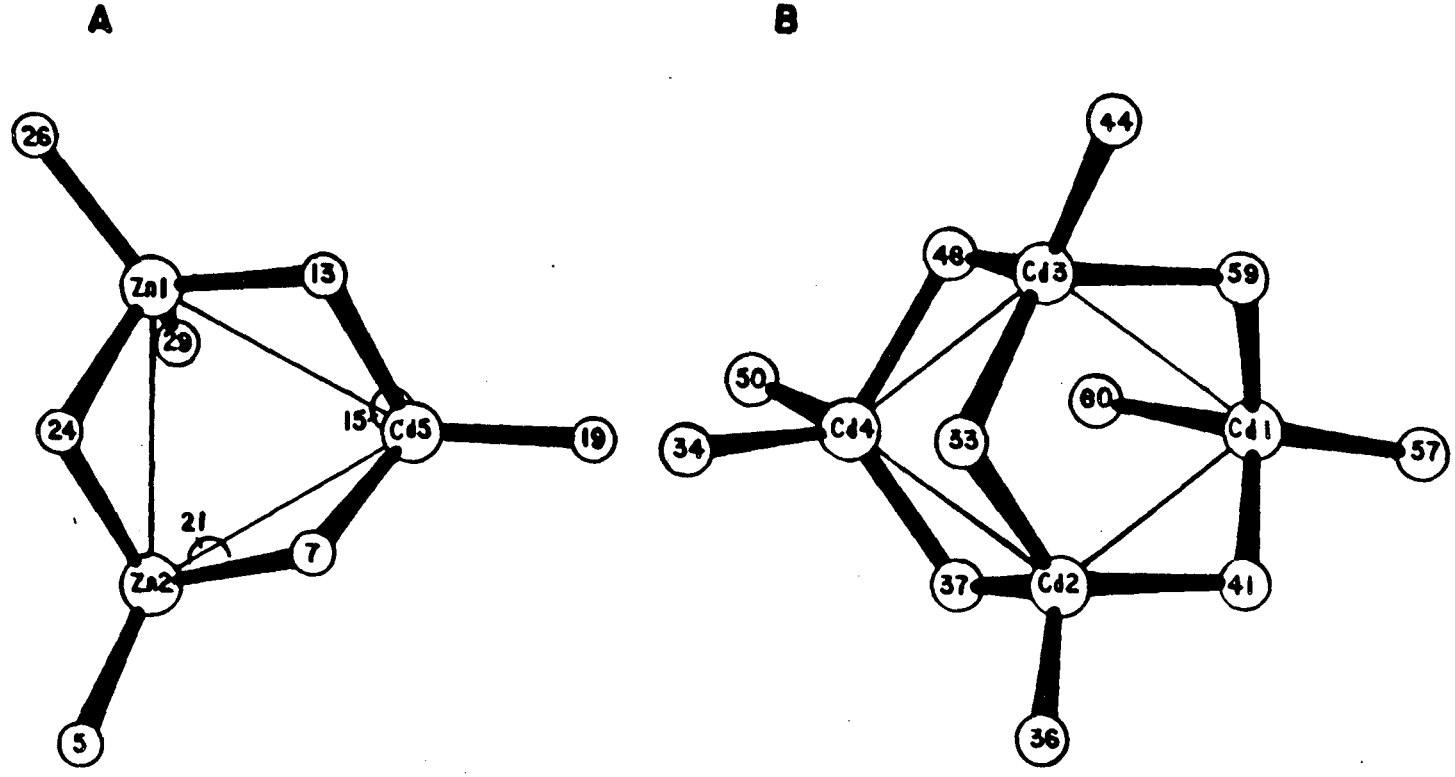
The purpose of this study is to establish that mammalian MTs are functional in microorganisms such as bacteria and yeast, i.e. expression of MT renders these microorganisms more resistant to a variety of heavy metal ions. The metal resistance phenotype of these clones will provide the basis for selecting mutants of MT with different specificities and binding strengths for various metal ions. These mutant MTs are useful for exploring the molecular basis for the conformational changes with different metal ions and for the site-selective metal-binding of MTs. Expression of a mammalian MT in bacteria or yeast has another advantage of producing a single isoform of MT. When MTs are isolated from mammalian tissues, they often contain microheterogeneity (Klauser *et al.*, 1983), which complicate the interpretation of results (Otvos and Armitage, 1980b). The results presented in this study are obtained by using a bacterial clone containing the mouse MT-I cDNA and a yeast clone containing the human MT-II cDNA. Several experiments show that the unique heavy metal-binding ability of eucaryotic MTs is successfully reflected in the phenotypes of both microorganisms.

Figure 1-1. Summary of amino acid sequence data of 11 mammalian MTs (Winge *et al.*, 1984). The extent of variation at each sequence position is shown. Cysteinyl residues are marked by filled squares. The metal binding domains as well as the three-exon coding regions in the mouse and human genes (Karin and Richards, 1982a; Durnam *et al.*, 1980) are indicated.


β Domain
α Domain
Exon 1
Exon 2
Exon 3
Number Of Residues
5
10
15
20
25
30
35
40
45
50
55
60
Sequence Number

- XBL 872-613 -

Figure 1-2. Structures of the three-metal (A) and four-cadmium (B) clusters of MT as depicted from X-ray crystal data of rat liver MT-II (Furey *et al.*, 1986). Only the cysteine and metal atoms are shown. The cysteine atoms are labelled with their cysteine residue numbers. Thin bonds represent metal-metal bonds.




- XBL 873-10808 -

# CHAPTER 2

## Materials and Methods

### 2.1. Strains

#### 2.1.1. Bacterial Strains

Except for plasmids containing a $P_L$ promoter, *Escherichia coli* K-12 strain HB101 (*gal*$^-$, *pro*$^-$, *leu*$^-$, *thi*$^-$, *Endo; I-hsm; hrs; recA, rpsl*) was used as the bacterial plasmid host. Long term storage of bacterial strains was in soft agar containing Luria broth (0.01% Bacto-tryptone, 0.05% Bacto-yeast extract, and 0.005% NaCl) and 0.75% Bacto-agar without antibiotics at room temperature. Short term storage was on Luria agar plates (Luria broth plus 1.5% agar) with antibiotics at 4°C. For plasmids containing a $P_L$ promoter, *E. coli* strain N99cI$^+$ (*Str*$^+$, *Su*$^-$) (obtained from Dr. M. Rosenberg) was used as the host for storage and plasmid purification. *E. coli* strain M5248 ($\lambda$ *bio275 cI857* $\Delta$*H1*) (Dr. M. Rosenberg) (Shimatake and Rosenberg, 1981) was used only for studying gene expression in bacteria and was normally stored as competent cells at -70°C.

#### 2.1.2. Yeast Strains

*Saccharomyces cerevisiae* strain AB103.1 (*MAT* $\alpha$ *leu2-3,112 ura3-52 his4-580 pep4-3*) was constructed by Dr. A. Brake (Chiron Corp.) and was used for studying gene expression in yeast.

*Saccharomyces cerevisiae* strain AB108 (*HMLα MATa* HMRα, Sir3-8**, leu2-3,112, ura3-52 ade6*) was also constructed by Dr. A. Brake and was used for studying gene expression using the yeast α-factor promoter. The strains were maintained on a slant of YEPD medium (1% Bacto-yeast extract, 2% Bacto-peptone, 2% Dextrose, 2% Bacto-agar) in a screw-cap tube at 4°C.

### 2.2. Culture Methods

Unless otherwise stated, Luria broth (LB) medium was used for growth of *E. coli* as described by Miller (1972) with the addition of 30 μg/ml ampicillin (Sigma A-6140) or 12.5 μg/ml tetracycline (Sigma T-3383) for selection of plasmid transformants. Yeast were cultured in the following media: YEPD (non-selective); ura$^-$ (2% glucose, 2% Bacto-agar, 0.7% yeast nitrogen base, 0.5% casamino acids, 0.005% adenine, 0.005% L-tryptophan, 50 μM $ZnSO_4$); YEPE (1% Bacto-yeast extract, 2% Bacto-peptone, 3% ethanol, 2% Bacto-agar). The transformed yeast were always maintained in selective media to insure the presence of the plasmid.

### 2.3. Plasmid DNA Isolation Procedures

#### 2.3.1. Alkaline Method for Small-Scale Plasmid DNA Preparations.

The method was modified from Ish-Horowicz and Burke (1981). A single colony was used to inoculate a 1.5 ml bacterial culture of LB with the appropriate antibiotics. The cultures were incubated at 37°C (30°C for M5248 and N99cI$^+$ cells) overnight and then transferred to an Eppendorf tube and centrifuged for 2 min. The cell pellet was resuspended in 100 μl of Solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0), vortexed to mix completely, and incubated at room temperature for 5 min. To it, 200 μl of Solution II (0.2 M NaOH, 1% SDS) was added, mixed gently, and placed on ice for 5 min. This was followed by neutralizing the mixture with 150 μl of ice-cold Solution III (3 M KOAc, pH 4.8) on ice for 5 min. The denatured chromosomal DNA was pelleted by centrifuging the tube in an Eppendorf for 2 min and the supernatant was transferred to a fresh tube. The supernatant was added 2 volumes of cold ethanol, vortexed, and incubated at room temperature for 2 min. Plasmid DNA in the supernatant was subsequently precipitated after a 2 min spin in an Eppendorf. The pellet was air dried and then dissolved in 50 μl of 0.1 M ammonium acetate, followed by phenol/chloroform

(1:1) and chloroform/isoamyl alcohol (24:1) extraction. Plasmid DNA was precipitated again by adding 3 volumes of cold ethanol, incubating the mixture at room temperature for 2 min, and then centrifuging for 2 min in an Eppendorf. The air-dried pellet was resuspended in 20 $\mu$l TE (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) and after adding 0.5 $\mu$l RNase A (1 mg/ml, Sigma R-5500, bovine pancreas) and was incubated at 37°C for 15 min. The plasmid DNA prepared by this protocol was clean enough to be analyzed by restrction enzyme digestion. The yield was usually 200 $\mu$g DNA from a 1.5 ml overnight culture.

### 2.3.2. Large-Scale Plasmid DNA Preparations

This protocol originated from Dr. R. Myers (University of California, San Francisco). A 10 ml LB overnight bacterial culture grown from a single bacterial colony was used to inoculate one liter of M9 medium (2% glucose, 2 $\mu$g/ml thionine, 1 mM $MgCl_2$, 0.5% casamino acid) (Maniatis *et al.*, 1982) with the appropriate antibiotics for selection. Bacterial growth at 37°C (30°C for N99cI$^+$ cells) was monitored by $OD_{595}$ values (optical density at 595 nm). Chloramphenicol (150 $\mu$g/ml) was added at $OD_{595}$ = 0.6-0.8 and the culture was incubated for an additional 16-18 hrs. Cells were centrifuged down in a Sorvall GSA rotor at 6000 rpm for 10 min, and pellets were washed in 0.15 M NaCl, combined and centrifuged again. Cells were resuspended in 10 ml lysis buffer (25% sucrose, 50 mM Tris-HCl, pH 8.0, 1 mM EDTA) containing freshly dissolved lysozyme (40 mg). Incubation with lysozyme was at 4°C for 5 min, followed by the addition of 4 ml EDTA (pH 8.0) at 4°C and continued for 10 min. Digestion was stopped by the addition of 2 ml triton buffer (3% tritonX-100, 50 mM Tris-HCl, pH 8.0, 62.5 mM EDTA) at 4°C for 15 min. The denatured chromosomal DNA was pelleted with a one hour spin at 17,000 rpm in an SW 27 rotor at 4°C, and discarded. The supernatant was transferred to a conical screw-cap tube, adjusted to 0.1 M NaCl, and was extracted with phenol/TE three times. Residual phenol was removed by ether extraction. Plasmid DNA was precipitated with 2.5 volumes of cold ethanol, chilled at -70°C for 1 hr, and centrifuged down in a Sorvall SS34 rotor at 18,000 rpm for 10 min. After vacuum-drying, the pellet was resuspended in 3.5 ml of TE with 100 $\mu$l of predigested pronase (Calbiochem, predigested at 37°C, 30 min) at 5 mg/ml and incubated for 1 hr at 37°C. The digestion was stopped with 100 $\mu$l of ethidium bromide (10 mg/ml, Sigma, E-8751) and the solution was adjusted to a refractive index of 1.4-1.5 by adding rougly 3.4 g of CsCl (BRL).

After a thorough mixing, the DNA solution was transferred into a 5 ml Beckman Quick-Seal tube with a pasteur pipet and mineral oil was used for balancing, if necessary. Supercoiled plasmid DNA was banded by a centrifugation at 55,000 rpm for 16-18 hrs at 4°C in a Beckman VTi65 rotor. The band was collected with a 1 ml syringe and a 22 gauge hypodermic needle. Ethidium bromide was then extracted with equal volumes of n-butanol until the pink color disappeared. The residual CsCl was removed by dialyzing the DNA solution against 1 liter of TE at 4°C overnight. Finally, plasmid DNA was recovered and aliquoted in Eppendorf tubes. Concentration was determined by measuring the $A_{260}$ and was checked by agarose gel electrophoresis. The yield of such a preparation was usually around 1 mg to 2 mg of plasmid DNA from 1 liter of cell culture.

### 2.3.3. Yeast DNA Miniscreen

A single colony was used to inoculate 5 ml of minimal medium or 1.5 ml of YEPD medium and the inoculum was cultured to saturation. Cells were spun down by centrifugation, resuspended in 1.0 ml of water and then transferred to a 1.5 ml Eppendorf tube and spun down again. The pellet was resuspended in 500 $\mu$l of sorbitol solution containing 1 M sorbitol, 100 mM EDTA, pH 8.0, 0.1% 2-mercaptoethanol and zymolase 60,000 at 200 $\mu$g/ml. After incubation at 37°C for 60 min, the cells were loosely dissociated from the broken cell wall and the spheroplasts were spun down in an Eppendorf for 15 sec. The pelleted spheroplasts were lysed upon adding 250 $\mu$l of 50 mM Tris-HCl, pH 7.5, containing 20 mM EDTA. The yeast chromosomal DNA was denatured by incubating the lysate with 25 $\mu$l 10% SDS at 65°C for 30 min. The lysate was subsequently neutralized with 100 $\mu$l 5 M KOAc and placed on ice for 30-60 min. The denatured chromosome was removed by centrifugation in an Eppendorf for 5 min and the supernatant containing plasmid DNA was transferred to a new tube and extracted with phenol/chloroform/isoamyl alcohol (25:24:1) as described above. The deproteinated DNA was incubated with 250 $\mu$l isopropanol at room temperature for 5 min, and pelleted by centrifugation in an Eppendorf for 2 min. The pellet was lyophilized and then dissolved in TE with the addition of RNase at 50 $\mu$g/ml at 65°C for 10 min. A 5 $\mu$l of sample was sufficient for restriction digestion and transformation.

## 2.4. Transformation

### 2.4.1. Bacterial Transformation with Plasmid DNA

This protocol was derived from Morrison (1979). Unless otherwise stated, all solutions and glassware were autoclaved and chilled at 4°C prior to use. A 2 ml overnight culture of bacterial cells was used to inoculate 200 ml LB in a 500 ml Erlenmyer flask and the $A_{595}$ was monitored. When the cells reached an $A_{595}$ = 0.6-0.8, they were centrifuged down at 4°C in a GSA rotor at 3000 rpm for 10 min. The cell pellet was resuspended by gently swirling in 100 ml of 10 mM NaCl. Pippetting cells in and out was avoided. The suspension was incubated at 4°C for 10 min and cells were pelleted as before. Cells were resuspended in 100 ml of 75 mM $CaCl_2$, incubated for 20 min on ice, and centrifuged as above. Cells were resuspended in 3.0 ml of 75 mM $CaCl_2$ and incubated for 30 min on ice. They were adjusted to a glycerol concentration of 14%, aliquoted in 200 $\mu$l portions, and incubated on ice for 2.5 hrs before they were stored at -70°C.

For each transformation 200 $\mu$l competent cells were thawed and added to 7.5 $\mu$l of 1 M $CaCl_2$ and the plasmid DNA was added to give a final volume of 300 $\mu$l. This mixture was placed on ice for 1 hr. A temperature shock was usually applied by placing the cells at 42°C for 1 min, then back on ice for 5 min. This step was omitted for the M5248 cells. The cells were then added to 3.0 ml LB and incubated at 37°C for 1 hr (30°C for M5248) without shaking. Aliquotes of 300 $\mu$l were then plated on LB plates containing appropriate antibiotics.

### 2.4.2. Yeast Transformation with Plasmid DNA

The protocol was modified from Hinnen *et al.* (1978) by Dr. A. Brake. A 100 ml fresh logarithmic phase culture was spun down, washed in water and resuspended in 5 ml 1 M sorbitol/15% glycerol. A 50 $\mu$l aliquot was treated with zymolase at 30°C for 1 hr to obtain spheroplasts. For cells that were not immediately used, aliquots of 1 ml in sorbitol/glycerol suspension were frozen in dry ice/ethanol and stored at -70°C. Spheroplasts were washed three times with 1 M sorbitol and resuspended in 500 $\mu$l of 1 M sorbitol, 10 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$. Plasmid DNA was added to a final concentration of 10-20 $\mu$g/ml and incubated for 5 min at room temperature. Then, 5 ml of 40% polyethylene glycol 4000 (Baker Chemical Co, Phillipsburg, NJ), 10 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$ was added to the mixture. After 10 min the spheroplasts

were centrifuged down and resuspended in 5.0 ml of the sorbitol/Tris/$CaCl_2$ mixture. Aliquots of 0.2 ml were added to 10 ml of regeneration agar and poured on minimal agar plates. Regeneration agar is Difco yeast nitrogen base without amino acids, supplemented with 1 M sorbitol, 2% glucose, 2% YEPD and 3% agar.

## 2.5. Cloning Techniques

### 2.5.1. Plasmids Used for Cloning

The following plasmids and DNAs were used for constructing MT clones during this study. The Rsa-12 plasmid was obtained from Dr. R. Palmiter at the University of Washington, Seattle. The phMT$_{II}$-3 plasmid was obtained from Dr. M. Karin at the University of Southern California. The bacterial expression vectors pAS1 and pNS3, respectively, were obtained from Dr. A. Shatzman at Smith Kline & French Laboratories and Dr. R. Scheuermann from University of California, Berkeley. The yeast $\alpha$-factor plasmid was obtained from Dr. A. Brake at Chiron Corp. (Emeryville, California.) The restriction fragment containing the alcohol dehydrogenase II promoter was provided by Dr. J. Schuster at Chiron Corp. The shuttle vectors pC1/1 and pAB18 were also provided by Dr. A. Brake. The EcoRI linker was prepared by K. Metchette in this laboratory. The XbaI-BamHI linker and the RsaI-SalI linker were prepared by Dr. G. Mullenback at Chiron Corp.

### 2.5.2. Restriction Enzyme Digestion and Gel Electrophoresis

Restriction endonucleases and T4 DNA ligase were purchased from BRL and used as recommended by the manufacturer. Agarose gel (0.7% to 1.0%) electrophoresis was conducted at 30 volts in 1X Tris-acetate buffer (40 mM Tris-HCl, 20 mM acetic acid, 2 mM EDTA, pH 8.0) until the running dye bromophenol blue reached the bottom of the gel. Nondenaturing 7% polyacrylamide gel electrophoresis on a minigel apparatus (Idea Inc.) was used to analyze short DNA fragments in 1X Tris-borate buffer (100 mM Tris-HCl, 100 mM boric acid, pH 8.3, 1.2 mM EDTA). DNA fragments were visualized by staining for 15 min in 2 $\mu$g/ml ethidium bromide and photographed with Polaroid type 107 film using a long wavelength ultraviolet light transluminator.

### 2.5.3. DNA Fragment Isolation

A slice of agarose or polyacrylamide gel containing a DNA fragment was placed in a piece of thin dialysis tubing with 2.0 ml of 1X Tris-acetate buffer. The DNA was electrophoresed out of the gel overnight at 30 volts in a horizontal gel box. Then the electrode poles were reversed and electrophoresis continued for 1 min. The buffer from the dialysis membrane was removed and the inside of the tubing was washed three times with 0.5 ml TE. After all the washes were combined, the DNA solution was adjusted to 0.1 M NaCl, phenol/chloroform extracted twice, chloroform/isoamyl alcohol extracted once, and the DNA precipitated with 2 volumes of ethanol. Addition of carrier RNA was often necessary because of low DNA concentration.

#### 2.5.3.1. Preparation of Dialysis Tubing

Dialysis tubing was soaked for 1 hr in 1% acetic acid, then rinsed in distilled water for several minutes. Distilled water was replaced with 1% $Na_2CO_3$, 1 mM EDTA and the tubing allowed to soak for an additional 30 min. The tubing was then soaked in fresh $Na_2CO_3$-EDTA and the solution was heated to a boil. This treatment was repeated once with the heat step reduced to 75°C. Liquid was poured off and the tubing was rinsed twice with distilled water, heated to 75°C, then washed one more time with distilled water. The prepared dialysis tubing was stored in 10 mM EDTA with 1 drop of chloroform at 4°C.

### 2.5.4. DNA Fragment Preparation

#### 2.5.4.1. Calf Intestine Alkaline Phosphatase Treatment

Calf intestine alkaline phosphatase (CIAP) was purchased from Boehringer Mannheim (Lot 1144107). The DNA fragment to be ligated or kinased was dissolved in TE and incubated with 1 $\mu$l of CIAP for 30 min under the recommended conditions. The reaction was stopped by extracting three times with phenol:chloroform followed by one extraction with chloroform:isoamyl alcohol. DNA was then precipitated with ethanol and NaOAc as usual. For the purpose of ligation, the CIAP treated linear vector was incubated with the fragment to be inserted at a 1:10 molar ratio. The ligation mixture contained 1 mM ATP, 1 $\mu$g/$\mu$l BSA (Fraction V, Sigma A-4503) and 1 unit of T4 DNA ligase in addition to ligase buffer. Ligation normally proceeded overnight at room temperature and at 14°C for sticky-end and blunt-end ligation, respectively.

### 2.5.4.2. 5'-End Labelling with T4 Polynucleotide Kinase

The CIAP-treated DNA fragments were dissolved in 10 $\mu$l TE and 5-10 $\mu$l of carrier free [$\gamma$-$^{32}$P]ATP (Amersham, PB. 10218, 5000 Ci/mmol) together with 1 $\mu$l of T4 polynucleotide kinase (Boehringer Mannheim). The reaction was incubated at 37°C for 30 min and was stopped by the addition of 250 $\mu$l 2.5 M $NH_4OAc$. The DNA was then precipitated with 2.5 volumes of ethanol and 1 $\mu$l tRNA (10 mg/ml, wheat germ, Sigma R-7876). After centrifugation in an Eppendorf tube, the nucleic acid pellet was dissolved in 250 $\mu$l 0.3 M NaOAc and precipitated with 750 $\mu$l ethanol again. Following the second precipitation and centrifugation the pellet was dissolved in 50 $\mu$l distilled water and precipitated by the addition of 1 ml of 95% ethanol.

### 2.5.4.3. Conversion of a Sticky End to a Blunt End by S1 Nuclease

The S1 nuclease was purchased from BRL (Lot 25311, 20,000 u/46 $\mu$l). Nuclease activity was titered with each DNA fragment individually. A total of 195 units of enzyme was incubated with 36 $\mu$g of linear Rsa-14 after a BglII digestion. The incubation was conducted at 5°C in the S1 buffer (BRL) for 10 min and stopped by adding 0.5 M EDTA and 2 M Tris-HCl, pH 8.6 to a final concentration of 24 mM and 100 mM, respectively. The DNA was subsequently phenol/chloroform extracted and precipitated with carrier. To estimate the extent of S1 digestion, the treated fragment was 5' end-labelled with T4 polynucleotide kinase and electrophoresed on a denaturing sequencing gel with molecular weight markers. The fragment was visualized by autoradiography.

### 2.5.4.4. Addition of Linkers to a DNA Fragment

The linkers were 5'-labelled with polynucleotide kinase (BRL). To make sure all linkers had a 5'-phosphate, the kinase reaction was supplemented with 1 mM ATP after 15 min at room temperature, and incubated an additional 30 min at 37°C. Linkers were added to restriction fragments in ligase buffer containing approximately 5 pmoles restriction fragment, 5 pmol kinased linkers and 6 units T4 DNA ligase. Reactions were incubated overnight at 4°C, phenol extracted, and ethanol precipitated. The reactions were subsequently digested with the proper restriction enzyme to prevent multiple linkage of the linkers. The restriction fragments were separated from unligated linkers by a preparative gel electrophoresis (see section 2.5.2.). Cherenkov counting

was used to determine the amount of radioactivity incorporated. The isolated fragments were ligated to the CIAP-treated vector as described in section 2.5.3.1.

### 2.6. DNA Sequencing Techniques by the Dideoxy Chain Termination Method

The following protocols were obtained from M. Alberti in this laboratory. The dideoxy sequencing reaction was modified from Sanger *et al.* (1977).

#### 2.6.1. Bacterial Transfection with Plasmid DNA

The DNA fragment to be sequenced was ligated into the EcoRI and BamHI restricted mp19 DNA (BRL). The ligation mixture was used to transfect *E. coli* strain JM101 (*Δlacpro, supE, thi, F'traD36, proAB, lacI$^q$, ZΔM15*) (Messing, 1983). An overnight culture of JM101 was diluted 20 fold into 10 ml fresh LB, grown at 37°C to mid-log phase, and was further diluted 50 fold into 40 ml LB. The culture was incubated at 37°C for 90 min and then chilled on ice for 2 hrs. Cells were pelleted down at 4°C in a Sorvall SS34 rotor (4000 rpm, 5 min) and resuspended in half volume of 50 mM $CaCl_2$ on ice for 1 hr. Cells were pelleted again and resuspended in 1/10 volume of 50 mM $CaCl_2$. An aliquot of 0.3 ml $CaCl_2$-treated cells was added to the ligation mixture in a sterile plastic tube. The incubation proceded at 4°C for 1 hr and then at 42°C for 2 min. Fresh JM101 cells at mid-log phase (200 μl) were immediately added into the mixture. This was followed by the addition of 10 μl of 100 mM IPTG (Isopropyl-β-D-thiogalactopyranoside, Sigma I-5502), 40 μl of 2% X-gal (5-bromo-4-chloro-3-indolyl-β-D-galatopyranoside, Sigma B-4252) and 3.0 ml soft agar B (1% bacto-tryptone, 0.8% NaCl, 0.75% bacto-agar) at 55°C. The contents of each tube were gently mixed and then poured onto a B plate (1% bacto-tryptone, 0.8% NaCl, 1.5% bacto-agar). After 10 min at room temperature, the plate was incubated at 37°C overnight. The appearance of milky, white plaques indicated the presence of an insert in the phage DNA.

#### 2.6.2. Isolation of Single-Stranded Phage DNA

A single white colony was picked up with a sterile pasteur pipet into 0.5 ml LB in a 1.5 ml Eppendorf tube. The phage particles were released into LB by heating the tube at 65°C for 20 min. The solution was then poured into 10 ml fresh mid-log JM101 cells and incubated on a Junior Orbit Shaker (Lab-Line Instruments, Inc.) (200 rpm) at 37°C for 5 hrs. Cells

were subsequently pelleted down in a SM24 rotor (10,000 rpm, 10 min) and the supernatant was transferred to a Sarstedt tube containing 2.0 ml of 25% (w/v) polyethylene glycol (PEG-6000) and 2.5 M NaCl. After incubation at 4°C overnight, the single-stranded viral DNA was precipitated by a centrifugation at 10,000 rpm for 10 min in a SM 24 rotor. The pellet was resuspended in 1.0 ml TEN (20 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 10 mM NaCl), transferred to an Eppendorf tube, and precipitated again after incubation with 100 $\mu$l of PEG/NaCl solution for 30 min. The DNA was dissolved in 10 $\mu$l TEN with 0.1% SDS, heated at 55°C for 10 min, and brought up to 100 $\mu$l volume with TEN. The solution was phenol extracted and ethanol precipitated. The pellet containing the single-stranded DNA was resuspended in 20 $\mu$l TE. An $OD_{260}$ was taken to determine the DNA concentration assuming 1 OD unit has 40 $\mu$g/ml DNA.

### 2.6.3. Dideoxy Chain Termination Reactions

In a 10 $\mu$l reaction volume, approximately 1 $\mu$g of single-stranded viral DNA was mixed with the universal primer (15 bases, PL Biochemicals, Lot 1516) at a ratio of one primer for every two templete DNAs in polymerase reaction buffer (70 mM Tris-HCl, pH 7.6, 50 mM NaCl, 5 mM 2-mercaptoethanol, 0.1 mM EDTA, 5 mM $MgCl_2$) containing 20 $\mu$Ci [$\alpha$-$^{32}$P]dATP (Amersham, PB. 10164, 400 Ci/mmol). The mixture was denatured at 55°C for 5 min and then slowly cooled to room temperature. Primer extension was initiated by adding 3 units of Klenow fragment (BRL, Lot 8012SB) to the reaction mixture. This was immediately followed by aliquoting 1/4 volume of the mixture into each dideoxy reaction cocktail in a separate tube. The various nucleotide (PL Biochemicals) concentrations for each dideoxy reaction were as follows: 5 $\mu$M dGTP, 180 $\mu$M ddGTP, 50 $\mu$M dTTP, 50 $\mu$M dCTP for the G reaction; 35 $\mu$M dGTP, 360 $\mu$M ddATP, 35 $\mu$M dTTP, 35 $\mu$M dCTP for the A reaction; 50 $\mu$M dGTP, 80 $\mu$M ddTTP, 5 $\mu$M dTTP, 50 $\mu$M dCTP for the T reaction; and 50 $\mu$M dGTP, 140 $\mu$M ddCTP, 50 $\mu$M dTTP, 5 $\mu$M dCTP for the C reaction. After 15 min at room temperature, each reaction was added 1 $\mu$l chase solution containing 1 mM each of the four deoxynucleotide triphosphates. After another 15 min at room temperature, each reaction was stopped by 100 $\mu$l stop buffer (200 mM NaCl, 0.5% SDS, 20 mM Tris-HCl, pH 7.2, 10 mM EDTA). The extended DNAs were subsequently precipitated by ethanol, dried, and dissolved in 10 $\mu$l loading dye (0.1% bromophenol blue, 0.1% xylene cyanol, 10 mM EDTA in ultra pure formamide). They were denatured at 95°C for 2 min, then rapidly

cooled on ice before being loaded onto a sequencing gel (40 cm x 20 cm x 0.4 mm) in the order of GATCG.

### 2.6.4. DNA Sequencing Gels

Sequencing gel plates were washed and siliconized with 1% tetramethylsilane before assembling. The combs and spacers were from a 0.4 mm Mylar sheet. The gels were from 5% to 10% (w/v) acrylamide, with an acrylamide:bis-acrylamide ratio of 1:20, in 8.3 M urea, 60 mM Tris-borate, pH 8.3 and 1.2 mM EDTA. The gels were pre-electrophoresed at 20 watts for 20 min and, after the samples were loaded, were run at 60 watts until either bromophenol blue or xylene cyanol migrated to the bottom. The gel was blotted to a piece of Whatman 3MM paper and was exposed to the Kodak X-Omat AR-5 film (35 cm x 43 cm, Merry X-ray) at -70°C overnight in the presence of a Dupont Cronex Lightning-Plus intensifying screen for the development of the autoradiogram.

## 2.7. Metal Resistance Tests

### 2.7.1. Metal Resistance of Bacterial Clone Harboring pYM101 or pNS3XY on Agar Plates Containing Heavy Metals

Agar plates containing heavy metals were prepared by adding ampicillin and a stock solution of heavy metal ions to sterilized LB broth containing 1.5% agar at 55°C to give the desired concentrations. These were 30 $\mu$g/ml for ampicillin and the critical concentration for each metal. The critical concentration was defined as the threshold concentration at which growth of the pAS1 plasmid transformed M5248 was inhibited by the heavy metal. After preparation, the plates were stored at 37°C overnight and then streaked with overnight cultures the next day. The streaked plates were incubated at 30°C for 2 hrs before being shifted to 37°C for 12 hrs. The experiments showed that the induction at 37°C gave better results than that at 42°C.

### 2.7.2. Metal Resistance of the Bacterial Clone Harboring pYM101 in Medium Containing $ZnCl_2$

A single colony of freshly transformed M5248 cells was used to inoculate 1.5 ml LB containing 30 $\mu$g/ml ampicillin and 0.1 mM $ZnCl_2$. The culture was grown at 30°C overnight and

the next day was diluted 30 fold into 6 ml of fresh LB medium containing 0.1 mM $ZnCl_2$ in a 250-ml side-arm flask. The freshly diluted culture was shaken vigorously at 30°C until an $OD_{595}$ of 0.25-0.3 as measured by a Spectronics 21 (Bausch & Lomb) was reached (usually within 2 hrs). At this point, the cultures were shifted to 42°C for 30 min and then the concentration of $ZnCl_2$ in the medium was raised to 1.1 mM. Growth of the culture was continued at 42°C for another 4 hrs while being monitored on an hourly basis. When cysteine (Sigma) was included, it was added at a concentration of 20 $\mu$g/ml at the beginning of the growth study.

### 2.7.3. Metal Resistance Test of the Yeast Clone Harboring pADH224 on Agar Plates

Yeast transformants were grown in selective media overnight. Cell culture (0.1 ml) was added to 3 ml of YEPD (0.1% glucose) top agar (0.75% agar) which was kept melted at 50°C. After mixing, this was overlayed onto a YEPD (2% glucose) agar plate and allowed to solidify for 5 min at room temperature. With sterile forceps, sterile Bacto-concentration disks containing different amounts of sterile metal solutions were placed on the top agar (5 disks/petri dish). The plates were incubated right side up overnight at 30°C and the zones of growth inhibition around the disks were measured. Bacto-concentration disks (1/4 inch) were purchased from Difco.

### 2.8. $^{109}CdCl_2$ Incorporation by the Bacterial Clone Harboring pYM101

The preparation of cultures before induction was the same as in the metal resistance test in medium containing 0.1 mM $ZnCl_2$ and 20 $\mu$g/ml cysteine (see 2.7.2. in this chapter). A 1.0 ml culture was quickly transferred to a separate tube containing 5 $\mu$l of $^{109}CdCl_2$ (Amersham, 119 $\mu$Ci/ml, 83 Ci/mmol) and 1.5 $\mu$l of 2 M Tris base at 42°C. The cells were induced for 100 min, roughly one generation, and after the $OD_{595}$ was measured, the induced cells were harvested in an Eppendorf centrifuge for 30 sec at room temperature. The pellet was washed twice with 1.0 ml saline and solubilized in 20 $\mu$l of Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% 2-mercaptoehanol) at 100°C for 5 min. The whole mixture was further diluted into 5.0 ml of Aquassure scintillation fluid (New England Nuclear) and counted in a liquid scintillation spectrometer using a $^{109}Cd$ channel (Packard, model 3385).

### 2.9. Pulse-Labelling Experiments

### 2.9.1. Pulse-Labelling of the Bacterial Clone Harboring pYM101 with [$^{35}$S]Cysteine

The procedure is a modification of that of Rosenberg *et al.* (1983). The culture was grown in complete M9 medium with 30 $\mu$g/ml ampicillin at 30°C until an $OD_{595}$ of 0.3-0.4 (Spectronic 21) was reached. The cells (3.0 ml) were spun down at 10,000 rpm for 3 min at 15°C in an SM24 rotor. The pellet was resuspended in 1.5 ml minimal medium without cysteine at 30°C and was quickly diluted with an equal volume of the same medium at 65°C in a 50-ml Erlenmyer flask for a rapid temperature shift. The cells were kept at 42°C for 30 min to deplete their internal pool of cysteine. A 200 $\mu$l aliquot of starved cells was labelled with 50 $\mu$Ci of [$^{35}$S]cysteine (Amersham, 1120 Ci/mmol, 11 $\mu$Ci/$\mu$l) for 30 sec at 42°C. The cells were then pelleted and immediately frozen in a dry ice/ethanol bath. When ready for analysis, the pellet was resuspended in 15 $\mu$l of buffer containing 60 mM Tris-HCl, pH 9.5, 2 mM EDTA, 2% SDS, and 5% 2-mercaptoethanol and boiled for 10 min. After cooling to room temperatue, the sample was incubated for 40 min with 40 mM dithiothreitol (DTT) (Sigma, D-0632) and 160 mM Tris-HCl, pH 8.0, at room temperature under nitrogen. The proteins were then subjected to carboxymethylation with 0.1 M sodium iodoacetate (Sigma), pH 7.0, for 1 hr under nitrogen in the dark. This reaction was stopped by the addition of 2 $\mu$l 2-mercaptoethanol, followed by precipitation with 10% trichloroacetic acid (TCA). The acetone washed pellet was lyophilized and analyzed by a SUDS polyacrylamide gel (see 2.10.4.) electrophoresis. The proteins were detected by fluorography with EN$^3$HANCE (New England Nuclear, NEF-966) (Laskey and Mills, 1975). Supplements of each of the 19 amino acids (Sigma) at 20 $\mu$g/ml in M9 formed the complete M9 medium. Eliminating cysteine and methionine from the complete M9 generated the minimal medium lacking cysteine.

### 2.9.2. Pulse-Labelling of A Yeast Clone Harboring pADH224 with [$^{35}$S]$H_2SO_4$

The [$^{35}$S]sulfate labelling of yeast followred the methods of Julius (1984) with modifications developed by P. Olsen (Chiron Corp.). The sulfate-free minimal medium was as described by Sherman *et al.* (1979) except that chloride salts were replaced by sulfate salts (low sulfate medium, LSM). Amino acid supplements lacking methionine and uridine were added for use with strain AB103.1. The methionine was left out because of the belief that extracellular methionine may repress the transport system for sulfate uptake. A 200 times concentrated sulfate free vitamin

solution contained biotin, 2 mg; D-panthothenic acid, 80 mg; inositol, 400 mg; p-aminobenzoic acid, 40 mg; pyridoxine (mono HCl), 80 mg; riboflavin, 40 mg; thiamine, 80 mg; folic acid, 0.14 mg; niacin, 28.6 mg dissolved in 1 liter of water.

Yeast clones were grown overnight in ura selective media at 30°C with aeration. They were diluted 1:100 into LSM media containing 100 $\mu$M $(NH_4)_2SO_4$ and 2% glucose and allowed to grow overnight until the $OD_{650}$ reached 1-2. The cells were collected by centrifugation, washed with labelling medium and resuspended in prewarmed media (LSM with 25 $\mu$M $(NH_4)_2SO_4$ and 3% ethanol) at 1-2 $OD_{650}$/ml. Following a 30 min pre-incubation at 30°C, cells were labelled with carrier-free sulphur-35 (as sulfate in aqueous solution, pH 6-8, Amersham, 1210 Ci/mmol) at 0.25 mCi/ml for the indicated times. An aliquot of 2 $\mu$l of cells were removed and spotted on a GF/A filter (Whatman). The filter was washed two times with 10% TCA and subsequently placed in Aquassure scintillation fluid for the determination of radioactive incorporation. An identical aliquot was centrifuged, and the pellet was boiled in 5 $\mu$l Laemmli sample buffer for 5 min. Samples were loaded on a 20% Laemmli-SDS polyacrylamide gel and the proteins were detected by fluorography.

### 2.10. Examination of MT Gene Expression by Polyacrylamide Gel Electrophoresis

#### 2.10.1. Preparation of Bacterial Lysates

##### 2.10.1.1. Preparation of Active Cell Lysate

LB with 0.1 mM $ZnCl_2$ was inoculated with M5248 bacterial cells harboring plasmids, from an overnight culture at 30°C with 30 $\mu$g/ml of ampicillin. Each 10 ml sample was incubated in a 50-ml Erlenmeyer flask at 30°C. When the culture reached an $OD_{595}$ of 0.5 on a Cary 118 spectrophotometer (about 3 hrs), the concentration of $ZnCl_2$ was raised to 0.4 mM and the culture was swirled vigorously in a 42°C water bath for 3 min. After 3 hrs of incubation at 42°C, the culture was spun in a Sorvall SM24 rotor at 4° at 10,000 rpm for 10 min. The pellet was resuspended in 5.0 ml ice-cold lysis buffer (10 mM $KPO_4$, pH 7.5, 300 mM KCl, 10 mM EDTA) with freshly added lysozyme (Calbiochem-Behring Corp. Lot 001838, hen egg white) (2 mg/ml) and incubated for 15 min at 37°C. Spheroplasts were obtained by incubating the pellet with low ionic strength buffer (10 mM Tris-HCl, pH 8.0, 0.2 mM $CdCl_2$) at 37°C for 10 min. Digestion

of the suspension with 8.5 $\mu$l of DNase (Millipore Corporation, Worthington) at 3 mg/ml and 1.5 $\mu$l of 1 M $MgCl_2$ at 37°C for 10 min cleared the lysates of most nucleic acids. Heating the lysate at 68°C for 10 min, followed by 2 min of centrifugation in an Eppendorf centrifuge, fractionated the heat-resistant proteins (the supernatant) from the heat-sensitive proteins (the pellet). For the purpose of dot-blot analysis, the pellet containing the heat-denatured protein was resupended in 100 $\mu$l of 10 mM Tris-HCl, pH 8.0, 0.2 mM $CdCl_2$ and 0.1% SDS. For the purpose of polyarylamide gel electrophoresis, both the supernatant and the pellet suspension were adjusted to 0.1% SDS, 5% 2-mercaptoethanol and boiled for 5 min.

### 2.10.1.2. Preparation of Triton-$Mg^{+2}$ Quick Lysates

Freshly transformed M5248 cells were cultured in LB media containing 0.1 mM $ZnCl_2$ until an $OD_{595}$ of 0.3 was reached. Induction of the culture was initiated by mixing a portion of the cells with an equal volume of the LB medium containing 0.5 mM $ZnCl_2$ at 65°C. After 45 min of incubation at 42°C, the cells in a volume of no more than 10 ml were harvested by centrifugation and frozen immediately in a dry ice/ethanol bath. The cell pelletes were resuspended in 500 $\mu$l of $Mg^{+2}$-buffer (15 mM Tris-HCl, pH 8.0, 1 mM PMSF, 0.2 mM $CdCl_2$, 10 mM $MgCl_2$). The PMSF (phenylmethyl-sulfonyl fluoride (Sigma P-7626) was freshly prepared in ethanol. The cells were opened by sonication at 50 watts on a Branson sonifier (model W185) for 10 sec. After sonication, the lysates were adjusted to 0.1% of triton X-100 and 2 ng/ml of DNase, respectively. The mixture was incubated for 5 min at 37°C and the digestion was stopped by heat-treatment at 68°C for 10 min, followed by a short spin in an Eppendorf. The proteins in the supernatant were TCA precipitated, acetone-washed twice and dried. The dry pellet was thus subjected to polyacrylamide gel analysis. The mouse MT-I seeded sample was prepared by adding the purified mouse MT-I to the cell suspension in the $Mg^{+2}$-buffer prior to sonication.

### 2.10.1.3. Isolation of MT from Lysates by a Preparative Laemmli-SDS Polyacrylamide Gel

The freshly transformed M5248 cells harboring a plasmid were cultivated and induced as described in section 2.10.1.1. A 13-ml portion of the induced cells were collected by a 5-min centrifugation at 10,000 rpm in a SM24 rotor. The cell pellet was freeze/thawed in 240 $\mu$l of 2X

Laemmli sample buffer and was boiled for 10 min. Unsolubilized debris was discarded after a 5-min spin in an Eppendorf. The supernatant was loaded onto a 15% polyacrylamide gel (13.5 cm x 17.5 cm x 1.5 mm) in Laemmli-SDS buffer together with 7 $\mu$l of BRL protein standard and 1 $\mu$g of mouse MT-I on the side lanes. The electrophoresis in the stacking gel was at 10 mA for 1 hr and that in the separating gel was at 20 mA for 5 hrs until the bromophenol blue reached the bottom of the gel. The protein standards were sliced out and stained by Coomassie brilliant blue. The various forms of mouse MT-I ran to positions corresponding to molecular weights of 6, 14, and 18 kilodaltons, respectively. The portion of the preparative gel covering proteins from 6 to 18 kilodaltons was cut out and transferred to a dialysis bag containing 10 ml of Laemmli running buffer with 0.1% DTT. The gel slice was electroeluted in a horizontal Bio-Rad gel apparatus at 65 volts for 2 hrs in the cold. The electroelution was reversed for 5 min under the same condition. The eluted proteins were precipitated by 12.5% TCA with 0.5 mg/ml of deoxycholate. The pellets were acetone washed and lyophilyzed. The contents of the eluted material were analyzed by a SUDS gel (see 2.10.4).

### 2.10.2. Preparation of Yeast Lysates Containing the ADHII Promoter

A single colony of the yeast transformants was used to inoculate a 2.0 ml culture in selective media. The culture was grown at 30°C overnight with aeration. The cells were then diluted 1:100 into YEPD (2% glucose) plus 1 mM $ZnSO_4$ and grown at 30°C with aeration for 30-48 hrs. $OD_{650}$ was monitored during this period of time. A 15-ml culture was pelleted in a 50 ml conical centrifuge tube at 2000 rpm for 10 min at 4°C in a Beckman JS 5.2 rotor. The cells were washed in 10 ml of cold lysis buffer (10 mM $Na_2HPO_4$, pH 7.5, 0.1% triton X-100, 50 $\mu$M $ZnSO_4$, 1mM PMSF) and spun again as above. The packed cell volume was measured and an equal volume of cold lysis buffer was added to it. After vortexing briefly, the cell suspension was transferred to a 1.5 ml Eppendorf tube and kept on ice. One-half volume of glass beads (0.45-0.5 mm) was added and the cells were vortexed six times for 1 min each time, keeping them on ice in between. The extract was spun at 4°C in an Eppendorf centrifuge for 15 min. The supernatant could then be used for polyacrylamide gel analysis.

### 2.10.3. Determination of Protein Concentration

The protein concentration in various lysates was determined by the Bradford assay (Bradford, 1976). The concentrated Bradford dye reagent was purchased from Bio-Rad (Lot 29172) and used as recommended for the microassay procedure. The sample was prepared in 0.8 ml of buffer at a protein concentration of less than 25 $\mu$g/ml and incubated with 0.2 ml of the concentrated dye reagent. After 5 min at room temperature, the $OD_{595}$ was measured. Each time the assay was performed, a serial dilution of BSA was included along with the samples to establish a standard curve. The concentration of the test sample was extrapolated from the standard curve.

### 2.10.4. SDS/Urea (SUDS) Polyacrylamide Gel Electrophoresis

The gel was a modification of the one used by Kyte and Rodriguez (1983) to resolve low molecular weight proteins. It consisted of a 20% polyacrylamide gel in 1.615 M Tris-HCl, 0.5% SDS (pH 3.9) as the separation portion and a 5% polyacrylamide gel in 1.47 M HCl, 1.8 M pyridine (pH 4.4) as the stacking portion, both with 8 M urea. The buffer in the upper reservoir was 0.213 M 2[N-Morpholino]ethanesulfonic acid (MES), 0.269 M pyridine, 0.1% SDS, pH 5.8 and that in the lower reservoir was 0.323 M Tris base, 0.323 M Tris-HCl, pH 8.2. The samples were prepared by boiling in 5% SDS, 5% 2-mercaptoethanol for 5 min, lyophilizing and then resuspending in sample buffer (0.37 M HCl, 0.34 M pyridine, 8 M urea, 0.1% SDS). Electrophoresis was conducted at a constant current of 30 mA for 12 hrs on a vertical BRL apparatus (21.3 cm x 17.8 cm x 21 cm). The dimensions of the gel was 17 cm x 15 cm x 0.75 mm.

### 2.10.5. Examination of the Secreted Gene Product Expressed by Yeast $\alpha$-Factor Promoter

Yeast cells harboring a recombinant plasmid containing the $\alpha$-factor promoter and the pC1/1 vector were grown in $leu^-$ medium to an $OD_{650}$ between 4-6. Cells were removed by a 15-min centrifugation on a table-top centrifuge and protein was precipitated from the supernatant with TCA and deoxycholate at final concentrations of 12% and 0.25 mg/ml, respectively. The TCA mixture was placed on ice for one hour and then centrifuged at 7000 rpm for 10 min at 4°C. The pellet was thoroughly washed by vortexing the pellet in 1 ml cold acetone and spun in an Eppendorf. The washing step was repeated once more to remove the residual TCA. After

lyophilization, the pellet was ready for polyacrylamide gel analysis and was treated as described.

### 2.11. Immunological Experiments

Elisa, dot blot, and Western blot were used in the process of identifying various MT clones. The antiserum directed against horse kidney MT-I was prepared by Antibody Inc. (Davis, California.) The antiserum directed against rat liver MT-II was prepared in the laboratory of Dr. F. Brady (University of South Dakota) who also provided horse kidney MT-I, MT-II and rabbit liver MT-I, MT-II. Mouse liver MT-I, MT-II and rat liver MT-I, and MT-II were provided by Dr. D. Winge (Utah Medical Center). The purified rabbit immunoglobulin (IgG) was prepared by Dr. G. Meng in this laboratory. The goat anti-rabbit peroxidase (GAR-POD) was purchased from Boehringer-Mannheim.

#### 2.11.1. Elisa

The enzyme-linked immunosorbent assay (Elisa) used here was first developed by Voller *et al.* (1980) and was modified by Dr. R. Kim in this laboratory. The test sample was directly applied to the sample well on a plastic titer plate (Fisher). The plate was placed in a tray partly filled with saline and covered by saran wrap to keep it moist. Applicaton of antigen to the titer plate was done without mechanical shaking at either room temperature for one day or at 37°C for 2 hrs. Liquid was subsequently sucked out and each well on the plate was washed by three incubations of 0.02% triton X-100 in borate buffer, which contains 6.18 g of boric acid, 9.54 g of Borax (sodium tetraborate) and 1.38 g of sodium chloride in a total volume of one liter. Each wash solution was incubated for 10 min on the plate and then dumped out afterwards. The sample well was further coated with 50 $\mu$l of 10% goat serum in borate buffer for one hour at room temperature. The interaction of antiserum with the coated antigen was conducted at 37°C for 2 hrs without shaking and was terminated by three washes of 0.02% triton X-100 as described above. The application of a peroxidase-linked secondary antiserum to the titer plate was repeated as in the antiserum reaction. The amount of peroxidase left on the plate was titered with 50 $\mu$l of o-phenylenediamine-2HCl (OPD) and quantitated by the optical density of the developed color with an Elisa Reader machine (Bio-Rad). The readings were taken in the presence of a 490 nm filter. The OPD solution was prepared immediately prior to use by dissolving one tablet of OPD

(Sigma, P-2781) in 5 ml of 50 mM citrate buffer, pH 5.5, to which was added 2 $\mu$l of 30% $H_2O_2$. The development of color was complete after 5 min in the dark and was terminated by adding 200 $\mu$l of 1 N $H_2SO_4$.

### 2.11.2. Dot Blot

The dot blot apparatus (Minifold) was purchased from Schleicher & Schuell (Keene, New Hampshire). The apparatus can analyze up to 96 samples on the same blotting membrane. The following membranes have been used in the process of identifying MT clones: nitrocellulose paper, zeta-probe paper, nytran paper, carboxymethyl cellulose paper, and cyanogen bromide (CNBr) activated paper.

#### 2.11.2.1. Dot Blot Using Zeta-Probe Paper

The zeta-probe blotting membrane was purchased from Bio-Rad (catalog number 162-0514) and was soaked in TBS buffer (20 mM Tris-HCl, pH 7.5, 500 mM NaCl) at room temperature for 5 min prior to use. The metallothionein proteins and various cell lysates were attached to the membrane by the Minifold apparatus. The unfilled protein binding sites on the zeta-probe membrane were subsequently blocked by incubating the membrane with 10% BSA in TBS buffer at 50°C overnight. All the incubations were done on a tilt shaker (Thermolyne Corporation, model M26125). The membrane was washed twice for 5 min in TBS and 0.05% Tween 20 (polyoxyethylene sorbitan monolaurate, Sigma P-1379) at room temperature. Incubation of the membrane with 10 $\mu$l of the first antiserum in 5.0 ml TBS and 1% BSA was in a sealed plastic bag at room temperature for 1-2 hrs. After three washes of 10 min each with TBS, 0.05% Tween 20 and 1% BSA at room temperature, the membrane was incubated with 20 $\mu$l $^{125}$I-labelled protein A (Amersham, IM. 112, 100 $\mu$Ci/ml) and 5 ml TBS containing 1% BSA in a sealed plastic bag for 1 hr at room temperature. This was followed by three washes as before. The washed zeta-probe membrane was air-dried and then autoradiographed as described in 2.6.4. with an intensifying screen.

Alternatively, the $^{125}$I-protein A was substituted by the peroxidase conjugated second antibody (Boehringer Mannheim). Development of the color reaction was started by adding 1 ml of 4-chloro-1-naphthol (0.5 mg/ml in 10 mM Tris-HCl, pH 7.5, freshly diluted from a stock of 20

mg/ml in DMSO) to the paper and then incubating the paper at room temperature for 20 min, followed by the addition of 40 $\mu$l of 30% $H_2O_2$ for 1 hr at room temprerature.

### 2.11.2.2. Dot Blot Using CNBr Activated Paper

The CNBr activated paper was prepared in this laboratory (see 2.11.2.2.1.). It was pre-wetted in Solution A (50 mM sodium acetate, pH 7.0, 0.025% sodium azide) before setting up the dot blot apparatus. The samples of protein were diluted in Solution A and then applied to the dot blot apparatus. Each sample was washed once with 50 $\mu$l of Solution A. The CNBr activated paper was then removed and put in a stop bath solution (1% BSA, 1 M glycine) and shaken for 30 min at room temperature. A similar incubation with hybridization buffer (0.127 M NaCl, 2.5 mM $NaH_2PO_4$, 7.5 mM $Na_2HPO_4$, 0.1% Tergitol NP40, 0.01% sodium azide, 0.1% BSA) for 30 min then followed. The paper was then placed in a sealed plastic bag containing 5 ml of hybridization buffer and 10 $\mu$l of the first antibody solution. After a 3-hr incubation at room temperature on a tilt shaker, the paper was removed from the bag and was washed with 10 ml of hybridization buffer three times for 15 min each. The paper was subsequently incubated with 20 $\mu$l of $^{125}$I-labelled protein A (Amershm) for 1 hr and washed three times as above. If $^{109}$Cd was used, the same protocol was followed except that 1 $\mu$Ci of $^{109}CdCl_2$ (Amersham, 0.162 $\mu$g/ml) was included in the hybridization buffer instead of the antiserum and that the second incubation with protein A was eliminated. After hybridization and washing, the paper was air-dried, and subjected to autoradiogram analysis as described in section 2.6.4..

#### 2.11.2.2.1. Preparation of CNBr Activated Paper

The protocol is modified from Clarke *et al.* (1979) by Dr. A. Kazuko in this laboratory. Twenty 15 cm x 22.5 cm Whatman No. 52 sheets (VWR, Whatman 1452-270) were first soaked in 0.1 M $NaHCO_3$ solution for 30 min, and then in water for 45 min at room temperature. Gentle shaking was provided during the soaking and washing procedures. In the meantime, 25 g of CNBr powder (Kodak, Cat No. 919) was dissolved in 1 liter of distilled water in a 2-liter baking dish inside a fume hood. To initiate the activation process, the washed paper sheets were placed in the CNBr solution and droplets of 2 N NaOH were added to the solution to bring the pH to 10. The pH was maintained near 10 by the occasional addition of 2 N NaOH while vigorously

shaking the dish on a Junior Orbit Shaker for 5 min. The reaction was stopped by decanting the CNBr solution, and the sheets were washed with 500 ml of 0.1 M $NaHCO_3$ for 15 min. Five sheets at a time were then washed twice with water for 10 min and once with acetone for 5 min. The paper sheets were vacuum-dried and stored over desiccant at 4°C. The treated paper was stable for several months.

### 2.11.2.3. Dot Blot Using Nitrocellulose, Nytran, and Carboxymethyl Cellulose Paper

The nitrocellulosse paper (BA85R597, Lot 4138/4, 0.45 $\mu$m), the nytran paper (41-01040, Lot 10104, 0.45 $\mu$m) and the carboxymethyl cellulose paper (NA49, 0.45 $\mu$m) were all purchased from Schleicher & Schuell. The dot blot procedure of using each of the papers was as described with the CNBr activated paper except for the following changes for each respective membrane.

The nitrocellulose and the carboxymethyl cellulose paper, respectively, were wetted in PBS (140 mM NaCl, 3 mM KCl, 8 mM $Na_2HPO_4$, and 1.5 mM $KH_2PO_4$, pH 7.3) and 1% acetic acid prior to sample application. Both were incubated at room temperature with 10% normal goat serum (Chiron Corp.) in PBS for 45 min to block the free binding sites. Hybridization with the first and the second antibodies were in the presence of blocking solution for 3 hrs at room temperature. After each hybridization, both membranes were washed twice for 10 min with PBS, 0.1% deoxycholate-triton X-100 and once with PBS, containing 1% normal goat serum.

The nytran paper was wetted in 40 mM $NaH_2PO_4$ buffer (pH 7.4) prior to use. Blocking of the free binding sites was done by incubating the paper in a solution containing 200 mM NaCl, 15 mM Tris-HCl, pH 7.4, and 10% BSA at 48°C for 4-10 hrs. Hybridizations with the first and the second antibodies were carried out in blocking solution which contained 2% BSA for 1-5 hrs and 1 hr, respectively. The first wash was in 200 mM NaCl, 15 mM Tris-HCl, pH 7.4, for 5 min, followed by three washes of 10 min each in the same buffer containing 0.15% (v/v) triton X-100 for 10 min each.

#### 2.11.2.3.1. Staining of Protein on Carboxymethyl Cellulose Paper

After samples were applied on the carboxymethyl cellulose paper, the paper was stained with 0.1% amido black in 10% acetic acid/50% methanol for 15 min, Destaining was accomplished in 10% acetic acid.

### 2.11.3. Western Blot

The test samples were resolved by electrophoresis on a polyacrylamide gel using appropriate buffer conditions. After electrophoresis, the gel was removed from the plates and was soaked twice in water for 15 min at room temperature with gentle shaking on a Junior Orbit Shaker. The rinsed gel was subsequently equilibrated in the transfer buffer (50 mM sodium acetate, pH 7.0) for 30 min at room temperature, again with shaking. The gel was then placed between the prewetted CNBr activated paper and 2 or 3 sheets of Whatman 3 MM paper, all wetted in the same acetate buffer. Transfer of the proteins from the gel to the CNBr activated paper was achieved by electrophoresing the sandwich overnight at 8 volts in the transfer buffer. Alternatively, electroblotting was achieved in 2 hrs using the same buffer at 15 volts with cooling. After the transfer, the CNBr activated paper was blocked of free binding sites and then hybridized to the antibody or the $^{109}Cd$ as in the dot blot procedure. The gel could then be silver stained for protein detection to assure complete transfer.

### 2.12. Stability Test

The crude extract was prepared from an induced M5248 culture harboring pAS1 grown in 0.4 mM $ZnCl_2$ as described in section 2.7.2.. A 1.3 ml crude extract was obtained by sonicating 13 ml of induced cells in 200 mM Tris-HCl, pH 9.5, 1 mM $CdCl_2$, 0.1% triton X-100 and 2 mM PMSF at 50 watts for two bursts of 10 sec each on a Branson sonifier. The sonicated extract was heat treated at 68°C for 10 min and the denatured proteins were removed by centrifugation. The aliquoted supernatant (130 $\mu$l each), containing the heat-resistant proteins from 1.0 ml of cells, was immediately frozen.

To test proteolysis, one vial of such an extract was thawed out on ice and 19 $\mu$l was incubated with 0.6 $\mu$g of purified rat liver MT-II for 20 min at 22°C. The incubation was stopped by mixing 12 $\mu$l of Laemmli sample buffer into the reaction and boiling for 5 min. Alternatively, 0.6 $\mu$g of rat MT-II was incubated with a serial dilution of crude extract at room temperature for 5 min. The presence of rat MT-II protein in the extract was detected by SUDS gel electrophoresis, followed by silver staining.

# CHAPTER 3

## Construction of MT Clones

### 3.1. Introduction

The mouse liver MT-I gene and the human MT-II gene, respectively, were cloned into bacterial and yeast expression systems. In order to search for a clone that effectively expressed MT, at least two expression vectors were used in the construction of each gene. This chapter describes the clones that showed evidence of gene expression. These are bacterial clones involving the pAS1 and the pNS3 vectors, both of which contain a $\lambda$ $P_L$ promoter, and yeast clones involving the $\alpha$-factor promoter and the alcohol dehydrogenase II (ADHII) promoter. The work on the construction of yeast MT clones was done by Dr. R. Kim in this laboratory in collaboration with Chiron Corp. (Emeryville, California).

### 3.1.1. Factors Considered for Constructing MT Clones

#### 3.1.1.1. Bacterial MT Clones

Three basic factors which determine the efficiency of expressin of a eucaryotic gene in bacteria were considered (Rosenberg and Court, 1979; Grunberg-Manago and Gross, 1977). They are the promoter, the ribosome binding site and the spacing between the ribosome binding site and the initiation codon of the gene. The phage $\lambda$ promoter $P_L$ has been shown to be 8-10 times

more efficient than the bacterial promoter of the lactose operon ($P_{lac}$) (McKenney *et al.*, 1981; Rosenberg *et al.*, 1981; Shatzman and Rosenberg, unpublished data). Previous studies of the $P_L$ promoter had indicated that overexpression of a foreign gene may inhibit the growth of host cells (Shimatake and Rosenberg, 1981). To regulate the $P_L$ promoter, a plasmid containing the gene downstream from the $P_L$ promoter is used to transform bacterial cells harboring a temperature-sensitive $cI^{857}$ repressor. In the bacterial strain M5248, which contains the $cI^{857}$ repressor (see 2.1.1.), $P_L$ transcription was inhibited at 30°C and activated at 42°C (Shimatake and Rosenberg, 1981).

The ribosome binding site, also known as the Shine and Dalgarno (SD) sequence (Shine and Dalgarno, 1975), facilitates the initiation of protein translation. For the majority of bacterial translation signals, it is composed of a tetra-nucleotide, AGGA, and is complementary to the 3′ end of the 16S rRNA. The SD sequence in the pAS1 vector is that for the λ cII protein (TAAGGA) and the SD sequence in the pNS3 vector is a synthetic product containing a hybrid AGGA (AGGAGGA).

The spacing between the SD sequence and the initiation codon of the gene is normally between 3 and 12 base pairs (Steitz, 1979). Within such a small distance, the variation of the spacing has been shown to have a profound effect, as much as 1000 fold difference, on the level of gene expression (Guarente *et al.*, 1980; Thummel *et al.*, 1981; Backman and Ptashne, 1978; Backman *et al.*, 1976). For a particular expression system, optimal spacing cannot be predicted and must be determined experimentally. In the pAS1 vector, the spacing is 10 base pairs between the SD sequence and the initiation codon of the cII protein. In the pNS3 vector, this spacing can be adjusted, as will be described in section 3.2.1.2..

In addition to the above factors, gene expression is also dependent on : (i) the stability of the mRNA; (ii) the stability of the gene product; and (iii) the potential lethality of that product to the host; which may in turn influence the expression of the cloned gene.

The bacterial expression vectors pAS1 (Rosenberg *et al.*, 1983) and pNS3 (Scheuermann, 1986) were used in parallel to study the MT gene expression. The major difference between these two vectors is that the N gene sequence and a site necessary for N utilization (Nut site) in the pAS1 vector is absent in the pNS3 vector. The N plus Nut system inhibits premature termination

within the $P_L$ transcription unit (Heinemann and Spiegelman, 1971; Rosenberg *et al.*, 1978) and within the coding sequence. Such anti-termination sequence can lead to a dramatic increase in gene expression if a termination sequence is present between the $P_L$ and the initiation codon of a gene, as is the case of the *galK* gene protein (Rosenberg *et al.*, 1983). However, if no termination sequence is present, the N plus Nut system interferes with cell growth and would be preferably deleted, as is the case of the *dnaQ* gene expression (R. Scheuermann, personal communication).

### 3.1.2. Yeast MT Clones

Two systems in which a large quantity of a foreign protein can be expressed by yeast were explored for MT expression. In one, by using the yeast α-factor promoter and leader sequence, MT was synthesized as part of a larger precursor including the α-factor peptide as the signal sequence. In the other, by using the yeast ADHII promoter, MT was synthesized as the mature gene product and remained inside the cytoplasm.

The yeast α-factor promoter and the yeast α-factor leader sequence direct the synthesis and secretion of the yeast α-factor protein (Thorner, 1981). The α-factor protein contains a 13-residue α-factor peptide, which acts as a mediator for the conjugation between the α mating type and the *a* mating type (Julius *et al.*, 1983; Brake *et al.*, 1983; Julius *et al.*, 1984). In the construction scheme using the α-factor promoter/leader sequence, the DNA sequence coding for MT was linked in phase to the DNA sequence coding for the α-factor peptide. During the process of secretion, the α-factor peptide should be cleaved by a membrane-bound protease and MT released into the medium. The α-factor promoter/leader sequence was chosen because of its efficient processing and secretion. In the case of expressing human epidermal growth factor, a yield of between 5 and 10 milligrams of protein per liter of yeast culture was reported (Brake *et al.*, 1984). One apparant advantage of this secretion system is that because MT does not accumulate inside the yeast cells, the potential lethal effect of MT on the organism is presumably reduced.

The yeast ADHII promoter directs the synthesis of isoform II of the three alcohol dehydrogenase isozymes in *S. cerevisiae*. The isoform II is involved in ethanol utilization and is a glucose-repressed enzyme (Young *et al.*, 1982). The ADHII promoter was chosen because the MT gene expression can be regulated by level of glucose at the transcription (Denis *et al.*,

1981). This has been shown by the observation that when yeast cells growing on 2% glucose are shifted to medium containing a non-fermentable carbon source, such as 3% ethanol or glycerol, the mRNA from the ADHII promoter is derepressed. The construction of MT using the ADHII promoter requires the presence of a terminator sequence for the proper transcription termination and polyadenylation. This was provided by the $\alpha$-factor terminator sequence. It has been demonstated by Hitzeman *et al.* (1982) that heterologous promoter and terminator sequences can be joined together. In the expression of the human leukocyte interferon D gene, the transcript from the yeast alcohol dehydrogenase I promoter was efficiently terminated by the terminator sequence of the yeast *TRP* I gene, which codes for the N-5′-phosphoribosyl anthranilate isomerase (Stinchcomb *et al.*, 1979; Tschumper and Carbon, 1980). However, it is likely that MT expression using this internal system will be more subject to factors such as the stability and the lethal effect of the protein product than expression based on the $\alpha$-factor system.

### 3.1.2. The MT Genes

#### 3.1.2.1. The Mouse Liver MT-I cDNA

The original construction of a cDNA clone containing the gene for mouse liver MT-I was conducted in the laboratory of Dr. R. Palmiter and was described by Durnam *et al.* (1980). The basic strategy consisted of the following steps: (i) isolation of a mRNA fraction enriched for MT mRNA activity from the liver of $Cd^{+2}$-induced Swiss Webster mouse, (ii) synthesis of double-stranded cDNA using the enriched MT mRNA as a template and insertion of the cDNA into pBR322, (iii) transformation of the recombinant plasmids into $\chi$1774 cells and screening for a MT clone which contained the largest piece of the insert and also hybridized to the MT mRNA. Using the $^{32}$P-labelled insert of the cDNA clone as a probe, Durnam *et al.* screened a myeloma library and an embryo library and obtained two genomic MT clones. After sequencing the inserts in the two genomic MT clones, the genomic sequence of the mouse MT-I gene and its flanking regions was established. Comparison of the genomic sequence with that of the cDNA clone showed that the coding region of the gene is interrupted by two introns. The complete DNA sequence indicating the locations of exons relative to those of introns was published by Glanville *et al.* (1981). With the first nucleotide in the promoter site of the mouse MT-I gene

designated as the -1 position, the three exons encompassed from positions -51 to 101, 585 to 650, and 862 to 1086, respectively.

Our laboratory received from Dr. G. Stuart a cDNA clone, Rsa-12, which was constructed in the laboratory of Dr. R. Palmiter (personal communication). Plasmid Rsa-12 was derived from the original cDNA construction with the following modifications: (i) Rsa-12 only encompasses the nucleotide sequence of mouse MT-I gene from position 26 to position 1055 excluding the intervening sequences; (ii) the cDNA sequence of mouse MT-I in Rsa-12 was flanked by two BamHI linkers of the decamer sequence, CGGGATCCCG, (iii) plasmid Rsa-12 contains two copies of the mouse MT-I cDNA inserted into the BamHI site of pBR322 and is devoid of the nucleotide sequence from the SalI site to the PvuII site of pBR322, and (vi) plasmid Rsa-12 has an extra piece for high copy mutant number inserted with two Xho linkers near position 3000 of pBR322. Figure 3-1 shows the DNA insert in Rsa-12, which contains the DNA sequence surrounding the cDNA and the coding sequence for the mouse MT-I protein. The entire insert of 355 base pairs contains a BglII restriction site 5′ to the initiation codon and an SstII restriction site located 161 nucleotides downstream from the ATG sequence.

### 3.1.2.2. The Human MT-II cDNA

Isolation of a cDNA clone for the human MT-II gene was accomplised by Dr. M. Karin (Karin and Richards, 1982). Briefly, total mRNA from HeLa cells, previously induced with cadmium in the presence of cycloheximide, was used as a template for the synthesis of cDNA. This DNA was cloned into *E. coli* and the individual clones were screened with a short DNA probe whose sequence coded for the N-terminal fragment of human MT-II. Of those clones that hybridized to the probe, the one with the longest insert was chosen and its plasmid designated phMTII-3. The clone was further shown to cross-hybridize with a restriction fragment of a cloned mouse MT-I cDNA (Durnam *et al.*, 1980). The insert in phMTII-3 was subsequently sequenced.

Our laboratory received the plasmid phMTII-3 from Dr. M. Karin. Figure 3-2 shows the nucleotide sequence of human MT-II cDNA in plasmid phMTII-3, in which the cDNA sequence is flanked by 5′ and 3′ untranslated regions of 57 and 128 nucleotides, respectively. Nucleotides are numbered from the ATG initiation codon. Figure 3-2 also shows the amino acid sequence

above the human MT-II protein from the nucleotide sequence. Except for residues 58 and 59, which are actually conserved with respect to the rodent MT sequence (Kägi and Nordberg, 1978; Huang *et al.*, 1977), the predicted peptide sequence is identical to that of the published human MT-II protein sequence (Kissling and Kägi, 1977).

## 3.2. Results

### 3.2.1. Bacterial MT Clones

#### 3.2.1.1. Construction of the pYM101 Plamsid

Plasmid pBRsa-14 was derived from plasmid Rsa-12. This was done by restricting plasmid Rsa-12 with BamHI and inserting the 355 base-pair (bp) mouse MT-I cDNA fragment into the BamHI site of plasmid pBR322. The construct with the insert in the same orientation as the tetracycline gene was named pBRsa-14. In this laboratory, plasmid pBRsa-14, instead of plasmid Rsa-12, is the main source of the mouse MT-I cDNA. This is because isolation of plasmid pBRsa-14 by the method of CsCl-ethidium bromide density gradient ultracentrifugation (see 2.3.2.) gave a 5 fold greater yield than that of plasmid Rsa-12. For unknown reasons, plasmid Rsa-12 did not accumulate as much as plasmid pBRsa-14 in *E. coli* strain HB101 when the cellular protein synthesis was inhibited by chloramphenicol.

To construct plasmid pYM101, the bacterial expression vector pAS1 was used. Plasmid pBRsa-14 was restricted with BglII and BamHI to release the mouse MT-I cDNA in a 300 bp fragment. The fragment was ligated into the pAS1 vector, which had been linearized at the BamHI site and treated with phosphatase to eliminate self-ligation of the vector (see 2.5.4.1. in Chapter 2). The ligation mixture was used to transform *E. coli* strain N99cI$^+$. Plasmid DNA from transformants were isolated by the alkali-SDS procedure (see 2.3.1.). The clone with the insert in the correct orientation was identified by the presence of an SstII site, present only in the mouse MT-I cDNA, and by the proper restriction fragments obtained after EcoRI and BamHI restriction (Figure 3-3). Figure 3-3 shows the structure of the pYM101 plasmid. The insert was integrated in phase into the cII coding frame so that the hybrid open reading frame had codons for four amino acids, met-asp-leu-gly, added in front of the initiation codon for the mouse MT-I structural gene.

### 3.2.1.2. Construction of the pNS3XY Plasmids

Plasmids pNS3XY were derived from the bacterial expession vector pNS3. In pNS3, the cloning sites (EcoRI, SmaI, BamHI) are placed in front of a truncated *lacZ* gene, which lacks the DNA sequence coding for the first 7 amino acids. The mouse MT-I cDNA was ligated into the EcoRI and BamHI sites of pNS3 to generate the pNS3XY plasmids. The pNS3XY plasmids are a family of constructions in which the number of base pairs between the SD sequence and the initiation codon for the mouse MT-I gene is different for each member of the family. Figures 3-4 and 3-5, respectively, shows the structural features of the pNS3 and pNS3XY plasmids.

The variation of spacing between the SD sequence and the initiation codon among the different pNS3XY plasmids was the result of an S1 nuclease digestion of MT-I cDNA prior to cloning. Plasmid pBRsa-14 was linearized by a BglII restriction and was treated by S1 nuclease to convert the sticky ends into blunt ends (see 2.5.4.3.). The linear pBRsa-14 was then restricted with BamHI and the 300 bp fragment containing the mouse MT-I cDNA was isolated. After an EcoRI linker, GGAATTCC, was ligated onto its 5′ blunt-ended terminus, the 300 bp fragment was inserted into the EcoRI and BamHI sites of pNS3. The events before and after the S1 nuclease digestion are illustrated in Figure 3-6. Because S1 can slowly digest blunt-ended termini, a population of spacings between the SD sequence and the initiation codon of the MT-I gene was anticipated.

A total of 56 transformants containing the mouse MT-I gene in plasmid pNS3 were obtained in *E. coli* strain N99cI$^+$. The presence of the insert was identified by detecting the 300 bp fragment on a vertical minigel apparatus after ethidium-bromide staining. As a whole, these 56 plasmids were named pNS3XY. Individual clones were designated by putting a number, representing the order in which the plasmid was examined, next to the name pNS3. For example, the first plasmid that was isolated from a transformants would be named as pNS301, the second would be pNS302, and so forth.

### 3.2.2. Yeast MT Clones

### 3.2.2.1. Construction of the pRK724 Plasmid

Plasmid pRK724 was derived from the yeast expression vector α-factor, which contains a XbaI restriction site within the coding sequence of the α-factor peptide (Figure 3-7). A 252 bp fragment containing the human MT-II cDNA was isolated by restricting plasmid phMTII-3 with BamHI and RsaI. This fragment did not contain the ATG triplet coding for the first amino acid in the human MT-II. Ligation of this 252 bp fragment with a XbaI-BamHI linker and a RsaI-SalI linker at the 5′ and 3′ termini, respectively, restored the missing ATG sequence (Figure 3-8). Figure 3-8 shows the schematic steps leading to the insertion of the human MT-II cDNA into the XbaI and SalI sites of the α-factor. The resultant plasmid is pRK724. When the gene product of plasmid pRK724 was secreted by the α-factor promoter/leader sequence, the signal peptide was cleaved in front of the methionine residue of the human MT-II protein.

The initial propagation and selection of transformants harboring the recombinant plasmid was in *E. coli* strain HB101 selected for ampicillin-resistance. For the purpose of examining gene expression in yeast, a BamHI fragment containing the α-factor promoter/leader sequence, the human MT-II cDNA, and the α-factor terminator sequence was isolated from plasmid pRK724 and subsequently ligated into the BamHI site of the shuttle vector pC1/1 (Figure 3-9). Transformants in pC1/1 were used to study gene expression.

### 3.2.2.2. Construction of the pADH224 Plasmid

Plasmid pADH224 contains the the yeast ADHII promoter and the yeast α-factor terminator sequence. Construction of plasmid pADH224 involved two phases. Figure 3-10 shows the first phase in which the BglII-XbaI fragment containing the yeast α-factor promoter/leader sequence in plasmid pRK724 was replaced by the BamHI-XbaI fragment containing the yeast ADHII promoter. The product was plasmid ADH-F which carried the human MT-II cDNA inserted between the yeast ADHII promoter and the yeast α-factor terminator sequence. Figure 3-11 shows the second phase of construction using plasmid ADH-F. A partial BamHI digestion on plasmid ADH-F resulted in a 1.2 Kb fragment containing the ADHII promoter, the human MT-II cDNA and the α-factor terminator sequence. This fragment was ligated into the BamHI site of the shuttle vector pAB18 (Figure 3-9). Figure 3-11 indicates the two possible orientations of the insert in pAB18. The one in which the transcription of the ADHII promoter is in the same direction as that of bacterial ampicillin gene was arbitrarily chosen and was designated as

pADH224. Maintenance of plasmid pADH224 and examination of gene expression were done in *S. cerevisiae* strain AB103.1.

## 3.3. Discussion

### 3.3.1. The M5248 and the N99cI$^+$ Cells

Plasmids carrying the $P_L$ promoter are often unstable (R. Rao, unpublished data). Therefore, all plasmids containing the $P_L$ promoter were maintained in N99cI$^+$ cells. In N99cI$^+$ cells, the $P_L$ transcription is completely inhibited by the phage $\lambda$ cI repressor protein, which is synthesized continuously and regulated autogeneously in the lysogen (Ptashne *et al.*, 1976). Only for the purpose of expression would the plasmid be transformed into freshly thawed competent M5248 cells. The transformants in M5248 cells were used immediately and discarded after each experiment. The plasmid DNA was purified from the N99cI$^+$ cells to ensure the stability of the $P_L$ promoter within the plasmid (P. Modrich, personal communication).

### 3.3.2. S1 Digestion

The generation of pNS3XY containing clones by S1 digestion raised the possibility of examining the effects of the spacing between the SD sequence and the first codon of MT-I on MT gene expression in bacteria. These clones were subjected to various tests described in the next chapter and the ones with the strongest indications of gene expression were then characterized for the actual base-pair numbers in the spacing. If the S1 enzyme had no activity on the double-stranded DNA, the spacing between the SD sequence and the ATG sequence of human MT-II cDNA would be 10 bp. If the S1 enzyme had a limited activity on the double-stranded DNA, e.g. removal of less than 5 bp (see Figure 3-6), then the number of total bacterial transformants (56) should fully cover the range of spacing variations. If the ATG triplet and the following sequence were not affected by the S1 enzyme, the gene product would have a protein sequence identical to that of the mammalian protein. This is in contrast to the gene product made by the pYM101 plasmid, which is a hybrid containing the partial sequence of the cII protein and the complete sequence of the mouse MT-I protein.

### 3.3.3. Constructions Involving Other Bacterial or Yeast Promoters

The bacterial lacUV5 promoter (Stefano and Gralla, 1980), the lpp promoter (Nakamura and Inouye, 1979) and the yeast 3PGK promoter (Hitzeman *et al.*, 1980; Hitzeman *et al.*, 1982) were also used for the MT gene expression. The bacterial lacUV5 promoter carries a mutation in the $P_{lac}$ promoter. This mutation is an up-mutation, which makes possible the initiation of transcription in the absence of the catabolic activator protein (CAP). The lpp promoter is another strong promoter which is responsible for the production of the outer membrane lipoprotein in *E. coli* as 5-10% of total protein. The yeast 3PGK promoter precedes the 3-phosphoglycerate kinase gene. Expression of hepatitis B virus surface antigen by the 3PGK promoter gave a very high yield (Hitzeman *et al.*, 1983). Unfortunately, constructions using these promoters failed to produce signals of gene expression by various tests as will be described in Chapter 4.

### 3.4. Conclusion

Table 3-1 summarizes the various plasmids constructed for bacterial and yeast MT gene expression. The characteristics of each plasmid and its respective vector are compared.

## Table 3-1

## Characteristics of Plasmids in MT Clones

| | Bacterial Clones | | Yeast Clones | |
|---|---|---|---|---|
| Plasmid in the clone | pYM101 | pNSXY | pRK724 | pADH224 |
| Vector used for construction | pAS1 | pNS3 | α-factor | pAB18 |
| MT gene | mouse-I | mouse-I | human-II | human-II |
| Host strain | M5248 | M5248 | AB103.1 | AB103.1 |
| Promotor for expression | regulated | regulated | constitutive | regulated |
| System of expression | internal | internal | secretion | internal |
| Gene product | hybrid | mouse-I | human-II | human-II |
| Size of clone plasmid (Kb) | 6.2 | 10.5 | 5.3 | 12.6 |

Figure 3-1. The DNA sequence of mouse liver MT-I cDNA (Glanville *et al.*, 1981) and its 5′ and 3′ flanking regions in plasmid Rsa-12. The sequences of restriction enzymes that recognize 6 bp sequences are underlined and named. Exons are shown in triplets. The mouse MT-I protein sequence is indicated above the exon sequences.

TACCTTCTCCTCACTTACTCCGTAGCTCCAGCTTCACCAGATCTCGGA

*Bgl II*

met asp pro asn cys ser cys ser thr gly gly ser cys thr cys
ATG GAC CCC AAC TGC TCC ACC TGC TCC GGC GGC TCC TGC ACT TGC

thr ser ser cys ala cys lys asp cys lys cys thr ser cys lys
ACC AGC TCC TGC GCC TGC AAG AAC TGC AAG TGC ACC TCC TGC AAG

lys ser cys cys ser cys cys pro val gly cys ser lys cys ala
AAG AGC TGC TGC TCC TGC TGT CCC GTG GGC TGC TCC AAA TGT GCC

gln gly cys val cys lys lys ala ala asp lys cys thr cys cys
CAG GGC TGT GTC TGC AAA GGC GCC GCG GAC AAG TGC ACG TGC TGT

*Sst II*

ala *Ter*
GCC TGA TGTGACGAACAGCGCTGCCACCACGTGTAAATAGTATCGGACCAACCCAGCG

TCTTCCTATACAGTTCCACCCTGTTTACTAAACCCCCGTTTTCTACCGAGTAC

Figure 3-2. The DNA sequence of human MT-II cDNA and its flanking 5′ and 3′ regions (Karin and Richards, 1982b).The amino acid sequence for the MT-II protein is indicated above the cDNA sequence (Kissling and Kägi, 1977; Kägi, unpublished data). The sequences of restriction enzymes that recognize 6 bp sequences and the RsaI site are underlined and named.

AGTCCCAGCGAACCCGCGTGCAACCTCTCCCGACTCTAGCCGCCTCTTCAGCACGCC

*Nc*

met asp pro asn cys ser cys ala ala gly asp ser cys thr cys

ATG GAT CCC AAC TGC TCC TGC GCC GCC GGT GAC TCC TGC ACC TGC

*ol Bam HI*

ala gly ser cys lys cys lys glu cys lys cys thr ser cys lys

GCC GGT TCC TGC AAA TGC AAA GAG TGC AAA TGC ACT TCG TGC AAG

lys ser cys cys ser cys cys pro val gly cys ala lys cys ala

AAA AGC TGC TGC TCC TGC TGC CCT GTG GGC TGT GCC AAG TGT GCC

gln gly cys ile cys lys gly ala ser asp lys cys ser cys cys

CAA GGC TGC ATG TGC AAA GGG GCG TCG GAC AAG TGC AGC TGC TGC

*Pvu II*

ala *Ter*

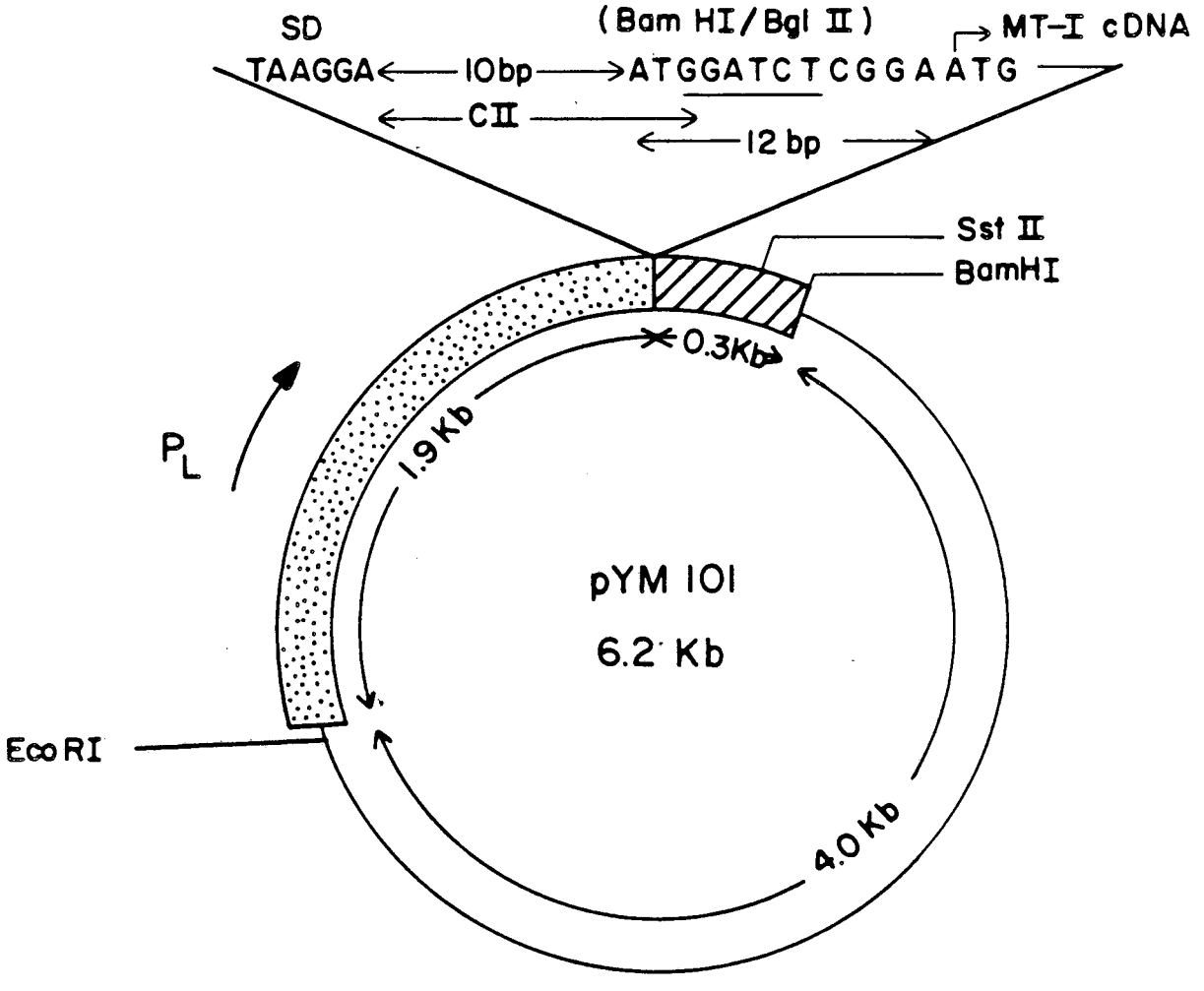
GCCTGATGCTGGGACAGCCCCGCTCCCAGAT GTAAAGAACGCGACTTCCACAAACCTG

GATTTTTTATGTACAACCCTGACCGTGACCGTTTGCTATATTCCTTTTTCTATGAAATAATGT

*Rsa I*

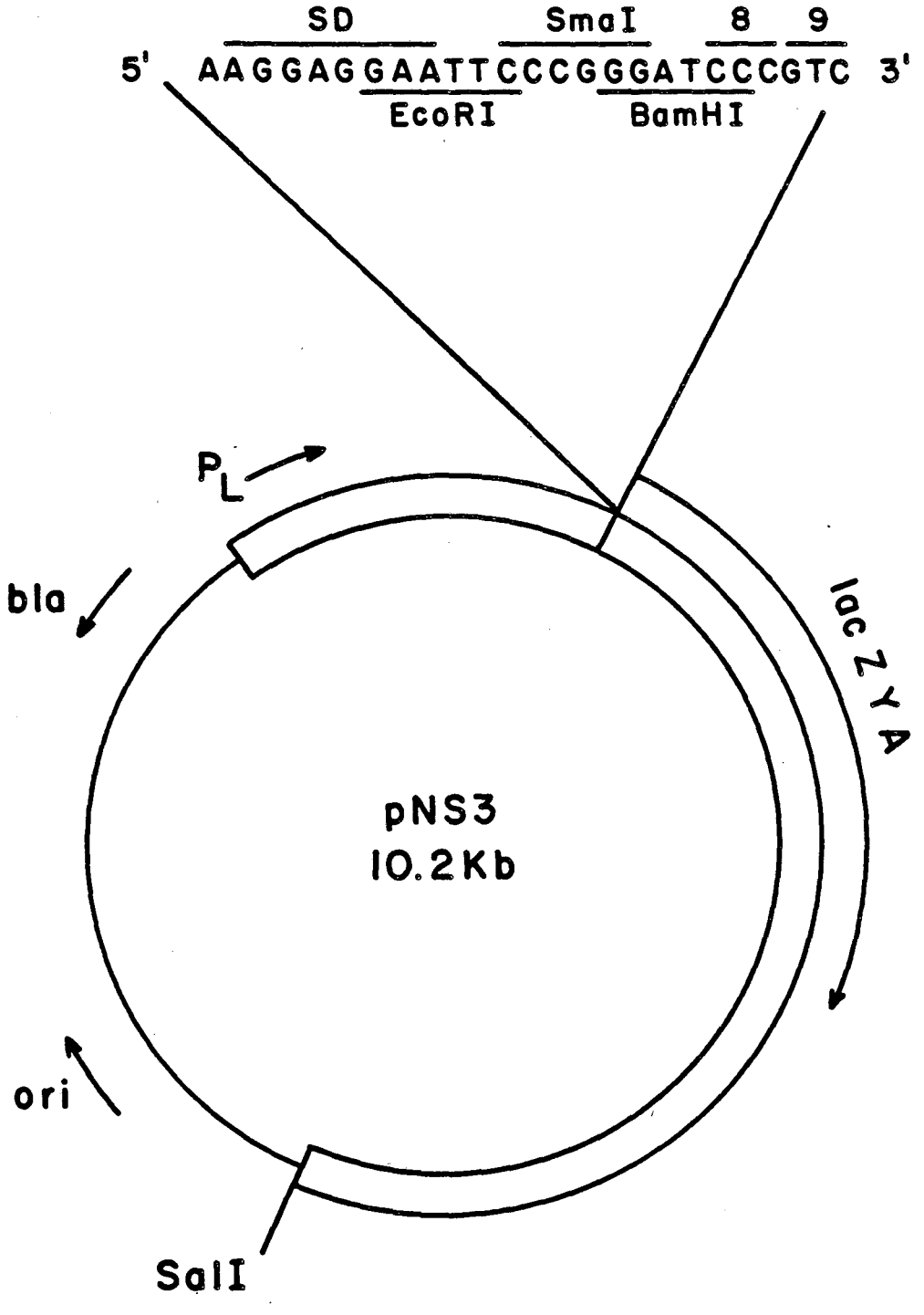
GAATGATAATAAAA

Figure 3-3. Structure of the pYM101 plasmid. This 6.2 Kb (kilobase pairs) plasmid was constructed by ligating a 0.3 Kb BglII-BamHI fragment (hatched region), which contains mouse MT-I cDNA, into the BamHI site of the 5.9 Kb pAS1 vector. The $P_L$ promoter, which is upstream from the ligated site, is contained in a 1.9 Kb EcoRI-BamHI fragment (dotted region) on the pAS1 vector. Upon insertion, the BamHI site immediately following the SD sequence in pAS1 is abolished due to the BglII/BamHI junction. This generates a 2.2 Kb EcoRI-BamHI fragment that contains the $P_L$ promoter, the cII sequence and a hybrid coding frame with a stretch of 12 base pairs fused to the mouse MT-I structural gene. The restriction site SstII is only present in the insert.


SD
(Bam HI/Bgl II)
MT-I cDNA
TAAGGA
10bp
ATGGATCTCGGAATG
CII
12 bp
Sst II
BamHI
0.3Kb
1.9Kb
$P_L$
pYM 101
6.2 Kb
EcoRI
4.0Kb

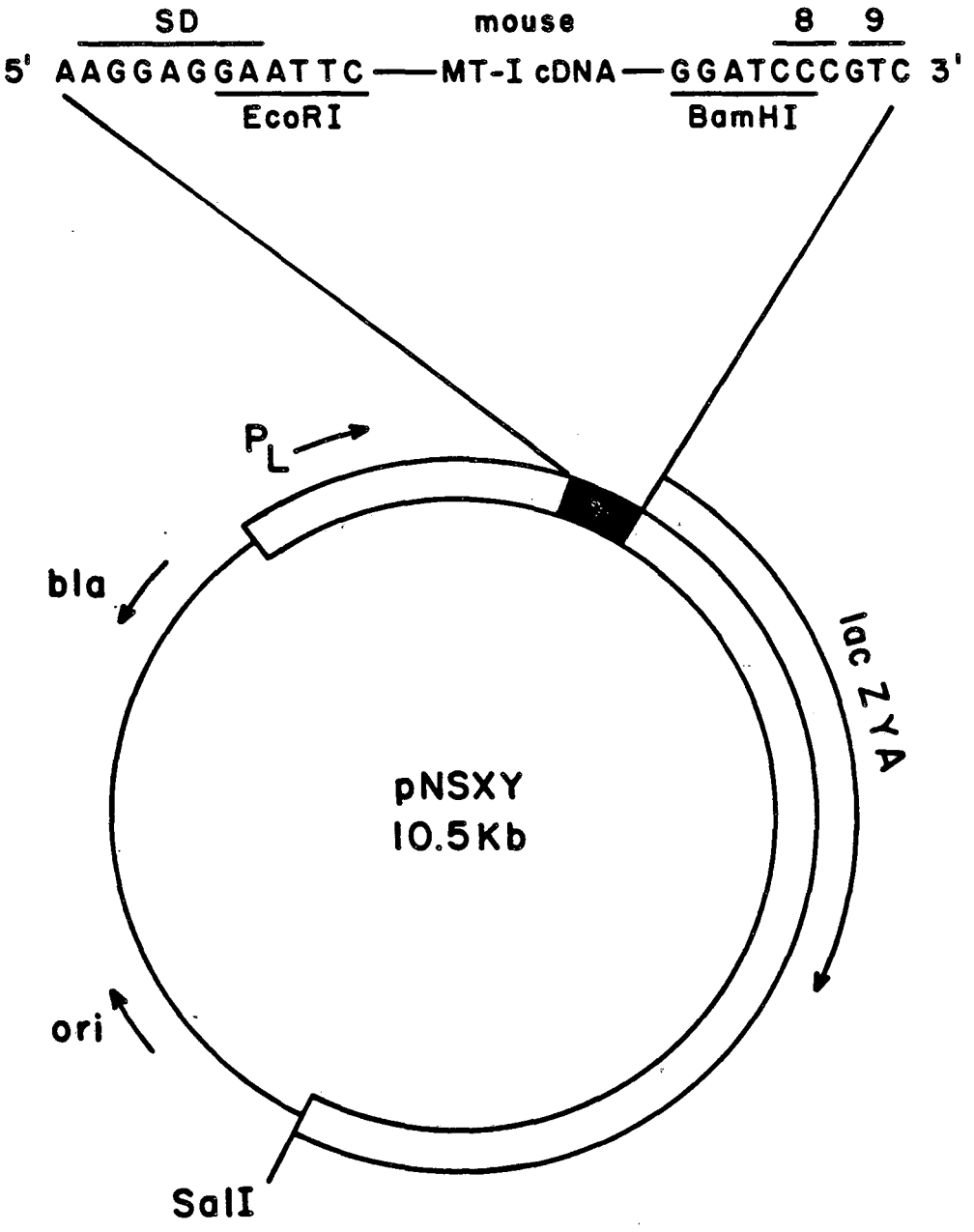
- XBL 872-614 -

Figure 3-4. Structure of the pNS3 vector (Scheuermann, 1986) showing the DNA sequence containing the synthetic SD site, the EcoRI, SmaI and BamHI restriction sites. The CCC and GTC triplets, respectively, are the eighth and nineth coding sequences in the *lacZ* gene (Kalnins *et al.*, 1983).



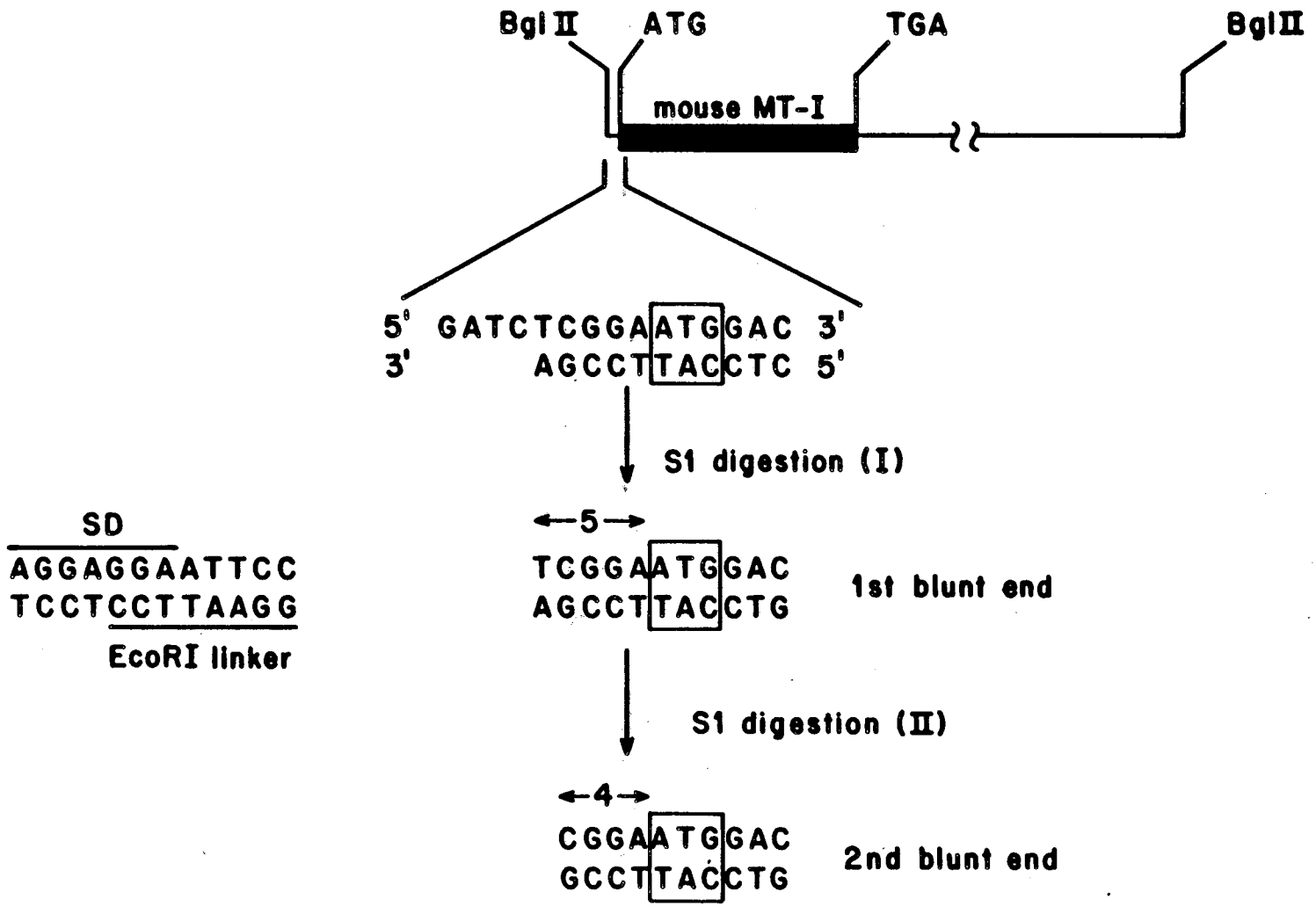

XBL 873-10809

Figure 3-5. Structure of the pNS3XY plasmid showing the insertion of the mouse MT-I cDNA in a 0.3 Kb fragment into the EcoRI and the BamHI restriction sites of the pNS3 vector (10.3 Kb, Figure 3-4). The expressed protein sequence will terminate within the 0.3 Kb fragment using the termination codon in the mouse MT-I cDNA and will not contain the sequence of the *lacZ* protein.



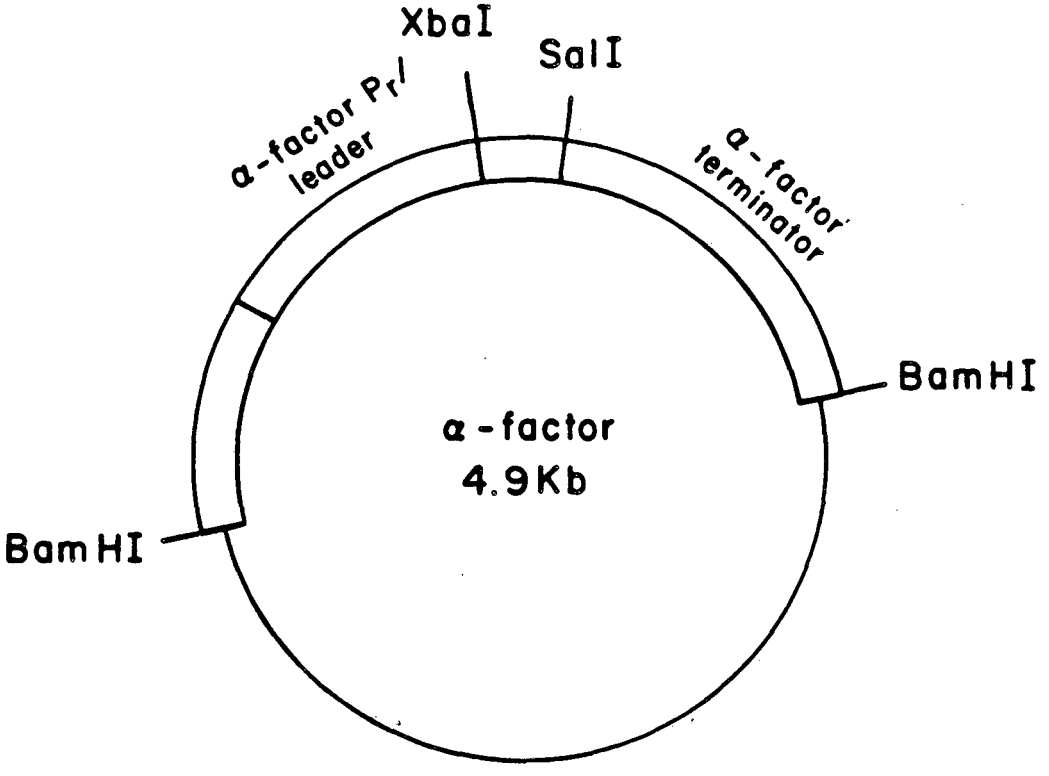

- XBL 873-10810 -

Figure 3-6. S1 nuclease conversion of the BglII sticky end to a blunt end. The first phase of the digestion is the complete removal of the single-stranded DNA, leaving 5 bp in front of the ATG triplet (1st blunt end). The second phase of the digstion is the progressive cleavage of the double-stranded DNA, leaving 4 bp or less in front of the ATG triplet (2nd blunt end). After S1 digestion, the blunt-ended DNA is ligated with the EcoRI linker (shown at left) and inserted into the EcoRI restriction site next to the SD sequence in the pNS3 vector.




- XBL 873-10811 -

Figure 3-7. Structure of $\alpha$-factor vector and cleavage of $\alpha$-factor peptide. (Top) The yeast $\alpha$-factor promoter/leader sequence and the yeast $\alpha$-factor terminator sequence are contained in a BamHI fragment. (Bottom) The DNA sequence around the XbaI restriction site is shown as well as the amino acid sequence for the last 7 residues of the $\alpha$-factor peptide. Cleavage of the $\alpha$-factor peptide during the process of secretion occurs at the arginine residue.




gly val ser leu asp lys arg

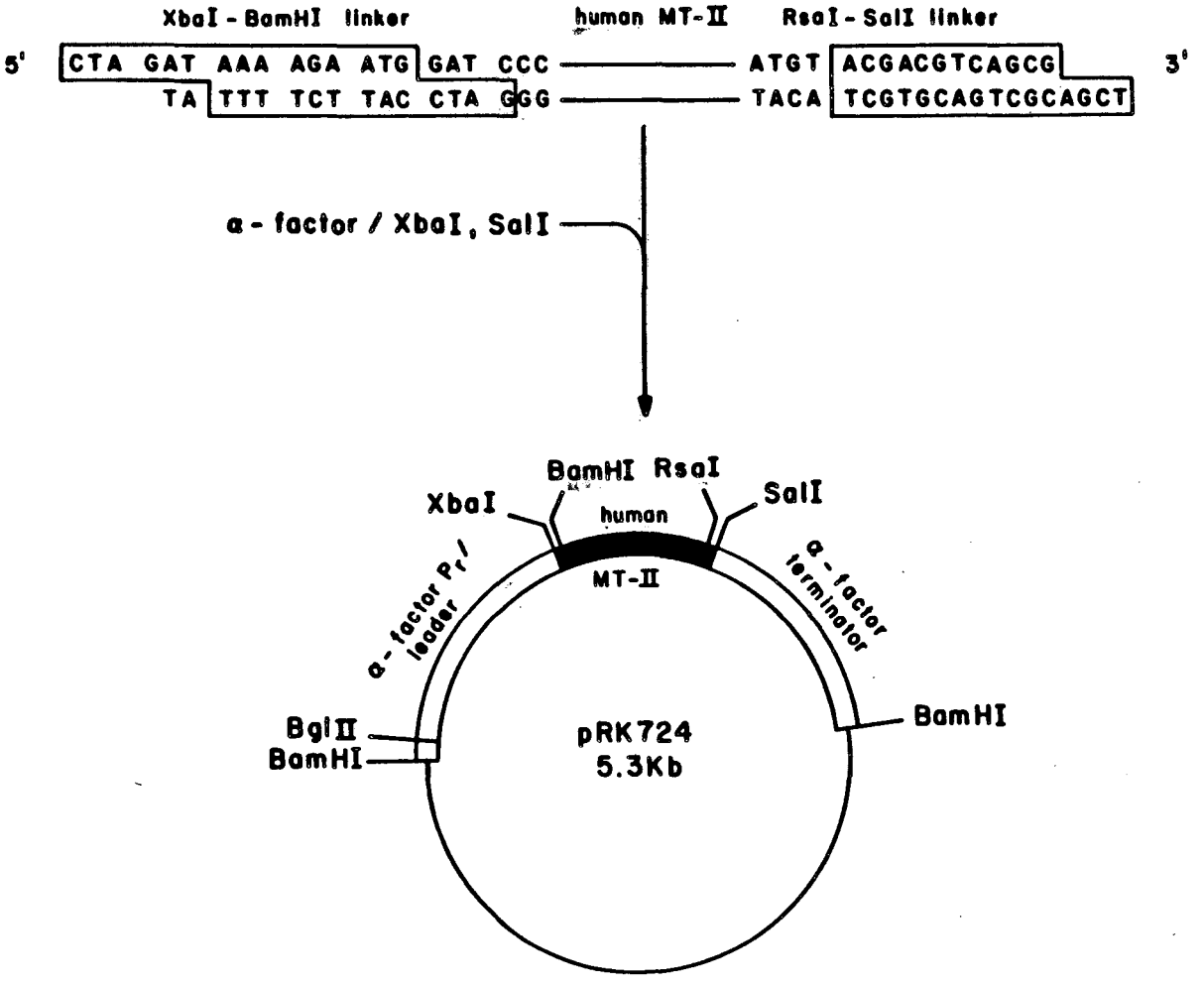
5'····· GGG GTA TCT CTA GAT AAA AGA ····· 3'

····· CCC CAT AGA GAT CTA TTT TCT ·····

XbaI

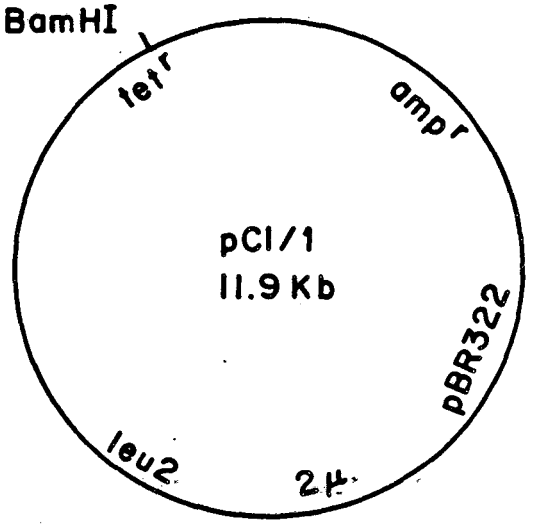
- XBL 872-615 -

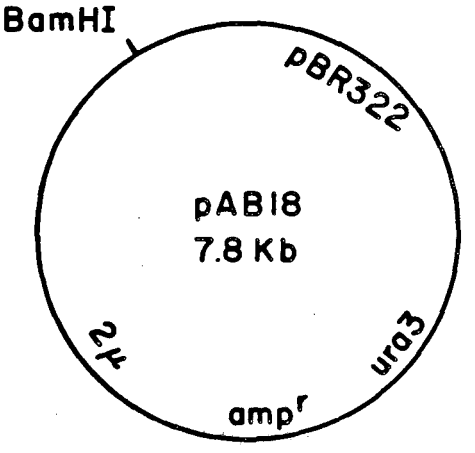
Figure 3-8. Structure of the pRK724 plasmid. (Top) This 5.3 Kb plasmid was constructed by ligating a 0.3 Kb fragment into the XbaI-SalI restriction sites of the 5.9 Kb $\alpha$-factor. (Bottom) Such a construction resulted in a hybrid reading frame for the $\alpha$-factor peptide and the human MT-II protein. Cleavage of the $\alpha$-factor peptide after secretion generated the complete human MT-II protein.




gly val ser leu asp lys arg ▼met—human MT-II

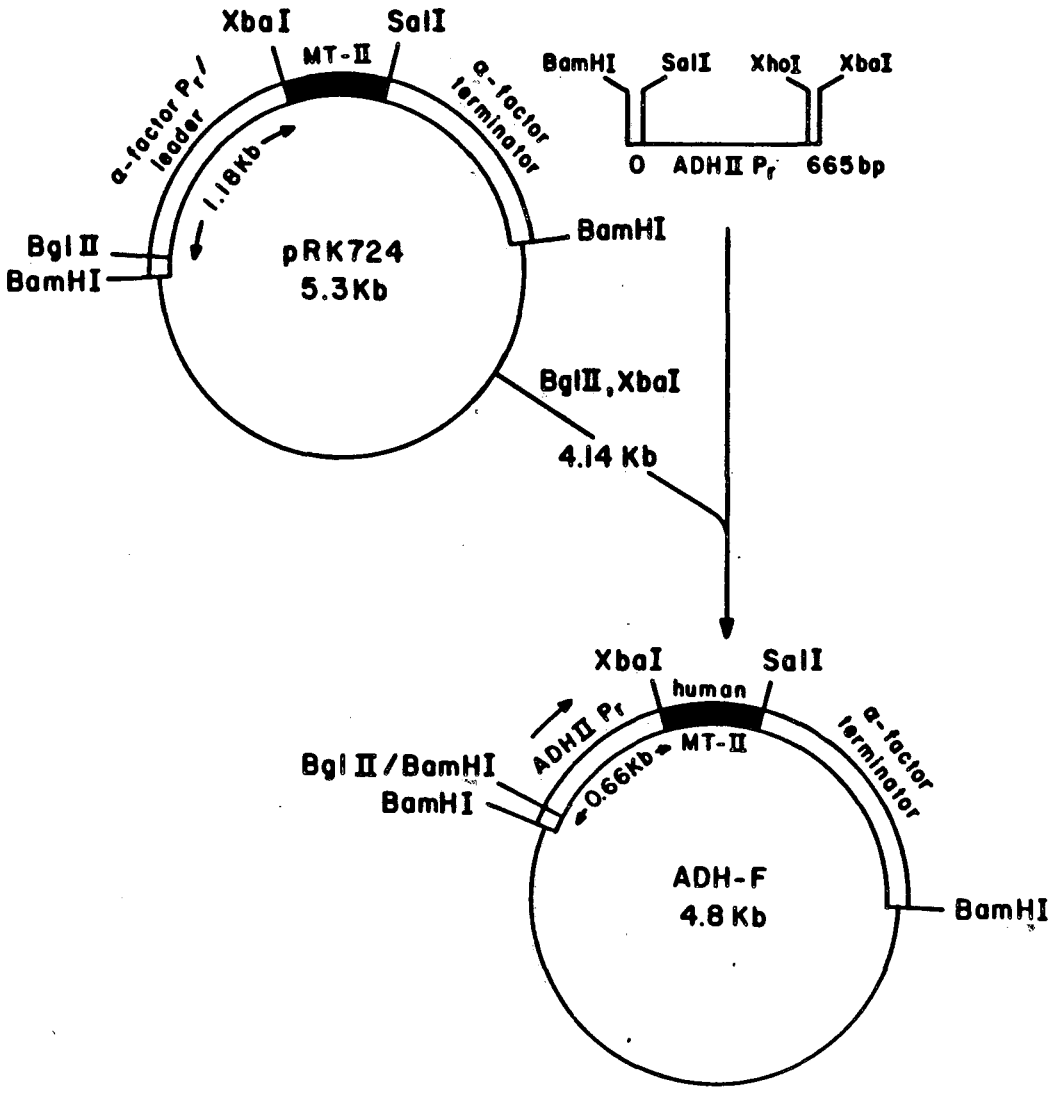
Figure 3-9. Structure of the pC1/1 and the pAB18 shuttle vectors. Both vectors contain the ampicillin-resistance gene and the replication origin of pBR322 for selection and propagation in *E. coli*. They also contain the yeast 2$\mu$ plasmid sequence for autonomous replication in yeast. The presence of the *leu2* gene in pC1/1 and the *ura3* gene in pAB18, respectively, allows for selection in yeast *leu-2* and *ura-3* mutants.


BamHI
tet^r
amp^r
pCI/1
11.9 Kb
pBR322
leu2
2μ


BamHI
pBR322
pAB18
7.8 Kb
2μ
ura3
amp^r

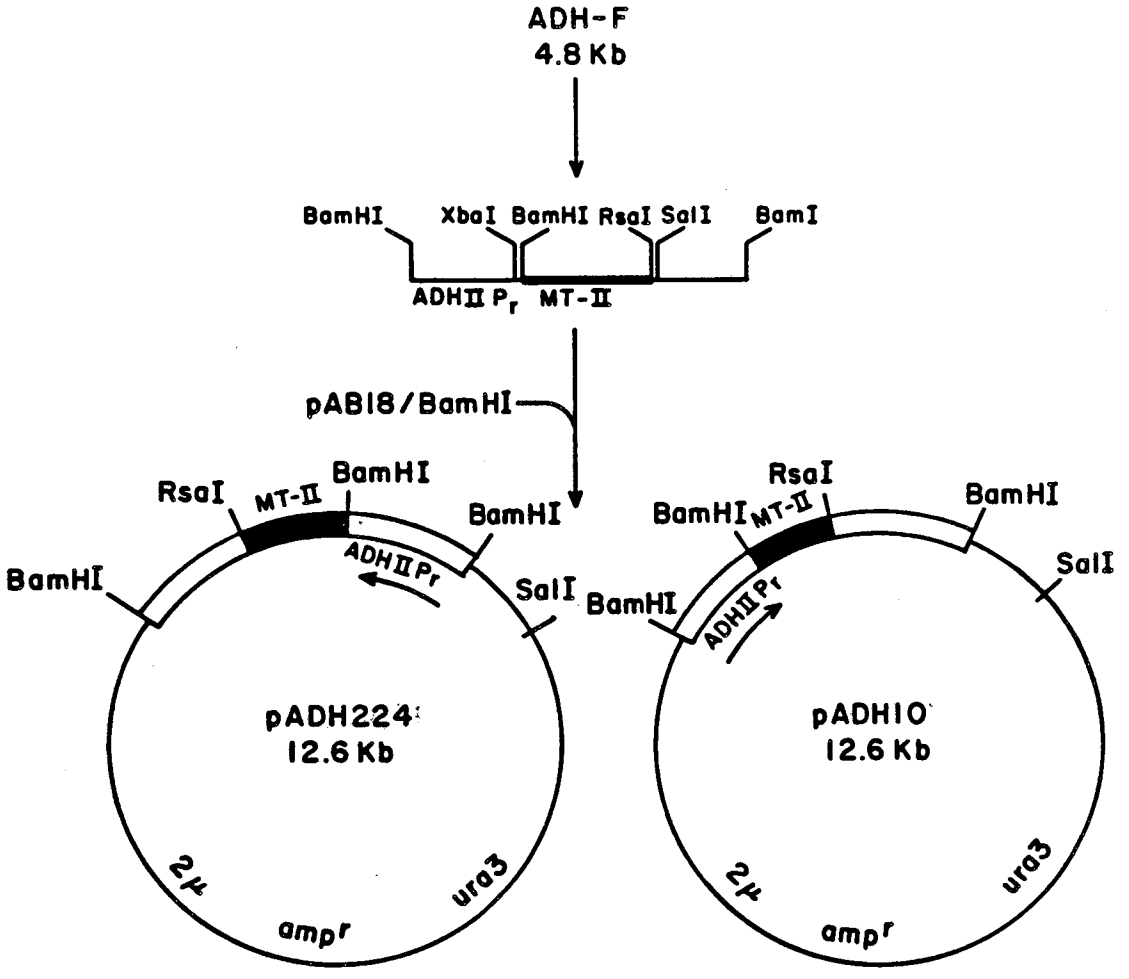
- XBL 872-616 -

Figure 3-10. Construction of the ADH-F plasmid. Plasmid ADH-F is an intermediate plasmid used in the construction of pADH224. This 4.8 Kb plasmid was constructed by ligating the 0.66 Kb fragment which contains the yeast ADHII promoter into the BglII and XbaI sites of the pRK724 plasmid.




- XBL 873-10812 -

Figure 3-11. Construction of the pADH224 plasmid. This 12..6 Kb plasmid was constructed by ligating a 1.2 Kb BamHI fragment which contains the yeast ADHII promoter, the human MT-II cDNA and the $\alpha$-factor terminator sequence into the BamHI restriction site of the pAB18 vector. The 1.2 Kb BamHI fragment was obtained from a partial BamHI digestion of the ADH-F plasmid. Plasmids pADH224 and pADH10 correspond to the two orientations of the recombinant plasmid.




- XBL 873-10813 -

# CHAPTER 4

## MT Gene Expression in Bacteria and Yeast

### 4.1. Introduction

Functional expression of the MT gene in bacteria and yeast is described in this chapter. A variety of assays were used to test whether bacteria and yeast clones harboring and expressing the gene were more resistant to heavy metals than the wild type. The experiments presented in this chapter confirmed that prediction. In addition, bacterial MT clones showed a prefential incorporation of $^{109}Cd$ during growth and were readily labelled by [$^{35}S$]cysteine, the major amino acid in MT. Yeast MT clones contained a protein species of 6 kilodalton molecular weight and showed various characteristics which indicated that expression of MT is harmful to the microogranism. Overall, the heavy metal-resistant phenotype of these clones serves as a useful basis for isolating mutants of MT with altered metal-binding affinities and specificities.

### 4.2. Results

#### 4.2.1. Bacterial MT Clones Are Resistant to Heavy Metals on Agar Plates

Bacteria harboring either the pYM101 plasmid or the pNS3XY plasmid were able to grow on agar plates containing a heavy metal at a concentration that would have been lethal for cells harboring either the pAS1 or the pNS3 plasmid. This is shown by making agar plates in various

concentrations of a wide range of heavy metals and using them to screen a large number of clones. As a reference, the lethal concentration for a specific heavy metal was chosen as the lowest concentration at which bacteria carrying the pAS1 plasmid would die. This concentration is defined as the critical concentration for that particular heavy metal. The critical concentrations for heavy metals, Ag(I), Hg(II), Cu(II), Cd(II), and Pb(II), were determined as follows: 0.13 mM $AgNO_3$, 40 $\mu$M $Hg(CH_3COO)_2$, 3.2 mM $CuCl_2$, 0.83 mM $CdCl_2$ and 1.9 mM $Pb(NO_3)_2$. The criteria for chosing a particular anion was that the heavy metal salt solution not precipitate during the formation of agar plates. For example, because $HgCl_2$ easily precipitated upon addition to the agar plates while $Hg(CH_3COO)_2$ did not, the acetate salt was used for the experiment.

Different clones were streaked on a plate containing the critical concentration of a metal ion. They were examined for growth under conditions in which the MT gene expression was turned on. Controls were provided by another plate containing the same metal but at a concentration much lower than the critical concentration. This was to ensure the viability of all cells under the test. An example of a set of Hg plates is shown in Figure 4-1. At the concentration of 30 $\mu$M $Hg(CH_3COO)_2$, all cells including those carrying plasmids pAS1 and pNS3 were alive, but at 40 $\mu$M $Hg(CH_3COO)_2$, only cells carrying the pYM101 plasmid and the pNS319 and pNS320 plasmids were alive. As would be expected, bacteria harboring the pYM101 plasmid and some of the pNS3XY plasmids grew on media containing the Hg(II) ions at an unusual concentration. Similar experiments were performed with other heavy metal ions. In all cases, the pYM101 containing bacterial cells were more resistant to heavy metals than the pAS1 containing cells. These results were reproducible and are summarized in Table 4-1.

Bacterial cells harboring the pNS3XY plasmid gave less reproducible results under the same experimental conditions. By setting the pYM101 harboring clone as the reference, this would indicate that the MT-I gene expression in these cells was weaker than in the pYM101 harboring cells. In order to compare the degree of gene expression among different clones, an arbitrary scoring system was formulated as follows. For each heavy metal ion, three tests were performed with each clone. A clone would have to be positive at the critical concentration at least two times in order to acquire a score. If so, it was given a score of 2 when passing twice and a score of 3 when passing all three tests. After all the tests were finished, the score obtained from each

heavy metal was summed up for each clone. Tests on $Pb(NO_3)_2$ were not included because none of the clones harboring the pNS3XY plasmid had a score. Of the 56 pNS3XY harboring clones, 80% showed positive growth once and thus were abandoned. Table 4-2 lists the remaining clones and their respective scores. Tests on bacteria containing the pYM101 plasmid were included as a positive control and those harboring the pAS1 and pNS3 plasmids were included as negative controls. On a scale of 0 to 12, the pNS3XY containing clones scored intermediate points with an average of 5.0 while the pYM101 containing clone scored 12. Neither of the negative controls gave any score. It should be noted that the scoring system treated each metal on an equal basis and did not take into account the different affinities of MT for each of the heavy metals.

### 4.2.2. Bacterial MT Clones Are Resistant to $ZnCl_2$ in Liquid Medium

Bacteria harboring the pYM101 plasmid grew at a faster doubling time than those harboring the pAS1 plasmid in liquid medium containing the critical concentration of $ZnCl_2$. The heavy metal Zn(II) was chosen for this experiment because it is required for many important enzymes and its transport into the bacteria is well characterized (Chlebowski and Coleman, 1976; Silver, 1977). Previously, the critical concentration of Zn (II) ions in liquid medium was determined as 1.1 mM for bacterial cells harboring either the pYM101 plasmid or the pAS1 plasmid under uninduced conditions. To test the effect of MT gene expression, both types of cells were grown at 0.1 mM $ZnCl_2$ initially to an equal cell density after which MT was induced for 30 minutes. Following induction, the $ZnCl_2$ concentration in the medium was raised to 1.1 mM and the growth rate for both cells was monitored for another four hours. Figure 4-2 shows the growth curves of the pYM101 and the pAS1 harboring cells. At 1.1 mM $ZnCl_2$, the control cells (harboring pAS1) were arrested in growth at 42°C while the pYM101 harboring cells preserved a slow but continued growth. The doubling time for each cell line was measured and is included in the same figure for comparison. The results showed that the pYM101 harboring cells grew twice as fast than the pAS1 harboring cells at 1.1 mM $ZnCl_2$ under induced conditions. Both cells, however, grew at the same rate at 0.1 mM $ZnCl_2$ under the same induction conditions.

Since overexpression of MT-I in bacterial cells may deplete the internal cysteine pool, experiments were also performed with an elevated amount of cysteine in the medium (Figure 4-2). Under the induction conditions, the addition of 20 $\mu$g/ml cysteine to the liquid medium con-

tributed very little to the growth of cells containing pAS1 but definitely improved the growth of cells containing pYM101 at 1.1 mM $ZnCl_2$. The cysteine supplement, however, did not alter the growth rate of either cell line at 0.1 mM $ZnCl_2$ under the induced conditions. Although the effect of cysteine on cells harboring pYM101 at 1.1 mM $ZnCl_2$ was small, the contrast between clone and control was clear.

Bacteria harboring the pNS3XY plasmids presented in Table 4-2 were similarly analyzed. Their doubling times are presented in Table 4-3. Table 4-3 shows that these bacterial cells had longer doubling times than the pYM101 harboring cells. Longer doubling time would indicate a lower level of MT gene expression. Nevertheless, their doubling times were still shorter than that of the controls. The two control cultures, harboring the pAS1 and the pNS3 plasmids, behaved the same.

### 4.2.3. Bacteria Harboring pYM101 Incorporated More $^{109}Cd$ Than the Control Cells

The pYM101 harboring cells incorporated 10 times as much $^{109}CdCl_2$ from liquid medium during growth than the pAS1 harboring cells upon induction. This value was obtained from cells grown in the presence of a high concentration of $ZnCl_2$ and a trace amount of $^{109}CdCl_2$ under the induction conditions. The radioactivity incorporated by the pYM101 harboring cells and the pAS1 harboring cells was then normalized for the same number of cells and listed in Table 4-4. From the results in Table 4-4, it is possible to estimate the minimal amount of MT present in the bacterial cells containing pYM101. Since the binding affinity of Zn(II) to MT is $10^4$ times weaker than that of Cd(II) to MT and since a vast excess of the Zn(II) ions in the medium (2 x $10^6$ times more concentrated than the $^{109}Cd(II)$) was used, one can assume that every molecule of $^{109}Cd(II)$ incorporated by the cells represents at least one molecule of the MT-I protein. This calculation leads to a minimum 40 ng of MT-I present in 100 $\mu$g of bacterial crude extract isolated from one milliliter of cell culture.

### 4.2.4. Bacteria Harboring pYM101 Are Labelled by [$^{35}S$] Cysteine

Pulse-labelling of bacteria cells harboring pYM101 showed a protein species that comigrated with the purified mouse MT-I. This is shown in Figure 4-3 in which the extracts from bacteria containing plasmid pAS1 and plasmid pYM101 were analyzed on a SUDS gel (see 2.10.4.).

The SUDS gel contains a 20% polyacrylamide matriz with 8 M urea. The highly denaturing conditions of the SUDS gel prevented MT from forming nonspecific disulfide bridges via its active sulfhydryls. The protein samples in Figure 4-3 were also carboxymethylated (see 2.10.4.) for the same reason. In this gel system, the standard mouse MT-I protein migrated with a mobility equivalent to an 8-10 kilodalton protein (Figure 4-3A). Within this same migration range on the autoradiogram (Figure 4-3B), a new protein species in the extract from the pYM101 harboring cells was found. This protein species was not present in the extract from the cells harboring the pAS1 plasmid. However, this 8-10 kilodalton protein species was not detected in the Coomassie blue stained gel from which the autoradiogram was developed, nor was it detected with an even more sensitive silver staining procedure.

### 4.2.5. Bacteria Harboring pNS3XY Belong to Two Types

Bacteria harboring pNS3XY plasmids with a score higher than 2 (Table 4-2) were further characterized. This was done by isolating the EcoRI-BamHI fragment containing the cloned mouse MT-I cDNA from each clone and subjecting each to a dideoxy sequencing analysis (see 2.6.3. and 2.6.4.). To better resolve the DNA spanning the region from the EcoRI site to the initiation codon of the mouse MT-I gene, the sequencing results were analyzed on a 10% polyacrylamide gel. Of the eight pNS3XY plasmids that were analyzed, only two types of sequences were found. Figure 4-4 shows the sequencing gels of the type I and type II constructions and Table 4-5 shows the members of the pNS3XY plasmids belonging to each type. In the type I construction, characteristic of most of the clones, a spacing of 10 bp was found between the SD sequence and the ATG triplet of the mouse MT-I gene. In the type II construction, observed in only 2 plasmids (pNS319 and pNS398), the DNA sequence immediately following the SD sequence was the third coding triplet for the mouse MT-I gene. There was no nucleotide sequence coding for the first two amino acids in the type II construction. The type I construction would result from S1 nuclease stopping in front of the double-stranded DNA (see 3.2.1.1.). This spacing is identical with that in plasmid pYM101. The type II construction would result from S1 nuclease hydrolyzing an additional 6 base pairs past the single-stranded region.

### 4.2.6. Yeast Harboring Plasmid pRK724

#### 4.2.6.1. Secretion of a 14-18 Kilodalton Protein in the AB108 Strain

Examination of medium, in which yeast cells harboring pRK724 were grown, on a 20% Laemmli-SDS polyacrylamide gel showed that a protein of 14-18 kilodalton was secreted by the AB108 strain but not by the AB103.1 strain (Figure 4-5). The major difference between the two strains was that the yeast $\alpha$-factor promoter was constitutive in the AB103.1 strain, but was regulated in the AB108 strain due to a temperature sensitive repressor. This 14-18 kilodalton protein species comigrated with the dimer of the purified mouse MT-I in the same polyacrylamide gel. In Figure 4-5, several yeast clones, all containing pRK724, were analyzed simutaneously. Each clone grew at a different rate even though all were derived from the same transformation experiment.

#### 4.2.6.2. Slower Growth Rate at Induction Temperature

In the AB108 cells, the $\alpha$-factor promoter was turned on at 34°C but turned off at 24°C. At 34°C, the doubling time for the AB108 cells harboring pRK724 was 12.0 hours while that for the control cells harboring the $\alpha$-factor was only 7.0 hours. At 24°C, however, both cells had the same doubling time of 7.7 hours.

### 4.2.7. Yeast Harboring Plasmid pADH224

#### 4.2.7.1. Yeast Harboring pADH224 Contained Dark Vacuoles

When the glucose concentration in the regular YEPD medium (with 2% glucose) was exhausted, the ADHII promoter was activated. This occurred after yeast cells containing the ADHII promoter had been grown for 48 hours. Examination of the induced yeast cells harboring the pADH224 plasmid under the light microscope showed that they had a smaller size and contained many dark vacuoles. They also stopped budding. Formation of vacuoles inside the yeast cells was an indication that the cells were under stress. This is supported by the fact that they stopped budding while control cells harboring pAB18 were mostly budding. Both the presence of vacuoles and the arrest of budding were in response to changes in the environment or to internal stress. Under the same experimental conditions, the control cells had no vacuoles.

#### 4.2.7.2. Growth of Yeast Harboring pADH224 Is Inhibited Upon Induction

The following two growth studies showed that yeast cells harboring pADH224 completely stopped growth upon induction. In one study, the growth of cells was examined in YEPD medium containing 2% glucose over a period of 4 generation times (Figure 4-6A). In the other study, the growth of cells was examined in YEPE medium containing 3% ethanol over a period of 3-4 generation times (Figure 4-6B). In both cases, when glucose was absent in the medium, the growth of cells harboring pADH224 was immediately blocked while that of cells harboring pAB18 was only slightly affected.

The morphological changes in yeast cells harboring pADH224 upon induction in YEPE included a smaller cell size and a cessation of budding. However, they did not appear to contain as many dark vacuoles as those cells described in the previous section. It is probably because cells grown in YEPE were never allowed to grow and thus no change in the internal stress was created.

#### 4.2.7.3. Yeast Harboring pADH224 Contained a 6 Kilodalton Protein

Examination of a yeast lysate on a 20% Laemmli-SDS polyacrylamide gel showed that a 6 kilodalton protein was present in the extract from the pADH224 transformed cells but almost absent in the extract from the pAB18 transformed cells (Figure 4-7). The lysates which showed this 6 kilodalton protein most vividly were prepared with a heat-treatment and a carboxymethylation reaction. If the lysates were only carboxymethylated but not heat-treated, this 6 kilodalton protein band was less obvious. This is expected because the heat-treatment removed the high molecular weight proteins and enhanced the low molecular weight proteins.

The amount of this 6 kilodalton protein in the yeast lysate is estimated as 0.02% of the total cellular extract. The calculation is as follows. Approximately 50 $\mu$g of lysate proteins was loaded onto the polyacrylamide gel in Figure 4-7. Because the lysate had been heated, this amount of protein represented 10% of the total cellular proteins. Assuming that the intensity of the 6 kilodalton protein was 10 times higher than the sensitivity of the silver stain, which is 10 ng (Merril *et al.*, 1981) the protein was present at around 100 ng in the 500 $\mu$g of lysate proteins.

#### 4.2.7.4. Yeast Harboring pADH224 Are Resistant to Heavy Metals

Filter disks containing different amounts of a heavy metal solution were used to show the metal-resistance of yeast harboring pADH224 on an agar plate. For this purpose, the disks were placed on top of a confluent layer of yeast cells grown on the top of an agar plate. The concentration of a heavy metal that was applied to the filter disk was thus radially diffused to the surrounding medium. Yeast cells that were sensitive to the amount of heavy metal in the environment were killed and a halo indicating a region of no cell growth would appear around the filter disk. The diameter of the halo is an index of the sensitivity of the cells to that particular metal concentration. The more sensitive the cells were, the larger the diameter was. Table 4-6 lists the measurement of halos around the disks. Comparing the halos of yeast harboring pADH224 with those of yeast harboring pAB18 shows that the clone was more resistant than the control to Ag(I), Cu(II), Cd(II) (Table 4-6A), and was as sensitive as the control to Hg(II) and Zn(II) (Table 4-6B). Figure 4-8 is a photograph of agar plates, showing the resistance of the clone to $CuSO_4$ (Figure 4-8A) and to $CdCl_2$ (Figure 4-8B). Figure 4-9 is another photograph of agar plates, showing the similar sensitivity of the clone and the control to $ZnCl_2$. In Figure 4-9, although the control cells appeared to have a second halo larger than the clone, it is not well-defined and thus not considered. The lack of difference between the clone and the control to $ZnCl_2$ and $Hg(CH_3COO)_2$ might be due to the poor transport of these metal ions across the cell wall or to the low affinity of the expressed MT to the particular metal ion.

### 4.2.7.5. Yeast Harboring pADH224 Are Not Labelled by [$^{35}$S]sulfate

To detect full expression of the MT under the control of the ADHII promoter, yeast cells harboring pADH224 were incubated with [$^{35}$S]sulfate in medium containing ethanol (2.9.2.). Surprisingly, the amount of radioactivity incorporated over a period of one hour was nearly zero. The incorporation by control cells, on the other hand, increased with the time of incubation (Figure 4-10). Figure 4-11 is an autoradiogram of various samples taken at different time points and resolved by a 20% Laemmli-SDS polyacrylamide gel. While the control had considerable labelled protein bands, the clone had absolutely none. The samples in Figure 4-11 were analyzed with an equal amount of total radioactivity, similar results were obtained for samples analyzed with an equal amount of total protein (data not shown).

## 4.3. Discussion and Conclusion

### 4.3.1. Expression of MT Renders Bacteria and Yeast Resistant to Heavy Metals

The metal resistance tests, either on agar plates or in liquid media, showed that bacteria harboring the pYM101 plasmid and yeast harboring the pADH224 plasmid were resistant to a variety of heavy metals. This effect is due to the expression of MT because control cells were sensitive under the same experimental conditions. The resistance to many different heavy metals that are known to interact with mammalian MTs argues that the resistance is not due to a spontaneous mutation of a transport protein gene for a particular metal ion. That the cysteine supplement to liquid medium containing the critical concentration of $ZnCl_2$ helped the metal-resistance of the bacterial MT clone (but not the control) adds more weight to the above notion.

### 4.3.2. MT Expression in the pNS3XY Containing Clones Is Weaker Than in the pYM101 Containing Clone

Several lines of evidence indicate that the MT gene expression in bacterial clones containing pNS3XY is not as efficient as in the clone containing pYM101. The evidence includes: (i) pNS3XY clones did not score as high as the clone containing pYM101 by the metal resistance tests on agar plates; (ii) pNS3XY clones had longer doubling times than the clone containing pYM101 in liquid medium with a critical concentration of $ZnCl_2$; and (iii) pNS3XY clones were sensitive to $Pb(NO_3)_2$ at the critical concentration while the clone containing pYM101 was resistant. For those bacteria containing the type I pNS3XY plasmid, the reason for the weaker expression is not clear at present.

The experiments reported here also showed that there are two types of pNS3XY plasmids, differing in the number of base pairs between the SD sequence and the ATG triplet of the mouse MT-I gene. The spacing of 10 bp in type I is the same as the spacing in pYM101. This spacing is probably a good distance for an efficient translation of a MT transcript. The type II pNS3XY plasmid has no ATG triplet adjacent to the SD site. It is not clear where the ribosome would start protein synthesis in this case. There are no other methionein residue in the protein sequence. Whether an unusual initiation codon was used by the bacterial ribosome because of the synthetic SD sequence has yet to be investigated.

### 4.3.3. Less Than 50 ng of MT Is Present in 100 $\mu$g of Cell Extract

The $^{109}CdCl_2$ incorporation study suggested that the amount of MT present in the bacterial lysate from the pYM101 transformed cells is less than 40 ng in 100 $\mu$g of bacterial lysate. The estimation of MT in yeast lysate from the pADH224 transformed cells suggested the presence of 20 ng per 100 $\mu$g of yeast lysate. The estimation of MT in yeast using the $\alpha$-factor promoter was not done because the expression was not as reproducible as that using the ADHII promoter. In all the clones examined, MT represents less than 50 ng protein present in 100 $\mu$g of bacterial or yeast lysates. This corresponds to approximately 0.05% of the total cellular proteins.

### 4.3.4. Expression of MT Is Lethal to Yeast

In an internal expression system, yeast cells harboring pADH224 showed a cessation of growth and a complete inhibition of protein translation upon MT gene expression (Figures 4-6 and 4-11). Such an effect was observed to a lesser extent in a secretion expression system using the $\alpha$-factor promoter. This is shown by the fact that yeast cells harboring pRK724 showed a retardation of growth upon MT gene expression (4.2.6.2.). That MT is only expressed by a regulated $\alpha$-factor promoter but not by a constitutive $\alpha$-factor promoter supported this analysis. The toxic effect, however, is not as dramatic on bacterial MT clones as on yeast MT clones.

## Table 4-1

## Metal Resistance of Bacteria Harboring pYM101 on Agar Plates

| | Critical Concentration of Heavy Metals | | | | |
|---|---|---|---|---|---|
| | $AgNO_3$ | $CuCl_2$ | $HgOAc_2$ | $CdCl_2$ | $Pb(NO_3)_2$ |
| Cells Harboring Plasmids | 0.13 mM | 3.20 mM | 40 $\mu$M | 0.83 mM | 1.9 mM |
| pAS1 | – | – | – | – | – |
| pYM101 | + | + | + | + | + |

+: resistant

–: sensitive

$HgOAc_2$: $Hg(CH_3COO)_2$

## Table 4-2

## Metal Resistance of Bacteria Harboring pNS3XY on Agar Plates

| | Metals in Agar Plates | | | | |
|---|---|---|---|---|---|
| Cells Harboring Plasmids | Ag | Cu | Hg | Cd | Scores |
| pNS3 | 0 | 0 | 0 | 0 | 0 |
| pNS319 | 3 | 3 | 2 | 2 | 10 |
| pNS320 | 3 | 0 | 3 | 2 | 8 |
| pNS321 | 2 | 0 | 0 | 0 | 2 |
| pNS332 | 2 | 2 | 0 | 0 | 4 |
| pNS375 | 2 | 0 | 3 | 2 | 7 |
| pNS390 | 0 | 2 | 0 | 0 | 2 |
| pNS398 | 0 | 3 | 3 | 2 | 8 |
| pNS399 | 0 | 0 | 2 | 3 | 5 |
| pNS3104 | 0 | 0 | 2 | 0 | 2 |
| pNS3106 | 0 | 0 | 3 | 0 | 3 |
| pNS3108 | 0 | 0 | 0 | 2 | 2 |
| pNS3130 | 2 | 3 | 0 | 2 | 7 |
| pAS1 | 0 | 0 | 0 | 0 | 0 |
| pYM101 | 3 | 3 | 3 | 3 | 12 |

The metal ions were included in agar plates at their critical concentrations. The scores were given to each clone from 0 to 3 based on reproducibility as described in the text. Summation of the scores are addition of all four scores for each clone.

## Table 4-3

## Doubling Times of Bacteria Harboring pNS3XY in Liquid Media Containing $ZnCl_2$

| Cells Harboring Plasmids | Scores | Doubling Time (min) |
|---|---|---|
| pNS3 | 0 | 830 |
| pNS319 | 10 | 600 |
| pNS321 | 2 | 560 |
| pNS332 | 4 | 530 |
| pNS375 | 7 | 480 |
| pNS398 | 8 | 570 |
| pNS399 | 5 | 500 |
| pAS1 | 0 | 810 |
| pYM101 | 12 | 450 |

The score for each clone was obtained from Table 4-2. The doubling time was measured at 1.1mM $ZnCl_2$ in liquid medium.

## Table 4-4

## Incorporation of $^{109}CdCl_2$ by Bacteria

| Cells Harboring Plasmids | cpm[a] | $OD^{b}_{595}$ | $cpm/OD_{595}$ |
|---|---|---|---|
| pAS1 | 2,700 | 0.502 | 5,300[c] |
| pYM101 | 37,300 | 0.649 | 57,500 |

a. Total cpm used in the medium was $5.5\times10^5$ for each experiment.

b. The $OD_{595}$ reading of each cell culture was taken just prior to harvest on a Cary 118 spectrophotometer.

c. The data given in the table were from a single experiment. The $cpm/OD_{595}$ values from a number of experiments gave averages of 5,200 ± 200 and 58,000 ± 200, respectively.

## Table 4-5

### Two Types of Metal Resistant Bacteria Harboring pNS3XY

| Type | Cells Harboring Plasmids | | Sequence after SD Site |
|---|---|---|---|
| I | pNS320 | pNS321 | AGGAATTCC TCGGA ATG GAC CCC |
| | pNS332 | pNS375 | |
| | pNS390 | pNS399 | |
| | pNS3104 | pNS3106 | |
| | pNS3108 | pNS3130 | |
| II | pNS319 | pNS398 | AGGAATTCC CCC |

The sequence AGGAATTCC, which contains the SD site (AGGA), was carried by the pNS3 vector. The EcoRI linker (GGAATTCC) was ligated to the fragment containing the mouse MT-I gene after the S1 digestion. The CCC triplet is the third coding sequence for the mouse MT-I.

## Table 4-6A

### Measurement of Halo Diameters of the pADH224 Clone Around Filter Disks Containing Heavy Metals

| Metal | Amount ($\mu$l) | Cells Harboring pAB18 Size of Halo (mm) | Cells Harboring pADH224 Size of Halo (mm) |
|---|---|---|---|
| 200 mM $AgNO_3$ | 2 | 2 | r |
| | 5 | 3 | r |
| | 10 | 4 | r |
| | 15 | 4 | r |
| 1M $CuSO_4$ | 3 | r | r |
| | 5 | 1 | 0.5 |
| | 7 | 1.5 | 0.5 |
| | 10 | 2 | 1 |
| | 15 | 3.5 | 2 |
| 10 mM $CdCl_2$ | 1 | 1 | r |
| | 3 | 4 | r |
| | 5 | 5 | r |
| 100 mM $CdCl_2$ | 2 | 7 | r |
| | 5 | 10 | s |
| | 10 | 11 | 1 |

r: resistant, no halo observed. s: slight halo observed.

## Table 4-6B

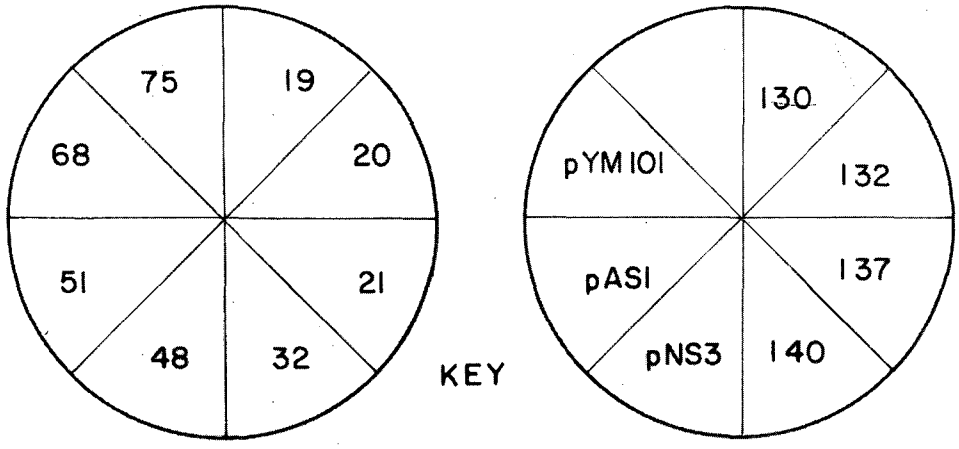
## Measurement of Halo Diameters of the pADH224 Clone Around Filter Disks Containing Heavy Metals

| Metal | Amount ($\mu$l) | Cells Harboring pAB18 Size of Halo (mm) | Cells Harboring pADH224 Size of Halo (mm) |
|---|---|---|---|
| 10 mM $HgOAc_2$ | 3 | r | r |
| | 5 | s | s |
| | 7 | s | s |
| | 10 | 0.5 | 0.5 |
| | 15 | 1 | 1 |
| 0.5M $ZnCl_2$ | 3 | 3 | 3 |
| | 5 | 4 | 4 |
| | 7 | 5 | 5 |
| | 10 | 7 | 7 |
| | 15 | 9 | 9 |

r: resistant, no halo observed.

s: slight halo observed.

Figure 4-1. Differentiation of bacterial cells harboring pYM101 and pNS3XY from those harboring pAS1 and pNS3 by growth on agar plates containing $Hg(CH_3COO)_2$. The critical concentration is 40 $\mu$M; 30 $\mu$M serves as a positive control for both types of cells. The key indicates the plasmids transformed into bacterial M5248 cells. The numbers shown on the plates are the numbers for the pNS3XY plasmids. For example, the number 19 represents plasmid pNS319. Bacteria transformed with pYM101, pNS319, pNS320 are more resistant to the critical concentration than their controls.

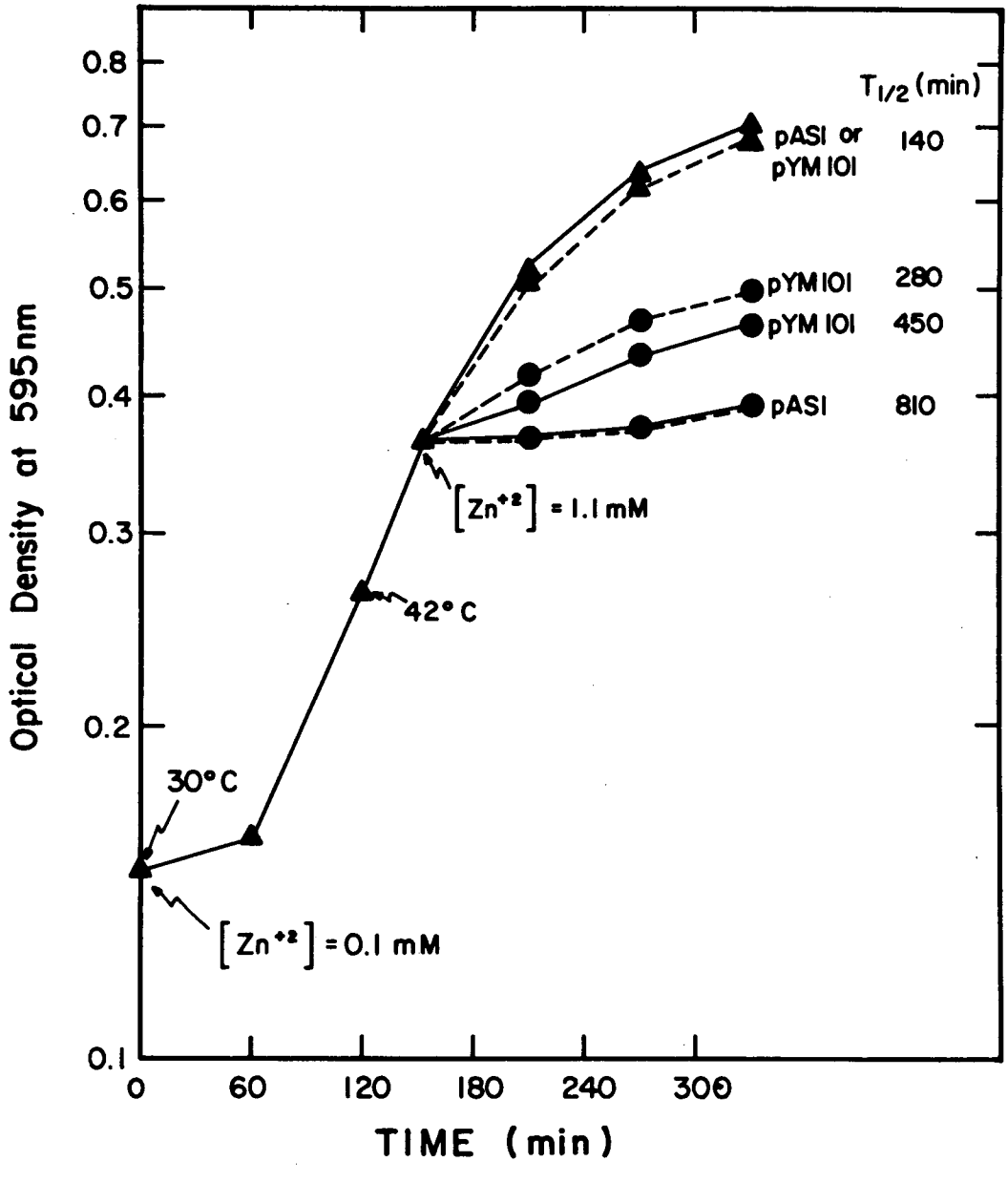



30 $\mu$M $Hg(CH_3COO)_2$

40 $\mu$M $Hg(CH_3COO)_2$

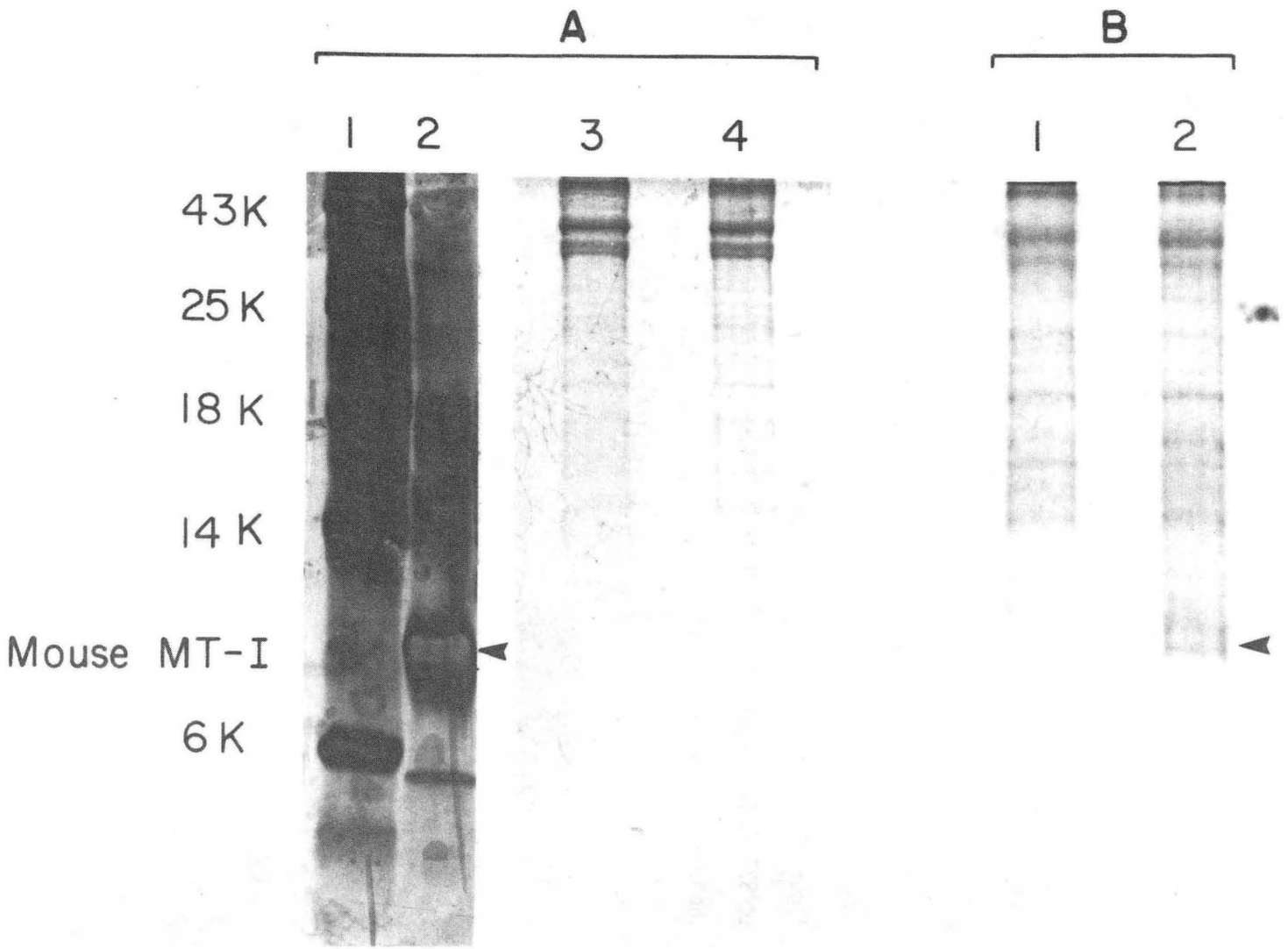
XBB 873-1554

Figure 4-2. Effect of 1.1 mM $ZnCl_2$ on the growth rate of bacteria harboring pYM101 versus bacteria harboring pAS1 in liquid media. The curves are plotted for growth at 1.1 mM $ZnCl_2$ (closed circles) or for growth at 0.1 mM $ZnCl_2$ (closed triangles). Each growth curve is designated by the name of the plasmid and its respective doubling time ($T_{1/2}$) is indicated at the end of each curve. Continuous lines are growth rates determined in regular LB medium. Broken lines are growth rates determined in LB containing a 20 $\mu$g/ml supplement of cysteine.


Optical Density at 595nm
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
60
120
180
240
300
TIME (min)
T1/2 (min)
pASI or pYM 101
140
pYM 101
280
pYM 101
450
pASI
810
[Zn+2] = 1.1 mM
42° C
30° C
[Zn+2] = 0.1 mM

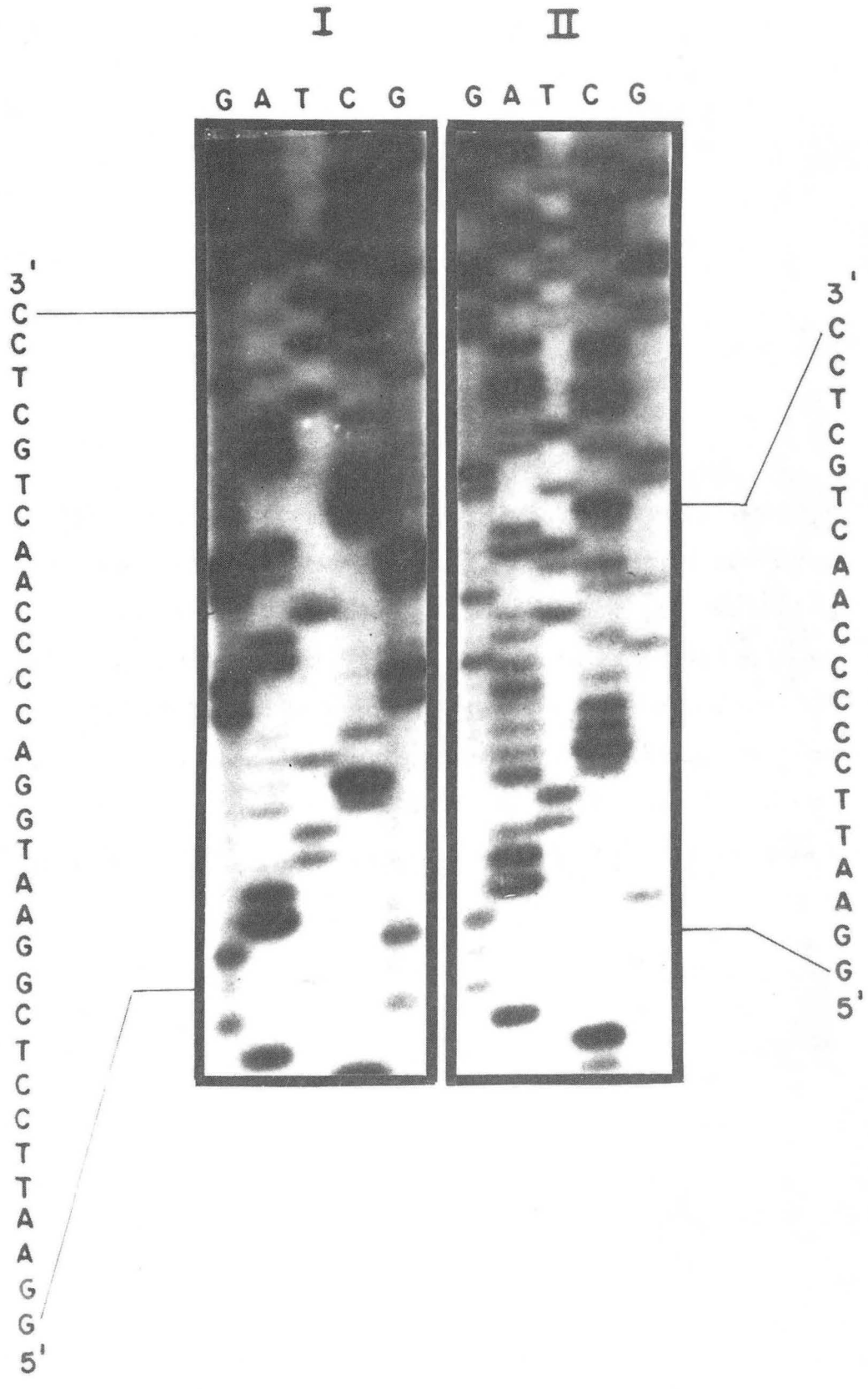
- XBL 872-617 -

Figure 4-3. A SUDS polyacrylamide gel analysis of crude bacterial extracts. Cultures of bacteria carrying either pAS1 or pYM101 were pulsed with [$^{35}$S]cysteine, lysed and carboxymethylated as described in 2.9.1.. After electrophoresis, the gel was stained by Coomassie brillant blue and fluorographed. (A) Coomassie brilliant blue stained gel of total cellular proteins. Lane 1, molecular weight markers of standard proteins: bovine trypsin inhibitor (6 Kd), lysozyme (14 kd), lactoglobulin (18 kd), chymotrypsinogen (25 Kd), and ovalbumin (43 kd). Lane 2, purified mouse MT-I protein. Lane 3, lysate from the pAS1 transformed cells. Lane 4, lysate from the pYM101 transformed cells. (B) An autoradiogram of pulse-labelled proteins. Lane1, lysate from the pAS1 transformed cells. Lane 2, lysate from the pYM101 transformed cells. Kd: kilodalton



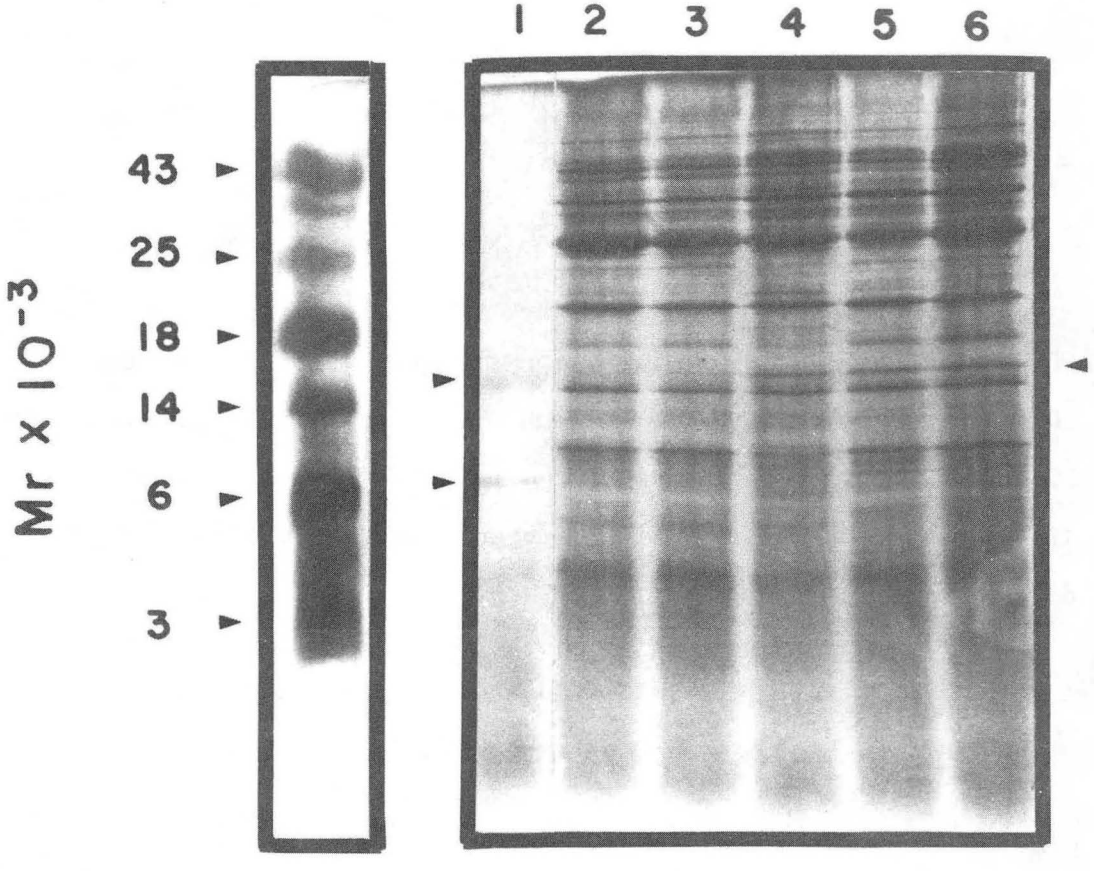

XBB 873-1555

Figure 4-4. DNA sequencing gel analysis showing the type I and type II constructions of pNS3XY. The reading starts from the EcoRI linker and extends to the sixth nucleotide triplet in the mouse MT-I coding sequence.


I
II
G A T C G
G A T C G
3'
C C T C G T C A A C C C C A G G T A A G G C T C C T T A A G G
5'
3'
C C T C G T C A A C C C C C T T A A G G
5'

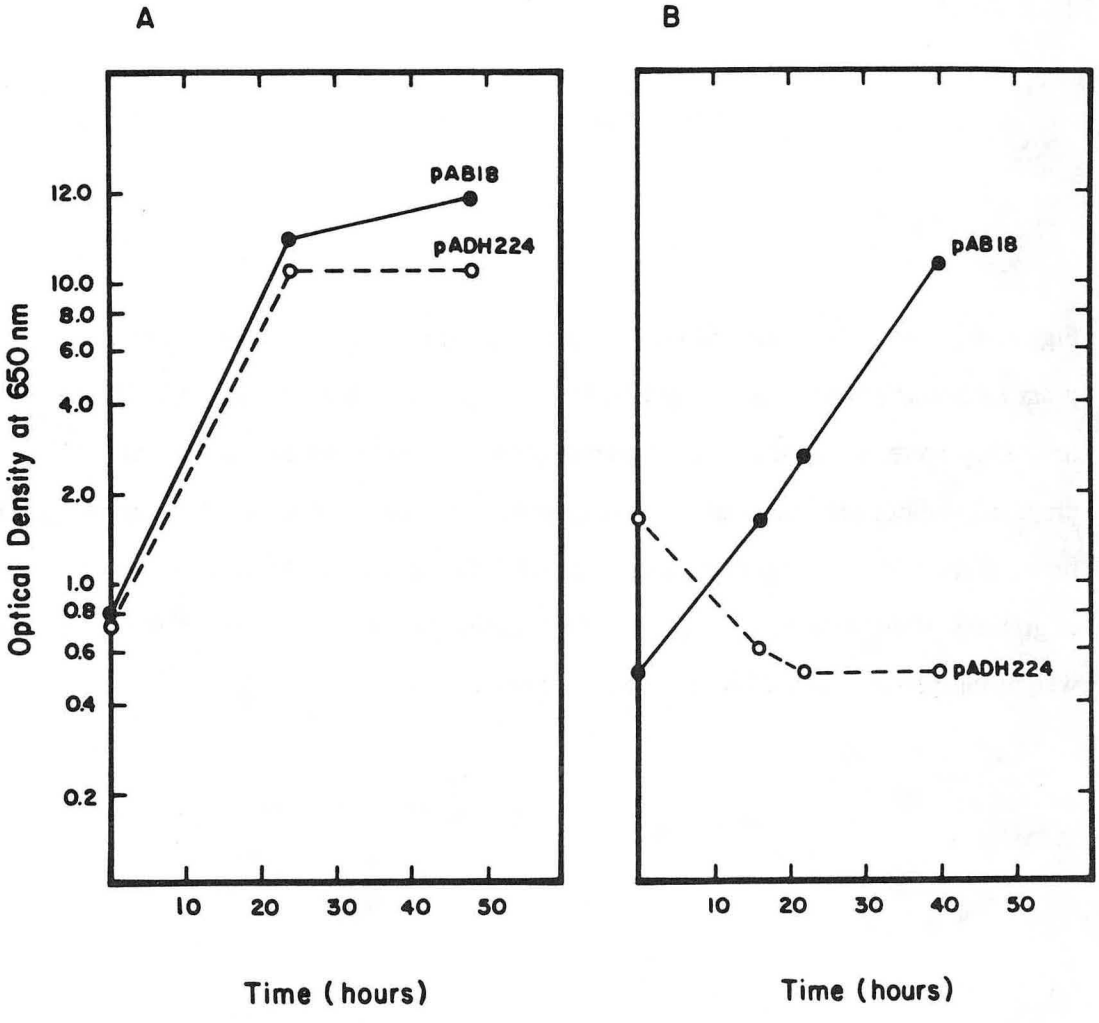
XBB 873-1556

Figure 4-5. A 20% Laemmli-SDS polyacrylamide gel analysis of human MT-II gene expression by the yeast $\alpha$-factor promoter. Cultures of yeast harboring either $\alpha$-factor or pRK724 were grown to saturation in the presencen of 50 $\mu$M $ZnSO_4$. The cells were spun down and proteins present in 2.5 ml of medium was precipitated with 12.5% TCA and 0.5 mg/ml deoxycholate. The precipitates were boiled in Laemmli buffer for 5 min and then applied to the gel. Left: molecular weight markers of standard proteins as in Figure 4-3A. Right: various yeast samples. Lane 1, purified mouse MT-I with dimer and monomer as indicated by the arrows. Lanes 2 and 3, samples of the $\alpha$-factor transformed cells. Lanes 4 to 6, samples of the pRK724 transformed cells.



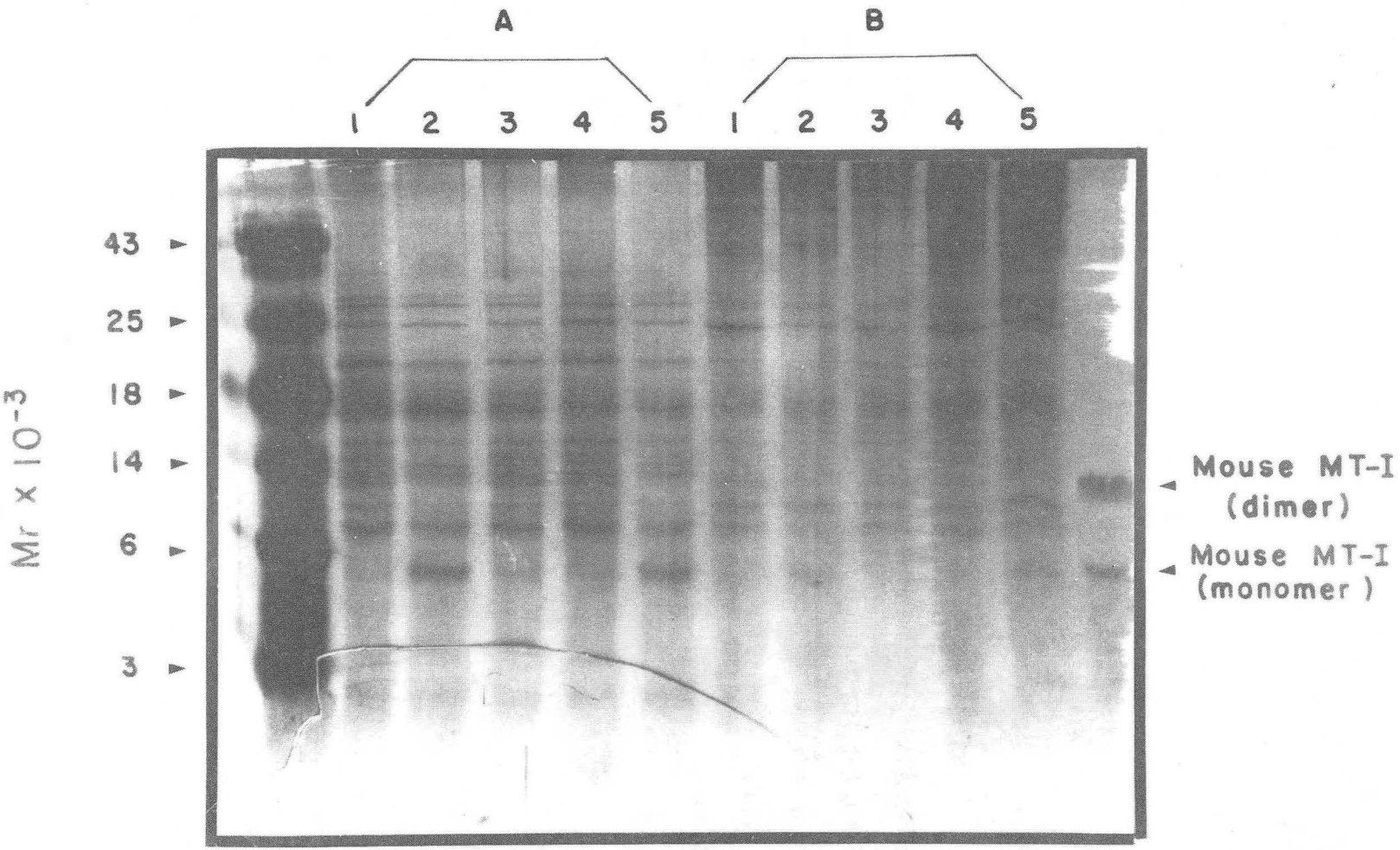

XBB 873-1557

Figure 4-6. Effect of human MT-II gene expression by the yeast ADHII promoter on the growth of yeast cells in (A) YEPD medium containing 2% glucose and in (B) YEPE medium containing 3% ethanol. Cells for YEPE were initially prepared in YEPD medium until mid-log phase. They were washed in YEP (no carbon source) and then diluted in YEPE. Each growth curve is designated by the name of the plasmid.




- XBL 873-10814 -

Figure 4-7. A 20% Laemmli-SDS polyacrylamide gel analysis of yeast extracts. Cultures of yeast harboring either pAB18 or pADH224 were grown in YEPD with 50 $\mu$M $ZnSO_4$ for 30-48 hrs. They were lysed, heated, carboxymethylated and resolved by the gel (A). They were also prepared without the heat-treatment but carboxymethylated and resolved by the gel (B). Lane 1, lysate of the pAB18 transformed cells. Lanes 2-5, lysates of the pADH224 transformed cells. The migrations of the dimer and monomer of the purified mouse MT-I are indicated. The molecular weight markers of standard proteins are as in Figure 4-3A.

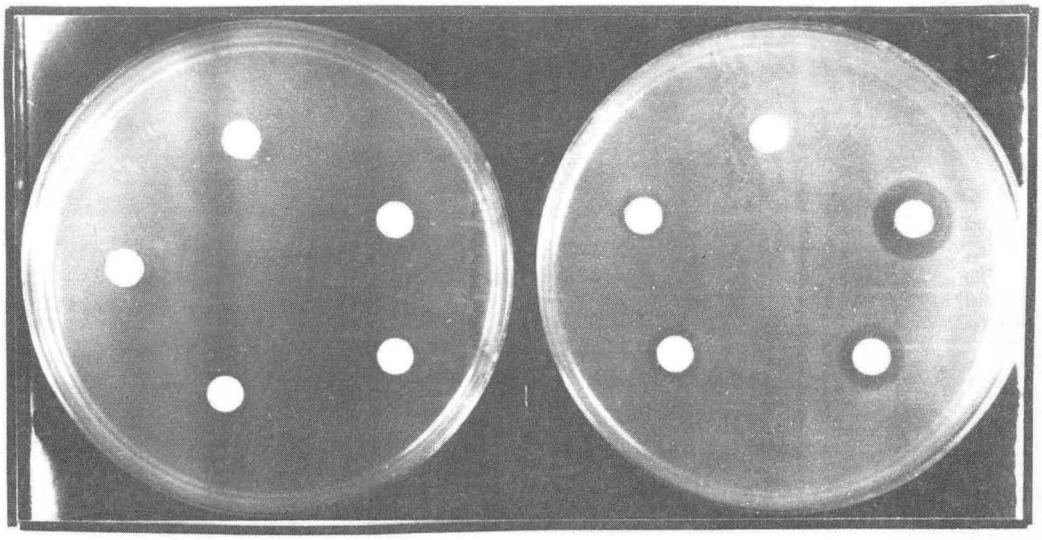

A
B
1 2 3 4 5 1 2 3 4 5
Mr x 10-3
43
25
18
14
6
3
Mouse MT-I (dimer)
Mouse MT-I (monomer)

XBB 873-1558

Figure 4-8. Metal resistance of yeast clone harboring pADH224 to 1 M $CuSO_4$ (A) and to 100 mM $CdCl_2$ (B). The control is the yeast clone harboring pAB18. (A) The amounts ($\mu$l) of 1 M $CuSO_4$ applied to the filter disks are (clockwise from the arrow): 3, 15, 10, 7, and 5. (B) The amounts ($\mu$l) of 100 mM $CdCl_2$ applied to the filter disks are (clockwise from the arrow): 1, 10, 5, 2, 5, and 3.

A

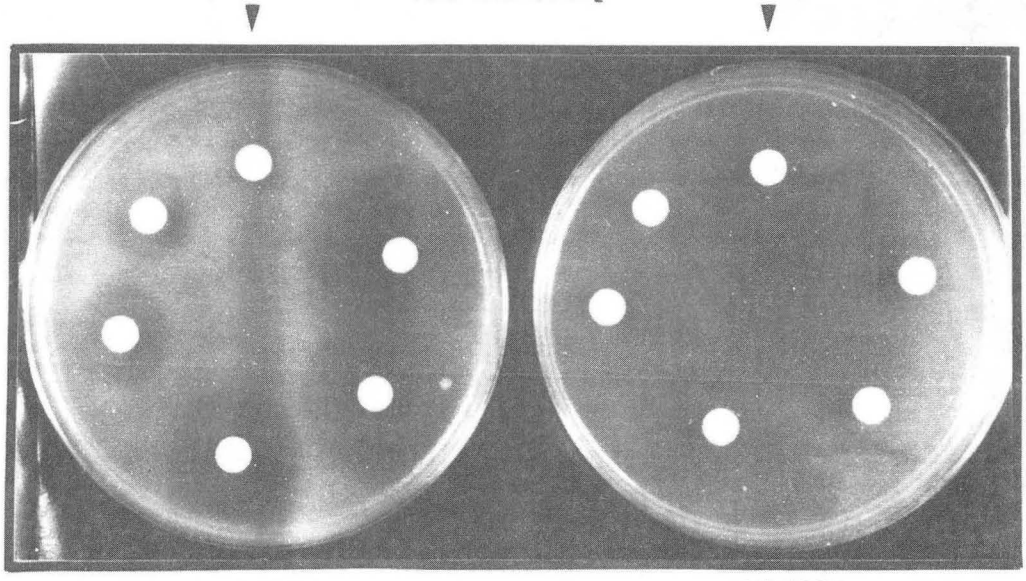
1 M $CuSO_4$



pADH224 pAB18

B

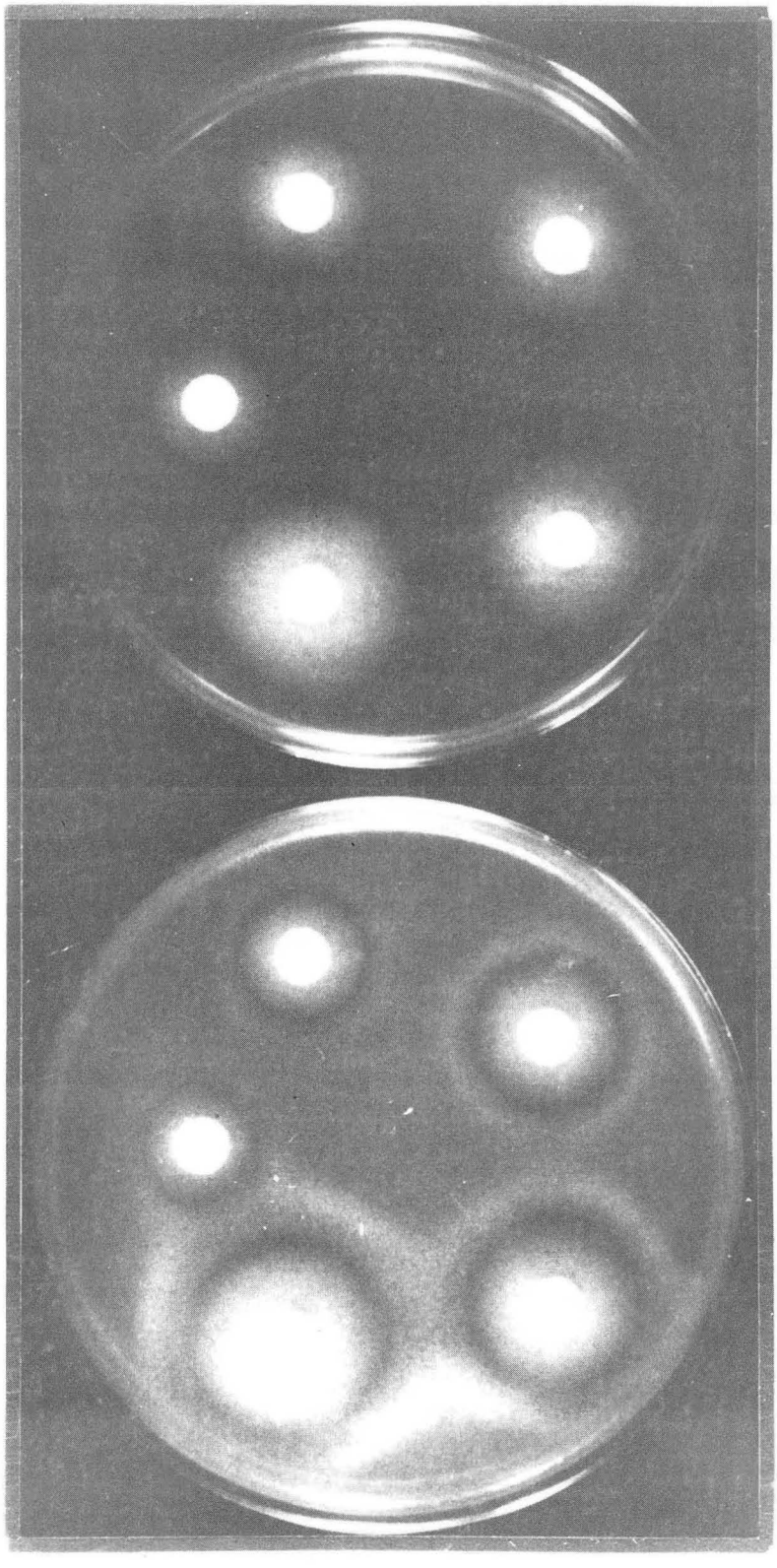
100 mM $CdCl_2$



pAB18 pADH224

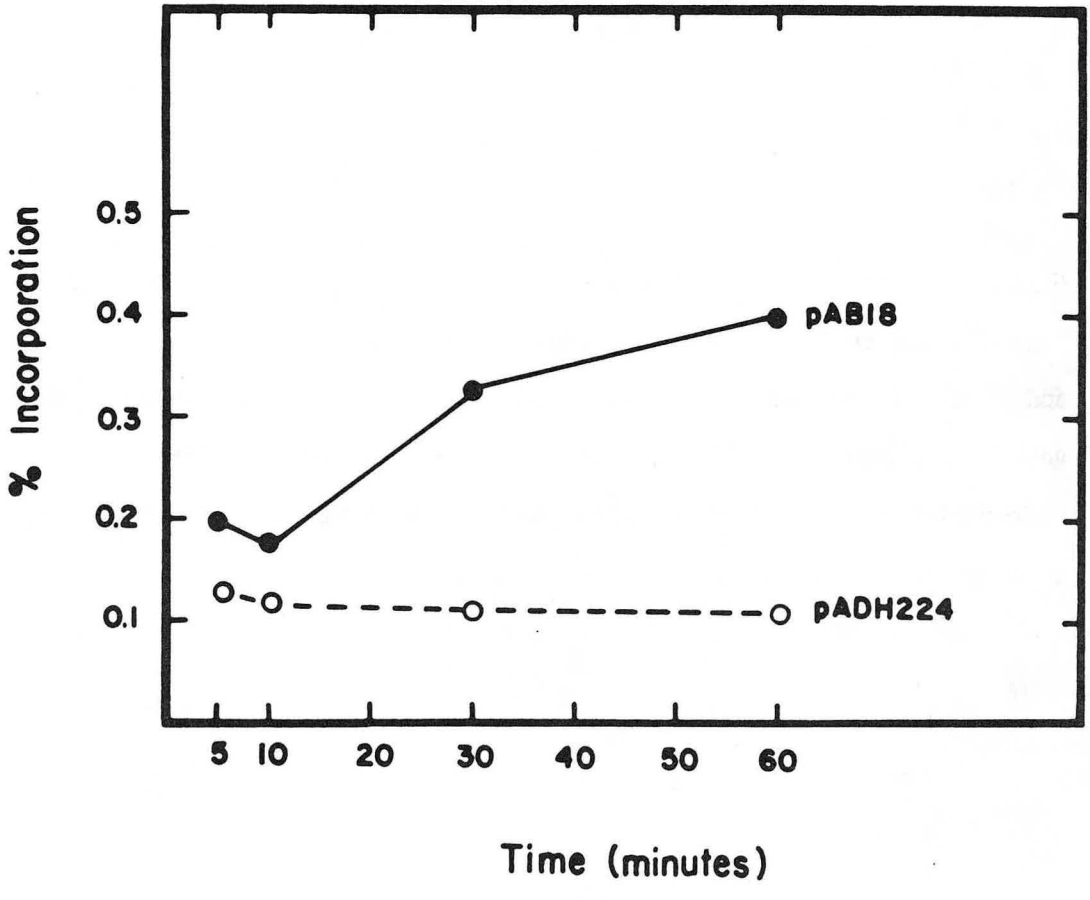
XBB 873-1559

Figure 4-9. Similar sensitivities of yeast clones harboring pADH224 and pAB18 to 500 mM $ZnCl_2$. The amounts ($\mu$l) of 500 mM $ZnCl_2$ applied to the filter disks are (clockwise fromt the arrow): 2, 5, 7, 10, and 15.



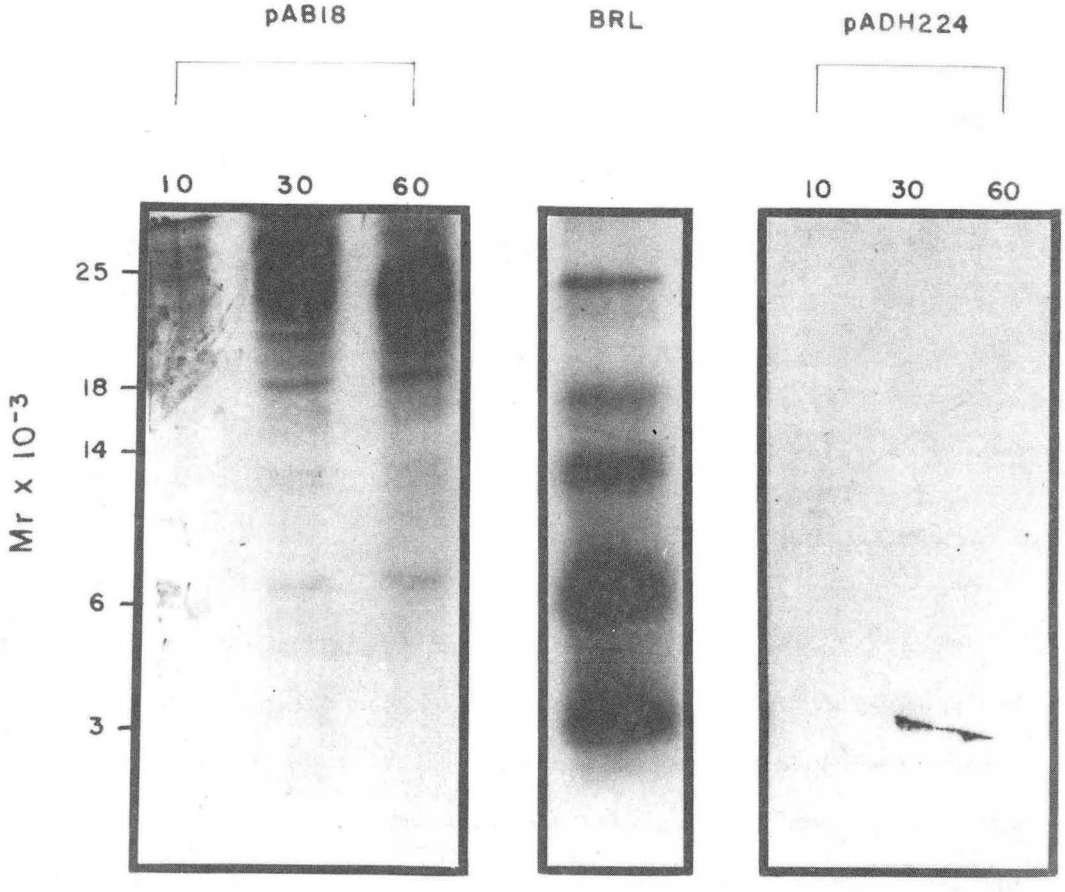

XBB 873-1560

Figure 4-10. Percentage of $^{35}S$ incorporation into yeast cells harboring pAB18 and pADH224 over a period of one hour. Closed circles: yeast cells harboring pAB18. Open circles: yeast cells harboring pADH224.


% Incorporation
0.5
0.4
0.3
0.2
0.1
pAB18
pADH224
5
10
20
30
40
50
60
Time (minutes)

- XBL 872-618 -

Figure 4-11. An autoradiogram of yeast extracts labelled with [$^{35}$S]sulfate. Samples of yeast extracts were prepared from yeast cells grown in the presence of [$^{35}$S]sulfate for 10 min, 30 min and 60 min as described in 2.9.2.. They were resolved on a 20% Laemmli-SDS polyacrylamide gel. Extracts from the pAB18 transformed cells and from the pADH224 transformed cells are indicated by the respective plasmids. The molecular weight markers of standard proteins are as described in Figure 4-3A.




XBB 873-1561

# CHAPTER 5

## The Search For Immunological Evidence

### 5.1. Introduction

Three methods, including Elisa, dot blot, and Western blot, were used to search for immunological evidence of the MT gene expression. While control experiments for each method showed a strong sensitivity of detection, all attempts failed to identify MT in the cell extract. The *in vitro* stability test showed that the MT is rapidly degraded in the presence of cell lysate. This suggests that mammalian MT is unstable in the microorganisms and that the amount of MT remaining after proteolysis is too low for detection by the three methods.

All three methods involve immobilizing the antigen to a solid phase as the target for a specific antibody. Immobilized antigen has an advantage over free antigen in that no specific one-to-one ratio for the antigen and the antibody is required to build an effective lattice for detection. This accounts for their ability to detect nanogram amounts of antigen. In Elisa, the antigen is covalently linked to a plastic titer plate. In dot blot, the antigen is fixed to a piece of blotting matrix. In Western blot, the antigen is first resolved by an electrophoresis and then blotted to a matrix. Both the dot blot and Western blot methods are more versatile than the Elisa method due to the variety of blotting matrice currently available. These blotting matrice differ in

pore size, protein-binding capacity and charged functional groups available for interacting with antigens. Technically, Western blot is often preferred because less background is detected in a biological sample after electrophoresis. The results of applying these methods to the bacterial and yeast MT clones and the results of their control experiments are described below.

## 5.2. Results

### 5.2.1. Elisa

#### 5.2.1.1. Titer of Goat Anti-Rabbit Peroxidase (GAR-POD) to Rabbit IgG is 1:800

The titer of GAR-POD to rabbit immunoglobulin (IgG) was determined as follows. On an Elisa titer plate, a serial dilutions of GAR-POD in 10% normal goat serum were added to sample wells containing a constant amount of purified rabbit IgG. A sample well without any rabbit IgG was included as a control to estimate the background. The extent of reaction in each sample well was judged by a color development reaction (see 2.11.1. in Chapter 2). The reaction was positive to a dilution of GAR-POD at 1:1600. Similar results were obtained when twice the amount of rabbit IgG was coated on the well. The GAR-POD was arbitrarily used at a dilution of 1:800 throughout the Elisa protocols.

#### 5.2.1.2. Titer of Rabbit Anti-Horse MT-I Antiserum to Mouse MT-I Is 1:120

With the titer of GAR-POD to rabbit IgG at 1:800, the amount of rabbit anti-horse MT-I antiserum necessary to produce the maximal signal with mouse MT-I was determined. This was done by coating a constant amount of purified mouse MT-I, together with a constant amount of bacterial lysate from the pAS1 transformed cells (see 2.10.1.1. in Chapter 2), to an Elisa titer plate. The quantities used for the purified protein and for the lysate were 200 ng and 5 $\mu$g, respectively. This was equivalent to a 4% yield of MT in the total bacterial cellular protein. The 4% yield was based on the reported overproduction of the $\lambda$ cII protein under the control of the $P_L$ promoter by Rosenberg *et al.* (1983). Phenylmethyl-sulfonyl fluoride (PMSF) at 1 mM concentration was included in each assay as a precaution against proteases. Previous experiments in this laboratory by D. Hoffman had shown that assays in the absence of PMSF were unsuccessful (data not shown). The titration curve of antiserum from dilutions of 1:1 to 1:500 with a constant

amount of antigen is presented in Figure 5-1. The series of reactions gave a bell-shaped curve which showed a peak at a 1:120 dilution of rabbit anti-horse MT-I antiserum. No Elisa reading was obtained from samples containing only the lysate. This indicates that the signal produced in Figure 5-1 was primarily due to the seeded mouse MT-I. The dilution, 1:120, of rabbit anti-horse antiserum was incorporated into the Elisa assay for the following experiments.

#### 5.2.1.3. The Sensitivity of Elisa Is 10 Nanogram of MT

Figure 5-2 shows the titration of rabbit MT-I by rabbit anti-horse MT-I antiserum at a dilution of 1:120, together with GAR-POD at a dilution of 1:800. Under these conditions, the amount of GAR-POD is in excess relative to that of antiserum. Except for the most concentrated antigen sample, the Elisa reaction is a function of the amount of rabbit MT-I present in the assay. The system is capable of detecting purified rabbit MT-I to at least 10 nanogram. This sensitivity was independently confirmed by substituting horse MT-I for rabbit MT-I in the assay (data not shown).

To address the possibility of lysate proteins interfering with the sensitivity of Elisa, rabbit MT-I was seeded into yeast lysates prepared from transformants harboring pAB18 and pADH224 respectively. The mixture was then coated to the sample well to serve as the antigen. Figure 5-3 shows the titration curves of rabbit MT-I in the presence of lysates (curves A and B) and in purified form (curve C). While the presence of lysate proteins increased the overall background readings, it did not alter the sensitivity of detection. All three curves, whether titrating a purified antigen or not, had a sensitivity of 10 nanogram. The lysates prepared from the pAB18 transformed cells and the lysates prepared from the pADH224 transformed cells behaved the same.

#### 5.2.1.4. No Signals in MT Clones by Elisa

The lysates of both bacterial and yeast MT clones were analyzed by Elisa over a wide range of protein concentrations. The results are shown in Table 5-1 and Table 5-2 for bacterial and yeast lysates, respectively. Over a range from 5 $\mu$g to 50 $\mu$g of total bacterial lysate proteins, the Elisa readings for the pAS1, the pYM101, and the pNS332 transformed cells were all near background level (Table 5-1). Similarly, no signal was evident from 2 $\mu$g to 200 $\mu$g of total yeast

lysate proteins from either the pAB18 or pADH224 transformed cells (Table 5-2). If anything, the readings from the control samples were slightly higher than those from the clones.

Also examined were lysates subjected to a heat treatment. Table 5-1 and Table 5-2 show that the heat treatment did not make a significant difference in the Elisa readings. There was no MT detected by Elisa.

### 5.2.2. Dot Blot

#### 5.2.2.1. Variations of MT Signals on Different Blotting Membranes

To characterize each blotting membrane, purified horse kidney MT-I, rabbit liver MT-I, mouse liver MT-II and rabbit IgG were blotted to each membrane and probed by rabbit anti-horse MT-I antiserum, followed by GAR-POD (see 2.11.2. in Chapter 2). The results are summarized in Table 5-3. A striking feature of Table 5-3 is that a substantial variation exists among different MTs on different blotting membranes. The following are two examples. First, rabbit MT-I gave no signal on nitrocellulose paper but quite strong signals on zeta-probe, nytran and carboxymethyl cellulose paper. Second, carboxymethyl cellulose paper gave a strong signal with rabbit protein, but did not give any signals with either the horse or the mouse protein.

The rabbit IgG served as a control for the assay. It was positive with all the membranes except the cyanogen bromide (CNBr) activated paper. Based on the reactivity of rabbit IgG, nitrocellulose paper is the least sensitive membrane and zeta-probe is the most sensitive membrane.

#### 5.2.2.2. $^{125}$I-Labelled Protein A Give Signals to MTs on CNBr Activated Paper

Among the five membranes, the CNBr activated membrane is the only one which forms a chemical linkage with the attached antigen; other membranes bind through either hydrophobic or hydrophilic interactions. The fact that CNBr activated paper did not give a signal to any of the proteins tested, including rabbit IgG, suggests an inherent incompatibility with the above assay. Using $^{125}$I-labelled protein A in place of GAR-POD rectified this problem. As demonstrated in Table 5-4, rabbit IgG and many MTs now gave signals on CNBr activated paper upon reacting with rabbit anti-horse MT-I antiserum and $^{125}$I-labelled protein A. Only isoform mouse MT-I remained inactive under these conditions. The effect of $^{125}$I-labelled protein A on the results of

zeta-probe was also investigated. Table 5-4 shows that as far as the horse MT-I and rabbit MT-I were concerned, the $^{125}$I-labelled protein A probe and the GAR-POD probe were equivalent on zeta-probe membrane.

### 5.2.2.3. Sensitivity to Mouse MT Is 25 Nanogram Using Rabbit Anti-Rat MT Antiserum or $^{109}CdCl_2$ as A Probe

The rabbit anti-rat MT antiserum was received after the dot blot study had been started. The purpose of using anti-rat MT antiserum in place of anti-horse MT antiserum was to take advantage of its stronger affinity for mouse MT. This is illustrated in Table 5-5, where the sensitivity of the two probes on CNBr activated paper are compared. Using the same $^{125}$I-labelled protein A detection method, the anti-horse antiserum only responded to mouse MT-II, but the anti-rat antiserum responded to both MT-I and MT-II of the mouse protein. The sensitivity of detection by the anti-rat antiserum using $^{125}$I protein A was 25 nanogram for both mouse proteins. For horse MT, rabbit MT, and rabbit IgG, the two probes were equally sensitive. For rat MT-I, the anti-rat MT antiserum was superior to the anti-horse MT antiserum, as had been expected. It is not clear, however, why the anti-rat MT antiserum did not react with the rat MT-II isoform.

Because of the high affinity of MTs in general for Cd, the approach of using $^{109}$Cd as a probe was explored. Table 5-5 shows the results of using $^{109}CdCl_2$ in replacement of antiserum and $^{125}$I labelled protein A in the dot blot assay. On the CNBr activated paper, this approach gave a sensitivities of 25 ng and 200 ng to the mouse MT-I and mouse MT-II respectively. For mouse MT-I, the sensitivity of $^{109}$Cd probe was the same as that of antiserum-$^{125}$I protein A probe. In addition to the mouse proteins, Table 5-5 also lists other mammalian MTs detected by the $^{109}$Cd probe. For proteins such as horse MT-I, horse MT-II, and rat MT-II, all of which either showed little or no signal with the antiserum-$^{125}$I protein A probe, the $^{109}$Cd was actually a better probe. Since using the $^{109}$Cd probe does not require the presence of antiserum, the absence of signal with the rabbit IgG was expected.

The use of $^{109}$Cd probe was extended to samples on zeta-probe, carboxymethyl cellulose, and nitrocellulose paper. Of the three membranes, zeta-probe and carboxymethyl cellulose paper had no signal. Nitrocellulose paper gave signals, but they were not as strong as on CNBr activated

paper. The comparison between CNBr activated paper and nitrocellulose paper, using $^{109}Cd$ as a probe, is made in Table 5-6.

Because the sensitiviy of the $^{109}Cd$ probe partly depends on the available $Cd^{+2}$-binding sites in the MT proteins, the effects of unfolding the proteins with SDS or by lowering the pH were examined (Table 5-7). The experiments were done by incubating the mammalian MT samples with SDS (at concentrations ranging from 0.1% to 2%) or at pH 3 to release the bound metal ions and then applying the denatured proteins to the CNBr activated paper under the conditions described in 2.11.2.2.. Table 5-7 shows that either SDS or low pH did not increase the sensitivity of $^{109}Cd$ probe with horse MT-I, mouse MT-I or rat MT-II. The negative result with rabbit MT-I in the presence of 2% SDS is probably not significant since the native protein does not give a very strong signal, either. Table 5-7 also shows that SDS alone did not produce a signal by the $^{109}Cd$ probe.

### 5.2.2.4. MTs Bind to Carboxymethyl Cellulose Paper

Membranes such as carboxymethyl cellulose, nytran, zeta-probe, and nitrocellulose bind to proteins via electrostatic and hydrophobic forces. The conclusion drawn from Table 5-3 that not all of the MTs gave signals on these membranes raised the question whether the protein stayed on these membranes throughout the detection procedure. To address this question, duplicate sets of horse MT-I, rabbit MT-I and mouse MT-II were applied to carboxymethyl cellulose paper. One set was probed by rabbit anti-horse MT antiserum and GAR-POD. The other set was subjected to protein staining with amido black (2.11.2.3.1.). The two sets of results are compared in Figure 5-4. While only the rabbit MT-I gave a signal with antisera, (as was shown in Table 5-3), all three MTs were shown to remain on the membrane. The control with rabbit IgG was positive with both methods. Similar staining procedures cannot be used with nytran paper, nitrocellulose paper and zeta-probe paper. However, we believe that MTs also bind to these other membranes. The signals of several MTs on nitrocellulose paper by the $^{109}Cd$ probe (Table 5-6) support this notion.

### 5.2.2.5. MT Clones Are Not Differentiated from the Control by Dot Blot

Zeta-probe paper and CNBr activated paper were used to examine gene expression in the

MT clones. After sample application, zeta-probe was analyzed by rabbit anti-rat MT antiserum, followed by either the $^{125}I$ labelled protein A or GAR-POD. CNBr activated paper was analyzed by either the antiserum-$^{125}I$ protein A method or the $^{109}Cd$ probe. Table 5-8 summarizes the results of two bacterial clones harboring pYM101 and pNS332 and the yeast clone harboring pADH224, together with the results of the respective controls. The amounts of total lysate protein investigated in the test covered a wide range. In almost all cases, both the clones and their controls had signals. These signals were similar in intensity and thus did not differentiate the clones from the controls. Two cases were found where the signals of the clones were weaker than those of the controls. The significance of such a phenomenon was not further assessed in this study. It was also noted that the signal intensity for the various samples did not change substantially as the test samples increased in protein concentration.

### 5.2.3. Western Blot

#### 5.2.3.1. The SUDS Gel Is Better Than The Laemmeli-SDS Gel for Western Blot

To characterize the conditions for Western blot, duplicate samples of mammalian MTs were electrophoresed in a SUDS gel and in a Laemmli-SDS gel, respectively. After electrophoresis, the proteins were electrotransferred to CNBr activated paper and then detected by rabbit anti-rat MT antiserum, followed by the $^{125}I$ labelled protein A (see 2.11.3. in Chapter 2). The results of the two gel systems are compared in Figure 5-5. The SUDS gel resolved mouse MT-I to a position corresponding to a molecular weight of 8-10 kilodalton. The Laemmli-SDS gel resolved the MT-I to a position equivalent to a 14 kilodalton molecular weight. For the purpose of performing Western blot on MT clones, the SUDS gel is better than the Laemmli-SDS gel for the mouse MT-I. This is because very few bacterial or yeast cellular proteins have monomeric molecular weights less than 14 kilodaltons, but quite a few have molecular weights around and above 14 kilodaltons. Both gels, however, resolved the rabbit MT-I protein to a 14 kilodalton molecular weight.

The system of Western blot demonstrated in Figure 5-5 gave signals only to MTs and carboxymethylated MTs. It gave no signal for trypsin or any other standard proteins that were applied to the gel with the MT samples. Approximately 90% of the total amount of protein was

transferred to the CNBr activated paper. This was estimated from the amount of residual proteins left on the gel by silver stain.

The signal of mouse MT-I in Figure 5-5 was generated from 300 ng of the purified protein. It should be noted that this does not represent the sensitivity of the method. Lower amounts were possible, but were not determined.

### 5.2.3.2. Triton-$Mg^{+2}$ Lysates for the SUDS Gel

The bacterial lysates previously examined by Elisa and dot blot were prepared by the method described under active lysate preparation (see 2.10.1.1. in Chapter 2). For Western blot analysis, lysates were prepared by the triton-$Mg^{+2}$ lysis method instead (see 2.10.1.2. in Chapter 2). These two methods differ in that the active lysis procedure used exogeneous DNase to remove the nucleic acids in the bacterial cell extract while the triton-$Mg^{+2}$ lysis procedure used sonication. Because the SUDS gel has a much lower capacity than the Laemmeli-SDS gel, bacterial lysates were prepared by the trion-$Mg^{+2}$ method so as not to add DNase. Except for the proteins associated with the bacterial periplasmic space, which were removed by the active lysis preparation, the two methods yielded the same fraction of cellular proteins.

Bacterial lysates were tested for the maximal amount of protein that could be applied to a SUDS gel. For this purpose, lysates were prepared from 0.5 ml, 1.5 ml, 2.0 ml, 3.0 ml and 5.0 ml of cell culture, heated at 68°C, and then electrophoresed on a SUDS gel. The gel was examined after silver staining for the quality of protein resolution. It was decided that 1.5 ml of cell culture gave the best results (data not shown). This amount of cell culture provided approximately 15 $\mu$g of total cellular protein to be loaded onto a SUDS gel, assuming that 1.0 ml of cell culture has 100 $\mu$g of protein and that 10% of the total cellular protein is preserved after the heat treatment.

### 5.2.3.3. No Signals from MT Clones by Western Blot

Figure 5-6 shows the autoradiogram of a Western blot examining 15 $\mu$g of heated bacterial lysates and 10 $\mu$g of heated yeast lysates. These lysates were prepared from bacterial cells harboring plasmids pYM101 and pAS1, and from yeast cells harboring plasmids pADH224 and pAB18. As an internal control, 300 ng of purified mouse MT-I was added as a spike to the preparation of bacterial lysates from the pAS1 transformed cells. While the spiked sample

showed a signal at the position where the purified mouse MT-I would run, none of the MT clones had a signal. In addition to the signal from the spiked MT-I, there were a few bacterial lysate proteins that cross-reacted with the antiserum. One of them migrated very close to where the signal of mouse MT-I was detected. No such cross-reaction was present in the yeast lanes.

#### 5.2.3.4. Spiked MT Is Partly Degraded During the Preparation of Lysates

A spiked sample containing 300 ng of mouse MT-I and 15 $\mu$g of bacterial cell lysate from the pAS1 transformed cells was prepared in a different way than that in Figure 5-6. The purified mouse MT-I was added to the lysate after the lysis protocol was completed and the sample was then immediately subjected to electrophoresis. Western blot analysis of such a spiked sample showed that the intensity of the signal from mouse MT-I was several times stronger than what was shown in Figure 5-6 (data not shown). The contrast between the two systems leads to the conclusion that mouse MT-I when spiked during the preparation of the bacterial lysate was partly degraded. A pertinent observation was that when the same amount of mouse MT-I was spiked during lysate preparation from more than 1.5 ml cell culture, it failed to even generate a signal under the same experimental conditions. Although a protease inhibitor, PMSF, was included throughout the lysate preparation at concentrations ranging from 1 mM to 5 mM, it did not prevent the loss of MT protein.

#### 5.2.3.5. No Signals from MT Clones Grown in Anaerobic Conditions

Anaerobic growth conditions were achieved by inducing the bacterial cells in the absence of shaking or aeration for the period of one generation time. Cells were subsequently harvested and the lysates were subjected to Western blot analysis. Upon examining the bacterial lysates from the pAS1 and pYM101 transformed cells, the spiked sample again had a strong signal at a position corresponding to the purified mouse MT-I, but the lysate from the pYM101 transformed cells did not (data not shown). Two volumes of cell lysates wer loaded onto the gel; the clone and the control looked identical in both cases.

#### 5.2.3.6. No Signals from MT Clones Prepared by Gel Isolation

A preparative denaturing polyacrylamide gel was used to isolate the cellular fraction that possibly contained MT. Figure 5-7 shows the Western blot autoradiogram of bacterial lysates

by such a preparation. The internal control was provided by spiking 3 $\mu$g of purified mouse MT-II during the preparation of the control lysate. At the region where the purified mouse MT-II migrated, bacterial lysate obtained from the pYM101 transformed cells had no signal while the spiked sample contained a very strong signal. A very weak signal was detected in the lysate from the pAS1 transformed cells. It is not clear what this weak signal originated from.

The bacterial lysates for Figure 5-7 were prepared by boiling the cells after harvest in the Laemmli sample buffer. Such a quick denaturation resulted in a high yield of spiked MT. By comparing the intensity of the spiked MT with that from the purified protein, Figure 5-7 demonstrated a greater than 90% recovery of the seeded protein following the workup and analysis. In a lysate normally prepared from 13 ml of bacterial cell culture, the amount of seeded protein represented only 0.2% of the total cellular protein. The high recovery of spiked MT confirms the specificity of the method.

The autoradiogram in Figure 5-7 has a much less background than the previous Western blots. This is because only that fraction of protein with molecular weights similar to that of MT was investigated.

#### 5.2.3.7. No Signals from MT Clone by $^{109}$Cd Probe

Western blot using $^{109}$Cd probe instead of antiserum-$^{125}$I labelled protein A probe was investigated. To promote the binding between MT and $^{109}$Cd, the samples were electrophoresed in a native polyacrylamide gel rather than in a SUDS gel. The purified rat MT-II and its spiked sample contained in a yeast lysate from the pAB18 transformed cells served as positive controls for this assay. Both positive controls gave strong signals in the area where the purified protein migrated under the native gel conditions. However, the yeast lysate prepared from the pADH224 transformed cells showed no signal and was indistinguishable from that prepared from the pAB18 transformed cells (data not shown). The overall background with $^{109}$Cd probe was higher than that obtained with antiseral probe, but this did not affect the above results.

### 5.2.4. MT Is Unstable in Bacterial Cell Lysate

The lysate of a bacterial crude extract containing plasmid pAS1 was incubated with purified rat MT-II at a ratio of 20 to 1 (w/w). At various time points, aliquots of the mixture were

removed and immediately denatured by boiling in Laemmli sample buffer. The presence of the rat MT-II in the lysate was analyzed by a SUDS gel and detected by silver stain. The results of such a stability test is shown in Figure 5-8. The intact rat MT-II was detected only at time 0; within 2 min, no MT-II was detected. Figure 5-11 also shows an increased stability of rat MT-II in the presence of a serial dilution of the bacterial extract. More protein was detected as the extract became less concentrated. Both lines of experiments suggest that the eucaryotic MTs can encounter severe proteolysis in a bacterial crude extract.

## 5.3. Discussion

### 5.3.1. Elisa

It should be noted that the antiserum used in this laboratory is polyclonal. In the limited space of an Elisa sample well, the presence of a variety of antibodies may pose a steric hindrance to the coated antigen. This is why it is necessary to titer both the first (rabbit anti-horse MT) and the second (GAR-POD) antisera in the Elisa assay. The bell-shaped curve of Figure 5-1 is not attributed to the lattice theory as in solution precipitation (Eisen, 1974), but is a result of the polyclonal nature of the antisera. When the rabbit anti-horse MT-I antiserum is very concentrated relative to the amount of coated MT-I, the overall Elisa reaction was inhibited by the presence of weak antibodies. As the antiserum was more and more diluted, the reaction was gradually enhanced due to the improved access of strong antibodies to the antigen. When the amount of strong antibodies became the limiting factor, the reaction again decreased correspondingly. This effect would also explain the initial irregular behavior in Figure 5-2, when the most concentrated sample was examined.

Although rabbit MT-I was primarily tested in the Elisa assay, it was assumed that mouse MT-I or human MT-II would behave similarly in the Elisa assay. This notion was supported by the same titration activity of rabbit MT-I and horse MT-I toward rabbit anti-horse MT-I antiserum.

The experiments in this study established that mammalian MT, whether purified or not, can be detected to at least the 10 nanogram level. However, bacterial or yeast MT clones failed to produce a signal under these same experimental conditions. This means that the level of MT was less than 10 ng in 50 $\mu$g of bacterial lysate or 10 ng in 200 $\mu$g of yeast lysate. Alternative Elisa

protocols have also been tried: for example, the titer plate was first coated with goat anti-rabbit MT-I and then allowed to interact with rabbit anti-horse MT-I and GAR-POD. These alternative protocols did not give any better results.

### 5.3.2. Dot Blot

Using the dot blot method, different mammalian MT samples have very different immunological properties. On the same blotting membrane, some MTs react strongly with a MT specific antibody (not necessarily directed against the same protein) while others do not react at all. For example, while both the rabbit and horse MTs gave signals to rabbit anti-horse MT antiserum on zeta-probe membrane, neither of the two mouse MT isoforms gave a signal (Table 5-4). In fact, the mouse proteins only reacted with the antiserum on CNBr activated paper. This finding is somewhat surprising because, by every physical means, MTs are conserved in both structure and function and therefore, one might expect that they would also be conserved in immunological activity. The unique behavior of mouse MTs indicates an intrinsic property of that particular MT species. It is conceivable that this variation in antigenic activity may have biological significance.

On different blotting membranes, the same MT species exhibits different reactivities toward a particular antibody. This can be attributed to factors such as the configurations of the protein and the orientation of charged groups on the membrane. Among the five blotting membranes used, CNBr activated paper worked the best for the mouse MT-I protein. With either the antiserum-$^{125}$I protein A probe or the $^{109}$Cd probe, 25 ng of the purified protein can be detected. MTs attached to CNBr activated paper are probably in a configuration independent of metal ions because neither SDS nor low pH had an effect on their reactivity to the $^{109}$Cd probe. It is likely that the protein has to release its metal ions in order to interact efficiently with the CNBr groups on the paper.

By a variety of assays, the dot blot study found that none of the MT clones produces a distinguishable signal over control. The prevelance of a high background in the assay indicates that bacterial or yeast cellular proteins interfere with detection. The background can be due to the nonspecific interaction between cellular proteins and antiserum in the antiserum-$^{125}$I protein A assay or due to the random mercaptolinkages of the $^{109}$Cd probe to the cysteine residues in general in the $^{109}$Cd assay. There is also speculation that overexpression of a foreign gene

can induce protease activities in procaryotes and in yeast (C.-T. Chin, personal communication). Although this hypothesis has not been vigorously proved, it might explain the observation that the background signals in MT clones sometimes are weaker than those in the control.

### 5.3.3. Western Blot

Western blot has the advantage over dot blot in that the interference of cellular proteins is significantly reduced. The present study shows that electrophoresing a cell lysate before subjecting it to hybridization with antiserum or $^{109}Cd$ probe allows one to focus on MT in a cell lysate. A spiked sample containing a mammalian MT and a bacterial cell lysate gave a discrete signal of MT above a low background level. The background interactions in bacterial lysates were present in Western blot, dot blot, and Elisa. These background interactions were not easily removed even if a prehybridization with a control bacterial lysate had been conducted. The absence of background in the yeast lysate (Figure 5-6) is a little surprising since the rabbit from which the antiserum was prepared would have elicited an immune reaction should an infection by yeast had occurred during its lifetime. The same sample had shown background in dot blot, and so it was probably degraded prior to the Western blot analysis.

Western blot can examine 15 $\mu$g of bacterial lysate and can detect at minimun 300 ng of purified MT. If MT clones had expressed 2% of their total proteins as MT, the expressed protein would have been detected. However, no evidence was obtained although a variety of lysates had been prepared and tested. In fact, this study shows that MT is prone to degradation in the presence of the lysate. Presumably, the longer a lysate preparation takes, the more likely the degradation. Since the protease inhibitor PMSF is included and since it only affects certain proteases in the bacterial or yeast lysates, other proteases probably account for the rapid degradation. The protease problem for an expressed protein in bacteria has been previously observed by Talmadge and Gilber (1982). Prouty *et al.* (1975) has suggested that abnormal proteins in *E. coli*, such as those foreign to the cell, tends to form an aggregate as a way to prevent degradation. This possibility of MT present as an aggregate in the cell lysate, however, has not been investigated.

## 5.4. Conclusions

Summarizing the results of Elisa, dot blot, and Western blot, the following conclusions were

made. An intensive search for the presence of a protein species in the MT clones that would specifically interact with an antibody directed against MT or with $^{109}Cd$ has been unsuccessful. However, all three approaches were able to detect mouse MT-I to a quantity in the nanogram range when the protein was spiked into a crude lysate. Rapid degradation of the newly synthesized protein in the presence of lysate is a likely explanation for the negative results. This is supported by the stability test *in vitro*.

## Table 5-1

## Elisa Readings of Bacterial Lysates[1]

| | Unheated Lysates | | | Heated Lysates | |
|---|---|---|---|---|---|
| Lysates of Transformed Cells | pAS1 | pYM101 | pNS332 | ΔpAS1 | ΔpYM101 |
| Amount of Protein Tested | | | | | |
| 5 μg | 0.11 | n.d.[2] | 0.09 | n.d. | n.d. |
| | 0.13 | n.d. | 0.09 | n.d. | n.d. |
| 10 μg | 0.11 | n.d. | 0.11 | 0.07 | 0.05 |
| | 0.13 | 0.09 | n.d. | n.d. | n.d. |
| 20 μg | 0.13 | n.d. | 0.12 | 0.06 | 0.07 |
| | 0.12 | 0.10 | n.d. | n.d. | n.d. |
| 25 μg | 0.16 | 0.12 | n.d. | n.d. | n.d. |
| 50 μg | 0.14 | 0.06 | n.d. | n.d. | n.d. |

1. Bacterial lysates were prepared from transformed bacterial cells by the procedures described in Chapter 2. The heated lysates, as indicated by the Δ sign prior to the plasmids, were supernatants obtained after denaturing the lysates at 68°C for 10 min.

2. n.d. Not Determined.

## Table 5-2

## Elisa Readings of Yeast Lysates[1]

| | Unheated Lysates | | Heated Lysates | |
|---|---|---|---|---|
| Lysates of Transformed Cells | pAB18 | pADH224 | ΔpAB18 | ΔpADH224 |
| Amount of Protein Tested | | | | |
| 2 μg | n.d.[2] | n.d. | 0.15 | 0.15 |
| 6 μg | n.d. | n.d. | 0.15 | 0.15 |
| 12 μg | n.d. | n.d. | 0.16 | 0.13 |
| 45 μg | 0.28 | 0.22 | n.d. | n.d. |
| 135 μg | 0.38 | 0.22 | n.d. | n.d. |
| 270 μg | 0.45 | 0.24 | n.d. | n.d. |

1. Yeast lysates were prepared from transformed yeast cells by the procedures described in Chapter 2. The heated lysates, as indicated by the Δ sign prior to the plasmids, were supernatants obtained after denaturing the lysates at 68°C for 10 min.

2. n.d. Not Determined.

## Table 5-3

## Reactivities of Various Metallothioneins to Rabbit Anti-Horse MT-I Antiserum on Different Blotting Membranes Using GAR-POD as a Probe

| | Blotting Membranes | | | | |
|---|---|---|---|---|---|
| Samples | Nitrocellulose | Zeta-Probe | Nytran | CMC | CNBr |
| Horse MT-I | ±50 ng | 50 ng | — | — | — |
| Rabbit MT-I | — | 100 ng | ±50 ng | 25 ng | — |
| Mouse MT-II | — | — | — | — | — |
| Rabbit IgG | 50 ng | 12.5 ng | 25 ng | ±25 ng | — |

The symbol ± indicates a very weak signal.

The quantity included in each column represents the least amount of protein tested that gave a signal for a particular membrane. The higher the quantity required, the less sensitive the detection.

The symbol "—" means no signal was detected.

The following blotting membranes were used: nitrocellulose paper (0.45 $\mu$m), zeta-probe paper (0.45 $\mu$m), nytran paper (0.45 $\mu$m), carboxymethyl cellulose paper (0.45 $\mu$m) and CNBr activated paper (0.45 $\mu$m). Carboxymethylated cellulose paper is abbreviated as CMC and cyanogen bromide activated paper is abbreviated as CNBr.

## Table 5-4

## Reactivities of Metallothioneins to Rabbit Anti-Horse MT-I Antiserum Analyzed by $^{125}$I-Labelled Protein A

| | Zeta-Probe | CNBr | Zeta-Probe Analyzed by GAR-POD |
|---|---|---|---|
| Horse MT-I | ±50 ng | 25 ng | 50 ng |
| Rabbit MT-I | 100 ng | 25 ng | 100 ng |
| Mouse MT-I | — | — | — |
| Mouse MT-II | — | ±25 ng | — |
| Rabbit IgG | 12.5 ng | 12.5 ng | 12.5 ng |

The symbols used in Table 5-4 are the same as those in Table 5-3.

## Table 5-5

## Reactivities of Various Metallothioneins to Antiserum and to $^{109}Cd$ on CNBr Activated Paper

| | Rabbit Anti-Horse MT-I $^{125}I$-protein A | Rabbit Anti-Rat MT-I $^{125}I$-protein A | $^{109}Cd$ |
|---|---|---|---|
| Mouse MT-I | — | 25 ng | 25 ng |
| Mouse MT-II | 25 ng | 25 ng | 200 ng |
| Horse MT-I | 25 ng | 100 ng | 25 ng |
| Horse MT-II | — | — | 200 ng |
| Rabbit MT-I | 50 ng | 25 ng | 200 ng |
| Rat MT-I | — | ±25 ng | ±100 ng |
| Rat MT-II | — | — | 100 ng |
| Rabbit IgG | 50 ng | 50 ng | — |

The symbols used in this table are the same as those in Table 5-3.

## Table 5-6

## Reactivities of $^{109}Cd$ to MTs on Nitrocellulose and CNBr Activated Paper

| | CNBr | Nitrocellulose |
|---|---|---|
| Mouse MT-II | 200 ng | ±200 ng |
| Horse MT-I | 25 ng | 50 ng |
| Horse MT-II | 200 ng | ±200 ng |
| Rabbit MT-I | 200 ng | 100 ng |
| Rat MT-II | 100 ng | 100 ng |
| Rabbit IgG | — | — |

The symbols used in this table are the same as those in Table 5-3.

## Table 5-7

## Effects of SDS and Low pH on the Reactivity of $^{109}Cd$ with MTs Attached to CNBr Activated Paper

| | No SDS pH = 7.0 | SDS pH = 7.0 | No SDS pH = 3.0 |
|---|---|---|---|
| Horse MT-I | 50 ng | 100 ng[a] | 100 ng |
| Mouse MT-I | 100 ng | 100 ng[a] | 100 ng |
| Rat MT-II | 100 ng | 100 ng[b] | 100 ng |
| Rabbit MT-I | — | —[c] | n.d. |
| SDS Alone | — | — | n.d. |

a. SDS at a concentration of 0.1-0.2%.

b. SDS at a concentration of 1%.

c. SDS at a concentration of 2%.

n.d. Not Determined.

The rest of the symbols are the same as those in Table 5-3.

## Table 5-8

## Analysis of Bacterial and Yeast Lysates by Dot Blot

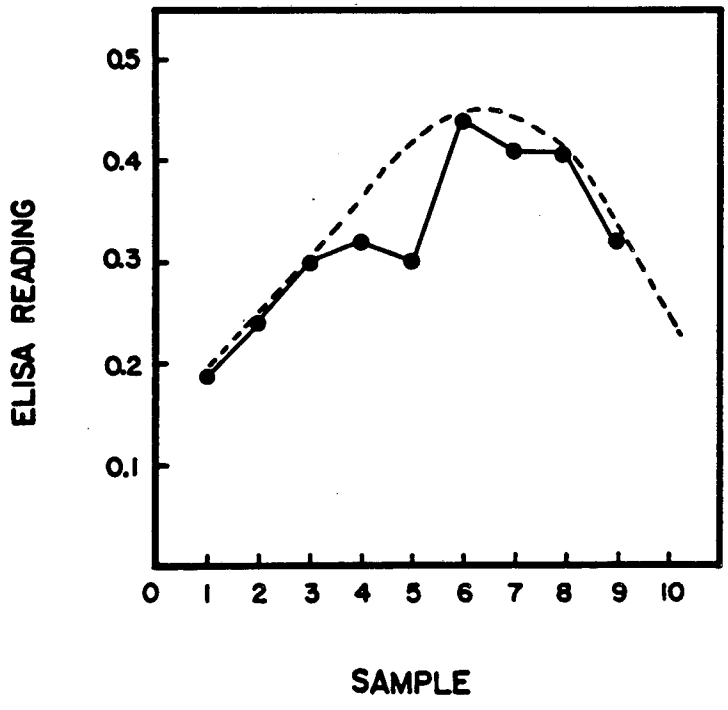
| | Zeta-Probe Membrane | | CNBr Activated Membrane | |
|---|---|---|---|---|
| Plasmids in Cells | $^{125}$I-protein A | GAR-POD | $^{125}$I-protein A | $^{109}$Cd |
| pAB18 | + (1) | + (2) | + (3) | + (4) |
| pADH224 | + (1) | ± (2) | + (3) | + (4) |
| pNS3 | + (5) | ± (6) | | + (7) |
| pNS332 | ± (5) | ± (6) | | + (7) |
| pAS1 | | + (8) | | |
| pYM101 | | + (8) | | |

The numbers in the parentheses refer to the following protein quantities ($\mu$g) that were tested. (1) 0.75, 1.5, 3.0, 6.0, 10, 100. (2) 90, 180. (3) 200. (4) 100. (5) 2, 6, 8, 10. (6) 10. (7) 80. (8) 10.

+: presence of a strong signal. ±: presence of a weak signal.

Figure 5-1. Titration of rabbit anti-horse MT-I antiserum against 200 ng of rabbit MT-I and 5 $\mu$g of bacterial lysate prepared from the pAS1 transformed cells. (Top) Elisa readings of samples. Samples 1-9 contained dilutions of antiserum at 1:1, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:236, and 1:512, respectively. Samples 10-12 were controls. +: the presence of material. -: the absence of material. (Bottom) The titration curve obtained from samples 1-9. The Elisa reaction with hydrogen peroxide alone gave 0.00.

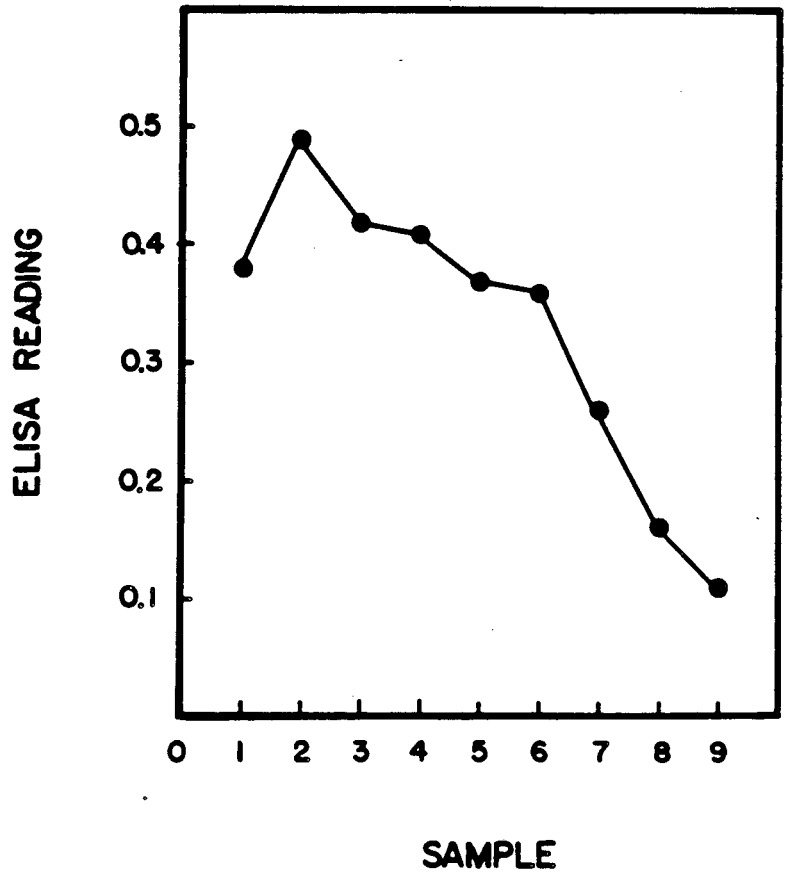
| Sample | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Lysate | + | + | + | + | + | + | + | + | + | − | + | − |
| MT-1 | + | + | + | + | + | + | + | + | + | − | − | + |
| Ab Diln | 1:1 | 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | 1:128 | 1:256 | 1:512 | 1:4 | 1:4 | 1:4 |
| Elisa | 0.19 | 0.24 | 0.30 | 0.32 | 0.30 | 0.44 | 0.41 | 0.41 | 0.32 | 0.12 | 0.30 | 0.28 |




- XBL 873-10815 -

Figure 5-2. Titration of rabbit anti-horse MT-I antiserum at a dilution of 1:120 against rabbit MT-I. (Top) Elisa readings of samples. Samples 1-9 contained various amounts of rabbit MT-I as the antigen. Sample 10 contained no antigen. Sample 11 contained rabbit IgG as the antigen. (Bottom) The titration curve obtained from samples 1-9.

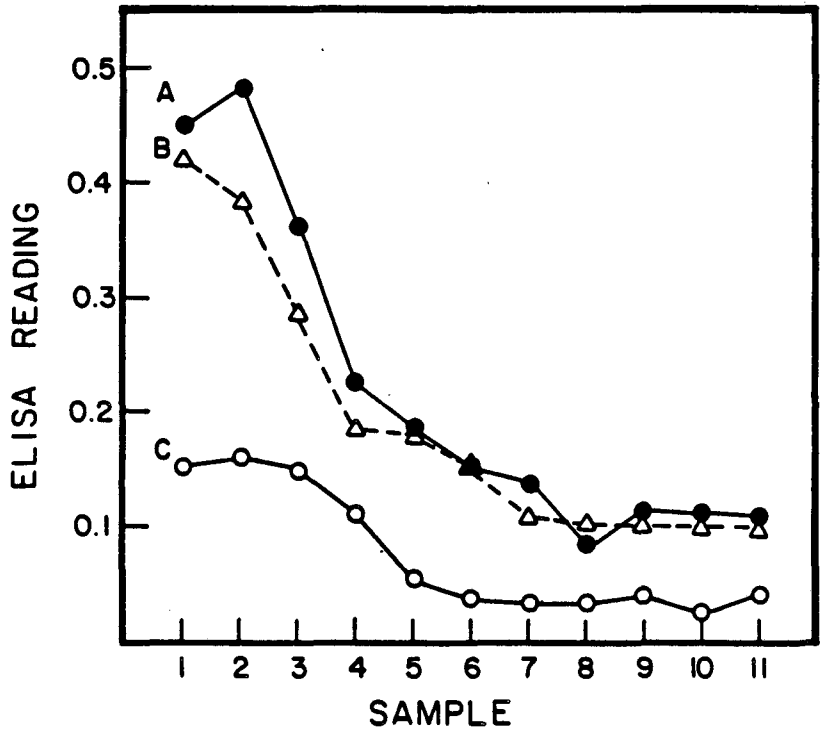
| Sample | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Antigen (ng) | 1000 | 500 | 250 | 125 | 62.5 | 31.2 | 15.6 | 7.8 | 3.9 | – | 250 |
| Elisa | 0.38 | 0.49 | 0.42 | 0.41 | 0.37 | 0.36 | 0.26 | 0.16 | 0.11 | 0.02 | 0.38 |




- XBL 873-10816 -

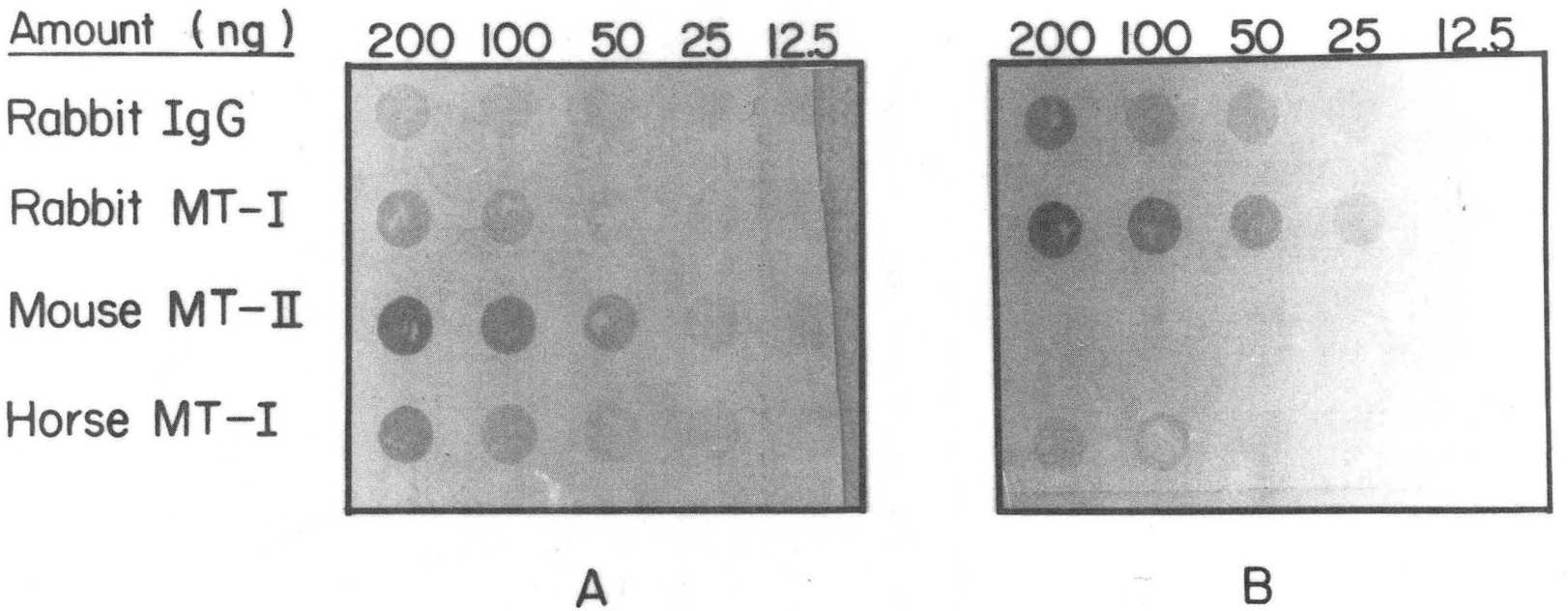
Figure 5-3. Titration of rabbit anti-horse MT-I antiserum at a dilution of 1:120 against rabbit MT-I in the presence of (A) 45 $\mu$g of yeast lysate from the pAB18 transformed cells, (B) 30 $\mu$g of yeast lysate from the pADH224 transformed cells, and (C) borate saline and 0.1% triton X-100.

| Sample | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| MT-I (ng) | 200 | 100 | 50 | 25 | 12.5 | 6.2 | 3.1 | 1.6 | 0.8 | 0.4 | 0.2 |



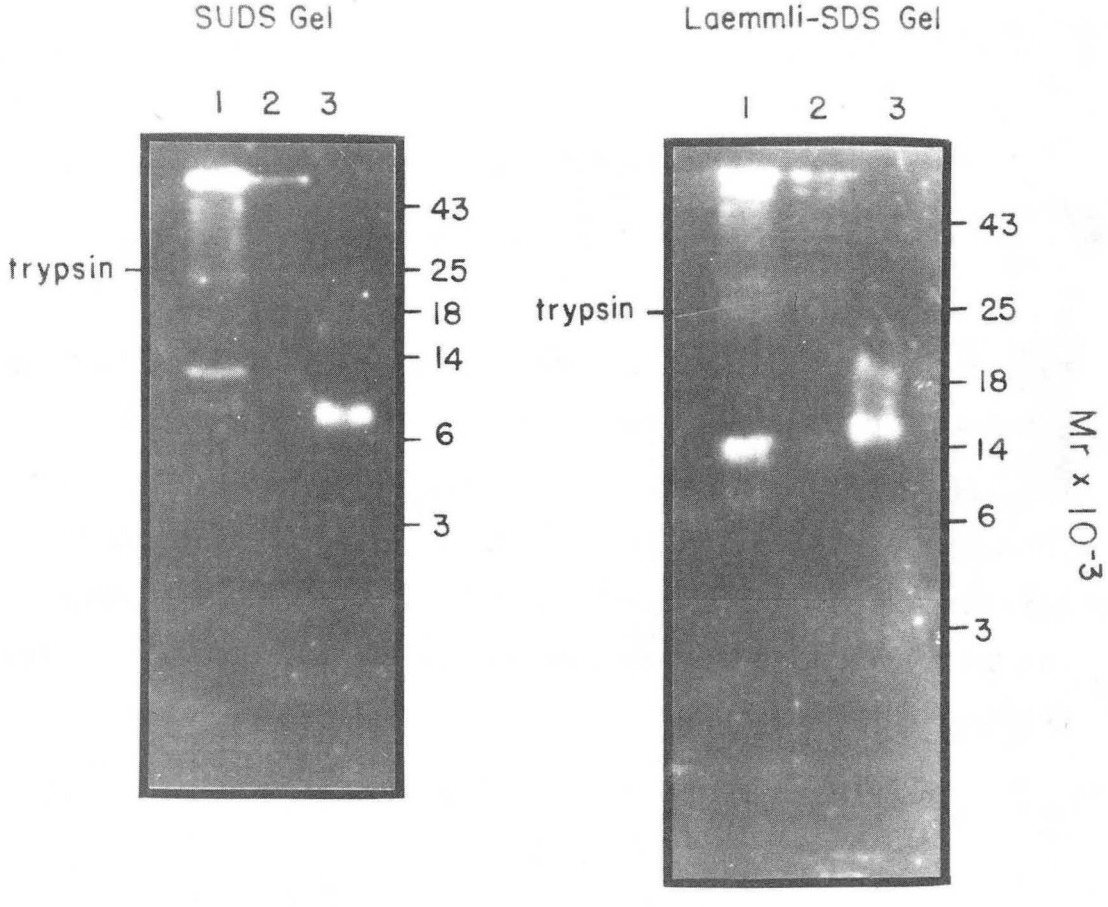

- XBL 873-10817 -

Figure 5-4. Contrast between MTs stained by amido black (A) and detected by antiserum (B) on carboxymethyl cellulose paper. Different proteins were dot-blotted to carboxymethyl cellulose paper and stained as described in 2.11.2.3.1.. Duplicate samples were subjected to antiserum-GAR-POD analysis (2.11.2.3.). The amounts of protein analyzed are indicated.


Amount (ng)
200 100 50 25 12.5
Rabbit IgG
Rabbit MT-I
Mouse MT-II
Horse MT-I
200 100 50 25 12.5
A
B

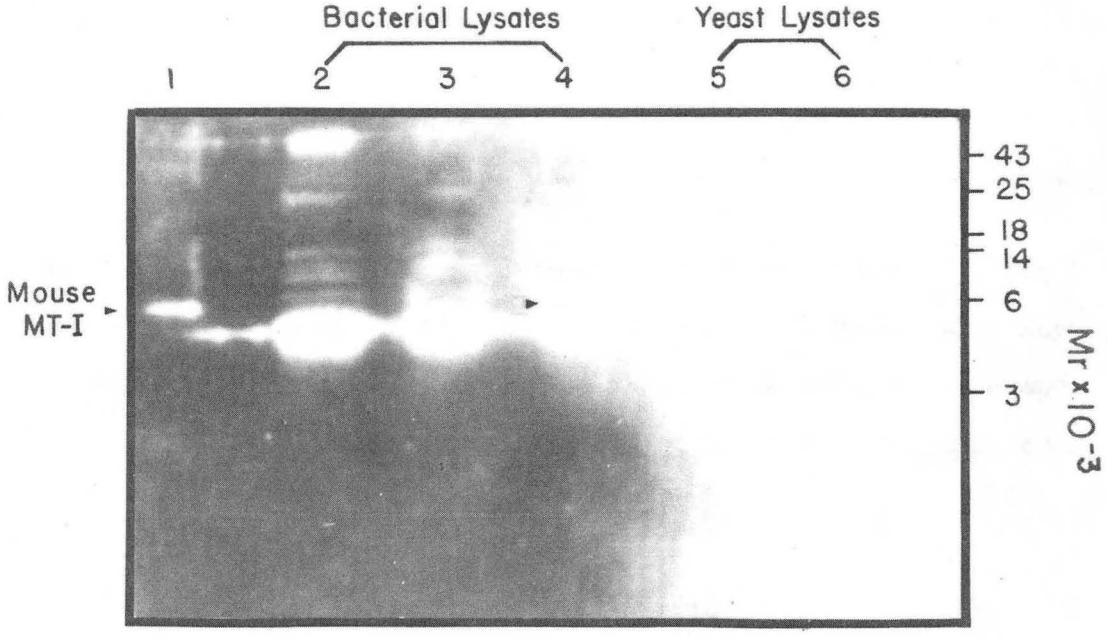
XBB 873-1562

Figure 5-5. Western blots of MTs resolved in (A) a SUDS gel and in (B) a 20% Laemmli-SDS gel. The two gels were loaded with identical samples and were blotted and analyzed by the antiserum-$^{125}$I labelled protein A procedure. Lane 1, trypsin (10 $\mu$g). Lane 2, carboxymethylated rabbit MT-I (2 $\mu$g). Lane 3, carboxymethylated mouse MT-I (300 ng). Lane 4, mouse MT-I (300 ng). Lane 5, molecular weight markers of standard proteins, as in Figure 4-3A, together with insulin (3 Kd). The standard proteins were superimposed onto the autoradiogram from the silver stained gels.


SUDS Gel
1 2 3
trypsin
43
25
18
14
6
3
Laemmli-SDS Gel
1 2 3
trypsin
43
25
18
14
6
3
Mr x 10-3

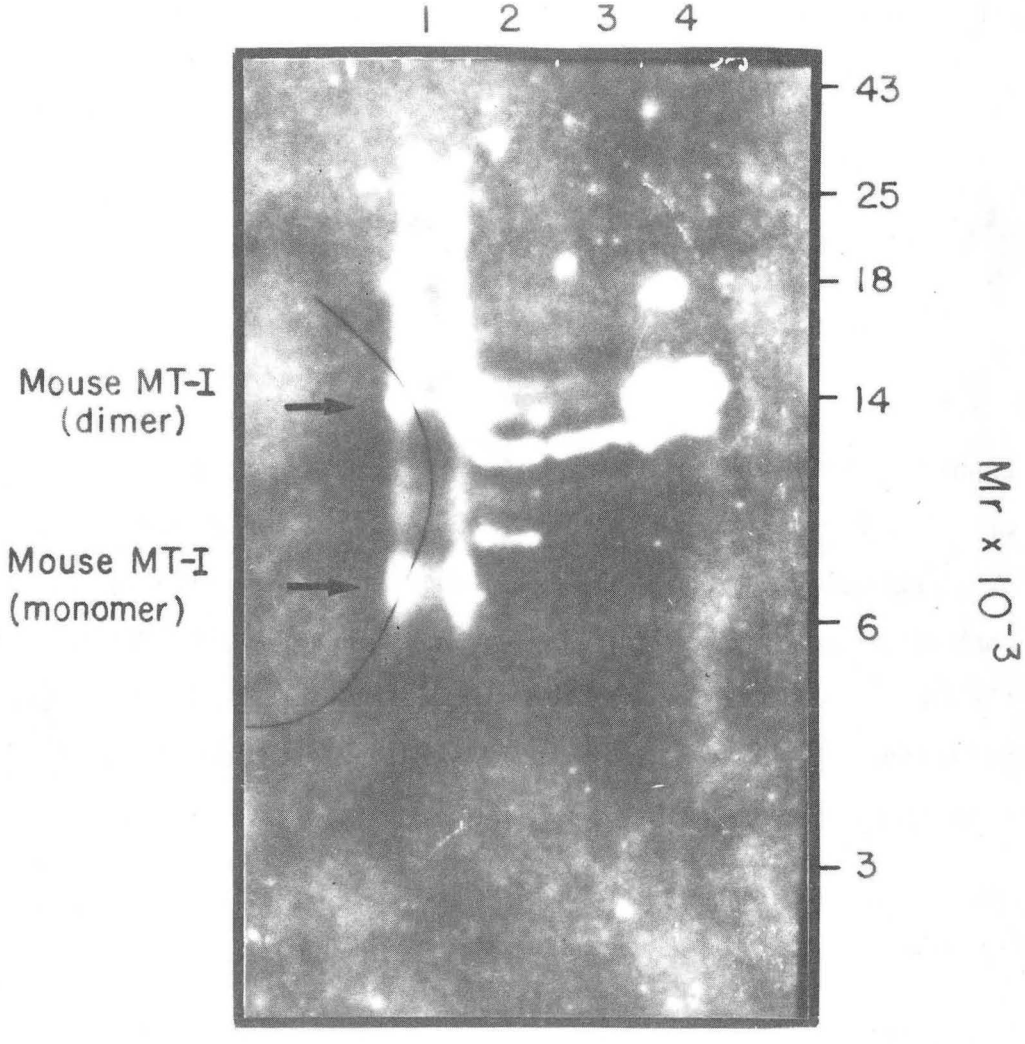
XBB 873-1563

Figure 5-6. Western blot of bacterial and yeast lysates hybridized to the rabbit anti-rat MT antiserum and developed with $^{125}I$ labelled protein A. Lane 1, purified mouse MT-I (300 ng). Lane 2, lysate from the pAS1 transformed bacterial cells (15 $\mu$g). Lane 3, lysate from the pYM101 transformed bacterial cells (15 $\mu$g). Lane 4, purified mouse MT-I (300 ng) seeded to lysate from the pAS1 transformed cells. Lane 5, lysate from the pAB18 transformed yeast cells. Lane 6, lysate from the pADH224 transformed yeast cells. The lysates were heated at 68°C prior to loading.


Bacterial Lysates
Yeast Lysates
1
2
3
4
5
6
Mouse
MT-I
43
25
18
14
6
3
Mr x10-3

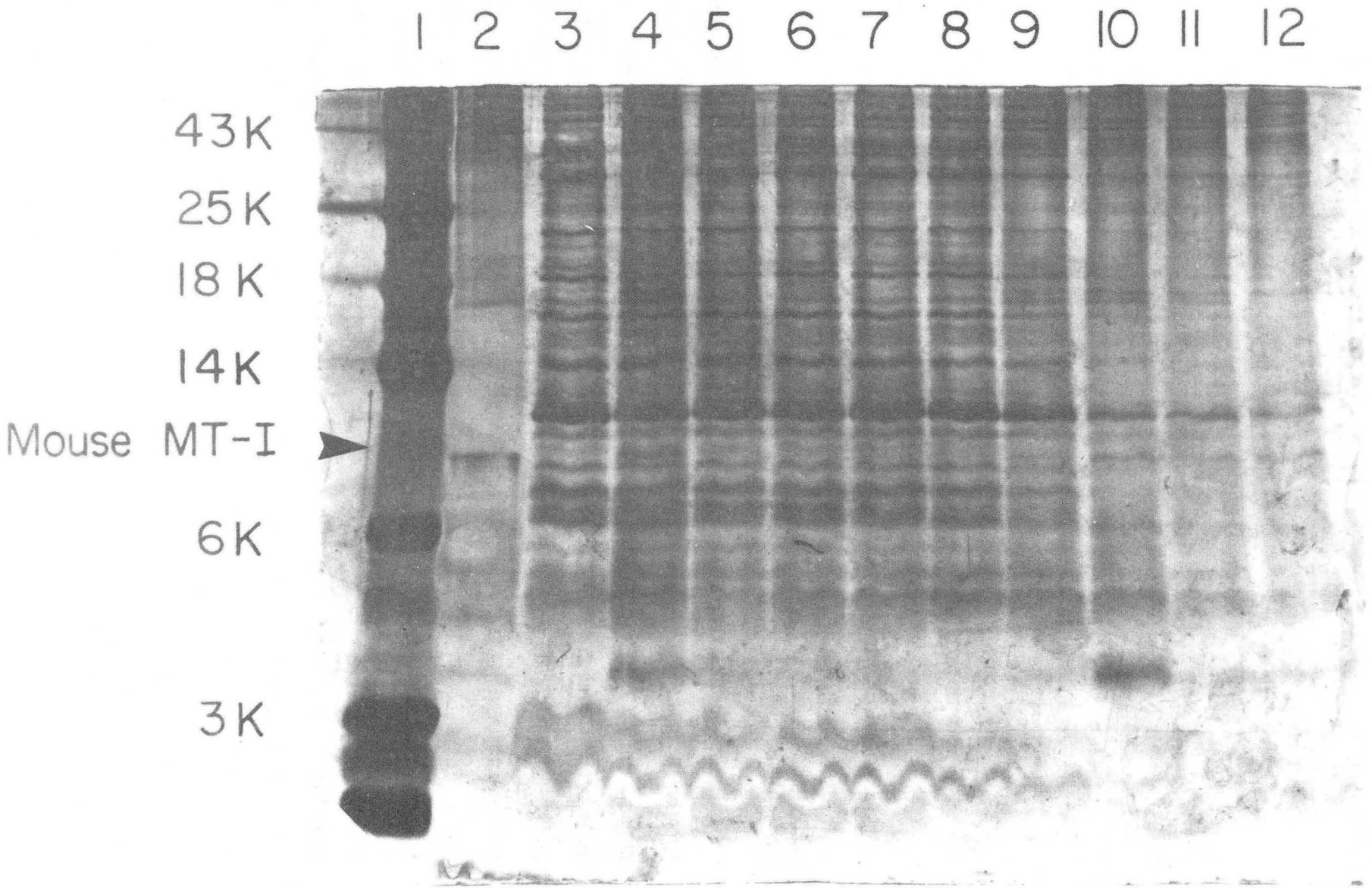
XBB 873-1564

Figure 5-7. Western blot analysis of bacterial lysates. Lane 1, mouse MT-I (1 $\mu$g). Lane 2, proteins isolated from the pAS1 trasformed cells. Lane 3, proteins isolated from the pYM101 transformed cells. Lane 4, proteins isolated from the pAS1 transformed cells and seeded with 3 $\mu$g of purified mouse MT-I during the gel isolation procedure.




XBB 873-1565

Figure 5-8. Stability of rat MT-II in a crude bacterial extract prepared from the pAS1 transformed cells. Samples were loaded onto a SUDS gel and visualized by silver stain. Lane 1, molecular weight markers as described in Figure 4-3A. Lane 2, purified rat MT-II, 0.6 $\mu$g. Lane 3, heated crude extract, approximately 20 $\mu$g. Lanes 4-8, incubation of rat MT-II (0.6 $\mu$g) and heated lysate (20 $\mu$g) for 0, 2 min, 5 min, and 20 min, respectively. Lanes 9-12, incubation of 0.6 $\mu$g of rat MT-II with 10 $\mu$g, 5 $\mu$g, 2.5 $\mu$g and 1.25 $\mu$g of heated lysate, respectively, for 5 min at room temperature.




XBB 873-1566

# CHAPTER 6

## Summary, Discussion and Future Perspectives

### 6.1. Summary

The cDNAs of the mammalian mouse MT-I gene and human MT-II gene have been cloned into bacteria and yeast, respectively. Using the bacterial $P_L$ promoter and the yeast ADHII promoter, both MT genes have been expressed. The following findings are the conclusions drawn from the gene expression study. The first and the most important finding is that expression of MT in bacteria and yeast renders them more resistant to a number of heavy metal ions than cells without the gene. The second finding is that the amount of MT gene product in both bacteria and yeast is relatively low despite the fact that the gene is expressed from a high copy number vector and under the control of a strong promoter. The third finding is that mouse or human MT produced in bacteria or yeast, respectively, is not recognized by the antibodies directed against horse MT-I and rat MT-II or the $^{109}Cd$ probe. The fourth finding is that expression of MT, particularly in yeast, has a lethal effect on the host cells. A discussion of each conclusion and the future perspectives of this study follow.

### 6.2. Discussion

#### 6.2.1. Metal Resistance of the MT Clones

The effect of metal resistance in bacteria and yeast is specific for MT. This is because either uninduced cells harboring the gene or induced cells without the gene did not show such an effect. Induction is the condition where MT gene expression is activated.

The heavy metal resistance of the bacterial MT clone is most apparent using the pYM101 plasmid and is observed to a lesser extent using the pNS3XY plasmids. The results of the pYM101 and the pNS3XY plasmids together provide the first conclusive data that a mammalian MT is functional in bacteria. This finding is contradictory to what was found by Mbikay *et al.* (1983) in which the synthesis of a fused protein, $\beta$-lactamase-metallothionein, did not make the host bacteria more resistant to cadmium. It is possible that because $\beta$-lactamase-metallothionein is mainly excreted into the periplasmic space, the sensitivity of bacterial cells containing the fusion protein to external heavy metal pressures may have been different. Whether the conformation of MT within the periplasmic space is different from that inside the cytoplasm is yet to be investigated.

In yeast, Thiele *et al.* (1986) had previously shown that the expression of monkey MT-II gene can complement the function of yeast copperthionein, which has little sequence homology to MT (Fogel and Welch, 1982). The ability of their clone to protect yeast cells against copper poisoning is consistent with the copper resistant characteristics of the yeast clone harboring the pADH224 plasmid. Although the endogeneous yeast copperthionein remains in the clone harboring the pADH224 plasmid, this does not seem to affect the functions of the expressed human MT-II in protecting the yeast cells against silver and cadmium poisoning. The yeast clone harboring the pRK724 plasmid is not resistant to heavy metal ions. Conceivably, the MT expressed by the pRK724 plasmid is secreted to the medium and does not function inside the cells as in the internal system.

### 6.2.2. Low Level of Detecting MT Gene Expression

Using the pYM101 plasmid, between 40 ng and 50 ng of bacterial MT is detected in 100 $\mu$g of bacterial lysate protein. The amount of MT is equivalent to 0.05% of the total bacterial cellular protein. Using the pADH224 plasmid, roughly 20 ng of yeast MT is detected in 100 $\mu$g of lysate protein, equivalent to 0.02% of the total cellular extract. Compared to the 3-5% yield of the $\lambda$

cII protein under the expression of the $P_L$ promoter (Rosenberg *et al.*, 1983) and the 2-5% yield of the hepatitis B surface antigen using the internal 3PGK promoter of yeast (Hitzeman *et al.*, 1983), respectively, MT gene expression is detected at a much lower level (by a factor of 100 or more). The low yield of MT gene product in bacteria is also reflected in the small effect of added cysteine on the pYM101 harboring clone during the growth study in liquid medium containing the critical concentration of $ZnCl_2$. Although a low level of the gene product is detected, the actual level of MT gene expression has not been directly assessed at the transcription level.

The stability test described in chapter 5 using a native MT protein as a probe suggests proteolysis as a likely cause for the low level of MT detection. One factor which can account for the sensitivity of the expressed MT to proteolysis is the scarcity of heavy metals inside the bacterial or yeast cells. Feldman *et al.* have reported that heavy metals stabilize the integrity of MTs in general (1978). Although all the MT gene expression experiments were done in the presence of a very high concentration of metal in the media, e.g. 0.4 mM to 0.8 mM $ZnCl_2$ for bacteria and 0.05 mM to 1.0 mM $ZnSO_4$ for yeast, perhaps only a small portion of the metal ions was actually transported into the cells. Consequently, the majority of nascent MT proteins, lacking metals, were quickly degraded. This applies to both bacteria and yeast. Using a pulse-chase labelling technique, Shatzman and Rosenberg (unpublished data) recently showed that the internal half life of monkey MT expressed in bacteria is less than 2 minutes. Furthermore, they showed that the half life of MT did not increase even if the MT gene expression was carried out in a protease defective strain. Their results thus further confirm the proteolysis problem in bacteria. In yeast, which is a unicellular eucaryotic organism, most of the protease activity has been associated with the vacuoles (L. Holbrook, personal communication). Presumably, in a stressful situation, yeast cells produce an elevated amount of protease and subsequently store the enzymes in newly formed vacuoles as the cells enter the dormant stage. One major morphological change for yeast cells expressing human MT-II is that they contain many dark vacuoles (see 4.2.7.1. in Chapter 4). This indicates that the nascent MT protein is very likely to encounter proteolysis. However, whether the protein is degraded inside the yeast cells, i.e. via a possible contact with a protease in the vacuoles, or degraded upon lysis of yeast cells cannot be easily determined at present.

### 6.2.3. MT Not Recognized by Antibodies or $^{109}Cd$ Probe

Efforts to detect bacterial or yeast expression of MT by antibodies or $^{109}CdCl_2$ probe failed. This, again, can be explained by four phenonema. First, the clones produced a low yield of MT which was quidkly degraded upon cell lysis. Second, the gene product in bacteria or yeast may be polymerized or otherwise altered in conformation as a result of low intracellular metal concentrations. This may alter the antigenicity of the MT and reduce its affinity for the $^{109}CdCl_2$ probe *in vitro*. Third, the antibodies used in this laboratory are polyclonal, and have relatively weak titers to the purified mouse MT-I and to other mammalian MTs. Fourth, the MT produced by bacteria or yeast may have a different metal-binding specificity than the MTs used as antigens. A different metal-binding specificity suggests that the cloned MTs exist in a different conformation than the mammalian MTs (Chapter 1) and thus may not be readily recognized by the antibody or interact efficiently with $^{109}CdCl_2$ *in vitro*.

The third possibility has been further examined by Shatzman and Rosenberg (unpublished data, 1985), using a variety of antibodies in a similar attempt to detect cloned MT. They have obtained both polyclonal and monoclonal antibodies, but have not reported any success with either. Because of the highly conserved structure of MTs, it is difficult to obtain antibodies with very high titers.

There are four extra amino acids at the N terminus of the expressed mouse protein. This should not interfere with antibody recognition. A recent study on the crystal structure of rat MT-II suggests that the additional amino acids at the N terminus of MTs are not likely to significantly alter the structure (Furey *et al.*, 1986) . The strong metal resistance phenotype of the mouse clone is consistent.

### 6.2.4. Toxic Effect of MTs on Host (Yeast)

Expression of MT in yeast cells leads to a cessation of cell division and an inhibition of protein translation. expression. The slower than regular growth rate of a bacterial MT clone in liquid media containing the critical concentration of $ZnCl_2$ may imply a similar effect on bacteria. One possibility for the toxicity is that the cloned MT sequester heavy metal ions required for essential cellular functions. Because MT has an unusually high affinity for metal ions and because

a saturation of the protein metal binding sites is unlikely, a depletion of essential metal ions such as $Zn^{+2}$ could lead to a failure of enzymes such as DNA polymerase and RNA polymerase. If the microorganism is unable to increase its transport of metal ions from the medium in response to MT synthesis, such a problem will continue to prevent high level of MT production. Solaiman *et al.* (1979a; 1979b) have found that a synthetic compound, 3-ethoxy-2-oxobutyraldehyde bis (N4-dimethylthiosemicarbazonate), and its derivatives are able to bind the heavy metal ion $Cd^{+2}$ tightly (on the order of $10^6$ to $10^{10}$ binding affinity) and also be transported across the bacterial cell wall. Use of these compounds for the transport of metal ions into cells may prove important in elevating the overall yield of MT production in bacteria and yeast.

### 6.3. Future Perspectives

The heavy metal resistance of the MT clones is a result of MT gene expression in the microorganisms. Such a phenotype will conceivably change if a mutation occurs which alters the metal-binding property of MT. Several experiments described in chapter 4 are useful in monitoring changes in the metal resistance phenotype of microorganisms. The followings are examples. First, the study of $^{109}Cd$ incorporation directly measures the number of $Cd^{+2}$-binding sites and the strength of $Cd^{+2}$-binding affinity in a cloned protein. If a mutation affects either of these two parameters, it should be easily detected by a change of $^{109}Cd$ incorporation. Second, the growth study in liquid medium containing a critical concentration of $ZnCl_2$ measures the interaction of a cloned protein with the heavy metal ion Zn(II). If a mutation affects either the number or the strength of $Zn^{+2}$-binding sites, it should be reflected in a change of the growth curve in the mutant clone. Third, examination of the growth pattern on agar plates containing various heavy metal ions establishes the metal-binding priority of a MT clone. This can be used to determine if a mutation which alters the metal-binding specificity of the wild type MT has occurred. In fact, a variation on this experiment is that one can increase the concentration included in the agar plates so as to select mutant clones that are able to grow at a concentration higher than the critical concentration for the wild type MT clone. It is recommended that the bacterial clone harboring plasmid pYM101 be chosen for the mutagenesis study because most of the above experiments have already been characterized for this clone.

The stategy of mutagenesis is a modification of that of Shortle and Nathans (1978), using

the plasmid encoded mouse MT-I cDNA sequence as the primary target. It involves nicking the plasmid pYM101 at the unique SstII site, which is roughly in the middle of the cDNA insert (Figure 3-2), creating a single-stranded gap from the nick with exonuclease III, and treating the single-stranded DNA in the gap with the mutagen bisulfite. Bisulfite attacks singel-stranded cytidines converting that residue into a U after deamination. Upon repair, the U is base-paired with an A residue, leading to a C-G to T-A transition after replication. Primary mutagenesis experiments have shown that this protocol is feasible. The mutagenized MT DNA gave rise to a number of mutant clones that grew at metal concentrations higher than what the wild type MT clone can tolerate. By keeping a strong selective pressure in any one of the three methods described above, it should be possible to select mutants of MT clones that have particularly strong affinities to a given heavy metal.

Each mutant will be subjected to DNA sequence analysis around and within the region of the MT gene in the plasmid and will subsequently be identified by the corresponding mutation(s) in the MT protein sequence. Additionally, each mutant will be characterized for its resistance to various heavy metals and compared with the wild type clone. The accumulation of a number of mutations will present a comparison of their effects on metal-cluster formation and binding affinity of MT. Correlation of these results will provide an insight to understanding unique metal-protein interaction at the molecular level. In the long run, such knowledge is necessary for detailed interpretation of X-ray diffraction analysis of the available MT crystals and other MTs containing different metal ions.

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