CLONING OF WINTER FLOUNDER (Pseudopleuronectes americanus) METALLOTHIONEIN cDNA AND ANALYSIS OF METALLOTHIONEIN GENE EXPRESSION IN WINTER FLOUNDER TISSUES

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CLONING OF WINTER FLOUNDER (*Pseudopleuronectes americanus*) METALLOTHIONEIN cDNA AND ANALYSIS OF METALLOTHICNEIN GENE EXPRESSION IN WINTER FLOUNDER TISSUES

by

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ABSTRACT

Cadmium chloride (Cd^{2+}) administration induces the production of metallothionein (MT) mRNA in the liver of winter flounder. Analysis of polyadenylated mRNA directed cell free translation products by polyacrylamide gel electrophoresis showed that hepatic mRNA from Cd^{2+}- injected flounder translated to yield MT. Polyadenylated RNA purified from liver samples of winter flounder after Cd^{2+}-injections was then used to construct a cDNA library. Several recombinant clones made complementary to MT mRNA were selected from this cDNA library by an oligonucleotide derived from the amino-terminal amino acid sequence of winter flounder MT. Sequence analysis of two of the cDNA inserts gave the structure of the entire 3'-untranslated region, a coding region corresponding to the flounder MT and 49 nucleotides of the 5'-untranslated region.

One of the flounder MT cDNAs, pWFMTC4, was subcloned into an RNA probe plasmid and transcribed to produce antisense MT RNA (MT cRNA) which was then used to detect the MT mRNA levels in tissues of the winter flounder after various treatments. The hepatic MT mRNA levels were found to be induced in the winter flounder following multiple injections of metal ions (Cu^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+}, and Hg^{2+}). The time required for the induction of hepatic MT mRNA by a single injection of Cd^{2+} was approximately 96 hours.

Dexamethasone did not induce any accumulation of MT mRNA in any of the tissues examined (liver, kidney, heart, brain, intestinal scrape, and gill filament), whereas Cd^{2+} induced MT mRNA in all of those tissues except brain, where the endogenous level of expression was high.

Southern blot analyses of winter flounder genomic DNA showed that there is a single MT gene in the winter flounder. These results together with the protein and cDNA sequence analysis establish that there is a single species of MT protein in the winter flounder. The complete amino acid sequence of flounder MT was derived from the cDNAs. It has 20 cysteine residues in a 60 amino acid polypeptide with a characteristic of Class I MT protein structure. It shows 50% to 57% amino acid sequence identity with chicken and pigeon MTs respectively, approximately 60% sequence identity with mammalian MTs and 85% sequence identity with rainbow trout MTs.

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LIST OF ABBREVIATIONS

Amino acids symbols:	A	Alanine (Ala)
	B	Asx, undefined, Asparagine (Asn) or aspartic acid (Asp)
	C	Cysteine (Cys)
	D	Aspartic acid (Asp)
	E	Glutamic acid (Glu)
	F	Phenylalanine (Phe)
	G	Glycine (Gly)
	H	Histidine (His)
	I	Isoleucine (Ile)
	K	Lysine (Lys)
	L	Leucine (Leu)
	M	Methionine (Met)
	N	Asparagine (Asn)
	P	Proline (Pro)
	Q	Glutamine (Gln)
	R	Arginine (Arg)
	S	Serine (Ser)
	T	Threonine (Thr)
	V	Valine (Val)
	W	Tryptophan (Trp)
	Y	Tyrosine (Tyr)

Amp^r	ampicillin resistant
ATP	adenosine triphosphate
bp	base pairs
BRL	Bethesda Research Laboratories
cDNA	deoxyribonucleic acid complementary to RNA
cRNA	ribonucleic acid complementary to RNA
Ci	Curie(s)
CNBr	cyanogen bromide
cpm	counts per minute
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate

dT_{12-18}	dephosphorylated oligodeoxythymidylic acids (12 to 18 bases long)
dTTP	thymidine triphosphate
DTT	dithiothreitol
Denhardt's	100X Denhardt's solution contains Ficoll, BSA (bovine serum albumin), and PVP (polyvinylpyrrolidone), each at 2%
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
EDTA	ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
GRE	glucocorticoid responsive element
GTP	guanosine 5'-triphosphate
HMW	high molecular weight
kb	kilobases
kbp	kilobase pairs
kDa	kilo-Dalton
M_r	relative molecular mass (dimensionless)
MLTF	major late transcription factor
MRE	metal responsive element
min	minute(s)
mRNA	messenger ribonucleic acid
MT	metallothionein
n.m.r. (NMR)	nuclear magnetic resonance
Ori	origin of replication
PAGE	polyacrylamide-gel electrophoresis

poly(A)$^+$	polyadenylated
PolIK	Klenow fragment of DNA polymerase I (*E. coli*)
R	G or A (guanine or adenine)
RNA	ribonucleic acid
RNase	ribonuclease
RTase	reverse transcriptase
S	sedimentation constant
SDS	sodium dodecyl sulfate
SSC	standard saline citrate, 1X= 0.15M NaCl, 0.015M Na_3 citrate, pH 7.0
SSPE	20X SSPE : 3.6M NaCl, 0.2M NaH_2PO_4 pH 7.4, 2mM EDTA
SV40	simian virus 40
TAE	Tris/acetate/EDTA buffer (Maniatis *et al.*, 1982)
TBE	Tris/borate/EDTA buffer (Maniatis *et al.*, 1982)
TCA	trichloroacetic acid
TMAC	tetramethyl ammonium chloride
TPA	12-o-tetradecanoylphorbol-13-acetate
Tris	tris(hydroxymethyl)amino methane
tRNA	transfer ribonucleic acid
UTP	uridine 5'-triphosphate
UV	ultra-violet (light)
Y	T or C (thymine or cytosine)

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PUBLICATIONS ARISING FROM THIS WORK

Full Papers

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Chan, K.M., Davidson, W.S., and Fletcher, G.L. (1989). Metallothionein messenger RNA: potential molecular indicator of metal exposure. *In*: Aquatic Toxicology and Water Quality Management. Nriagu, J.A. (ed.) John Wiley & Sons, Inc. pp.89-109.

Chan, K.M., Davidson, W.S., Hew, C.L., and Fletcher, G.L. (1989). Molecular cloning of metallothionein cDNA and analysis of metallothionein gene expression in winter flounder tissues. Canadian Journal of Zoology 67:2520-2527.

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Fletcher, G.L., Chan, K.M., Shears, M.A. and Hew, C.L. (1986). Metallothionein in a marine flatfish. *In*: Proceeding of the 7 th Society of Environmental Toxicology and Chemistry (SETAC) Annual Meeting, Radisson Mark Plaza Hotel, Alexandria, Virginia, November 2-5, 1986. Abstract Number 228, pp.119.

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Chan, K.M., and Fletcher, G.L. (1989). Molecular cloning of

metallothionein (MT) cDNA and analysis of MT gene expression in winter flounder tissues. *In*: 10 th Annual Meeting of the Society of Environmental Toxicology and Chemistry, Royal York Hotel, Toronto, Ontario, Canada, October 28- November 2, 1989. Abstract Number 53. (Oral Presentation).

The sequence of pWFMTC69 reported in this thesis has been deposited in the EMBL/ GenBank data base, accession number: X13584.

CHAPTER ONE

INTRODUCTION

INTRODUCTION

1.1. STRUCTURE OF METALLOTHIONEIN (MT) AND ITS POSSIBLE FUNCTIONS

1.1.1. Discovery of MTs

A cadmium (Cd^{2+}) and zinc (Zn^{2+}) binding protein was first discovered and isolated from normal kidney by Margoshes and Vallee in 1957. Characterization of this protein revealed its two unique and unusual properties: it is cysteine rich and has a high metal-binding capacity. This protein was subsequently named "metallothionein" (Kagi and Vallee, 1960; Vallee, 1979). It was unexpected that Cd^{2+} could be enfolded inside a protein *in vivo*, and it was believed that this protein caused the accumulation of Cd^{2+} in the kidney (Kagi and Vallee, 1960; Kagi *et al.*, 1974; Nordberg and Kojima, 1979).

1.1.2. General properties of MTs

Since MT was first isolated from equine kidney more than 30 years ago (Margoshes and Vallee, 1957), it has been established that it is ubiquitous among animals, with related proteins occurring in lower eukaryotes, some plants, and prokaryotes (Nordberg and Kojima, 1979; Hamer, 1986;

Kagi and Kojima, 1987). The properties and characteristics of MTs were reviewed by Nordberg and Kojima (1979), and used as criteria for the identification of MTs. The following are the general properties of MTs: (1) MTs are cytosolic metal-binding proteins that have a high cysteine content (30%) and do not normally contain aromatic amino acids or histidine. Thus, they do not absorb at 280 nm. (2) Their relative molecular mass (M_r) is in the range of 6,000 to 10,000 and they usually consist of about 60 amino acid residues. (3) They have a very high affinity and capacity to bind heavy metal ions such as copper (Cu^+), Zn^{2+}, and Cd^{2+}. A total of seven atoms of divalent metal ions can be bound in a single MT molecule through mercaptide bonds provided by the cysteine residues. (4) They show spectroscopic features characteristic of metal-thiolate clusters, with a maximum absorbance 254 nm (A_{254}) for Cd^{2+}-bound MT. (5) The cysteine residues are usually distributed along the polypeptide chain. (6) They show heat stability (65°C to 70°C, for 5 to 10 minutes (min)) and (7) metal inducibility in most biological tissues.

Isoforms of MTs are also well documented in mammalian cells (Hamer, 1986). Based on ion-exchange chromatography, two major MT isoforms (MT-I and MT-II) have been identified in most mammalian cells (Hamer, 1986). Each major form also contains isoproteins which are separable by reverse phase High Pressure Liquid Chromatography (HPLC) (Hunziker and

Kagi, 1985; Richards and Steele, 1987). Human MTs exhibit the most complex polymorphism, with at least six isoforms of MT-I and a single major form of MT-II being expressed and further resolved by reverse phase HPLC analyses (Hunziker and Kagi, 1985).

1.1.3. Primary structure of MTs

Since the cysteine residues in MT are essential for the binding of heavy metal ions, they can be aligned to facilitate comparisons between the different molecules from diverse organisms. Such an alignment was reported by Fowler *et al.* (1987a) who divided MTs into three classes.

Class I comprises vertebrate MTs, some invertebrate MTs, and other MT-like sequences which have highly conserved cysteine residues at specific positions (Fig. 1). The highly conserved central sequence, KKSCCSCCP, found in all mammalian MTs (Kagi *et al.*, 1984) is underlined in the human MT-II sequence in Fig. 1. Invertebrate MTs do not have the highly conserved central sequences of the mammalian MTs. However, Nemer *et al.* (1985) proposed a so called " central sequence", CXCXXXCXC (where X represents any non-cysteine amino acid), as the evolutionary conserved sequence found in most MTs.

Class II consists of MTs from sea urchin, wheat, yeast, and others which show less sequence homology than found in

Figure 1. Amino acid sequences of representative MTs.

Gaps in the Class I MT sequences are introduced to maximize structural alignments. The highly conserved central sequence (KKSCCSCCP) found in all mammalian MTs is underlined in the human MT-II sequence. The numbers on top of human MT-II refer to the sequences of major mammalian MTs. Mould MT sequence is the *Neurospora* MT and mushroom MT sequence is from *Agaricus bisporus*. For yeast MT, the amino-terminal (leading) sequence (MFSELINF) is not included in this figure. These sequences were adapted from Kagi and Schaffer, 1988. Nematode MT sequence is the *Caenorhabditis elegans* MT (Slice *et al.*, 1990).

CLASS I METALLOTHIONEINS

```
                  1         10         20         30         40         50         60
human MT-II       MDP NCSCAAGDSCTCAGS CKCKE CKCTSCKKSCCSCCPVGCAKCAQGCICKG ASDKCSCCA
horse MT-I        MDP NCSCPTGGSCTCAGS CKCKE CRCTSCKKSCCSCCPGGCARCAQGCVCKG ASDKCSCCA
rat   MT-I        MDP NCSCSTGGSCTCSSS CGCKN CKCTSCKKSCCSCCPVGCSKCAQGCVCKG ASDKCTCCA
chicken           MDPQDCTCAAGDSCSCAGS CKCKN CRCRSCRKSCCSCCPAGCNNCKQGCVCKEPASSKCTCCH
crab  MT-I          PGPC C  NDKCVCKEG  GCKEGCQCTSCRCSPCEKCSSGC KCANKEECSKTCSKACSCCPT
mould             M  GDCGCSGASSCTCGSG CSCSN CGSK
mushroom          M  GDCGCSGASSCTCASGQCTCSG CGK
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CLASS II METALLOTHIONEINS

```
sea urchin MPDVKCVCCTEGKECACFGQDCCVTGECCKDGTCCGICTNAACKCANGCKCGSGCSCTEGNCAC
nematode   MVCKCDCDCKNQNCSCNTGTKDCDCSDAKCCEQYCCPTASEKKCCKSGCAGGCKCANCECAQAAH
yeast                QNEGHECQCQCGSCKNNEQCQKSCSCPTGCNSDDKCPCGNKSEETKKSCCSGK
cyanobacterium     TSTTLVKCACEPCLCNVDPSKAIDRNGLYYCCEACADGHTGGSKGCGHTGCNC
```

CLASS III METALLOTHIONEINS

phytochelatins $(\tau\ \text{Glu-Cys})_n\text{-Gly}$ n=2-8

Class I. Two unique features of the yeast MT (CUP1) sequence should be noted. (1) There is an amino-terminal sequence of eight residues in CUP1 (not shown in Fig. 1) that is not found in any of the other MTs described. (2) There is a sequence of KKSCCS close to the carboxyl end which is homologous to the conserved central sequence found in mammalian MTs (KKSCCSCCP) (Fig. 1) (Butt *et al.*, 1984a; Karin *et al.*, 1984c). This CUP1 protein contains only 12 cysteine residues (22 mol%) in the 53 residue mature (cleaved) protein, and is capable of binding only four Cd^{2+} ions or eight Cu^{2+} (Butt *et al.*, 1984b; Winge *et al.*, 1985; Byrd *et al.*, 1988).

Class III MT is a group of polypeptides containing glutamylcysteinyl units which can bind metals (Grill *et al.*, 1985). These MTs are known as plant phytochelatins which are converted from glutathione by phytochelatin synthase (Grill et al. 1987, 1989) and the prokaryotic oligopeptides (*e.g.* $[\gamma$ Glu-Cys$]_n$-X, where n= 2-8 and X is a glycine or alanine). Their sequences are much shorter than Class I and Class II MTs (Fowler *et al.*, 1987a; Kagi and Schaffer, 1988).

1.1.4. Tertiary structure of MTs

MTs (Class I) are non-globular proteins which behave like prolate ellipsoids on gel-filtration (Kagi *et al.*,

1974). Their Stokes' radius has been estimated to be 16 Å and does not change upon the removal of the metal ions (Kagi *et al.*, 1974). Secondary structure predictions based on the method of Chou and Fasman (1978) and spectroscopic analysis have shown that the MT molecule consists of 55% random coil, nearly 40% reverse turn and β-turn conformation, and very little α-helical structure (Vasak and Kagi, 1983; Vasak, 1986; McCormick *et al.*, 1988). To date, no apo-thionein has been isolated from any organism. The molecule contains no disulfide bridges or free sulfhydryl groups as determined by spectroscopic analysis (Kagi *et al.*, 1974; Vasak *et al.*, 1981; Hunt *et al.*, 1984).

The tertiary structures of three Class I MTs from rabbit, rat and crab, have been well characterized by nuclear magnetic resonance (n.m.r.) spectrometry and X-ray crystallographic studies (Winge and Miklossy, 1982; Hunt *et al.*, 1984; Nielson and Winge, 1985; Furey *et al.*, 1986, 1987; Schultze *et al.*, 1988). The molecule consists of two independent globular domains (α and β) with clusters of Cys-Cys and Cys-X-Cys sequences which together chelate six or seven atoms of Zn^{2+} or Cd^{2+} (Hunt *et al.*, 1984; Braun *et al.*, 1986; Furey *et al* ., 1986, 1987). Cadmium 113 n.m.r. and ^{1}H-^{1}H n.m.r. studies using crab or rabbit MTs showed that three or four metal ions are organized into each of the two independent domains (α and β)(Otvos and Armitage, 1979; Otvos *et al.*, 1982; Boulanger *et al.*, 1983; Wagner *et al.*,

1986). Domain α is at the carboxyl end and comprises amino acid residues 31 to 61 in rabbit MT-II; domain β is at the amino terminus and comprises amino acid residues 1 to 29 (Braun *et al.*, 1986; Wagner *et al.*, 1986).

Furey *et al.* (1986) studied a rat MT-II crystal structure that showed domain β enfolding a three metal cluster of one Cd^{2+} and two Zn^{2+} coordinated by six terminal cysteine thiolate ligands and three bridging cysteine thiolates. The α domain enfolded a four-Cd^{2+} cluster coordinated by six terminal and five bridging cysteine thiolates. All seven metal sites had tetrahedral geometry. Schultze *et al.* (1988) reported similar results from the three dimensional structure of rat liver Cd_7-MT-II in aqueous solution using n.m.r. spectrometry and distance geometry calculations. Kagi *et al.* (1984) compared the spectroscopic analyses of MTs bound with a single metal species, i.e. Zn^{2+}, Cd^{2+}, Ni^{2+}, Co^{2+}, Hg^{2+}, Pb^{2+} or Bi^{2+}, prepared *in vitro*. They found that all cysteine residues participated in metal binding; each metal ion was bound to thiolate ligands, and that the symmetry of each complex in the domain was close to a tetrahedron (Kagi *et al.*, 1984).

Copper binds to all MTs as a monovalent ion (zinc and cadmium bind as divalent ions) so that 11 to 12 cuprous ions can be bound within a single polypeptide in a trigonal geometry (Nielson and Winge, 1985; Nielson *et al.*, 1985).

1.1.5. Possible function of MTs

MTs are cytosolic cysteine-rich metal-binding proteins that can be induced by, and can bind to heavy metal ions (Hamer, 1986; Kagi and Schaffer, 1988). However, there is a paucity of information on the interactions among metal ions, MT and other metal binding proteins. Therefore, the precise *in vivo* mechanism of action of MT is not yet known (Karin, 1985; Hamer, 1986; Dunn *et al.*, 1987; Kagi and Schaffer, 1988). It is possible that they may serve in a variety of physiological and biochemical roles. The two major functions which have been suggested for MT are intracellular Cu^{2+} and Zn^{2+} homeostasis, and heavy metal (mainly Cd^{2+}) detoxification (Brady, 1982; Webb and Cain, 1982; Cherian and Nordberg, 1983; Karin, 1985; Hamer, 1986; Bremner, 1987; Dunn *et al.*, 1987; Kagi and Schaffer, 1988).

The presence of MT in normal tissues suggests that it is involved in metal homeostasis during normal cell proliferation and growth, possibly by supplying Zn^{2+} and Cu^{2+} to metal- dependent enzymes and providing Zn^{2+} to RNA polymerases and their associated protein factors (Cousins, 1979; Cherian *et al.*, 1987; Webb, 1987). This suggestion is consistent with the results of other studies which demonstrate ontogenetic changes in MT gene expression during the early development of sea urchins, chickens and mammals (Ouellette, 1982; Andersen *et al.*, 1983a; Andrews *et al.*,

1984; Nemer *et al.*, 1984; Cherian *et al.*, 1987; Wei and Andrews, 1988). For example, Andersen *et al.* (1983a) found that fetal and neonatal rat livers had higher MT levels than did those of adults. Similar observations have been made in chickens where hepatic MT mRNA levels increased significantly following hatching and returned to basal levels by the time they were adults (Wei and Andrews, 1988). The precise reasons for these developmental changes in MT gene expression are unknown.

Although the above studies favour the hypothesis that MT is important for normal cellular growth and development, the existence of cell lines which have lost their ability to synthesize MT has been used as evidence against this involvement (Hamer, 1986; Dunn *et al.*, 1987). However, this argument is a weak one since cell lines are unlikely to be representative of an intact organism.

There is a considerable body of literature concerning the homeostatic control of Cu^{2+} and Zn^{2+} absorption by the mammalian gastrointestinal tract and the role of MT in the regulatory process. Since Cu^{2+} and Zn^{2+} are essential trace elements, the impetus behind these studies is their nutritional importance and the fact that dietary levels in humans may be marginal. This literature has been reviewed extensively by Cousins (1985) and summarized more recently by Bremner (1987). Although there appears to be some agreement that MT is involved in the homeostatic regulation

of the gastrointestinal uptake of these metals, its precise role is far from clear.

Richards and Cousins (1975a,b, 1976, 1977) were the first to demonstrate that injections of stable Zn^{2+} and the feeding of high Zn^{2+} diets resulted in the synthesis of MT by the intestinal mucosa of rats. They concluded that the efflux of Zn^{2+} from the mucosal cells into the blood was inversely related to the level of MT in the mucosal cytosol. Cousins (1979) proposed that the control of Zn^{2+} absorption, in response to the body's needs, was mediated through changes in the production of MT. According to their model, when the Zn^{2+} status of an animal is elevated, MT synthesis is induced in the intestinal mucosa. The MT then competes for newly absorbed Zn^{2+} with the normal "carrier" protein in the cell, thereby reducing the amount of Zn^{2+} that is available for transfer into the body. Zinc ions bound to MT is then eliminated during desquamation of the mucosal cells (Menard *et al.*, 1981). However, as Cousins (1985) and Bremner (1987) point out in more recent reviews, the inverse relationship between MT synthesis and Zn^{2+} absorption has only been observed after the administration of fairly large doses of Zn^{2+} or a sudden increase in the dietary supply of Zn^{2+} to animals previously fed a Zn^{2+} deficient diet. The role that MT plays at normal intake levels of Zn^{2+} is not clear. No major changes in mucosal MT content have been found in rats or sheep when their dietary Zn^{2+} intake is

increased over a wide range, even though the efficiency of Zn^{2+} absorption is greatly reduced (reviewed by Bremner, 1987).

Evidence that MT plays a role in metal detoxification comes from the fact that MT production can be induced in most organisms and cultured cell lines by the administration of heavy metal ions (Hamer, 1986; Grady *et al.*, 1987). This evidence is further strengthened by the fact that MT production cannot be induced in cultured mouse lymphoid cells, a cell line which is very sensitive to the toxic effects of Cd^{2+} (Compere and Palmiter, 1981). The sensitivity of lymphoid cell to Cd^{2+} appears to be due to an inability to produce MT. Further evidence for a role for MT in metal detoxification stems from experiments using tissue culture, where cell lines selected for increased metal resistance, amplified their MT genes (Beach and Palmiter, 1981; Crawford *et al.*, 1985; Hamer, 1986).

Although the cultured cell line studies present persuasive evidence for a metal detoxification role for MT, experiments using intact vertebrates are less convincing. Since an understanding of the physiological role of MT is of importance to human health, most of the research on intact vertebrates has been confined to mammals. Cd^{2+} causes renal proximal tubule cell damage, tubule proteinuria and ultimately renal failure in vertebrates. Therefore, when Cd^{2+}-MT was discovered in horse kidney, the question arose

concerning the role of MT, if any, in preventing renal failure. Experiments designed to answer this question illustrate the complexity of the mammalian heavy metal detoxification system and serve to demonstrate why it is difficult to come to firm conclusions about the role of MT in the process.

The current hypothesis is that Cd^{2+} is taken up through the lungs (inhalation) or from the gastrointestinal tract (ingestion) and transported to the liver where the production of MT is induced (Kagi and Nordberg, 1979; Piscator, 1986). For reasons that are not yet known Cd^{2+}-MT is released or lost from the liver (possibly by damaged cells) and transported to the kidney via the circulatory system (Squibb *et al.*, 1982, 1984; Squibb and Fowler, 1984; Suzuki, 1984; Nordberg and Nordberg, 1987). The Cd^{2+}-MT then passes through the glomerulus to be reabsorbed by the renal proximal tubules (Cherian *et al.*, 1976; Sato and Nagai, 1982; Squibb *et al.*, 1982, 1984; Nordberg, 1984; Dudley *et al.*, 1985; Abel *et al.*, 1987). It has been shown that, in tubular cells, MT can accumulate inside the lysosomes where it is degraded to produce free Cd^{2+} (reviewed by Goering *et al.*, 1987). This free Cd^{2+} causes nephrotoxicity, and also induces the production of renal MT, thus promoting the accumulation of Cd^{2+}-MT in the kidney (Cherian *et al.*, 1976; Cain and Holt, 1983; Suzuki, 1984; Fowler *et al.*, 1987b; Nordberg and Nordberg, 1987).

One of the key questions concerning MT as a mechanism of detoxification is what happens to the bound metal. The MT turnover rate in mammals is quite rapid, in the order of 10 to 20 hours (Bremner, 1987). Since there is no good evidence for the excretion of MT or its bound heavy metal from the body, this relatively high MT turnover rate indicates that the animal must continue to synthesize MT in order to detoxify the accumulated metal ions. This suggests that MT is functioning as a storage protein and serves no role in the excretory process.

1.2. MOLECULAR BIOLOGY OF MT GENES

Knowledge concerning the regulation of MT gene structure and expression far surpasses our poor understanding of the precise function of MT itself. This is because the MT genes constitute one of the best known models for the study of eukaryotic gene expression. In addition, the recent advent of genetic engineering and the production of transgenic organisms for basic and applied research has relied heavily on the promoter region of several MT genes to construct a large array of fusion genes.

1.2.1. MT gene organization

The first MT gene to be isolated was from mouse DNA

libraries (Durnam *et al.*, 1980). Since then, a number of functional vertebrate MT genes have been cloned and characterized. These include mouse MT-I (Durnam *et al.*, 1980) and MT-II (Searle *et al.*, 1984), up to ten human MT genes including MT-IIA (Karin and Richards, 1982a) and MT-IA (Richards *et al.*, 1984), a rat MT-I gene (Andersen, R.D. *et al.*, 1986), a sheep MT-IA gene (Peterson and Mercer, 1986), and a chicken MT gene (Fernando and Andrews, 1989). These genes all show a similar three exon structure separated by two introns, with exon I encoding the first nine amino acids at the amino-terminal end, exon II encoding the remainder of the β-domain, and exon III encoding the α-domain. Processed MT pseudogenes have also been well documented (Karin and Richards, 1982a; Varshney and Gedamu, 1984; Schmidt *et al.*, 1985).

In *Drosophila*, there is a single copy MT gene which has only two exons (Maroni *et al.*, 1986). This *Drosophila* MT is regarded as a Class I MT, although it has only 40 amino acid residues (Maroni *et al.*, 1986; Kagi and Kojima, 1987).

1.2.2. Regulation of MT gene expression

In mammals, the MT mRNA levels are increased by the administration of various metal ions (i.e. Cu^{2+}, Zn^{2+}, and Cd^{2+}), dexamethasone, lipo-polysaccharides, interferon, and interleukin-I (Durnam and Palmiter, 1981; Hager and

Palmiter, 1981; Mayo *et al.*, 1982; Durnam *et al.*, 1984; Karin *et al.*, 1985; Morris and Huang, 1987). Evidence suggests that these increases in MT mRNAs levels are the result of increased transcription of the MT genes (Durnam and Palmiter, 1981; Hamer, 1986).

Other inducers of MT synthesis and/or accumulation of MT mRNA in mammals include ultra-violet (UV) irradiation, mitomycin-C (a growth inhibitor), surgical wounds (skin excision in mice), a tumor promoter, 12-0-tetradecanoylphorbol- 13 acetate (TPA), lymphokines, glucagon, estrogen, progesterone, vitamin D3 and inflammatory agents (Lieberman *et al.*, 1983; Angel *et al.*, 1986; Karasawa *et al*, 1987; Fornace *et al.*, 1988). At present, it is not known whether these inducers act at the level of gene transcription.

There is very little known about the functional significance of individual MT genes within the cell, although it has been suggested that each MT gene has a specific function (Richards *et al.*, 1984; Searle *et al.*, 1984; Winge *et al.*, 1984; Yagle and Palmiter, 1985; Karin, 1988). These genes show tissue specific gene expression in mammals and sea urchins (Schmidt and Hamer, 1986; Wilkinson and Nemer, 1987). Human MT genes also show significant differential regulation. For example, in human skin fibroblasts (GM 969) and HeLa cells, the hMT-IIA gene is highly responsive to Zn^{2+}, Cd^{2+} or dexamethasone;

whereas hMT-IA gene is mainly responsive to Cd^{2+}, but not to Zn^{2+} (except at higher concentrations) or dexamethasone (Richards *et al.*, 1984). In the human hepatoma cell line (HepG2) the hMT-IIA gene responds equally well to Cu^{2+}, Zn^{2+}, and Cd^{2+}. However, the hMT-IF gene is induced poorly by Cu^{2+} (relative to Zn^{2+} and Cd^{2+}) and the hMT-IG gene is induced poorly by Cd^{2+} (relative to Cu^{2+} and Zn^{2+}) (Sadhu and Gedamu, 1988). The picture in mice differs from that of human where similar levels of MT-I and MT-II mRNAs were observed following induction by the same heavy metal (Yagle and Palmiter, 1985).

Mammalian MT genes can also be regulated by the level of DNA methylation (Compere and Palmiter, 1981; Hildebrand *et al.*, 1982). In cultured mouse thymoma (W7) cells and chinese hamster ovary cells, expression of MT-I gene becomes Cd^{2+}- inducible following administration of azacytidine (a compound that causes hypomethylation), resulting in these cell types switching from a Cd^{2+}- sensitive phenotype to a Cd^{2+}- resistant one. In human cell lines, Heguy *et al.* (1986) demonstrated that the hMT-IB gene in HeLa cells is highly methylated and does not respond to metals, whereas in a hepatoma cell line, HepG2, the same gene which is not methylated, can be induced by metals. For dexamethasone, Jahroudi *et al.* (1987) reported that the expression of MT genes by this inducer can be altered by changing the degree of methylation. They took a human lymphoblastoid cell line

(WI-L2) containing a hMT-IIA gene promoter which was not inducible by dexamethasone, treated it with azacytidine and demonstrated the expression of the gene following dexamethasone treatment.

1.2.3. Promoters of MT genes

It has been suggested that the mechanism of regulation of differential MT gene expression can be attributed to the *cis*-acting elements in the 5' promoter region of the gene and their related *trans*-acting protein factors (transcription factors) (Dynan and Tjian, 1985; Johnson and McKnight, 1989). From sequence and functional analysis of the cloned human, mouse and rat MT genes, multiple *cis*-regulatory elements have been identified in their 5' promoter-enhancer regions (Searle *et al.*, 1984; Haslinger and Karin, 1985; Serfling *et al.*, 1985; Karin *et al.*, 1984a,b, 1987; Andersen *et al.*, 1987). These elements include the metal regulatory elements (MREs), the glucocorticoid regulatory elements (GREs), and the basal level expression elements (BLEs) (Hamer, 1986). Some MREs also contain a "GC box" sequence (GGCGGG), which is a binding site for transcription factor Sp1 (Lee *et al.*, 1987; Harrington *et al.*, 1988). Not all MT genes contain all of the regulatory elements (Karin *et al.*, 1987). For example, hMT-IA promoter does not contain any BLEs and also does not

show any enhancer activity when fused to a heterologous reporter gene (thymidine kinase gene of herpes simplex virus)(Richards *et al.*, 1984).

A number of studies have characterized the MREs of the human MT genes. Karin *et al.* (1984a,b) using deletion mutants of the hMT-IIA gene promoter found that a pair of MRE sequences, distal and proximal to the TATA box, were responsible for Cd^{2+}- inducibility. The consensus sequence of these two MREs was suggested to be 5'TGCGCCCGGCYC3' (Karin *et al.*, 1984b; Karin *et al.*, 1987).

Many MREs have been identified in the other MT gene promoters. For instance, there are six MREs distributed inside a region of 40-421 bp upstream from the transcription start site of the sheep MT-I gene (Peterson and Mercer, 1986), and six MREs in the rat MT-I gene, 50-170 bp upstream from the transcription start site (Andersen, R.D. *et al.*, 1986). Stuart *et al.* (1985) compared the MREs of several mammalian MT genes and proposed a consensus sequence of 5'CTNTGCRCNCGGCCC3'. The nucleotides essential for MRE function were determined using a series of oligonucleotides linked to a heterologous reporter gene (thymidine kinase gene of herpes simplex virus). Comparison of the metal inducibility of these nucleotides suggested that the core MRE motif was 5'TGCRCYC3' (Stuart *et al.*, 1984, 1985; Searle *et al.*, 1985).

A number of studies have been carried out to determine

how many MRE elements are required for the MT gene to be fully inducible. Karin *et al.*, (1984b) using deletion mutants found that a single proximal MRE confers full metal inducibility of the hMT-IIA gene. A similar result was also obtained using the chicken MT promoter (Fernando and Andrews, 1989). However, studies using MT genes other than hMT-IIA indicate that a pair of MREs are required (Carter *et al.*, 1984; Stuart *et al.*, 1984, 1985).

The glucocorticoid responsive element (GRE) has also been characterized in hMT-IIA by deletion assays and receptor binding analysis (Karin *et al.*, 1984a). This GRE sequence, 5'GGTACACTGTGTCCT3', located about 250 bp upstream from the start site, shows homology to the glucocorticoid responsive sequences of mouse mammary tumor virus- long terminal repeat (MMTV-LTR)(Karin, 1988). The hMT-IA and hMT-IB gene do not have GRE sequences and hence do not respond to glucocorticoid (*e.g.* dexamethasone) administration (Richards *et al.*, 1984; Heguy *et al.*, 1986).

1.2.4. *Trans*-protein factors that interact with MT gene promoters

One of the major subjects of MT gene regulation is whether metal ions are the primary gene inducers, and if they are, how do they activate the transcription of the MT genes. Recent studies on a yeast MT gene transcription

factor suggested that it had a cysteine rich region which could bind with metal ions. This *trans*-acting regulatory protein (ACE1) that activates yeast CUP1 gene transcription has been identified and sequenced (Thiele, 1988; Furst *et al.*, 1988; Szczypka and Thiele, 1989). The ACE1 gene encodes a protein of M_r 24,000 which has a MT-like primary structure in its amino-terminal domain. It is hypothesized that Cu ions (cuprous or cupric) bind to the amino-domain of ACE1, change the conformation of ACE1 and hence activate the CUP1 gene promoter by binding to the upstream activating sequences (Furst *et al.*, 1988).

In higher eukaryotic cells, it is believed that the MREs are binding sites for transcription factors which mediate or activate transcription of MT genes by RNA polymerase II. Current thoughts on the interactions between human or mouse MT gene promoters and their transcription factors are as follows. (1) The existence of multiple *cis*-acting elements in the human hMT-IIA and the mouse mMT-I 5' flanking promoter regions suggest that the activation of a MT gene requires a factor to bind to the MRE(s) and to interact with other factors that bind to the adjacent *cis*-elements (Imbra and Karin, 1987; Lee *et al.*, 1987; Chiu *et al.*, 1988). (2) In mouse cells, it has been shown, by *in vivo* and *in vitro* footprint analyses, that the MREs had no protein bound with them whereas the BLEs were bound with other transcription factors in the absence of heavy metal

ions (Andersen *et al.*, 1987; Mueller *et al.*, 1988). The same experiments also showed that in the presence of heavy metal ions, a new set of metal-dependent footprints were observed on all of the MREs of the mouse MT-I promoter (Mueller *et al.*, 1988).

Recently, three research groups reported the identification of MRE binding proteins from mouse cell lines. The first report was from Seguin and Prevost (1988) who showed that in heavy metal- resistant mouse L cells, a nuclear protein p108 (M_r= 108,000) was capable of binding with the mouse MT-I MREd oligonucleotide. This MREd oligonucleotide is a strong wild type DNA sequence which contains both the core MRE motif sequence- TGCRCYC, and the binding site for transcription factor Sp1 (CCGCCC), as 5'-CTCTGCACTCCGCCCG-3'. Another research group (Westin and Schaffner, 1988) analyzed the *in vitro* binding of Sp1 and a zinc-inducible factor (MTF-1) to a synthetic mMT-I MRE (MREd). They have not reported the size of the protein but they did show that the Sp1 and MTF-1 complexes had an overlapping binding site in MREd. However, there is no evidence to show that the MTF-1 is responsible for metal-induced transcription of the mMT-I gene (Westin and Schaffner, 1988). The relationship of this MTF-1 to the p108 protein is not known. Neither p108 nor MTF-1 binds to the Sp1 binding site, 5'CCGCCC3', although the search for the transcription factor(s) responsible for the binding and

activation of MT genes has been complicated by the presence of the binding site for Sp1. Using MRE sequences which do not contain the Sp1 binding sequence of 5'CCCGCC3' has led to the purification of another nuclear protein (M_r =74,000) from uninduced mouse cells (Imbert *et al.*, 1989). However, this protein does not show any metal inducibility, nor metal-induced binding of wild-type MREs (Imbert *et al.*, 1989).

The basal level of human MT gene expression can be regulated by transcription factors AP-1, AP-2 and Sp1 (Lee *et al.*, 1987; Mitchell *et al.*, 1987; Scholer *et al.*, 1986; Imbra and Karin, 1987). The AP-1 is a well studied phorbol ester-inducible, enhancer binding protein which is also the gene product of proto-oncogene c-*jun* (Angel *et al.*, 1987, 1988; Bohmann *et al.*, 1987; Imbra and Karin, 1987). Since AP-1 and AP-2 interact with the promoters of a number of genes that are stimulated by serum growth factors, hMT-IIA might be involved in cellular growth and proliferation (Imbra and Karin, 1987; Angel *et al.*, 1988; Piette *et al.*, 1988).

In the human hMT-IIA gene, the functional binding site for Sp1 is located in a "GC" box between two proximal MREs (Lee *et al.*, 1987). Transcription factor AP-1 (c-*jun*) has been shown to activate the hMT-IIA gene by binding to the BLEs (Angel *et al.*, 1987, 1988; Piette *et al.*, 1988). The binding activity of this AP-1 is independent of the "GC"

boxes and Sp1 (Angel *et al.*, 1988; Piette *et al.*, 1988).

In conclusion, multiple factors have been reported to be responsible for the binding of the MT gene promoter sequences and activation of the MT genes. The lack of consistency of purifying a factor or factors which can bind with the MREs suggests that more complicated protein-protein and protein-DNA interactions might be involved in the regulation of MT gene transcription.

1.3. FISH, MT AND HEAVY METALS

1.3.1. Fish MTs and their relationship to heavy metals

Research on MT in fish is still in its infancy with almost all of the studies following up on the MT detoxification hypothesis forwarded for mammals. Very few experiments have been carried out to examine MT's possible role in intracellular homeostasis of essential element (Cu^{2+} and Zn^{2+}) and the detoxification of non-essential heavy metals such as Cd^{2+}. Winge *et al.* (1974) hypothesized, from experiments using rats, that pathological effects of heavy metals such as Cd^{2+} occur when the amount of metal entering the animal exceeds the ability of MT to bind and detoxify it. At low doses Cd^{2+} binds to MT, whereas at higher doses the Cd^{2+} "spills over" to bind to high molecular weight proteins and cause tissue damage. Although we do not know

what the high molecular weight proteins are, several studies on fish tend to support this hypothesis (reviewed by Klaverkamp *et al.*, 1984; Hamilton and Mehrle, 1986; Hodson, 1988). Since the relationship between metals and other metal binding proteins is not well studied, it is difficult to come to firm conclusions about the validity of the hypothesis. The rate of MT biosynthesis would seem likely to be very important to the function of MT as a metal chelater for metal detoxification. Obviously the faster MT can be produced, the less chance there is for the trace metal to "spill-over" to other physiologically important intracellular proteins.

1.3.2. Fish MTs

MTs have been isolated and characterized from eel (*Anguilla anguilla*) (Noel-Lambot *et al.*, 1978), plaice (*Pleuronectes platessa*) (Overnell and Coombs, 1979), Pacific staghorn sculpin (*Leptocottus armatus*) (Ridlington *et al.*, 1981), carp (*Cyprinus carpio*) (Kito *et al.*, 1982, 1984, 1986), coho salmon (*Oncorhynchus kisutch*) (McCarter *et al.*, 1982), skipjack tuna (*Katsuwonus pelamis*) (Takeda and Shimizu, 1982), winter flounder (*Pseudopleuronectes americanus*) (Shears and Fletcher, 1985), rainbow trout (*Salmo gairdneri*, now *Oncorhynchus kisme*) (Olsson and Haux, 1985), roach (*Rutilus rutilus*) and stone loach (*Noemacheilus*

barbatulus) (Brown *et al.*, 1987). The amino acid composition of these fish MTs are essentially identical to those of mammalian MTs. Table 1 shows a comparison of several of the better characterized fish MTs along with those of the horse and mouse.

In contrast to mammals where the existence of two major isoforms of MT appears to be the rule, some fish species appear to possess only a single MT isoform. For example, independent studies of the plaice and winter flounder, two closely related marine genera, have found evidence for only one major MT isoform (Overnell and Coombs, 1979; Shears and Fletcher, 1985). Similar evidence for one isoform has been presented for the Pacific staghorn sculpin, a marine species (Ridlington *et al.*, 1981), and the roach and stone loach, two freshwater species (Brown *et al.*, 1987). Two major MT isoforms have been clearly identified in equally diverse fish taxa: eel (Noel-Lambot *et al.*, 1978), carp (Kito *et al.*, 1982), skipjack tuna (Takeda and Shimizu, 1982), and rainbow trout (Olsson and Haux, 1985). Species differences in the number of major MT isoforms does not appear to be restricted to fish. In amphibians, two forms of MT have been detected in the bullfrog (*Rana catesbeiana*) (Suzuki and Akitoma, 1983), but only one form has been found in the frog (*Xenopus laevis*) (Suzuki and Tanaka, 1983). However, since these amphibian MTs have not been as well characterized as those of the fish and mammals, further studies will have to

Table 1. Amino acid composition (residues per molecule) of hepatic MT isolated from Cd^{2+}- injected fish (1-4), mouse (5), and normal horse (6).

AMINO ACID	(1) Flounder	(2) Plaice	(3) Carp MT1	Carp MT2	(4) Rainbow Trout MT1	Rainbow Trout MT2	(5) Mouse MT1	Mouse MT2	(6) Horse MT1	Horse MT2
Asx	6	6	5	6	6	8	4	4	3	3
Thr	7	6	4	5	4	4	5	1	3	1
Ser	6	6	7	6	8	10	9	10	8	8
Pro	4	4	3	3	2	2	2	2	3	2
Glx	3	3	2	2	3	2	1	3	2	3
Gly	6	7	6	7	6	6	5	4	7	5
Ala	2	2	3	3	3	3	5	6	5	7
Cys	18	19	20	20	20	17	20	20	20	20
Val	1	1	1	1	1	1	2	1	1	3
Met	1	2	1	1	1	1	1	1	1	1
Ile	-	-	-	-	-	-	-	1	-	-
Leu	-	1	-	-	-	-	-	-	-	-
Tyr	-	-	-	-	-	-	-	-	-	-
Phe	-	-	-	-	-	-	-	-	-	-
Lys	6	6	7	6	6	6	7	8	6	7
His	-	-	-	-	-	-	-	-	-	-
Arg	-	-	-	-	-	-	-	-	2	1

Adapted from Shears and Fletcher, 1985.
(1) Shears and Fletcher, 1985.
(2) Overnell and Coombs, 1979;
calculated by Shears and Fletcher, 1985.
(3) Kito *et al.*, 1982; expressed to nearest integer.
(4) Olsson and Haux, 1985.
(5) Sequence analysis from Huang *et al.*, 1979.
(6) Sequence analysis from Kojima *et al.*, 1979.

be carried out to confirm the author's conclusions. For avians, only one MT isoform has been identified in white-leghorn chicken (McCormick *et al.*, 1988; Wei and Andrews, 1988), whereas evidence for two isoforms has been presented for quail (Yamamura and Suzuki, 1984) and pigeon (Lin *et al.*, 1990).

1.3.3. Quantification of fish MT

In order to evaluate the function of MT, it is essential to choose an appropriate method for its quantification. This presents a difficult choice for most investigators because rapid, sensitive and accurate methods have yet to be developed for fish tissues.

Many investigators have limited their methodology to the crude fractionation of cytosol extracts using standard chromatographic procedures, such as gel filtration and the determination of the relative amounts of heavy metals bound to the MT-like low molecular weight proteins (10,000 to 14,000 M_r range) eluting from the columns. These methods are inexpensive and straightforward and can be adapted to process relatively large numbers of samples, a common requirement of environmental toxicology studies. However, such methods suffer from being very non-specific, since there are invariably a number of proteins eluting from the column in the low molecular weight range even after heat

treatment of the crude cytosol preparation.

A number of investigators improved their MT quantification procedures by using polarography, particularly when combined with heat treatment of the cytosol followed by gel filtration column chromatography. These polarographic techniques, which can be used to determine the amount of metal-thiol groups, are specific for cysteine-rich metal binding proteins (Olafson and Sim, 1979). However, the occurrence in fish tissues of low molecular weight cysteine-containing proteins other than MT should caution us that polarographic methods may also be non-specific (Thomas *et al.*, 1983a,b, 1985; Stone and Overnell, 1985; Pierson, 1985a,b; Andersen, R. *et al.*, 1986; Kay *et al.*, 1986). This criticism is particularly relevant for winter flounder, where a relatively high (13%) cysteine-containing metal binding protein, which is not MT, can be found in the liver throughout the year (Shears and Fletcher, 1985).

1.3.4. MT and metal detoxification in fish

It is evident from the literature that fish toxicologists look upon MT as a promising and powerful means by which to study mechanisms of sub-lethal and ultimately lethal metal toxicity. A few reviews have been published on fish MTs advocating their potential as a general indicator

of heavy metal exposure in the environment (Klaverkamp *et al.*, 1984; Hamilton and Mehrle, 1986; Engel and Roesijadi, 1987; Chan *et al.*, 1989).

Apart from demonstrations that MT levels in fish tissues can be increased following the administration of heavy metals such as Cd^{2+} and Zn^{2+}, the evidence that MT plays an important detoxification role in fish is indirect and largely based on correlations between MT and heavy metal concentrations in their tissues or in the water in which they reside. In view of the fact that the role of MT in the metal detoxification process in mammals is unclear, this is not surprising.

As indicated in the preceeding section most studies on the relationship between heavy metal exposure and MT concentrations in fish have used non-specific methods to identify and quantify MT. Therefore in the ensuing discussion it should be kept in mind that although the investigators may be correct in assuming that they are indeed measuring MT, there is, in most cases, insufficient evidence to be certain of this fact.

A number of investigations have clearly demonstrated that hepatic MT levels in fish can be correlated with heavy metal concentrations in the water. In an extensive series of laboratory and field experiments using coho salmon and rainbow trout, McCarter *et al.*, (1982), Roch *et al.*, (1982), and Roch and McCarter (1984a,b) found a direct correlation

between hepatic MT levels, as measured by polarography, and the heavy metal (Zn^{2+}, Cu^{2+} and Cd^{2+}) concentrations in the water. Similarly field studies on perch (*Perca fluviatilis*) revealed a positive correlation between the hepatic MT level, measured using polarography, and hepatic Cd^{2+} levels (Olsson and Haux, 1986). The data presented by these authors strongly support their conclusion that the levels of hepatic MT provide a sensitive and reliable indicator of the biological response of fish to heavy metals in their environment (Roch *et al.*, 1986).

Despite the large body of literature supporting the hypothesis that hepatic MT concentrations reflect environmental metal levels, recent equally well designed studies on lake trout (*Salvelinus namaycush*) and white sucker (*Catostomus commersoni*) inhabiting metal contaminated and uncontaminated regions of the Great lakes found no relationship between hepatic and renal MT levels, as measured by polarography, and environmental or tissue levels of Cu^{2+}, Zn^{2+}, Cd^{2+} and Pb^{2+} (Hodson *et al.*, 1989). The reason(s) for the differences between the findings of these studies and those of the Roch and McCarter group are unknown.

It has long been known that pretreatment of an animal with low or sublethal doses of metals prevents or reduces the acute toxic effects of subsequent larger doses (Cherian and Nordberg, 1983). A number of studies have demonstrated

this phenomenon in fish. Dixon and Sprague (1981) pre-exposed rainbow trout for three weeks to water containing 94 μg/L Cu^{2+}, a sub-lethal concentration, before challenging them with a lethal concentration (570 μg/L). All of the control fish died within two days whereas 70% of the pre-exposed fish were alive after six days. A similar experiment was carried out on coho salmon by Buckley *et al.* (1982). These authors found that pre-exposure to Cu^{2+} resulted in up to two-fold increases in the 168-h medium lethal concentration (LC50) of Cu^{2+} depending on the pre-exposure concentration. Experiments by Roch and McCarter (1984a) demonstrated increases in the lethal resistance (96-h LC50) of rainbow trout to mixtures of Zn^{2+}, Cu^{2+}, and Cd^{2+} which were directly proportional to the pre-exposure concentrations of the same mixture.

All of the foregoing studies suggest that fish can respond to metal exposure by developing detoxification mechanisms that prevent or reduce the lethal effects of high metal concentrations. A number of experiments have been carried out to determine whether this detoxification mechanism involves the production of MT. Kito *et al.* (1982) demonstrated that the reduced toxicity of Cd^{2+} to carp following acclimation to low concentrations of Cd^{2+} or Zn^{2+} was accompanied by increased hepatic MT levels. McCarter and Roch (1983), using coho salmon, found a strong positive correlation (r=0.97) between the 168-h LC50 for Cu^{2+} and the

concentrations of hepatic MT. Similar studies carried out on rainbow trout found that both 96-h LC50 and hepatic MT had increased in direct proportion to the metal mixture (Zn^{2+}, Cu^{2+}, and Cd^{2+}) concentrations that they were exposed to in the water (Roch and McCarter, 1984a,b). In an experiment using fathead minnows (*Pimephales promelas*), Benson and Birge (1985) found that increased tolerance to Cd^{2+} was accompanied by increased gill MT levels and that this increased tolerance along with the elevated MT levels was lost when the fish were returned to clean, uncontaminated water.

The results from all of the above experiments are consistent with the hypothesis that MT detoxifies heavy metals. However, they do not establish a cause and effect relationship between MT and increased metal tolerance. In addition, changes in metal tolerance and tissue MT levels can occur independently. For example, McCarter and Roch (1983) found that coho salmon could acclimate to Cu^{2+} before any increase in hepatic MT could be detected. Furthermore when these fish were exposed to uncontaminated water their sensitivity to the lethal effects of Cu^{2+} increased, despite the fact that hepatic MT levels remained elevated (McCarter and Roch, 1984). Experiments by the same group demonstrated that rainbow trout, exposed to metal ions (Zn^{2+}, Cu^{2+}, and Cd^{2+}) in contaminated lakes, exhibited elevated hepatic MT levels, but no change in their tolerance to these metals

(Roch and McCarter, 1984a,b). Although these experiments do not argue against a role for MT in the detoxification process, they do suggest that MT may have biochemical roles other than detoxification.

Some of the evidence that MT is important to physiological processes other than detoxification comes from recent studies on normal (non-metal exposed) rainbow trout (Olsson *et al.*, 1987) and plaice (Overnell *et al.*, 1987a). Both of these groups found significant differences in hepatic MT levels which were attributable to the sex of the fish and to the season. In addition to showing that MT levels can vary without exposure to excess metals, these studies emphasize that caution must be exercised when interpreting the results of toxicological studies on metals.

It is clear that the evidence relating MT to the detoxification of excess heavy metals in fish is weak. As indicated earlier some of the problems with the research conducted to date may be with the identification and quantification of MT itself. In other cases the possible complicating factors of sex and season may not have been taken into account. Another criticism of the toxicological studies on MT in fish is with the use of lethal exposure to determine levels of metal tolerance. Since MT is an intracellular protein functioning within the protected and stable environment of the cell, its ability to detoxify metals will be dependent on the integrity of the cell

itself. Fish exposed to lethal or near lethal concentrations of metals may be suffering from many pathological changes which would disturb the functional integrity of the cell. In other words, lethal levels of metals may interfere with normal physiology and biochemistry of the fish to such an extent as to mask any possible detoxification process by MT. What is needed in fish is a better understanding of heavy metal metabolism and the intrinsic and extrinsic factors controlling MT.

1.3.5. MT and Zn^{2+} metabolism in the winter flounder

Very few studies have examined Zn^{2+} homeostasis in fish *per se*; little is known about the extent of regulation or the mechanisms involved. However, within a species consistent differences in tissue Zn^{2+} concentrations occur due to the sex of the fish (Fletcher and King, 1978; Chernoff and Dooley, 1979), the body size (Eisler and LaRoche, 1972; Cross *et al.*, 1973; Northcote *et al.*, 1975; Wiener and Giesey, 1979; Milner, 1979) and the season when sampled (Fletcher and King, 1978; Milner, 1979), implying some form of physiological control.

Evidence to date does suggest that marine fish obtain Zn^{2+} from food rather than by direct accumulation from the water (Hoss, 1964; Pentreath, 1973a,b; Renfro *et al.*, 1975). Willis and Sandra (1984) estimated that food sources

represented about 80% of the total accumulation of ^{65}Zn in the body of two species of marine fish. Marine fish also obtain their Cd^{2+} from food and accumulate most of it in the liver (Pentreath, 1977). These studies indicate that the gastrointestinal tract should be an important site for the regulation of the essential trace metal Zn^{2+} and the uptake of environmental Cd^{2+} (a non-essential and toxic metal).

Shears and Fletcher (1983, 1984) carried out several experiments to assess the role of the gastrointestinal tract in Zn^{2+} regulation in the winter flounder and the involvement of MT. In common with mammals (Becker and Hoekstra, 1971; Cousins, 1979), Zn^{2+} absorption from the intestinal lumen of the flounder appears to involve at least two steps: (1) accumulation of Zn^{2+} by the intestinal tissue, followed by (2) transfer of Zn^{2+} into the body. However, artificially elevating the Zn^{2+} status of the flounder by intravenous injections of stable Zn^{2+} (25% of the estimated total body Zn^{2+}) did not alter the intestinal accumulation of ^{65}Zn (or ^{65}Zn in the presence of a Zn^{2+} load) or its transfer into the body. As discussed previously, (section 1.1.5.), Cousins and his co-workers found that absorption of ^{65}Zn was reduced in rats which were previously injected with a Zn^{2+} load (Richards and Cousins 1975a, b; 1976; 1977; Cousins, 1985). They attributed the decrease in transfer to the binding of ^{65}Zn to MT and theorized that homeostatic control of Zn^{2+} absorption was modulated through

changes in MT content in the intestinal tissue. MT was detected in the intestinal cytosols of the Zn^{2+} injected flounder but the presence of the protein was not associated with any depression of Zn^{2+} uptake (Shears, 1983; Shears and Fletcher, 1984).

1.4. AIM OF THESIS

It is evident from the foregoing discussion that if we are to understand the role MT plays in metal metabolism in fish we must know more about MT and MT gene regulation in fish. The winter flounder was chosen as the model fish for these studies because of the considerable existing knowledge about its biology, physiology and biochemistry.

The specific objectives of this thesis were to (a) demonstrate whether or not MT genes are inducible by metals or glucocorticoids at the level of its mRNA, (b) elucidate the winter flounder MT amino acid sequence using cDNA sequencing techniques, (c) develop sensitive molecular probes (cDNA, cRNA), and analyze the level of MT gene expression in various tissues of the winter flounder in response to heavy metal ions and dexamethasone (a synthetic glucocorticoid), and (d) determine, using genomic Southern blotting, whether the flounder contains a single or multiple MT genes.

CHAPTER TWO

HEPATIC METALLOTHIONEIN mRNA IN THE WINTER FLOUNDER

2.1. INTRODUCTION

It is well documented in mammals that MT production is regulated at the level of transcription (Durnam and Palmiter, 1981; Hamer, 1986). While one can hypothesize that MT production in fish is also regulated at the level of transcription, at the onset of this study little direct evidence for this was available. Shears and Fletcher (1984, 1985) found that MT accumulated in the intestine and liver cytosols of winter flounder following parenteral administration of Zn^{2+} or Cd^{2+}. That this was possibly due to *de novo* synthesis of the protein was suggested by findings observed when the flounder were injected with Zn^{2+} and ^{35}S-cystine (used as a measure of MT synthesis, Richards and Cousins, 1975a,b). A peak of radioactivity, coinciding with the elution position of MT, was detected when the tissue cytosols were chromatographed on a gel filtration column. The present study addresses the question as to whether elevated levels of heavy metals in the flounder result in elevated levels of MT mRNA.

2.1.1. Induction of MT and MT mRNA

Since neither an antibody against winter flounder MT nor a cDNA probe to detect MT mRNA in winter flounder tissues were available at the onset of this study, the

approach taken to confirm the induction of MT mRNA in flounder tissues was to compare cell free translation products directed by mRNA purified from liver samples of saline- injected (control) and Cd^{2+}- injected (induced) fish. Purified winter flounder MT was used as a protein standard for polyacrylamide gel electrophoretic analyses of the translation products.

While the time course of MT mRNA induction in live fish had not been documented, available information on the isolation of the protein suggested that it could be considerably slower than that observed in mammals (Shears, 1983). In rat liver, the peak amount of MT mRNA was induced five hours (h) following subcutaneous injection of Zn^{2+} and Cd^{2+} (Andersen and Weser, 1978). In mouse, Durnam and Palmiter (1981) assayed maximal transcription rates of MT-I genes and MT mRNA levels at one and four hours respectively, in both the liver and the kidney, following Cd^{2+}-administration. In contrast, Shears (1983) presented evidence to suggest that it could take up to seven days for peak levels of MT to accumulate in winter flounder intestinal tissue. Therefore, in order to enhance the probability that MT mRNA was present, the cell free translation studies were conducted five days following the initiation of injection.

It is well documented that multiple injections of sublethal levels of metal ions such as Cd^{2+} result in much

higher levels of MT production in mammals than does a single injection (Ohi *et al.*, 1981). Investigators working on fish have generally adopted this procedure. For example, Bonham and Gedamu (1984) used a four- day injection scheme of increasing concentrations of Cd^{2+} (injected daily) to induce MT mRNA in rainbow trout and Overnell and Coombs (1979) administered multiple injections of Cd^{2+} over a two week period to examine the production of MT in plaice liver. A similar injection strategy was used in the isolation of MT and MT mRNA from winter flounder tissues.

2.2. MATERIALS AND METHODS

2.2.1. Winter Flounder

Winter flounder (*Pseudopleuronectes americanus*) were caught during August by SCUBA divers in Conception Bay, Newfoundland. The flounder were maintained in laboratory aquaria (250-500 L.) supplied with continuously flowing seawater under seasonally ambient conditions of temperature (10-11 °C) and photoperiod (Fletcher, 1977).

2.2.2. Induction of MT and MT mRNA

All injections were intraperitoneal.

Solutions of $CdCl_2$ were prepared in saline (1.1% NaCl)

such that the final concentration of Cd^{2+} was 2 or 4 mg/mL. The induction protocol for the preparation of MT was similar to the schedule described by Shears and Fletcher (1985): Day 1, 0.2 mg Cd^{2+}/kg body weight; Day 3, 0.6 mg/kg; Day 7, 2.0 mg/kg. The fish were killed on Day 14.

The induction protocol for the isolation of MT mRNA was similar to that of Bonham and Gedamu (1984): Day 1, 0.4 mg/kg body weight; Day 2, 0.6 mg/kg; Day 3, 1.0 mg/kg; Day 4, 2.0 mg/kg. The fish were killed 24 h after the final injection. Flounder of both sexes were used for control (injected with saline) and Cd^{2+}- treatment.

2.2.3. Purification of MT

Hepatic MT was purified from Cd^{2+}- treated winter flounder using a procedure modified from Cherian (1974) as outlined by Shears and Fletcher (1984, 1985). Livers were pooled and homogenized in 0.1 M ammonium bicarbonate, pH 8.5, containing 2 mM β-mercaptoethanol. A cytosolic extract, prepared by ultracentrifugation (65,000 x g for 1 h), was heat-treated at 70°C for 60 seconds and then centrifuged. The resulting supernatant was treated with ammonium sulfate at a concentration of 40% of saturation and centrifuged to remove high molecular weight proteins. The proteins remaining in the supernatant were then precipitated with 100 % ammonium sulfate. The precipitated proteins were

dissolved in 0.1 M ammonium bicarbonate, pH 8.5, containing 2 mM β-mercaptoethanol. This fraction was desalted by dialysis against the same buffer before $^{109}Cd^{2+}$ (New England Nuclear) was added to it. The sample was then incubated on ice for 30 min to 90 min and then subjected to gel filtration on Sephadex G-75 (Pharmacia). The ^{109}Cd-binding proteins were pooled and applied to an anion-exchange column of Whatman DE52. Flounder MT was eluted using a linear salt gradient (50-300 mM Tris HCl, pH 8.6). That this fraction contained MT was confirmed from analysis of its amino acid composition (Table 1), molecular weight, and spectral properties.

2.2.4. Total RNA extraction

Hepatic RNA was purified according to the procedure of Davies and Hew (1980) which was modified from the methods of Palmiter (1974) and White and De Lucca (1977). Ten grams of frozen liver tissue pooled from the controls or Cd^{2+}-treated fish were homogenized with a Sorval omnimixer (on ice) in a mixture containing 100 mL phenol (saturated with 0.1 M Tris HCl, pH 8.0, as described by Maniatis *et al.*, 1982) and 100 mL of water containing 0.5% diethylpyrocarbonate (DEPC), 0.5% sodium dodecyl sulfate (SDS), 25 mg/mL polyvinyl-sulfate, and 35 mg/mL spermine. The homogenate was centrifuged at 8,000 x g for 10 min at

4°C and then the supernatant was extracted with equal volumes of chloroform until no white interphase was visible. The RNA was precipitated from the aqueous phase by adjusting the NaCl concentration to 0.2 M and adding two volumes of redistilled ethanol (-20°C). The solution was mixed and stored overnight at -70°C. The RNA was precipitated by centrifugation at 10,000 x g for 35 min at 4°C. The pellet was washed twice with 3 M sodium acetate, pH 6.0 (150 mL each time), dissolved in water (12 mL) and centrifuged at 100,000 x g for 45 min at 4°C to pellet glycogen and DNA-protein complexes. The RNA in the supernatant was precipitated in the presence of NaCl and ethanol as described above. The RNA was washed twice with 70% ethanol, dried and dissolved in 10 mL of water. Proteinase K digestion was carried out by incubating the RNA in a solution containing RNA (25 $O.D._{260}$/mL), 0.5 M NaCl, 0.5 mg/mL proteinase K (Boehringer Mannheim), 0.5% SDS, 5 mM EDTA, and 10 mM Tris HCl, pH 7.4 for 1 h at 37°C. After incubation, the solution was extracted with an equal volume of phenol/chloroform (1:1). The aqueous phase was further extracted twice with an equal volume of chloroform-isoamylalcohol (49:1). The RNA was precipitated as described above, washed with 70% ethanol, dried, and then stored frozen in DEPC treated water at -70°C. All glassware and water used in the purification steps were treated with 0.5% DEPC and then autoclaved.

2.2.5. Isolation of polyadenylated RNA

Polyadenylated (poly(A)$^+$) RNA was isolated by affinity chromatography on oligo-(dT)-cellulose (Collaborative Research, Type III) following the procedure of Aviv and Leder (1972) with the following modifications. Total RNA was incubated with oligo-(dT)-cellulose for 3 h at 37°C in a high salt buffer (0.2 M NaCl, 10 mM Tris HCl, pH 7.4, 1 mM EDTA, 0.1% SDS). The unbound RNA (poly(A)$^-$ RNA) was removed by washing with this buffer at room temperature after the oligo-(dT)-cellulose was packed in a 10mL syringe. Poly(A)$^+$ RNA was eluted from the oligo-(dT)-cellulose with a low salt buffer (10 mM Tris HCl, pH 7.4, 1 mM EDTA, 0.1% SDS) at 65°C. Poly(A)$^+$ RNA and poly(A)$^-$ RNA were ethanol precipitated and stored as described above. The integrity of the RNA samples was determined by agarose gel electrophoresis in the presence of methylmercury (Bailey and Davidson 1976).

2.2.6. Sucrose gradient centrifugation

Poly(A)$^+$ or poly(A)$^-$ RNA (as marker) (100-200 μg) in water was heated at 65°C for 5 min and then quickly chilled on ice before being loaded on a 5-30% linear sucrose (RNase free, Bio-Rad) gradient made up in a buffer consisting of 0.1 M NaCl, 1 mM EDTA, and 10 mM Tris HCl, pH 7.5.

Centrifugation was for 15 h in a Beckman SW41 rotor at 250,000 x g. Thirty fractions (400 μL each) were collected. The RNA in each fraction was precipitated as above and dissolved in 10 μL of water containing ribonuclease inhibitor (Boehringer Mannheim). Poly(A)$^-$ RNA, yeast tRNA (Boehringer Mannheim) and globin mRNA (BRL) were used as sedimentation markers.

2.2.7. Cell free translation and analysis of the translation products

Cell free translations (Pelham and Jackson, 1976) were carried out using [^{35}S]-cysteine (Amersham) and a nuclease-treated rabbit reticulocyte lysate translation kit (N90 or N150, Amersham). Reactions were allowed to proceed for 2 h at 32°C or for 1.5 h at 37°C. The incorporation of [^{35}S]-cysteine into trichloroacetic acid (TCA) precipitable material was linear with respect to time under these conditions. A salt concentration of 80 mM KCl was found to be optimal for these batches of winter flounder mRNA. Approximately 5 μg of poly(A)$^+$ RNA or 1 μg of the sucrose gradient fractionated poly(A)$^+$ RNA were added to each cell free translation reaction.

Translation products were carboxymethylated by incubation in the dark for 1 h at 37°C in 0.2 M iodoacetate (Sigma) titrated to pH 8.6 with Tris base (modified from

Heikkila *et al.*, 1982). SDS- polyacrylamide gel electrophoresis (SDS-PAGE) was carried out following the procedure of Laemmli (1970). Native-PAGE (non-denaturing polyacrylamide gel electrophoresis) was carried out using the Laemmli system without any SDS present. Samples were diluted (1:5) with sample loading buffer containing 12.5% glycerol, 62.5 mM HCl pH 6.8, 0.18 M β-mercaptoethanol, 0.00125% (w/v) bromophenol blue, with (denaturing gel) or without (native gel) 1% SDS.

The purified flounder MT (see section 2.2.3.) was used as a protein marker on polyacrylamide gels. Since the behaviour of flounder MT on polacrylamide gels had not been previously demonstrated, purchased rabbit MT standards, MT-I and MT-II (Sigma), were used for comparison.

Gels were stained for protein with 0.1% Coomassie blue R250 (Bio-Rad) in a solution of isopropanol/acetic acid/water (25:10:65) containing 0.1% cupric acetate. Excess stain was removed by washing the gels with methanol/acetic acid/water (30:10:60) followed by soaking in 5% methanol and 7% acetic acid. If protein staining was not required, the gel was treated with trichloroacetic acid/acetic acid/methanol/water (20:10:30:40) for 1 h to 2 h. Low molecular weight protein standards (Gibco/BRL Canada) used were: insulin (α and β chains) (M_r= 3,000), bovine trypsin inhibitor (M_r= 6,200), lysozyme (M_r= 14,300), β-lactoglobulin (M_r= 18,400), α-chymotrypsinogen (M_r=

25,700), and ovalbumin (M_r= 43,000).

For fluorography, the gels were treated with Enhance™ (New England Nuclear) or Amplify™ (Amersham) according to the manufacturer's instructions and then dried under vacuum at 60°C for 2 h followed by 2 h at room temperature. Kodak X-Omat AR or AGFA CURIX RP-1 film was used and exposures were carried out at -70°C between intensifying screens (Dupont Cronex).

2.3. RESULTS & DISCUSSION

2.3.1. Hepatic MT in the winter flounder

Heat-treated and ammonium sulfate-fractionated liver cytosols obtained from Cd^{2+}- treated flounder were chromatographed on Sephadex G-75 (Fig. 2). As some proteins had been removed by the heat treatment and salt-fractionation steps, the major peak of $^{109}Cd^{2+}$ binding activity was observed in the position of low molecular weight proteins (M_r= 10,000 to 14,000). This peak was characterized by its high absorbance at 250 nm (metal-thiolates) and corresponding low absorbance at 280 nm (lack of aromatic amino acids). Anion exchange chromatography resulted in a pure preparation of Cd^{2+}-MT (Shears and Fletcher, 1984, 1985). This preparation was used as the winter flounder MT standard in the PAGE analyses (Fig. 3).

Figure 2. Gel filtration profiles of heat treated and ammonium sulfate fractionated liver cytosol obtained from Cd^{2+}- injected winter flounder.

(A) Sephadex G-75 fractionation shows a peak of MT containing proteins which is indicated by the high absorbance at 230 nm, the low relative absorbance at 280 nm, and the associated radioactivity of $^{109}Cd^{2+}$. The column size was 2.5 x 90 cm, fraction size 4 mL, and the buffer was 0.1 M ammonium bicarbonate, pH 8.5, containing 2 mM β-mercaptoethanol. The molecular weight markers were in order of elution: bovine serum albumin (M_r= 67,000), ovalbumin (M_r= 43,000), chymotrypsinogen A (M_r= 25,000), ribonuclease A (M_r= 13,500), cytochrome C (M_r= 12,000), aprotinin (M_r= 6,500), and insulin b (M_r= 3,000) (all purchased from Sigma).

(B) Ion-exchange (Whatman DE-52) chromatographic profile of the Sephadex G-75 MT containing peak. The column size was 1 x 15 cm, fraction size 4 mL, and the buffer was 50 mM to 300 mM Tris HCl, pH 8.6 linear gradient. The arrow indicates the beginning of the salt gradient.

A
A_{280} (●-●), A_{254} (○-○), A_{230} (●-●)
^{109}Cd, cpm 10^{-5} (--)
M_r x10^3
67
43
25
13.5
12
6.5
3
FRACTION NUMBER
B
^{109}Cd (cpm X 10^5) (--)
A_{230} (—)
FRACTION NUMBER

The relative molecular mass (M_r) of winter flounder MT was estimated to be approximately 7,100 based upon its amino acid composition (Shears and Fletcher, 1985). However, when this protein was run on a HPLC gel filtration column, it had an apparent M_r of 14,000, possibly due either to the formation of dimers or its ellipsoid conformation (Shears and Fletcher, 1985).

2.3.2. Purification of RNA

The yields of total RNA were approximately 3 mg per gram of liver from saline treated or from Cd^{2+}- treated flounder, approximately 2.4% and 3.9% of the total RNA was poly(A)$^+$ RNA respectively. Cd^{2+}- injections did not appear to affect the yield of total RNA or poly(A)$^+$ RNA. The integrity of RNA samples was examined by methylmercury agarose gel electrophoresis according to Bailey and Davidson (1976).

2.3.3. Analysis of cell free translation products and MTs on polyacrylamide gel electrophoresis (PAGE)

Poly(A)$^+$ RNA purified from Cd^{2+}- treated flounder liver was translated to yield a protein that co-migrated with the flounder MT standard in both native and denaturing gels (Fig. 3).

When MT was subjected to PAGE, it gave diffuse bands spread over a wide range of molecular weights suggesting the formation of polymerized MTs. This problem was resolved by carboxymethylating the protein and using a native PAGE system (Fig. 3.A). This observation has been noted for other MTs (Andersen and Weser, 1978; Karin and Herschman, 1980; Koizumi *et al.*, 1982; 1985; Sone *et al.*, 1987).

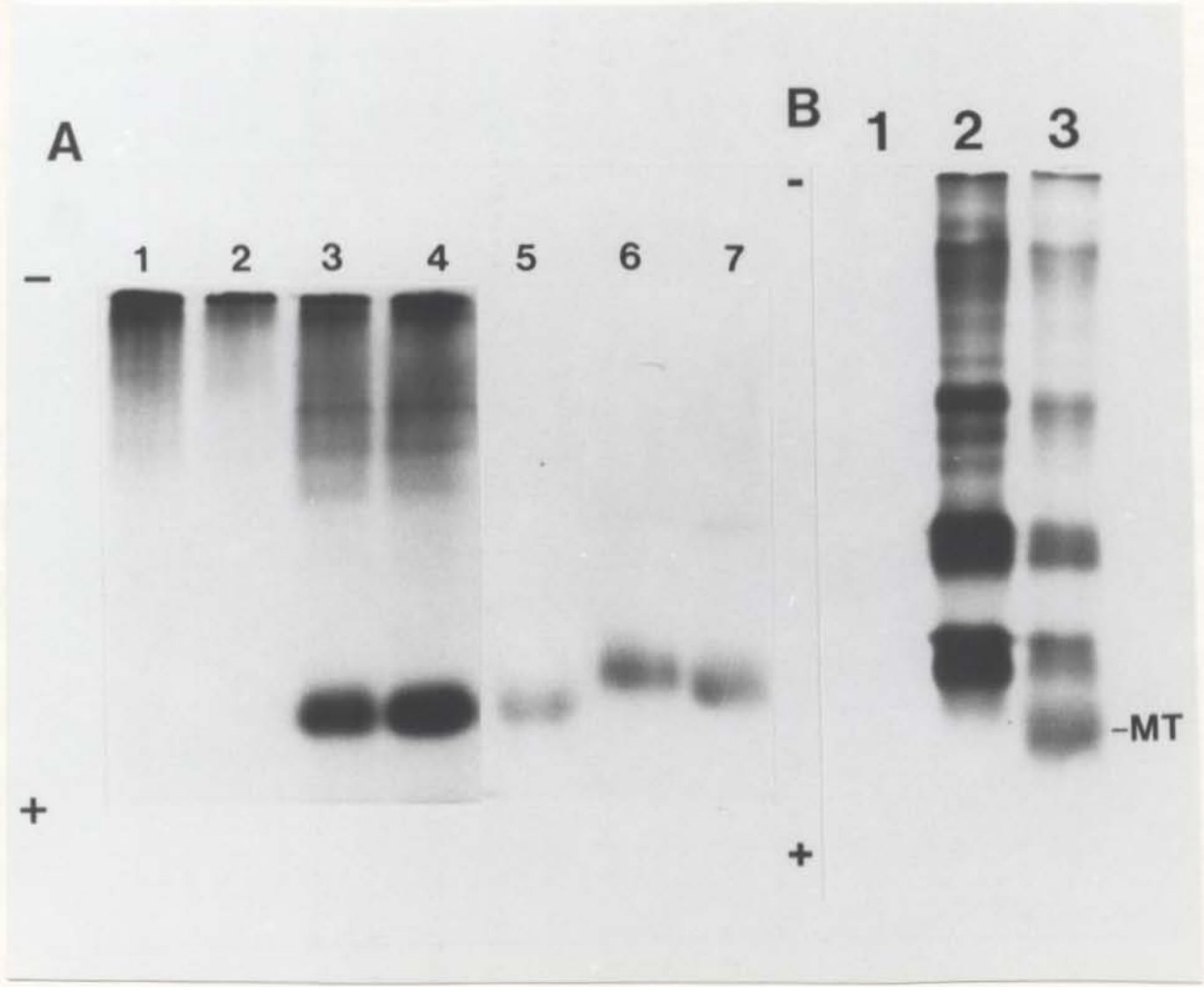
Figure 3.B shows the results of a fluorographic analysis of the carboxymethylated [^{35}S]-cysteine-labelled cell free translation products of poly(A)$^+$ RNA from Cd^{2+}- and saline-treated flounder liver after electrophoresis in a native 15% polyacrylamide gel. Only a few proteins entered the gel and MT was easily recognized because it migrated close to the dye front. No band corresponding to MT was observed in the translation products of saline-treated flounder.

Native PAGE separates proteins primarily according to their net charge and size. The carboxymethylated MT migrates rapidly and close to the dye front as a readily definable band. MTs are characterized by their high cysteine content. Iodoacetic acid carboxymethylates cysteine residues and for every cysteine that is modified the protein gains an additional negative charge (Light, 1974). Therefore, carboxymethylated MT has a very fast migration rate in native PAGE because of its small size and large negative charge (Andersen and Weser, 1978; Karin and

Figure 3. Fluorographic analysis of cell free translation products (carboxymethylated) directed by poly(A)$^+$ RNA.

(A) Native PAGE (15% polyacrylamide). Hepatic poly(A)$^+$ RNA from saline- treated flounder (lanes 1 and 2) and Cd^{2+}- treated flounder (lanes 3 and 4). Lanes 2 and 4 had 15 μM Cd^{2+} in the translation reaction mixtures. Lanes 5, 6, and 7 are flounder MT, rabbit MT-I and rabbit MT-II (Sigma), respectively, stained with Coomassie blue.

(B) 15-20% polyacrylamide linear gradient SDS-PAGE. Lane 1, without RNA added (background); lane 2, same sample as lane 2 in (a); lane 3, same sample as lane 4 in (a). MT indicates where the standard flounder MT migrates in this system. For all cell free translation samples, 20,000 cpm was loaded on each lane, except for lane 1 and 2 on gel (B). Lane 2 of gel (B) contained 40,000 cpm in order to show if there was any MT translated in this sample.


A
–
1
2
3
4
5
6
7
+
B
1
2
3
-
+
-MT

Herschman, 1980; Koizumi *et al.*, 1985).

SDS-PAGE is usually a reliable method for estimating the molecular weight of polypeptides. However, carboxymethylated MT has a rapid migration rate for its size. Andersen and Weser (1978) suggested that this is due to the additional negative charges on the carboxymethylated (reduced) amino acid residues of this small molecule. A slower migration rate (M_r= 12-14,000) is observed for MTs which have been carboxymethylated with iodoacetamide (Sone *et al.*, 1987). Although the relative molecular mass cannot be determined, the anomalous migration rate of the carboxymethylated MT allowed identification of the presence of MT, or an MT-like cell free translation product directed by poly(A)$^+$ RNA only from the liver of Cd^{2+}- treated flounder (Fig. 3).

Based on the results of SDS-PAGE analyses, several low molecular weight cysteine-containing proteins were found as cell free translation products in addition to MT (Fig. 3.B). MT could not be resolved from these other low molecular weight cysteine-containing proteins unless the carboxymethylated samples were loaded on a gradient gel (Fig. 3.B). As shown in Fig. 3.B, no MT band could be observed among the cell free translation products of the control flounder (Lane 2).

2.3.4. Sucrose density gradients

RNA samples were fractionated on a linear 5-30% sucrose gradient by ultracentrifugation. Fig 4.A shows the sedimentation profiles of hepatic poly(A)$^+$ and poly(A)$^-$ RNAs from saline and Cd^{2+}- treated flounder. The poly(A)$^-$ RNA contained primarily 28S, 18S and 4S RNAs whereas the poly(A)$^+$ RNAs were enriched in the range from 4S to 18S. The 9S globin RNA peaked in fraction 9 and ranged from fractions 8 to 10.

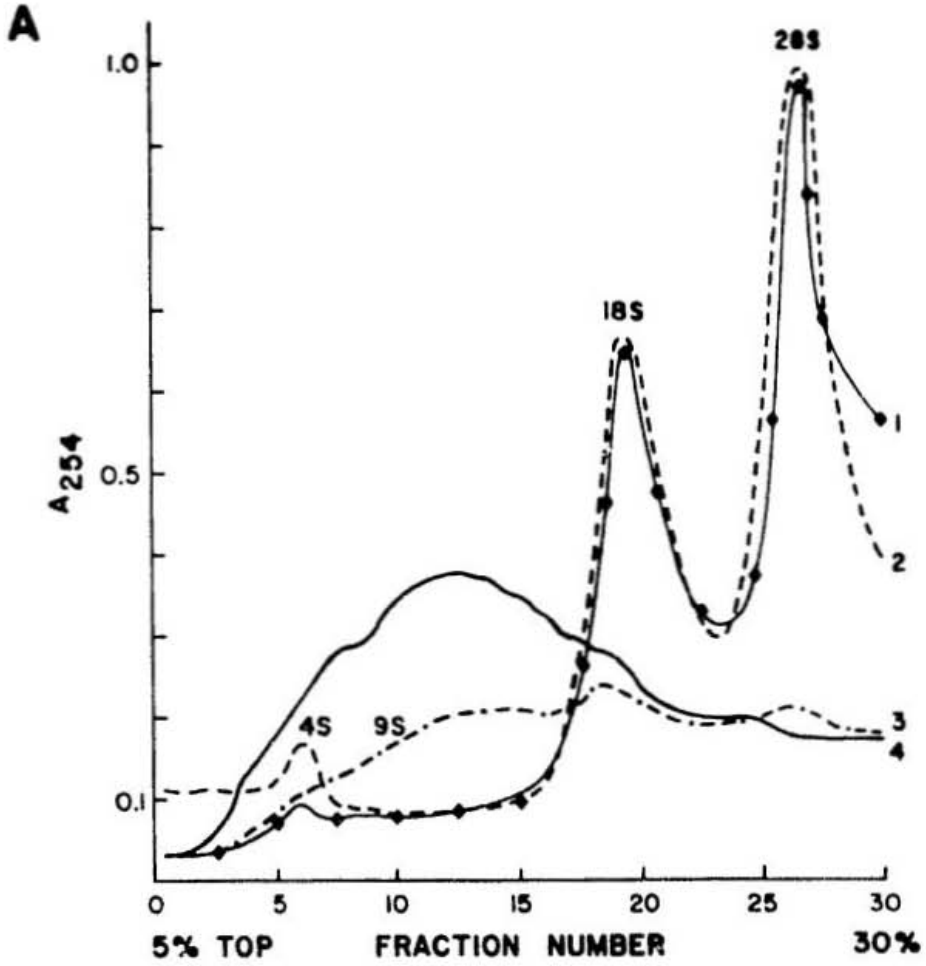
RNA from the sucrose gradient fractions was analyzed by cell free translation and PAGE analysis of the products. Only fractions 8 to 10 of the poly(A)$^+$ RNA from Cd^{2+}-treated flounder translated to yield MT as shown by fluorography of the native PAGE in Fig. 4.B. Fractions 8 to 10 correspond to the 9S globin mRNA region. Therefore, it is concluded that the mRNA for MT in the liver of the winter flounder is also 9S.

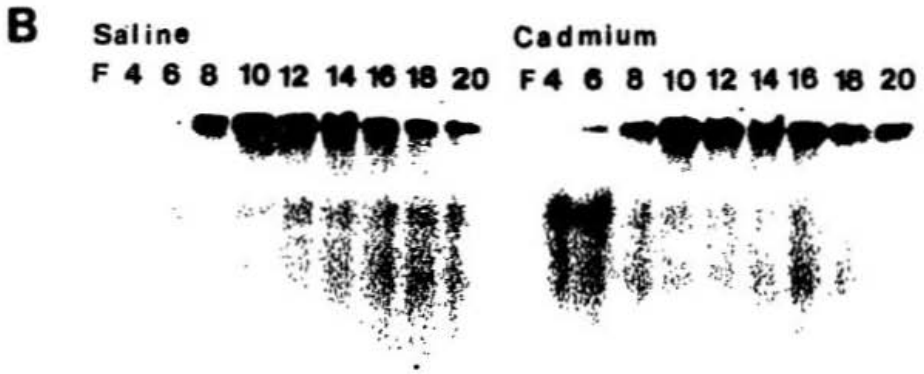
Cell free translation products were further analyzed on a 15-20% polyacrylamide SDS-PAGE. Fig. 5 shows the translation products from fractions 8, 9 and 10 from typical sucrose gradients of poly(A)$^+$ RNA from both saline- treated and Cd^{2+}- treated flounder. Fractions enriched for an RNA giving MT were only found in fractions 8, 9, and 10 in the gradient containing the poly(A)$^+$ RNA from Cd^{2+}- treated flounder. Some other low molecular weight cysteine-

Figure 4. Identification of the size of MT mRNA on cell free translation products of size fractionated RNA samples.

(A) Separation of RNA by centrifugation in a linear 5 to 30% sucrose density gradient. Line 1, 200 μg of poly(A)$^-$ hepatic RNA from saline- treated flounder; line 2, poly(A)$^-$ RNA (200 μg) from liver of Cd^{2+}- treated flounder; line 3, poly(A)$^+$ RNA (150 μg) from liver of Cd^{2+}- treated flounder; line 4, 250 μg of poly(A)$^+$ hepatic RNA from saline treated flounder. The size markers for 4S and 9S were yeast transfer RNA and globin mRNA, respectively.

(B) [^{35}S]-cysteine labelled cell free translation products directed from RNA isolated from individual fractions were carboxymethylated, loaded on a 20% polyacrylamide native PAGE system, and analyzed by fluorography. Fraction numbers refer to the gradients shown in (A).


A
1.0
0.5
0.1
A_{254}
28S
18S
4S
9S
1
2
3
4
0
5
10
15
20
25
30
5% TOP
FRACTION NUMBER
30%

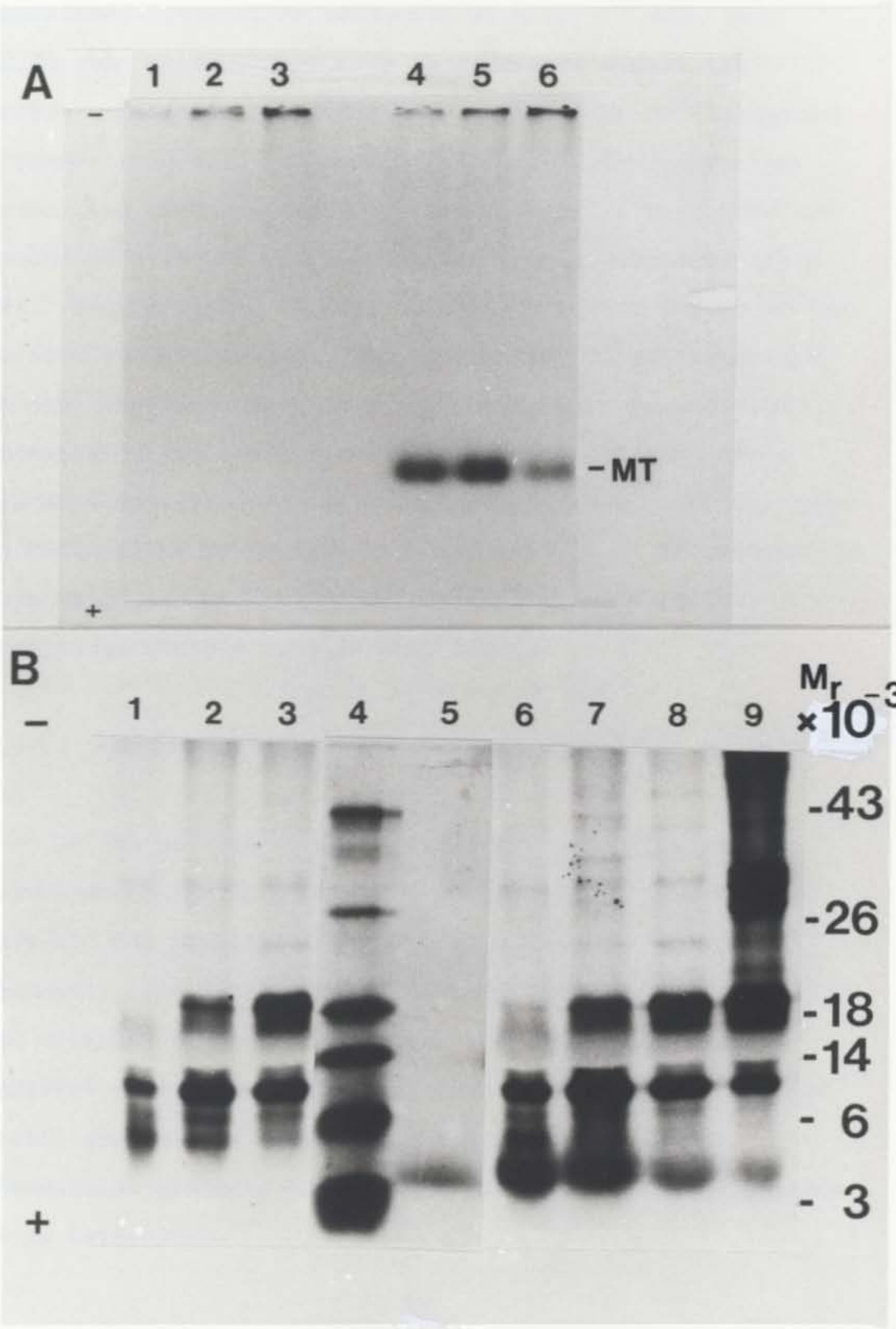

B
Saline
Cadmium
F 4 6 8 10 12 14 16 18 20
F 4 6 8 10 12 14 16 18 20

- MT

Figure 5. Fluorography of translation products of poly(A)$^+$ RNA fractionated as shown in Fig 4(A).

(A) 15% polyacrylamide native PAGE. Lanes 1-3, fractions 8, 9, 10 of saline- treated liver RNA; lanes 4-6, fractions 8, 9, 10 of Cd^{2+}- treated liver RNA.

(B) 15-20% polyacrylamide SDS-PAGE. Lanes 1-3, samples as in (A) lanes 1-3, respectively. Lane 4 is Coomassie blue stained protein markers (BRL), lane 5 is the winter flounder MT standard (Coomassie blue stained). Lanes 6-8, samples as in (A) lanes 4-6, respectively, and lane 9 contains translation products of total poly(A)$^+$ RNA from Cd^{2+}- treated liver. All samples were carboxymethylated except the protein markers.


A
1 2 3 4 5 6
–
+
–MT
B
–
1 2 3 4 5 6 7 8 9
+
M_r $\times 10^{-3}$
-43
-26
-18
-14
- 6
- 3

containing translation products, with M_r= 6-8,000, were identified in fractions 8-10 in gradients containing poly(A)$^+$ RNA from either saline- injected or Cd^{2+}- injected flounder (Fig. 5). These low molecular weight cysteine-containing proteins would overlap with MT if they were not loaded on a 15-20% polyacrylamide gradient SDS-denaturing gel. They migrated closely to MT on SDS-PAGE but could not be seen on native- PAGE (Fig. 4 and Fig. 5.A). Therefore, it was concluded that the carboxymethylated translation products of the control (saline-treated) did not have a cysteine labelled protein which had the same migration rate on PAGE as the MT protein standard (Fig. 5). If present, it must be in amounts below the detection limits of the analytical methods used in this study.

2.3.5. Low molecular weight cysteine-containing proteins

In the present study, several low molecular weight cysteine-containing proteins were translated from hepatic poly(A)$^+$ RNA purified from both saline and Cd^{2+}- treated flounder. Low molecular weight Zn^{2+}- binding proteins which had cysteine contents lower than that of MT have been isolated from winter flounder liver by Shears and Fletcher (1985) (Table 2). Whether the low molecular weight translation products noted above are these proteins remains to be determined.

Table 2. Amino acid composition (% total residues or mol/100 mol) of MTs and cysteine containing metal binding proteins from winter flounder tissues. These proteins all elute at the 10-14,000 (M_r) region on Sephadex G-75 and were then purified by ion-exchange (DE52) chromatography.

	Cd^{2+}-treated	Zn^{2+}-treated		Normal (control)	
Amino Acid	MT	Major peak MT	Minor peak non-MT	Major peak non-MT	Minor peak non-MT
Asx	9.4	10.6	9.8	10.9	11.9
Thr	12.7	11.7	7.3	8.8	6.8
Ser	9.8	9.5	7.5	8.1	7.0
Pro	6.9	7.4	6.4	6.8	6.3
Glx	4.3	4.9	11.6	10.3	13.0
Gly	10.7	10.1	9.4	9.3	9.4
Ala	2.5	2.9	6.8	5.6	7.7
Cys	31.2	29.3	9.2	13.0	2.1
Val	2.3	2.3	6.3	5.4	7.4
Met	1.7	1.7	2.4	2.1	2.3
Ile	0.4	0.6	4.1	2.4	4.4
Leu	0.7	1.0	5.7	4.3	6.6
Tyr	0.0	0.0	0.0	0.1	0.4
Phe	0.2	0.4	1.9	1.6	2.6
Lys	10.0	9.6	8.4	8.5	8.4
His	0.2	0.5	1.3	1.2	1.3
Arg	0.3	0.8	2.9	2.9	3.5

(1) Shears and Fletcher, 1985.

George and Young (1986) estimated that the time course of MT appearance in the liver of plaice was about six days after Cd^{2+} injection, although the Cd^{2+} content in liver increased immediately after injection. They also estimated that the hepatic MT levels could account for 30% of the hepatic Cd^{2+} at day 2, 40% at day 6 and 84% at day 14. Therefore, there is reason to believe that the other proteins might involve in sequesting Cd^{2+} during the early stages of Cd^{2+} administration.

2.3.6. Conclusion

The present investigation confirmed that MT is produced in the liver of the winter flounder in response to Cd^{2+}-injection. Moreover, these results indicate that administration of Cd^{2+} induces the accumulation of MT mRNA in the liver of winter flounder. Although this is consistent with the hypothesis that MT genes are regulated at the transcriptional level in flounder as they are in mammals (Durnam and Palmiter, 1981), other hypotheses, such as changes in mRNA or heterogenous RNA turnover rates cannot be excluded.

The conclusion that MT was a product of the cell free translation directed by winter flounder poly(A)$^+$ RNA is based on (1) the co-migration of the carboxymethylated derivative with similarly treated flounder MT on native PAGE

and SDS-PAGE systems, (2) the observation that the size of the MT mRNA was similar to those of mammalian MT mRNAs (Andersen and Weser, 1978; Ohi *et al.*, 1981), and (3) the fact that MT is found only in the cell free translation products directed by hepatic poly(A)$^+$ RNA from Cd^{2+}- treated flounder.

CHAPTER THREE

CLONING AND SEQUENCING OF WINTER FLOUNDER METALLOTHIONEIN cDNA

3.1. INTRODUCTION

It has been shown in Chapter Two that Cd^{2+}-administration induced the accumulation of hepatic MT mRNA in the winter flounder. Therefore it is believed that a cDNA library made from hepatic poly(A)$^+$ RNA of Cd^{2+}-injected flounder would contain cDNAs coding for the flounder MT polypeptide. In order to select a MT cDNA from the cDNA library, probes made specific to winter flounder MT are required. One approach to make such a specific probe for screening of the cDNA library is to obtain a partial amino acid sequence for flounder MT, and to synthesize oligonucleotides complementary to the predicted mRNA sequences. Subsequently, a MT cDNA can be isolated and nucleotide sequence determination will allow the elucidation of the flounder MT polypeptide sequence. In addition, the cloned flounder MT cDNA will enable MT mRNA levels to be measured with a high degree of specificity and precision.

3.1.1. Synthesis of cDNA

Because of the polyadenylated nature of the purified mRNA, the most direct method of synthesizing a cDNA is to use reverse transcriptase (RTase), primed with $dT_{12\text{-}18}$, to make the first strand cDNA. The efficiency of this step is usually low and ranges from 30% to 50%. The next step is to

remove the RNA template so that DNA polymerase I (*E. coli*) can synthesize the second strand of the cDNA. Prior to 1983, S1 nuclease was then added to digest the hair-pin loop formed on the double stranded cDNA. Since S1 nuclease is very reactive and difficult to control, the use of S1 nuclease was criticized because it can damage the cDNAs. As a result, full length cDNA synthesis was difficult to achieve.

In 1982 and 1983, three strategies were developed to overcome the above problem. The first strategy was developed by Land *et al*. (1983). They added dC-tails to the 3' ends of the first strand cDNA, so that full length double stranded cDNA could be made if the second strand synthesis was primed by a dG primer.

The second strategy was to prepare the first strand of the cDNA using an oligo(dT)- tailed plasmid primer. Investigators differed in the approach taken to achieve this. One method, the least popular, was developed by Heidecker and Messing (1983). In the first step of their method, a linearized plasmid is dT-tailed at the 3' ends and then primed for first strand cDNA synthesis. Tne second step is dG-tailing at the 3' end of each strand of the first strand of the cDNA-plasmid made in the first step. The two strands of the dG-tailed cDNA-plasmid are then separated by alkaline sucrose centrifugation, the mRNA are denatured and the products are single stranded cDNA consisting of plasmid

plus dG-tail at their 3'ends. Meanwhile, the same kind of plasmid used for first strand cDNA synthesis is linearized, dC- tailed and the two strands are separated by alkaline sucrose centrifugation. Annealing of the one strand of the dC-tailed plasmid and the dG-tailed cDNA-plasmid would put the first strand cDNA into a double stranded plasmid. Subsequently, DNA polymerase I (*E. coli*) makes the second strand of the cDNA on the plasmid.

Another variant of the second strategy, of using oligo(dT)- plasmid primer, was developed by Okayama and Berg (1982). Okayama and Berg (1982) prepared a plasmid primer with a dT-tail at one end (3') and a restriction enzyme linker at the other (5') end, which can lead to priming of first strand cDNA synthesis using the dT-tail. Further dC-tailing and removal of the dC-tail from the 5' end by restriction enzyme digestion allowed the plasmid with the dC-tailed first strand cDNA to anneal to an oligo dG-tailed linker of the same restriction enzyme. Finally, the RNA strand was replaced by DNA with RNase H (*E. coli*), DNA polymerase I (*E. coli*) and DNA ligase (*E. coli*).

The major advance of the above "Okayama-Berg" procedure was the utilization of RNase H to nick the RNA on the RNA-DNA hybrids and the use of DNA polymerase I (*E. coli*) to synthesize the second strand of the cDNA simultaneously. The third strategy was synthesis of double stranded cDNA, using these two enzymes (together with DNA ligase), directly

on the purified first strand cDNA-RNA hybrid (Gubler and Hoffman, 1983). First strand cDNA synthesis is still primed with dT_{12-18}, using revese transcriptase (RTase). The enzymatic reaction of second strand synthesis in this method is analogous to that used in nick translation and if the reaction is extended to a longer period (Citri *et al.*, 1987), full length, long cDNAs can be achieved. Many other modifications of the Gubler and Hoffman (1983) procedure have been reported. For example, Rutledge *et al.* (1988) reported the use of random primers (hexadeoxyribonucleotides) and *in situ* ligation in a cDNA synthesis procedure to achieve full length, long double stranded cDNA synthesis without using dT_{12-18} to prime for first strand cDNA synthesis.

3.1.2. Construction of cDNA libraries

In general, the efficiency of ligation of double stranded cDNA to the vector has to be high enough to create a high efficiency cDNA library. Tailing or addition of linkers would achieve the goal of joining cDNA to the plasmid efficiently. If the transformation efficiency of the competent cells is high (10^7 to 10^8 per μg of control plasmid), blunt end ligation is usually good enough for making a high efficiency cDNA library (Upcroft and Healey, 1987; Rutledge *et al.*, 1988). However, the orientation of

the insert cannot be controlled in this blunt-end ligation protocol.

Lambda phages are also very efficient for library construction, and are especially good for carrying extra long cDNA inserts and for immunoblotting (Huynh *et al.*, 1985). Since the length of insert and the abundance of MT mRNA were not major concerns here, a plasmid vector was judged to suffice for this study.

3.2. MATERIALS AND METHODS

3.2.1. Amino acid sequencing

Purified winter flounder MT (as described in Chapter Two) was carboxymethylated with [^{3}H]-iodoacetate (Amersham) in guanidine hydrochloride buffer (6 M guanidine HCl (Gibco/BRL Canada), 0.5 M Tris HCl, pH 8.6, 2 mM EDTA) at 37°C for 45 min; iodoacetate (Sigma) (titrated to pH 8.0 with Trizma base) was then added to a final concentration of 0.2 M and the reaction continued for 1 h (modified from Konigsberg, 1972; Heikkila *et al.*, 1982).

For automated Edman degradation, the carboxymethylated sample was further desalted on a Sephadex G-50 (Pharmacia) gel filtration column twice, purified on 15% polyacrylamide native gel by electro-elution and finally lyophilized. The purified sample was then sent to the Protein/Peptide

Sequencing Facility, The Hospital for Sick Children, Toronto, for sequence analysis. One nmol of the carboxymethylated sample (treated with cyanogen bromide, CNBr) was loaded into a Beckman 890C protein sequencer using a 0.1 Quadrol program in the presence of polybrene (Pierce). After conversion, the phenylthiohydantoin amino acids were analyzed by reverse phase High Pressure Liquid Chromatography (HPLC).

3.2.2. MT oligonucleotide probes

The 32 possible sequences for the first 17 nucleotides of mRNA corresponding to amino acids 1 to 6 of winter flounder MT are shown in Fig. 6c. Mixed oligonucleotide probes, complementary to these sequences, were synthesized on a Pharmacia Gene Assembler, purified by PAGE and SEP-PAK C-18 cartridges (Millipore, Water Associates).

3.2.3. Northern blot analysis

Total RNA was isolated from the kidney or liver of winter flounder that had been injected intraperitoneally with saline or Cd^{2+} as described in Chapter Two. The RNA samples were heat denatured (70°C, 10 min) and subjected to

(a) **PARTIAL PROTEIN SEQUENCES**

```
                  1       10        20
plaice            MDPCECSKTGTCNCGGSCTCKNCGCT----
winter flounder   MDPCECSKTGTCNCGGSCTCKNCSC-----
```

(b) **AMINO ACID RESIDUES**

```
                  1                5
                  Met-Asp-Pro-Cys-Glu-Cys
```

(c) **PREDICTED mRNA SEQUENCES**

```
          5' AUG GAU CCA UGU GAA UG 3'
                   C   C   C   G
                       G
                       U
```

(d) **PREDICTED cDNA SEQUENCES**

```
          3'  TAC CTA GGT ACA CTT AC 5'
                   G   G   G   C
                       C
                       A
```

Figure 6. Sequences of MT oligonucleotide probes used in the present study. Partial amino acid sequences of winter flounder and plaice MT (Overnell *et al.*, 1981) (a,b); potential sequences of part of the corresponding mRNA (c); and sequences of the mixture of oligonucleotides used as probes (d).

electrophoresis in a 1.8% agarose gel with TAE buffer (Tris-acetate-EDTA). The RNA was transferred to Hybond-N nylon membrane (Amersham) and probed with the ^{32}P-end labelled MT oligonucleotides as described by Zeff and Geliebter (1987). For autoradiography, the Hybond filter was exposed to Kodak X-Omat AR film at room temperature, after overnight hybridization (42°C, 5X SSC, 10X Denhardt's, 7% SDS, 10% dextran sulfate and 20 mM sodium phosphate, pH 7.0) and a final wash using 1X SSC, 1% SDS (42°C, 1 h) (Zeff and Geliebter, 1987).

3.2.4. Construction of cDNA library

Poly(A)$^+$ RNA was extracted from liver of Cd^{2+}- treated winter flounder as described in Chapter Two. Double stranded cDNA was prepared according to the method of Gubler and Hoffman (1983) as modified by Citri *et al.* (1987).

In brief, after oligo-(dT)-cellulose chromatography, 5 μg of poly(A)$^+$ RNA was primed with dT_{12-18} for the first strand cDNA synthesis using avian myeloblastosis virus reverse transcriptase (RTase) (Life Sciences). The reaction was carried out for 45 min at 42-43°C and terminated by the addition of EDTA to a final concentration of 50 mM, and phenol-chloroform extraction. The products were then precipitated in the presence of 5 μg of yeast tRNA as carrier, 2 M ammonium acetate and 80% ethanol, overnight

at -70°C. Second strand synthesis was carried out using 500 ng single strand cDNA. The reaction was incubated in the presence of RNase H (Pharmacia) and DNA Polymerase I (*E. coli*) (Pharmacia) at 15°C overnight followed by 2 h at 23°C. The products of single strand and double strand reactions were inspected by alkaline agarose gel electrophoresis followed by autoradiography (Maniatis *et al.*, 1982).

The double stranded cDNA was purified on a Sephadex G-50 spin column (Maniatis *et al.*, 1982) and ligated to dephosphorylated, *Sma* I digested pUC13 (Pharmacia).

E. coli (JM109) competent cells were transformed with the ligation products by the heat shock method (42°C, 2 min) (adapted from Hanahan, 1983). The transformed cells were plated on Luria-Bertani agar plates containing ampicillin (100 mg/L), isopropyl-β-D-thiogalactopyranoside (IPTG, 0.5 mM), and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal, 0.05%) (Davis *et al.*, 1986).

3.2.5. Isolation of cDNA clones

A combination of the colony hybridization procedures of Wood *et al.* (1985) and Cannon *et al.* (1987) was used to screen the cDNA library. Duplicate filter lifts (Hybond-N membranes, Amersham) were probed with ^{32}P-end labelled MT oligonucleotide probes for 16 h at 37°C in 6X SSC, 10X Denhardt's, and 50 mM sodium phosphate, pH 7.0. The filters

were washed three times for 10 min at 4°C in 6X SSC and once more for 30 min. TMAC buffer (3 M tetramethyl ammonium chloride (Sigma), 50 mM Tris HCl, pH 8.0, 2 mM EDTA, 0.1% SDS) (Cannon *et al.*, 1987) was used to wash the filters at 42°C for 15 min and once at 49°C for 20 min (Wood *et al.*, 1985). The locations of the positive colonies were identified by autoradiography of the filters using Kodak X-Omat AR film.

Plasmid DNA was purified as described by Birnboim (1983). The insert sizes of the positive clones were determined by agarose gel electrophoresis after *Eco* RI and *Hind* III digestions.

3.2.6. DNA sequencing

Alkaline denatured, double stranded plasmids were sequenced by the dideoxy chain termination method using RTase or PolIK (Mierendorf and Pfeffer, 1987). Sequencing was started at one of the ends of the cDNA insert by priming with either the reverse or the universal M13/pUC primer. All reagents and protocols were from New England Biolabs or Promega, except the radioisotope which was either [α^{32}P]-dATP (Amersham) or [α^{35}S]-dATP (New England Nuclear).

3.3. RESULTS AND DISCUSSION

3.3.1. Amino acid sequencing

The amino-terminal amino acid of winter flounder MT is blocked, probably by acetylation as is the case in plaice MT (Overnell *et al.*, 1981). There is only one methionine residue in winter flounder MT (Shears and Fletcher, 1985) and, as methionine has only been found as the amino-terminal amino acid in MTs that have been sequenced (Hamer, 1986; Kagi and Kojima, 1987), the carboxymethylated winter flounder MT was treated with CNBr. This released the amino-terminal methionine residue and allowed the sequence of the next 24 amino acids to be determined (Fig 6.a). The partial sequence of winter flounder MT is identical to the partial sequence of plaice MT (Overnell *et al.*, 1981) except at postion 24 where there is a serine residue in winter flounder and a glycine in the plaice (Fig. 6.a).

3.3.2. MT oligonucleotide probes

Potential mRNA sequences for winter flounder MT were deduced from the partial amino acid sequence (Fig. 6.c). This information was used to prepare a mixture of oligo-nucleotide probes with degenerate sequences (Fig. 6.d), one of which would be complementary to the mRNA coding for the

first six amino acids of the winter flounder MT.

The specificity of this mixture of MT oligonucleotides was determined by Northern blot analysis (Fig. 7). The size of the RNA that hybridized to the probe was about 500-600 nucleotides in length which corresponds to the reported size of winter flounder MT mRNA (Chapter Two). The response was greater from RNA isolated from liver rather than kidney and there was much more of the hybridizing species in tissues from Cd^{2+}- treated fish than in saline- injected controls. These results indicate that at least one of the mixture of oligonucleotides was capable of detecting winter flounder MT mRNA.

3.3.3. Construction of cDNA library and screening for MT cDNA

Double stranded cDNA was prepared from hepatic poly(A)$^+$ RNA and found to range mainly from 0.2 - 4 kbp, as determined by alkaline agarose gel. After ligation to the plasmid cloning vector, pUC13, and transformation of *E. coli* (JM109) cells, a cloning efficiency of 1.7 X 10^5 white colonies per μg of double stranded cDNA was obtained.

Approximately 900 colonies of the cDNA library were screened in duplicate with the oligonucleotide probes (Fig. 8). Sixteen positive clones were detected. Most of the cDNA inserts of these clones were approximately 400 bp.

Figure 7. Northern blot analysis of total RNA samples showing the specificity of the mixed oligonucleotide MT probes.

After hybridization and washing, the Hybond filter was exposed to X-ray film for (A) 10 hours or (B) 24 hours. The positions of the RNA molecular size markers (kilobases, kb) as indicated in the margins were visualized by ethidium bromide staining while still in the gel. Lanes 1 to 4, 50 μg total RNA isolated from Cd^{2+}- treated kidney (1); saline-treated kidney (2); Cd^{2+}- treated liver (3); saline- treated liver (4); lanes 5 to 8, 10 μg total RNA isolated from Cd^{2+}-treated kidney (5); saline- treated kidney (6); Cd^{2+}-treated liver (7); saline- treated liver (8).

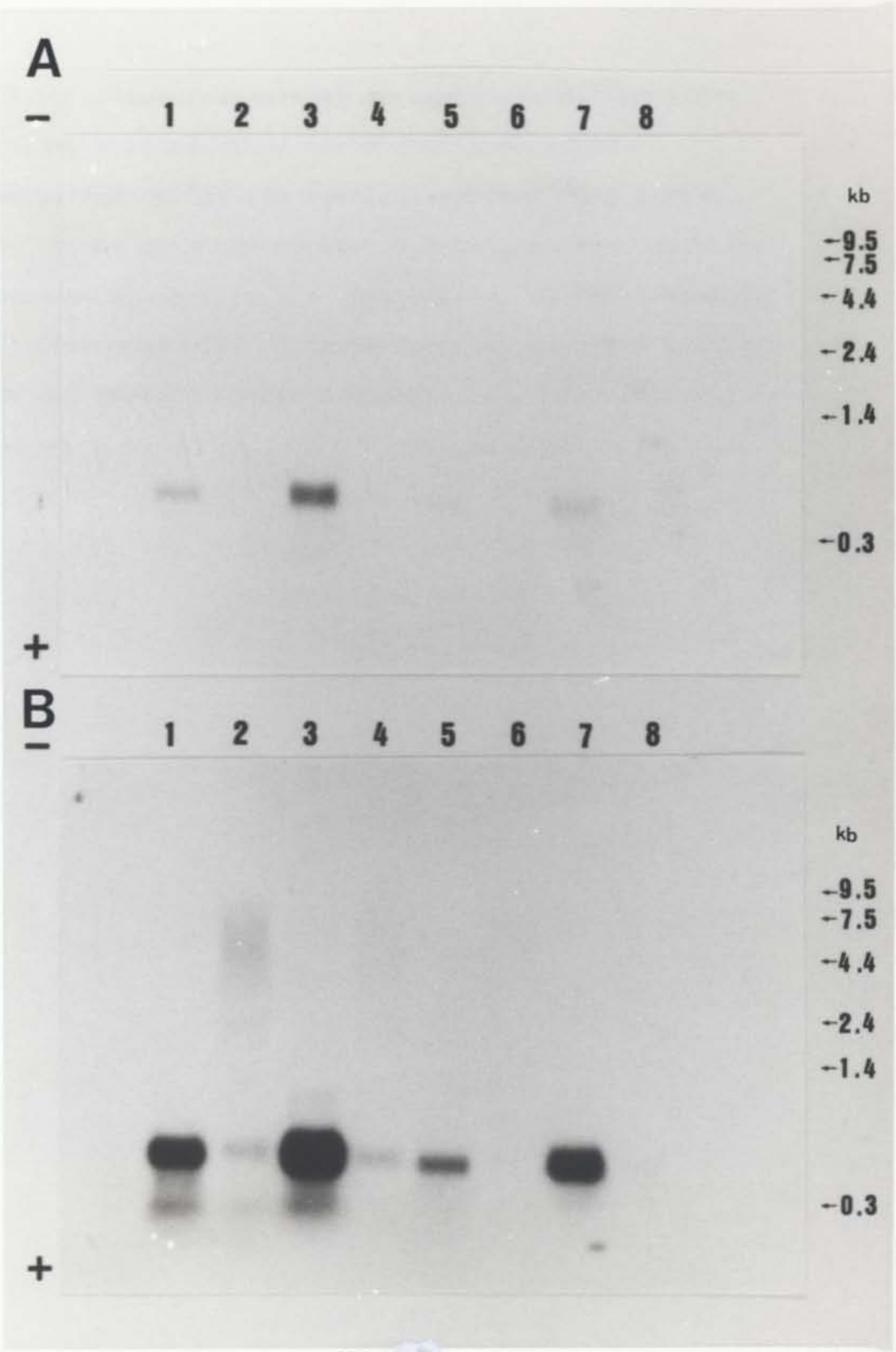
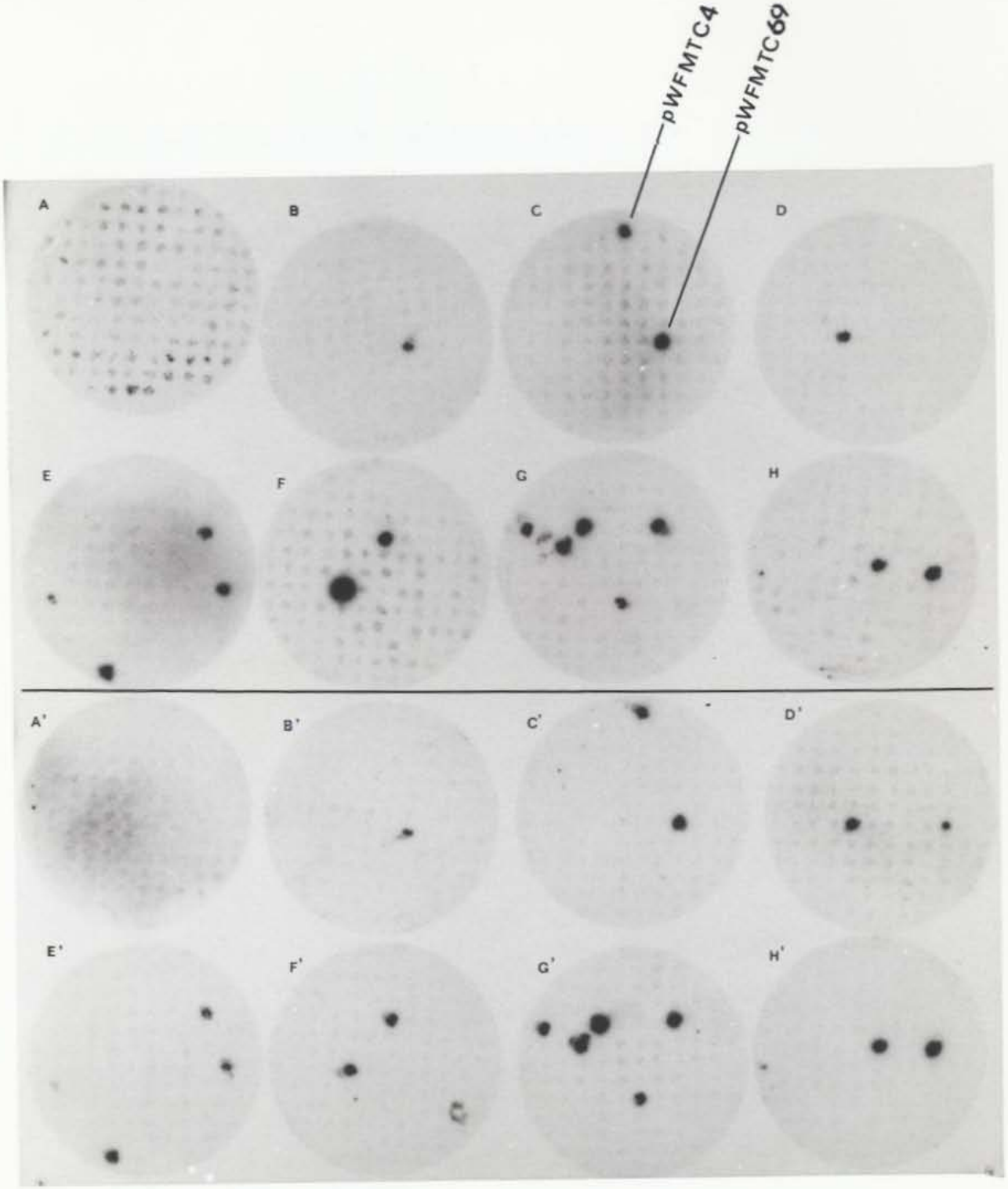

A
–
1 2 3 4 5 6 7 8
kb
9.5
7.5
4.4
2.4
1.4
0.3
+
B
–
1 2 3 4 5 6 7 8
kb
9.5
7.5
4.4
2.4
1.4
0.3
+

Figure 8. Autoradiography of duplicate filter lifts after colony hybridization to ^{32}P-end labelled MT oligonucleotide probes and washing with the TMAC buffer.

Eight grid plates were screened at the same time, labelled A to H, whereas the duplicate lifts were labelled A' to H', respectively. Inserts from two positive clones (pWFMTC4 and pWFMTC69) were sequenced.


pWFMTC4
pWFMTC69
A
B
C
D
E
F
G
H
A'
B'
C'
D'
E'
F'
G'
H'

3.3.4. Nucleotide sequence of flounder MT cDNAs

The insert of pWFMTC4 was sequenced by using reverse transcriptase (RTase). Both strands were sequenced. Direct sequence analysis revealed that the 338 bp (1-338) (Fig. 9) insert of pWFMTC4 encoded winter flounder MT mRNA. The insert of another plasmid, pWFMTC69, was found to have a complete 3' untranslated region as judged by the presence of a poly(A)$^+$- tail in addition to the same sequence as pWFMTC4. The insert of pWFMTC69 was sequenced by using RTase with the universal pUC/M13 primer and PolIK with reverse pUC/M13 primer (both strands were sequenced). The inserts in these plasmids were in the opposite orientation to one another with respect to the cloning vector pUC13.

Figure 9 shows the nucleotide sequence of winter flounder MT cDNA and the derived amino acid sequence. The cDNA contains the entire 3' untranslated region of 124 nucleotides, a coding region of 183 nucleotides including the termination codon and 49 nucleotides of the 5' untranslated region.

According to these cDNA sequences, the winter flounder MT is a 60 amino acid polypeptide that contains 20 cysteine residues and has a Class I MT primary structure. The G+C content of the coding region is high (59%) and this is reflected in a biased codon usage. Most notable is the avoidance of U in the third position except in one codon for

Figure 9. Nucleotide sequence of winter flounder MT cDNA. This is a complete sequence of pWFMTC69 insert (the whole sequence). The insert of pWFMTC4 contains nucleotides 1 to 338.

The complete coding region and the corresponding amino acid sequence of winter flounder MT is shown and denoted by capital letters. The last "a" at the 3' end was the first of 23 "a"s and was presumed to be the beginning of a poly(A)$^+$- tail. The putative polyadenylation signal attaaa is underlined as is the sequence tttgta which may have some functional significance in MT mRNAs (Peterson *et al.*, 1984). Major restriction sites are also indicated.

```
agagactcac ctgctccact gaggaagaac cagacaaccg ctgagagacA  50


  Bam HI
TG GAT CCC TGC GAA TGC TCC AAG ACT GGA ACC TGC AAC TGC  91
M   D   P   C   E   C   S   K   T   G   T   C   N   C  14

                                  Pst I/Alu I/Pvu II
GGA GGA TCT TGC ACC TGC AAG AAC TGC AGC TGC ACC ACC     130
 G   G   S   C   T   C   K   N   C   S   C   T   T      27

                Alu I                       Bgl I
TGC AAC AAG AGC TGC TGC CCA TGC TGC CCA TCC GGC TGC     169
 C   N   K   S   C   C   P   C   C   P   S   G   C      40

CCC AAG TGC GCC TCT GGC TGC GTG TGC AAA GGG AAG ACA     208
 P   K   C   A   S   G   C   V   C   K   G   K   T      53

TGC GAC ACC ACT TGC TGT CAGt gagaggagcc tgatgcactg      250
 C   D   T   T   C   C   Q                              60

                                                  Xba I
tggagccgtg cgccctacac tgattaaact gtagccgcta atgtctagaa 300

                    Rsa I
tgagaataat ggctttgta cttgtctttc aatattaaaa tcaacatctt  350

tcctc(a)23
```

aspartic acid, one for threonine, one for cysteine and two for serine. Of the 20 codons for cysteine, 19 are UGC rather than UGU. A similar codon preference has been reported for mammalian MTs (Karin and Richards, 1982b; Andersen *et al.*, 1983b; Schmidt and Hamer, 1983; Peterson *et al.*, 1984) but this is not as pronounced in the MTs from rainbow trout (Bonham *et al.*, 1987).

In the translated region of the fish MT cDNAs, 21% of nucleotides differ between flounder and trout, half of which do not alter the amino acid (silent mutations). The two trout MT cDNAs differ from each other by eight nucleotides (4.4%), six (3.3%) of which are silent mutations.

Two putative polyadenylation signals (AAUAAU and AUUAAA) were found in the 3' untranslated region of the winter flounder MT mRNA. The polyadenylation signal in most mammalian MT mRNAs and in eukaryotic mRNAs in general (80%) is AAUAAA (Wickens and Stephenson, 1984). However, in sheep MT mRNA this sequence is AGUAAA (Peterson *et al.*, 1984). Based on its relative proximity to the poly(A)$^+$- tail, AUUAAA is the more likely candidate to be the polyadenylation signal for winter flounder MT mRNA. This sequence is also found in a similar location in the rainbow trout MT-B cDNA (Bonham *et al.*, 1987).

An interesting feature of mammalian MT mRNAs is the highly conserved sequence UUUCUA that is located 13-19 nucleotides upstream from the start of the polyadenylation

signal (Peterson *et al.*, 1984). In winter flounder MT mRNA, rainbow trout MT-A mRNA (Bonham *et al.*, 1987) and chicken MT mRNA (Wei and Andrews, 1988), the corresponding sequence is UUUGUA and in rainbow trout MT-B mRNA it is UUUGUC. Whether or not this sequence has any functional importance for MT mRNA remains an open question, but this sequence is specific to MT mRNA (Peterson *et al.*, 1984, Peterson and Mercer, 1986).

The insert in pWFMTC4 contains a shorter 3' untranslated region and lacks a poly(A)$^+$- tail, as compared to the pWFMTC69 insert, in spite of the fact that the mRNA for library construction was purified on an oligo-(dT)-cellulose column and the first strand cDNA synthesis was primed with a $dT_{12\text{-}18}$ primer. However, cDNA clones may have an incomplete 3' end as in pWFMTC4 for unknown reasons. One explanation is that cleavage happens after first strand cDNA synthesis but before second strand synthesis reaches the 3' end. Several other examples of an incomplete 3' end have been documented (Lomedico *et al.*, 1979; Heidaran and Kristler, 1987).

3.3.5. Comparison of vertebrate MTs

The overall amino acid sequence identity (%) of winter flounder MT with that of other vertebrate MTs ranges from 50% for pigeon MT-II to 85% for trout MT A and B (Fig. 10,

Table 3). The alignment of the amino acid sequences of MTs from winter flounder, rainbow trout (Bonham *et al.*, 1987), chicken (Wei and Andrews, 1988), pigeon (Lin *et al.*, 1990), mouse and human (Kagi and Kojima, 1987) is shown in Fig. 10. A comparison of all of the known vertebrate MTs (Kagi and Kojima, 1987 and references above) reveals that there are a total of 25 invariant amino acid residues, 19 of which are cysteine. The invariant cysteine at position 60 (numbering as in Fig. 10) in chicken, pigeon and mammalian MTs is replaced by a threonine in fish MTs. However, the fish MTs have a cysteine at position 58 whereas the MTs of higher vertebrates do not. Relative to mammalian and avian MTs, fish MTs studied to date lack an amino acid residue at position 4 whereas chicken and pigeon MTs have an additional amino acid in this region. One of the rainbow trout MTs (MT-A) has an additional amino acid at position 33 but this is unique to this protein. Avian MTs appear to have an additional amino acid at position 54 (numbering as in Fig. 10) compared to fish and mammalian MTs.

As MT sequences for more organisms become available it is tempting to try to construct an evolutionary history for this protein. However, this has largely been precluded by the lack of a clear relationship between isoforms of MTs found in divergent vertebrate species, most notably between different species of mammals (Hamer, 1986; Kagi and Kojima, 1987). The sequence identity of MT isoforms within a

Figure 10. Comparison of MT amino acid sequences from winter flounder, rainbow trout, chicken, pigeon, mouse and human (Bonham *et al.*, 1987; Kagi and Kojima, 1987; Wei and Andrews, 1988; Lin *et al.*, 1990). Invariant amino acid residues in vertebrate MTs, including the 19 cysteines are listed at the bottom. Empty positions in the figure are included to maximize structural alignments. "-" means the amino acid residues is the same as the flounder residue listed on the top.

```
                1   5   10   15   20   25   30   35   40   45   50   55   60
Flounder        MDP  CECSKTGTCNCGGSCTCKNCSCTTCNK SCCPCCPSGCPKCASGCVCK GKTCDTTCCQ
Trout-A         ---  -------S-------K-S--A--S-K-A---D------S--------- ------S---
Trout-B         ---  -------S-------K-S--A--S-K- --------D-S--------- ------S---
Chicken         ---QD-T-AAGDS-S-A---K----R-RS-R- ---S---A--NN--K-----EPASSKCS--H
Pigeon-I        --SQD-P-AAG---T--DN-K----K----K- G--S---A--A---Q-----GPPSAKCS--H
Pigeon-II       ---QD-T-AAGDS-S-A---K----R-QS-R- ---S---AS-SN--K-----EPSSSKCS--H
Mouse-I         ---N -S--TG-S-T-TS--A--N-K--S-K- ---S---V--S---Q--V-- -AADKC---A
Mouse-II        ---N -S-ASD-S-T-A-A-K--Q-K--S-K- ---S---V--A--SQ--I-- QASDKCS--A
Human-IA        ---N -S-ATG-S-T-T---K--E-K--N-K- ---S---MS-A---Q--I-- -ASEKCS--A
Human-IIA       ---N -S-AAGDS-T-A---K--E-K--S-K- ---S---V--A---Q--I-- -ASDKCS--A

INVARIANT       MD   C C     C C   C C  C C  C K  CC CCP C  C   GC CK        CC
```

Table 3. Percent of sequence identity (%) and numbers of amino acid substitutions (indicated in brackets) between MT sequences from fish, bird, mouse and human, as listed in Figure 10. (Insertions and deletions are not taken into account).

	Flounder	Trout-A	Trout-B	Chicken	Pigeon-I	Pigeon-II	Mouse-I	Mouse-II	Human-IA
Flounder	-----								
Trout-A	85% (9)	-----							
Trout-B	85% (9)	97% (2)	-----						
Chicken	57%(26)	62%(23)	60%(24)	-----					
Pigeon-I	57%(26)	62%(23)	60%(24)	68%(20)	-----				
Pigeon-II	50%(30)	62%(23)	60%(24)	94% (4)	68%(20)	-----			
Mouse-I	62%(23)	67%(20)	65%(21)	61%(24)	64%(22)	62%(23)	-----		
Mouse-II	55%(27)	63%(22)	58%(25)	67%(20)	69%(19)	64%(22)	79%(13)	-----	
Human-IA	58%(25)	60%(24)	60%(24)	67%(20)	70%(18)	64%(22)	80%(12)	84%(10)	----
Human-IIA	58%(25)	65%(21)	62%(23)	75%(15)	72%(17)	72%(17)	84%(10)	89% (7)	89%(7)

species is often less than that of MT isolated from two species within the same order. This phenomenon has been observed for some primate, rodent, ruminant and avian MTs (Hamer, 1986; Kagi and Kojima, 1987; Winge *et al.*, 1984; Lin *et al.*, 1990).

The mammalian and avian isoforms of MTs are probably related to one another by gene duplication events. It has been suggested that the isoforms emerged independently during mammalian radiation (Hamer, 1986), and avian radiation (Lin *et al.*, 1990), and they probably emerged at various time during evolution (Kagi *et al.* 1984). For example, for the avian MT sequences reported to date, the chicken MT sequence has a much higher sequence identity (94%) with one of the pigeon MTs (MT-II), whereas pigeon MT-I shows similar sequence identity (68%) with both the chicken MT and the pigeon MT-II (Lin *et al.*, 1990) (Table 3).

However, for fish MTS, the trout MT-A and MT-B sequences show similar sequence identity (85%) when compared with the flounder MT sequence. These two trout MTs show 97% sequence identity between each other. Since tetraploid evolution occurred in salmonids whereas all flat fish of the order *Heterosomata* are diploid (Ohno, 1970), the trout MTs probably evolved from their ancestral form via a genome duplication event.

3.3.6. Conclusion

Several recombinant clones containing cDNA inserts made complementary to MT mRNA were selected from a cDNA library by oligonucleotide probes derived from the amino-terminal amino acid sequence of winter flounder MT. Two of the cDNA inserts were sequenced, giving the structure of the entire coding region of 183 nucleotides for flounder MT, together with 5' and 3' untranslated regions. The complete amino acid sequence of flounder MT was inferred from the cDNAs. It has 20 cysteine residues in a 60 amino acid polypeptide with a characteristic Class I MT primary structure. It shows 60% amino acid sequence identity with mammalian MTs and 85% identity with the trout MTs.

CHAPTER FOUR

ANALYSIS OF METALLOTHIONEIN GENE EXPRESSION IN WINTER FLOUNDER TISSUES

4.1. INTRODUCTION

To gain a full understanding of MT gene expression in the winter flounder, sensitive and specific methods must be available for its determination. The cloning of MT cDNA (described in Chapter Three) opened the way for the development of an assay for the analysis of MT gene expression. In this study the subcloning of MT cDNA into a dual promoter RNA plasmid and the production of antisense MT RNA (cRNA) are described. The cRNA probe was used to examine the relative amounts of MT mRNA in different tissues of the winter flounder following administration of two different types of inducers. The probe was also used to determine: (1) the ability of various metal ions to induce MT mRNA in the liver (2) the time course required for a single dose of Cd^{2+} to induce MT mRNA in the liver, and (3) the dose of Cd^{2+} which resulted in a maximum level of induction in the liver in a given time period.

4.1.1. RNA analysis

An RNA assay is essential for the analysis of regulation of gene expression. For MT, it has been shown that the steady state concentration of MT mRNA increases when an organism is exposed to heavy metal ions and that this increase is the result of the activation of

transcription (Durnam and Palmiter, 1981; Hamer, 1986). The results presented previously indicate that the administration of Cd^{2+} induces the accumulation of MT mRNA in the liver and the kidney of winter flounder. I have also shown that it is possible to monitor the increase in the amount of MT mRNA in the winter flounder exposed to Cd^{2+} by isolating total mRNA and analyzing the translation products produced in a cell free translation system (Chapter Two). However, this is a lengthy procedure which does not lend itself to routine analysis of RNA levels in biological tissues.

Routine analysis of gene expression is commonly performed by dot blot hybridization or Northern blot hybridization. Dot blot hybridization can be non-specific and the hybridization conditions have to be determined empirically to increase the signal to noise ratio. Northern blot analysis is more precise, but it requires electrophoresis and further transfer of RNA samples to a solid support. Recently, it has been established that the most sensitive and quantifiable assay of specific transcripts is through the use of a cRNA probe (antisense RNA probe) and solution hybridization (Nobrega *et al.*, 1983; Krieg and Melton, 1987; Lee and Costlow, 1987; Firestein *et al.*, 1987). Solution hybridization was applied by Durnam and Palmiter (1983) to measure MT mRNA transcripts using a specific, single stranded cDNA probe. However, RNA probes

have been shown to be more effective for RNA detection than DNA probes labelled by nick translation or random priming (Krieg and Melton, 1987). The advantages of solution hybridization are its sensitivity (requires less RNA input), accuracy (minimizes sample manipulation in order to avoid RNA degradation) and specificity (i.e. DNA does not interfere, and nuclease digestion is used to remove non-specifically bound probe).

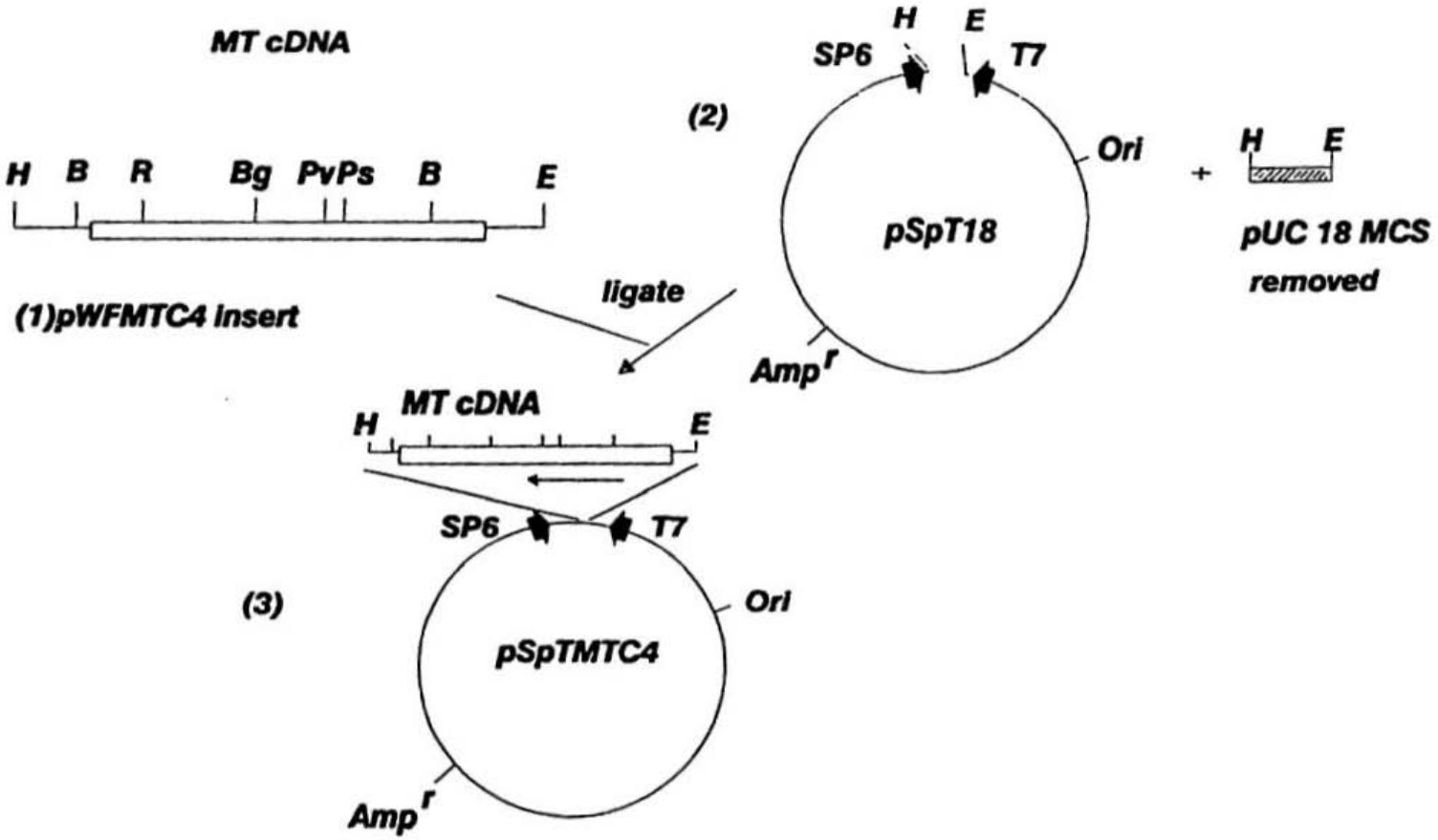
4.2. MATERIALS AND METHODS

4.2.1. Subcloning and *in vitro* transcription of cRNA

In order to produce antisense MT RNA (cRNA), the MT cDNA (pWFMTC4 insert) (Chapter Three, Fig. 9) was subcloned into a dual promoter (SP6 and T7) RNA probe plasmid, pSpT18 (Pharmacia). The MT cDNA insert was released from pWFMTC4 by digestion with *Hind* III and *Eco* RI restriction enzymes which cut only at the multiple cloning site in pWFMTC4 (Fig. 11 (1)). The insert, with the remaining pUC13 multiple cloning sites, was further purified on PAGE and then ligated to *Hind* III and *Eco* RI digested and purified pSpT18 (Fig. 11. (2)). This reconstructed plasmid is called pSpTMTC4 (Fig. 11. (3)). As a result, pSpTMTC4 has the pWFMTC4 insert at the *Sma* I site together with the remaining pUC13 multiple cloning sites between the SP6 and

Figure 11. Illustration of flounder MT RNA probe plasmid construction.

pWFMTC4 insert (1) was purified by PAGE and then ligated (2) into the *Hind* III/*Eco* RI site of the plasmid pSpT18 (Pharmacia). This pWFMTC4 insert (1) was inserted 3' to 5' with respect to the SP6 bacteriophage promoter (3). As a result, the pUC13 multiple cloning site (MCS) with the pWFMTC4 insert (still at the *Sma* I site) replaced the pUC18 multiple cloning site (MCS) in the pSpT18 plasmid (2). The MT RNA probe plasmid is called pSpTMTC4. Abbreviations used for restriction sites are: B, *Bam* HI; Bg, *Bgl* I; E, *Eco* RI; H, *Hind* III; Ps, *Pst* I; Pv, *Pvu* II; and R, *Rsa* I.


MT cDNA
H B R Bg PvPs B E
(1)pWFMTC4 insert
(2)
H E
SP6
T7
Ori
pSpT18
Amp r
+
H E
pUC 18 MCS
removed
ligate
MT cDNA
H
E
SP6
T7
(3)
Ori
pSpTMTC4
Amp r

T7 promoters (Fig. 11. (3)).

In vitro transcription was carried out according to Krieg and Melton (1987) using SP6 RNA polymerase (Pharmacia), and [α ^{32}P]-GTP (New England Nuclear). In general, ^{32}P-labelled MT cRNA (2 x 10^8 cpm/ μg) was synthesized from the SP6 promoter of pSpTMTC4 after *Eco* RI digestion (Fig. 11.). Following DNase I (Pharmacia) digestion, and phenol-chloroform extraction, the ^{32}P-labelled MT cRNA was purified from unincorporated nucleotides by ethanol precipitation. ^{32}P-labelled MT cRNA was further inspected after electrophoresis through a 1.8% agarose gel in TEA buffer, or an 8% polyacrylamide/ 7 M urea gel (in TBE buffer) by autoradiography (Maniatis *et al.*, 1982).

4.2.2. Ribonuclease (RNase) protection assay

The RNase protection assay was carried out according to Krieg and Melton (1987). Forty μg of total RNA or one μg of poly(A)$^+$ RNA purified from liver or kidney of saline-injected or Cd^{2+}- injected winter flounder was used for the RNase protection assay.

The hybridization was performed in a 30 μL reaction containing 80% formamide, 0.4 M NaCl, 1 mM EDTA, 50 mM Tris HCl pH 7.0 and excess purified ^{32}P-labelled MT cRNA probe (50 ng, 5 x 10^6 cpm)(4.2.1.). The samples were heated at

85°C for 5 min, slowly cooled down to 48°C (approximately 2 h) and then kept at 48°C for 18-20 h. After hybridization, 360 µL RNase A buffer was added to the samples and digestion was carried out for 1 h at 37°C. The RNase A buffer contained 50 µg/mL RNase A (Pharmacia), 300 mM NaCl, 10 mM Tris HCl pH 7.5, and 5 mM EDTA. Five µL of proteinase K (Boehringer Mannheim) (10 mg/mL) and 10 µL of 20% SDS was then added and the incubation was continued for 30 min at 37°C. Finally, the samples were phenol-chloroform extracted, ethanol precipitated with 5 µg of yeast tRNA (Pharmacia) as a carrier, and then resuspended in a formamide dye solution. The samples were loaded on an 8% polyacrylamide/ 7 M urea gel (Maniatis *et al.*, 1982). Size markers were *Hpa* II digested pBR322 plasmid fragments end-labelled with [α ^{32}P]-dCTP (Amersham) using PolIK (Pharmacia).

4.2.3. Winter flounder experimental procedures

Winter flounder were maintained in laboratory aquaria (200-500 L.) supplied with continuously flowing seawater under seasonally ambient conditions (Fletcher, 1977). All injections were done intraperitoneally. Analytical grade chemicals were purchased from Fisher Scientific Company and the metal stock solutions were prepared freshly in 1.1 % NaCl with distilled, deionized and millipore filtered water.

4.2.3.1. Experiment I: Tissue specificity of MT mRNA induction following administrations of Cd^{2+} or dexamethasone.

Two different types of inducers were examined in this test. $CdCl_2$ was dissolved in saline (1.1% NaCl), and dexamethasone (Sigma), a synthetic glucocorticoid, was dissolved in peanut oil (10 mg/ml). Cd^{2+} was injected as described in Chapter Two (2.2.2.): at Day 1, 0.4 mg/kg; Day 2, 0.6 mg/kg; Day 3, 1.0 mg/kg; Day 4, 2.0 mg/kg body weight. Tissues were removed on Day 5. Dexamethasone was injected at a daily dosage of 2.5 mg/kg for 4 days and the tissues were removed on Day 5. Water temperature was at 2°C (January) in these experiments.

4.2.3.2. Experiment II: Induction of hepatic MT mRNA after administrations of various metal ions (i.e. Cu^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+} and Hg^{2+})

All metals were injected as their chlorides. Zn^{2+} was injected at Day 1, 1 mg/kg; Day 2, 2 mg/kg; Day 3, 4 mg/kg; Day 4, 8 mg/kg body weight. Tissues were removed on Day 5. Cu^{2+}, Cd^{2+}, Pb^{2+}, and Hg^{2+} were injected using the procedure and dosages as described above (4.2.3.1.). This experiment was done in October when the average water temperature was 10°C.

4.2.3.3. Experiment III: Time course study

Cd^{2+} was injected as a single dose (1 mg Cd^{2+}/kg body weight) into each of four flounder. Fish were killed at 12 h, 24 h, 48 h or 96 h following the injection and the livers were assayed for MT mRNA. This experiment was carried out in January when the average water temperature was 1°C.

4.2.3.4. Experiment IV: Dose response test

Cd^{2+} (as the chloride) was injected into three separate flounder using different single doses (0.11, 0.56 or 1.1 mg Cd^{2+}/kg body weight) into each flounder. The fish were killed four days following the injection and the livers assayed for MT mRNA. This experiment was carried out in September when the average water temperature was 12°C.

4.2.4. RNA extraction

Total RNA was purified using the rapid acid guanidine thiocyanate phenol chloroform method of Chomczynski and Sacchi (1987). In brief, 100-200 mg of frozen tissue was homogenized in a Duall tissue grinder (Mandel Scientific) with 1 mL of extraction buffer (4 M guanidine isothiocyanate (Sigma), 25 mM sodium citrate pH 7, 0.5% sarcosyl (Sigma), 0.1 M β-mercaptoethanol), extracted with phenol:chloroform

(5:1), and precipitated twice with isopropanol (Anachemia, HPLC grade). RNA was resuspended in the extraction buffer for the second isopropanol precipitation. Finally, the samples were dissolved in 200 µL of 0.5% SDS (prepared with DEPC treated H_2O), heated at 65°C for 10 min and then stored at -70°C. The integrity of RNA was judged by inspection after electrophoresis in a 1.8% agarose gel containing 2.2 M formaldehyde and EtBr (Davis *et al.*, 1986). Forty µg of total RNA (determined by EtBr staining) was used for each RNase protection assay as described above (4.2.2.).

4.2.5. Northern blot analysis

Hepatic total RNA (40 µg) was transferred to a Nylon membrane (Hybond N, Amersham) in 20X SSPE after electrophoresis in a 1.8% agarose gel containing 2.2 M formaldehyde and EtBr (Davis *et al.*, 1986). Pre-hybridization was carried out at 60°C for 13 h, in a solution containing 50% formamide, 5 X SSC, 5 X Denhardt's, 500 µg/mL of yeast tRNA, 100 µg/mL of denatured DNA, and 1% SDS, without MT cRNA probe. Hybridization was performed at 68°C for 22 h in the same solution with 1.2 X 10^6 cpm/mL of ^{32}P-labelled MT cRNA. The post-hybridization washing procedure was as follows: (1) 2 X SSC, 65°C for 30 min, (2) 0.1 X SSC, 65°C for 30 min, (3) 1 X SSC with 100 µg/ mL of RNase A, room temperature for 30 min, and (4) 0.1 X SSC,

65°C for 10 min, twice. Finally, the membrane was exposed to Kodak X-Omat AR film at -70°C for 12 h between intensifying screens (Dupont Cronex).

4.2.6. Densitometry

Densitometric scanning of the autoradiograms from RNase protection assays and Northern blot analyses was performed using a GS 300 Dual Speed Scanning Densitometer (Hoefer Scientific Instruments). The values obtained are expressed as fold induction over saline- treated samples.

4.3. RESULTS AND DISCUSSION

4.3.1. Construction of the RNA probe plasmid

Figure 11 illustrates the construction of pSpTMTC4 (MT RNA probe) plasmid. Since the MT cDNA insert is in a different orientation relative to the plasmid, *Eco* RI digested pSpTMTC4 contains the pSpTMTC4 insert 5' linked to the SP6 promoter and thus can be transcribed by SP6 polymerase to produce winter flounder MT cRNA (antisense RNA probe).

4.3.2. RNase protection assay of MT mRNA levels in winter flounder tissues

The RNase protection assay (RNase mapping assay) utilizes RNase to specifically hydrolyze single strand RNA after solution hybridization with a specific complementary probe. Since the antisense MT cRNA probe is complementary to MT mRNA, the double stranded RNA-RNA hybrid formed after solution hybridization becomes resistant to RNase digestion.

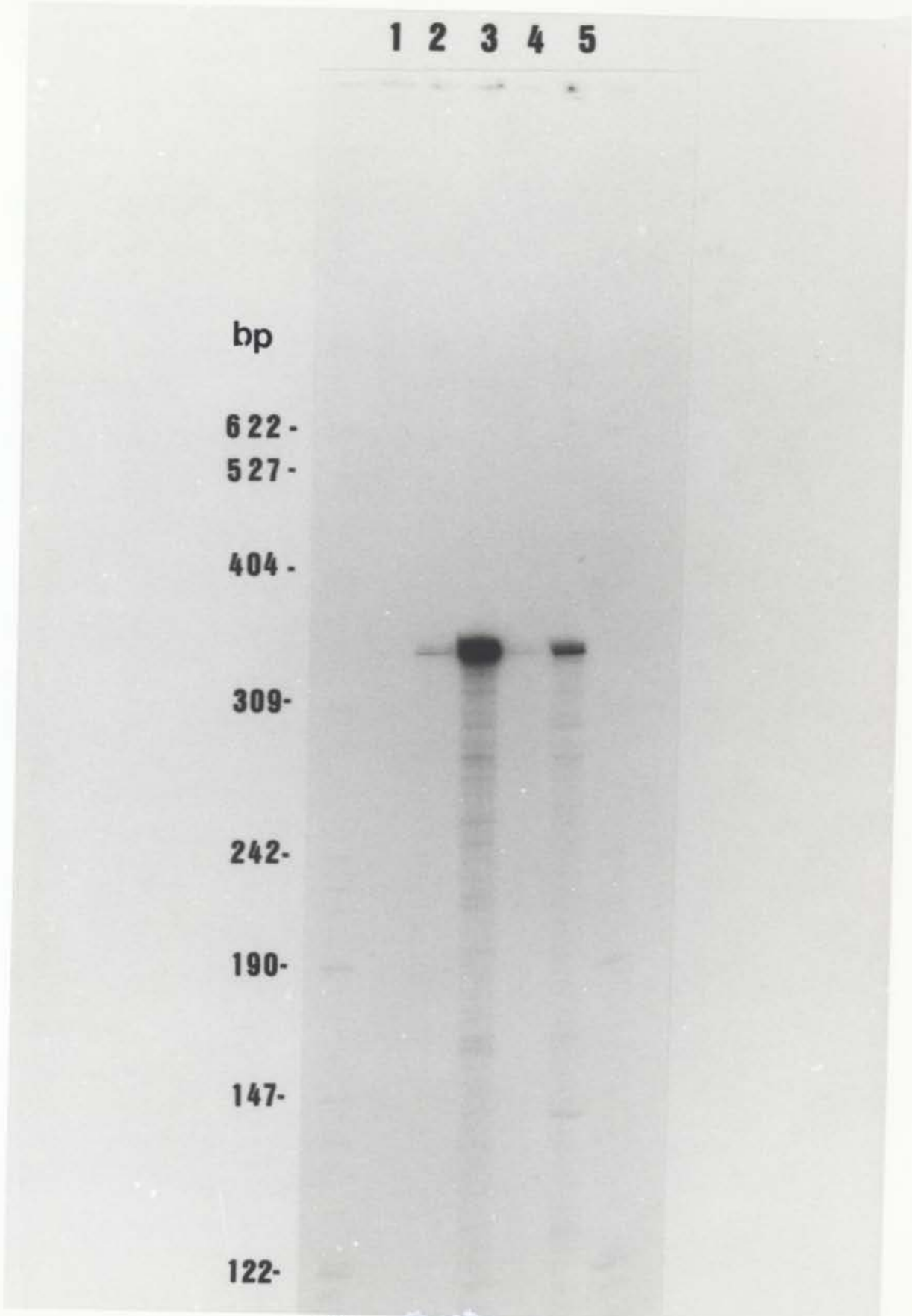
Figure 12 shows an autoradiogram of an RNase protection assay using the [α ^{32}P]-GTP labelled MT cRNA probe. The protected RNA was abundant in the poly(A)$^+$ RNA samples from Cd^{2+}- treated winter flounder tissues and was approximately 340 bases long (same size as the pWFMTC4 insert, without the multiple cloning sites).

The RNase mapping analysis technique was used to detect the presence of MT mRNA in three different experiments (I, II and III), and Northern blot analysis was used in the fourth experiment (4.3.3.).

In the first experiment, two different types of known MT inducers, Cd^{2+} and a glucocorticoid hormone (dexamethasone), were examined. The RNase protection assay showed that Cd^{2+}- induced the production of MT mRNA in all of the tissues examined except brain, where the level of MT mRNA appears to be relatively high in the control (saline-

Figure 12. Ribonuclease protection assay of poly(A)$^+$ RNA samples.

The protected RNA samples were separated on an 8% polyacrylamide gel (7 M Urea) and visualized by autoradiography (8 h exposure at room temperature). Sample 1 is a control, without addition of any RNA except ^{32}P-labelled MT cRNA; the others are 1 μg of poly(A)$^+$ RNA purified from liver of saline- (2) and Cd^{2+}- (3) injected flounder. Lanes 4 and 5 contain 1 μg of poly(A)$^+$ RNA purified from kidney of flounder treated with saline and Cd^{2+} respectively. Injections and RNA purification were done as described in Chapter Two. In brief, tissues were removed 24 h after the final injection of Cd^{2+} in a multiple injection scheme (Day 1, 0.4 mg/ kg; Day 2, 0.6 mg/ kg; Day 3, 1.0 mg/ kg; Day 4, 2.0 mg/ kg). Poly(A)$^+$ RNA samples were prepared from oligo-(dT)-cellulose chromatography of total RNA. *Hpa* II digested pBR322, end labelled with [α ^{32}P]-dCTP was run as size markers. The sizes of the fragments (in bp) are indicated on the figure.


1 2 3 4 5
bp
622-
527-
404-
309-
242-
190-
147-
122-

injected) flounder (Fig. 13A). MT mRNA is also found in normal mouse brain tissue and similar to the results observed for the flounder, the levels do not increase tremendously (as they do in liver) following Cd^{2+} administration (Duram and Palmiter, 1981).

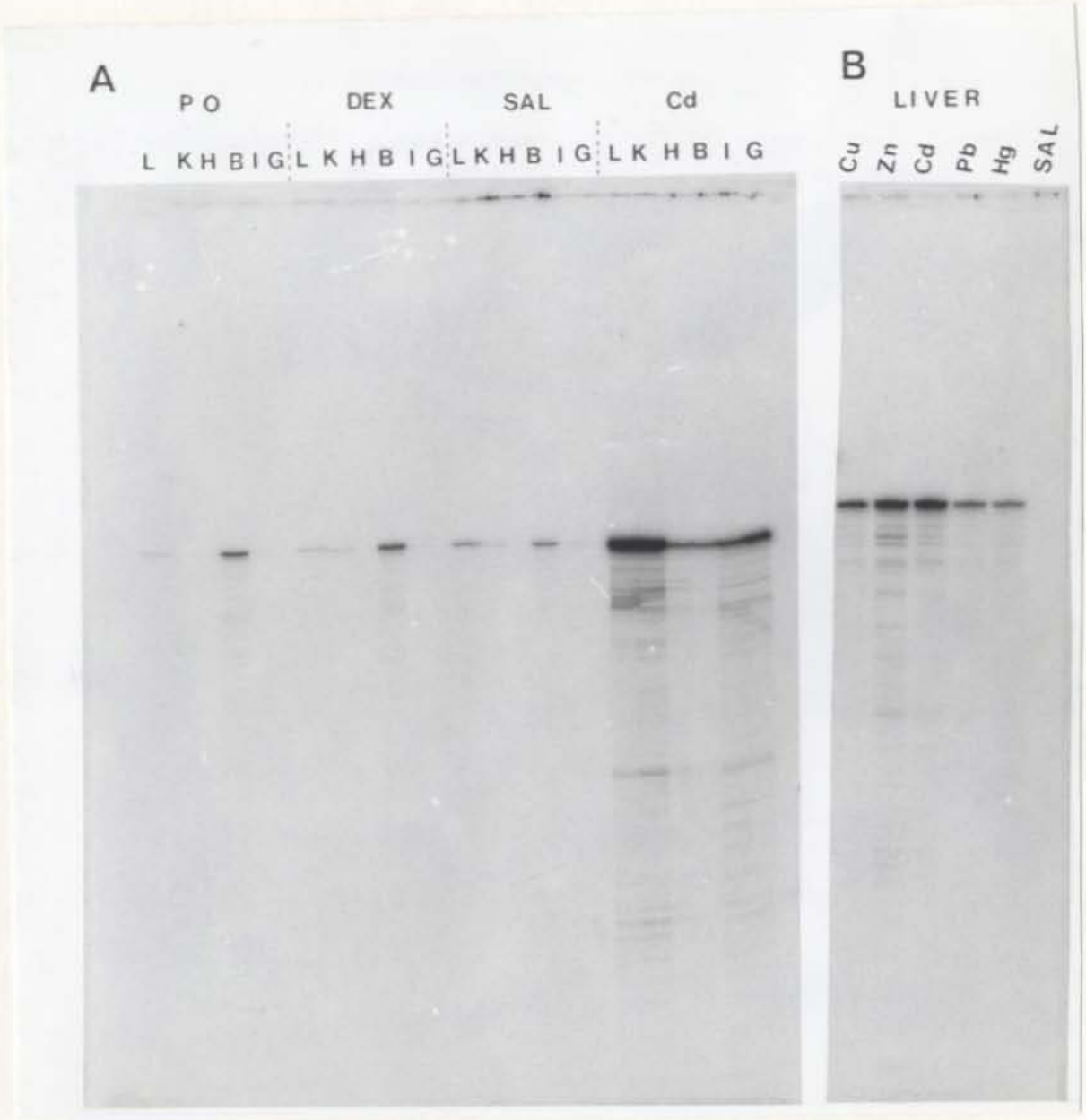
According to the densitometric analysis, the induction of MT mRNA in the Cd^{2+}- versus saline- injected flounder was 1-, 11-, 18- and 25- fold in the brain, liver, heart and gill filaments, respectively. While the background level of MT mRNA in the gill filaments was minimal, much higher levels were observed in the liver. This may be indicative of an involvement of MT in normal metal homeostasis in the flounder liver. Using MT cRNA probes Zafarullah *et al.* (1989) detected significant levels of MT mRNA in several tissues (i.e. brain, liver, kidney, spleen, gills, gut, testis, ovaries and eggs) of rainbow trout maintained in clean water. This apparent constitutive expression of MT gene(s) in several tissues of the rainbow trout is very suggestive of an involvement of MT in normal metal homeostasis in these fish. The presence of endogenous hepatic MT mRNA levels is of particular interest since it has been suggested that the MT levels are related to vitellogenesis and may regulate the hepatic Zn^{2+}-distribution during the annual reproductive cycle in rainbow trout (Olsson *et al.*, 1987, 1989). A similar investigation done on plaice also reported a higher hepatic MT level prior

Figure 13. Autoradiograms of the ribonuclease protection assay.

Forty μg of total RNA was used for the RNase protection assay. After hybridization with the cRNA probe overnight, samples were digeated with RNase A and then purified by proteinase K digestion and phenol chloroform extraction. The samples were precipitated with 5 μg of yeast tRNA in ethanol and resuspended in a formamide dye solution. The protected RNA samples were separated on an 8% polyacrylamide gel (7 M urea) and visualized by autoradiography (20 h exposure at room temperature).

(A) MT mRNA levels in the tissue tested (L, liver; K, kidney; H, heart; B, brain; I, intestinal scrape; G, gill filament) following administration of dexamethasone (DEX), peanut oil (PO), saline (SAL) and Cd^{2+} (Cd).

(B) Hepatic MT mRNA levels in flounder after multiple injections of metal ions (as indicated) and saline (SAL).


A
PO
DEX
SAL
Cd
L K H B I G L K H B I G L K H B I G L K H B I G
B
LIVER
Cu
Zn
Cd
Pb
Hg
SAL

to development of the gonads; this increased MT level correlated with a higher Zn^{2+} concentration in the liver (Overnell *et al.*, 1987a).

In contrast to the results with Cd^{2+}, dexamethasone did not induce an increase in MT mRNA in any of the winter flounder tissues tested (Fig. 13.A). This agrees with the results of Overnell *et al.* (1987b) where they found no evidence for increased MT levels in plaice liver following dexamethasone treatment. Dexamethasone is an inducer of MT and MT mRNA in mammals and chicken, but the induced levels are not as high as those following metal administration (Hamer, 1986; Dunn *et al.*, 1987; Wei and Andrews, 1988). Some of the cloned mammalian MT-I genes and the cloned trout MT-B gene do not respond to dexamethasone induction (Zafarullah *et al.*, 1988). Although no GRE homologous sequences have been identified in the trout MT gene promoters sequenced so far (Zafarullah *et al.*, 1988; Murphy *et al.*, 1990), the *cis*- acting elements might exist further upstream in the fish MT genes. Other factors, such as the lack of a glucocorticoid receptor or DNA methylation might be responsible for the failure of dexamethasone induction of fish MT genes. Recent studies also suggested that cortisol instead of dexamethasone could increase the MT mRNA levels (by two fold) in primary cultures of trout hepatocytes (Olsson *et al.*, 1990).

In the second experiment, Cu^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+} and Hg^{2+}

were tested for their ability to induce MT mRNA in the liver (Fig. 13.B). Since the time course of MT mRNA induction may not be the same for each metal ion, in this study multiple injections were used to give high levels of induction. Zafarullah *et al.* (1989) found when rainbow trout were subjected to single intraperitoneal injections of Cd^{2+}, Zn^{2+} or Cu^{2+}, the induction kinetics of MT mRNA differed between the metals injected and the tissue examined. All of the heavy metal ions known to induce MT in mammals were effective in inducing increased levels of MT mRNA in flounder liver (Fig. 13B).

Cd^{2+} appeared to be the most potent inducer (12.8 fold induction over saline-injected control) when compared with Cu^{2+} (9.5 fold), Pb^{2+} (7.5 fold) and Hg^{2+} (7.4 fold). However, since dose response curves were not generated, a quantitative comparison of the effectiveness of individual metal ions is not justified from this data. The different levels of MT mRNA observed in this study may be a result of differential uptake of the individual metal ions by the liver. In the future, regulation of MT mRNA levels by the different metal ions (Cu^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+}, and Hg^{2+}) should be done in the flounder controlling for such variables as age, sex, season, and time course.

Differential regulation of MT mRNA levels by Cu^{2+}, Zn^{2+}, and Cd^{2+} has also been reported for the trout system (Zafarullah *et al.*, 1988, 1989; Misra *et al.*, 1989).

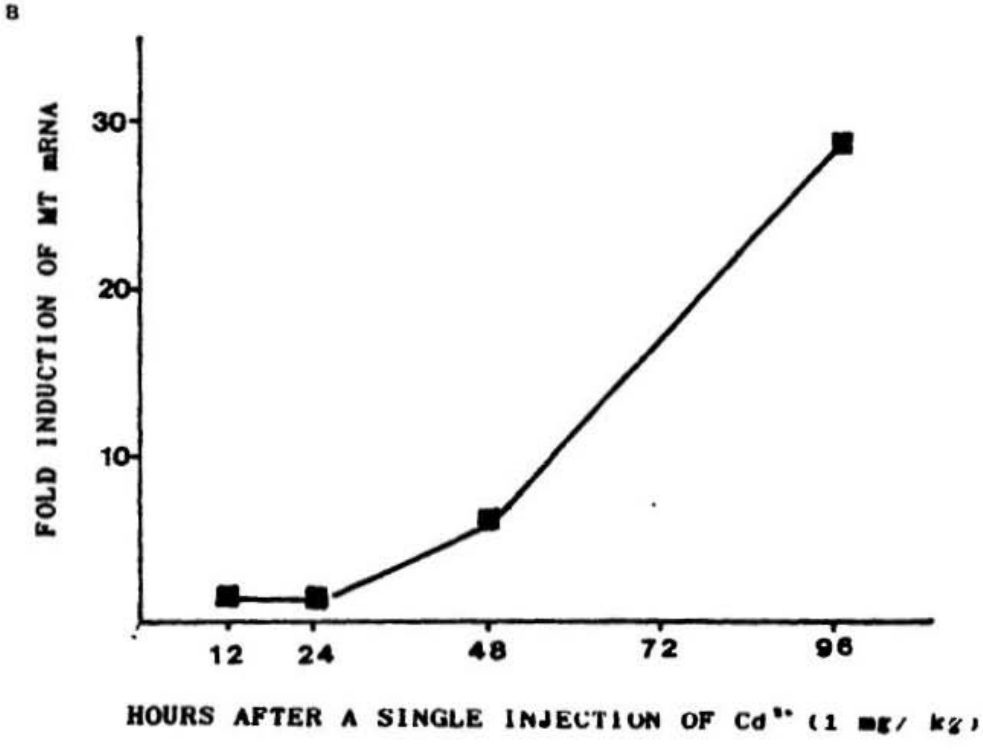
However, the time course of MT mRNA induction and de-induction following administrations of different doses of metal ions might also vary considerably. For example, Durnam and Palmiter (1981) reported that Cu^{2+}, Zn^{2+}, Cd^{2+}, and Hg^{2+} differed considerably in their ability to induce mouse MT-I (mMT-I) mRNA in the liver when measured 3 h after metal administration. Both Cu^{2+} and Hg^{2+} administration gave a lower mMT-I mRNA induction compared with Cd^{2+} and Zn^{2+} when administered at 0.5 mg/kg body weight, whereas higher concentrations (5 mg/kg body weight) resulted in similar levels of induction for Cd^{2+}, Cu^{2+} and Zn^{2+}, but not for Hg^{2+}. Further analysis on the time course of mMT-I induction in response to Cd^{2+} and Hg^{2+} showed that the mMT-I mRNA had a similar maximum level of expression at 4 h after the injection of Cd^{2+} (5 mg/kg body weight) and at 24 h after the injection of Hg^{2+} (5 mg/kg body weight) (Durnam and Palmiter, 1981).

The third experiment studied the timing of MT mRNA accumulation in the liver of flounder following a single injection of Cd^{2+} (1 mg Cd^{2+}/kg body weight). Hepatic MT mRNA started to rise at 96 h after the injection of Cd^{2+} (Fig. 14). This result is in general agreement with those of George and Young (1986) who reported that the increase in plaice MT levels occurred four to six days after Cd^{2+}-injection (1 mg Cd^{2+}/kg body weight, at a water temperature

Figure 14. Time course study of hepatic MT mRNA after a single injection of Cd^{2+}.

(A) Autoradiogram of the ribonuclease assay was done as described in Figure 13. (B) Fold induction of MT mRNA levels was measured using normal fish liver RNA (time= 0 h) as background.


A
0
12
24
48
96h


B
FOLD INDUCTION OF MT mRNA
30
20
10
12
24
48
72
96
HOURS AFTER A SINGLE INJECTION OF Cd++ (1 mg/ kg)

of 10°C).

The slow response of MT mRNA induction observed in flounder is in contrast to the mammalian systems and the rainbow trout systems (Durnam and Palmiter, 1981, 1984; Zafarullah *et al.*, 1989, 1990) which have a faster time course for MT mRNA induction. Such comparisons suggest that temperature might play an important role in the time course of hepatic MT mRNA induction as those experiments done on flounder, trout and mammals were at 1°C, 8-10°C and 37°C respectively. For example, Durnam and Palmiter (1981) found a significant induction of MT-I mRNA in mouse liver 6 h following Cd^{2+} administration, and Zafarullah *et al.* (1989) recently reported a fairly rapid induction (12 h) in rainbow trout liver following Cd^{2+}- injection. At low water temperatures (1°C), the flounder liver may exhibit a slower metabolic rate which could result in a direct reduction in the rate of MT gene transcription or have an indirect effect by reducing the rate of metal ions entering the cells.

However, at a water temperature of 10°C, plaice were found to start producing MT in four to six days after Cd^{2+} injection (1 mg Cd^{2+}/kg body weight) (George and Young, 1986). The more rapid rate of induction in trout compared to that in flounder and plaice may be related to the higher dose of Cd^{2+} injected into the trout (7.3- fold higher than that for plaice and flounder). Alternatively, this difference could be due to a species- specific response to

Cd^{2+}- induction. In this regard, it should be noted that plaice and flounder belong to the Family *Pleuronectidae*.

In conclusion, the timing of metal ion induction of MT mRNA could be affected by temperature in relation to metabolic rate, by the dosage of metal ions administered, or by the specific response of a particular species.

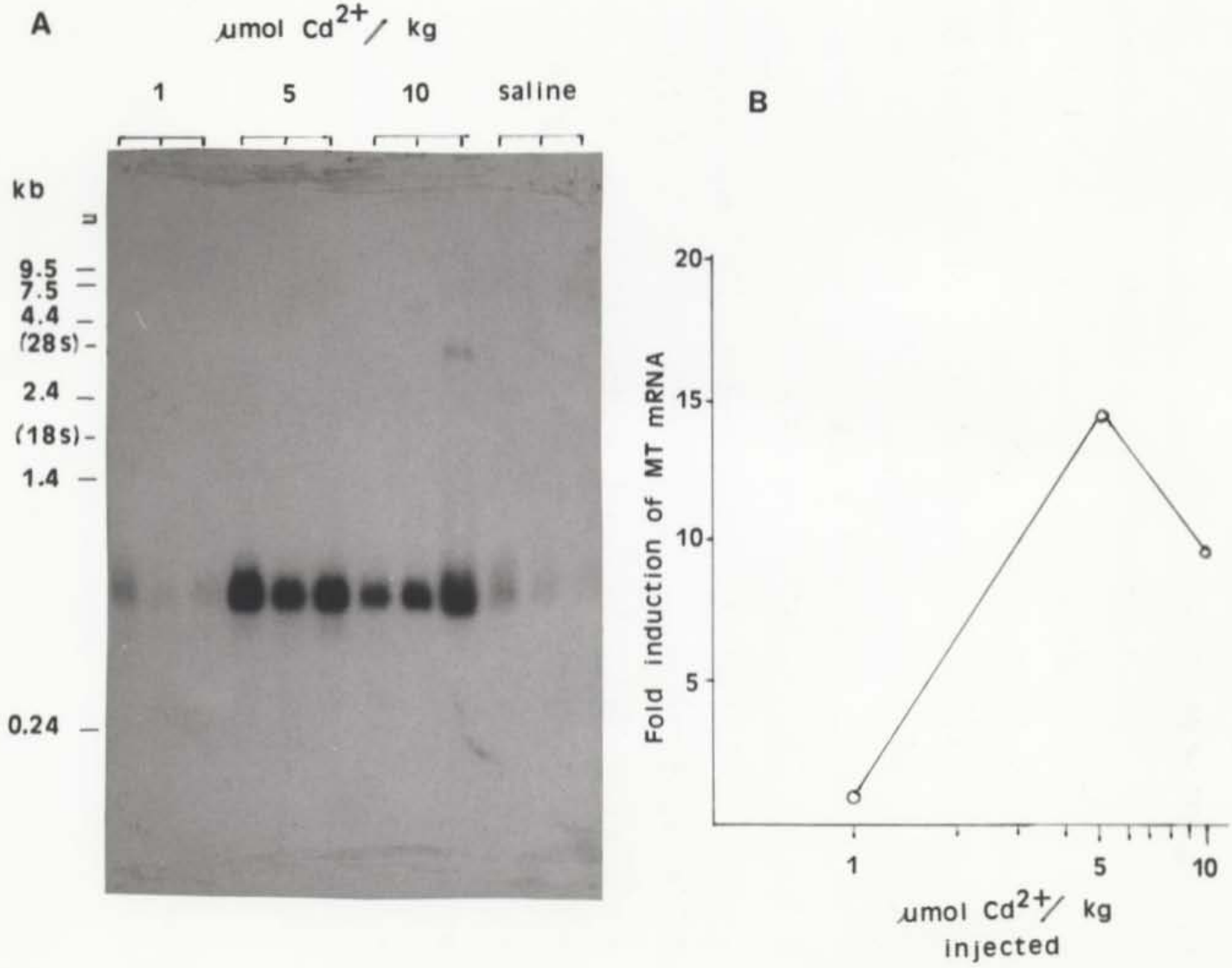
4.3.3. Northern blot analysis of the dose response tests

Based on the time course study, the fish were injected with different single doses of Cd^{2+} and their livers removed for MT mRNA measurement 96 h after the injection. Northern blot analysis using the MT cRNA probe showed that there was a relationship between the dose of Cd^{2+} injected into the flounder and the level of hepatic MT mRNA produced. A single injection of 5 μmol Cd^{2+} (0.56 mg Cd^{2+}/kg body weight) resulted in the highest level of MT mRNA induction (15.5-fold over the saline control), whereas a single injection of 10 μmol Cd^{2+} (1.1 mg Cd^{2+}/kg body weight) resulted in a lower level than that observed for the 5 μmol injection (Fig. 15). This result is in agreement with George (1989) for plaice. The hepatic MT concentration increased to the highest level of 223 μg/g following an injection of 0.5 mg Cd^{2+}/kg body weight (assayed at day six after the injection). A lower hepatic MT level (109 μg/g) was observed in plaice after the injection of 1 mg Cd^{2+}/kg body weight in the same set of

Figure 15. Northern blot analysis of MT mRNA levels in liver of flounder which had been injected with different single doses of Cd^{2+}.

(A) Autoradiogram of the Hybond-N membrane after hybridization with the ^{32}P-labelled MT cRNA probes and post-hybridization washes. RNA markers (Gibco/BRL Canada) were marked after EtBr staining before transfer onto the Hybond-N membrane. Forty μg of total RNA was loaded into each lane of a 1.8% agarose gel containing formaldehyde and EtBr.

(B) Fold induction of hepatic MT mRNA levels following Cd^{2+}- injections. The intensities of the MT mRNA signals were determined by densitometric scanning of the autoradiogram (Panel A). The values obtained are expressed as fold induction over saline treated samples and represent the average of three individuals tested (as shown on Panel A).


A
µmol Cd2+/ kg
1
5
10
saline
kb
9.5
7.5
4.4
(28s)
2.4
(18s)
1.4
0.24
B
Fold induction of MT mRNA
20
15
10
5
1
5
10
µmol Cd2+/ kg
injected

experiments (George, 1989).

It was suggested that a general inhibition of protein synthesis occurred in the fish which received the highest dose of Cd^{2+} and thus gave a limited synthesis of MT (George, 1989). In the present study, the highest Cd^{2+} dose of 10 μmol/kg body weight might also have caused liver cell damage that brought the MT mRNA levels below peak values. However, it is also possible that a higher dose of Cd^{2+} induces MT mRNA faster than a lower dose. In a different study on rainbow trout MT-B gene expression, Zafarullah *et al.* (1989) observed that in trout which received a single dose of 12 mg $CdCl_2$ (or 7.3 mg Cd^{2+})/kg body weight, the hepatic MT-B mRNA level increased to its peak at 12 h and then declined at 26 h.

4.3.4. Conclusion

The present study provides qualitative analyses of MT gene expression in the tissues of the winter flounder. (1) Cd^{2+} induced MT mRNA in all of the tissues tested (liver, heart, kidney, intestinal scrape) except brain, where the normal level of MT mRNA was relatively high. (2) Dexamethasone did not increase MT mRNA in any of the tissues tested. (3) All of the metal ions tested (Cu^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+} and Hg^{2+}) were able to induce hepatic MT mRNA levels. (4) The time required to induce MT mRNA was 96 h

after a single injection of 1 mg Cd^{2+}/kg of body weight. (5) A single injection of 0.56 mg Cd^{2+}/kg body weight gave the highest MT mRNA induction as tested on day four (96 h) following injection.

CHAPTER FIVE

METALLOTHIONEIN GENE ORGANIZATION IN THE WINTER FLOUNDER

5.1. INTRODUCTION

Multiple isoforms of MT have been documented in mammals (Hamer, 1986 and see section 1.1.2.). The observed heterogeneity of mammalian MT is also reflected by the number of genes found, especially in humans, and may be related to differential regulation of gene expression (Karin and Richards, 1982 a,b; Richards *et al.*, 1984; Sadhu and Gedamu, 1988 and see Section 1.2.2.). In other vertebrates there are examples of a simpler MT genomic organization with only one or two genes detected. For example, during the course of this study, a single MT gene was reported for chicken (Wei and Andrews, 1988; Fernando and Andrews, 1989) and two MT genes were detected in rainbow trout (Bonham *et al.*, 1987; Zafarullah *et al.* 1988; Murphy *et al.*, 1990).

Since only one MT was identified in liver cytosol of Zn^{2+}- or Cd^{2+}- injected flounder by ion exchange chromatography (Chapter Two), and the two MT cDNAs encode the same MT mRNA sequence (Chapter Three), it can be hypothesized that there is a single copy MT gene in the winter flounder. However, there may be more than one copy of an identical MT gene in the winter flounder genome or more than one MT gene but only one of them expressed following induction. In the present study, as an initial step in examining the single gene hypothesis, the cloned MT cDNA (described in Chapter Three) and the cRNA probe

(described in Chapter Four) were used to look at the genomic organization of MT genes in the winter flounder.

5.2. MATERIALS AND METHODS

High molecular weight DNA was purified from flounder (Conception Bay, Newfoundland) liver using a guanidine isothiocyanate cesium chloride ultracentrifugation method (Davis *et al.*, 1986). Ten μg of genomic DNAs were completely digested with excess amounts of restriction enzymes for 4 h at 37°C.

After agarose gel (1%) electrophoresis (in TBE buffer), alkali transfer to Hybond-N nylon membrane (Amersham) was performed overnight in 1.5 M NaCl and 0.25 M NaOH (modified from Reed and Mann (1985) for Hybond-N membrane as described in a protocol from Amersham). DNA size markers were purchased from Boehringer Mannheim and located by EtBr staining before alkali transfer. After hybridization (50% formamide, 5X SSPE, 5X Denhardt's, 1% SDS, 100 μg/mL tRNA and denatured DNA with the ^{32}P-labelled MT cRNA probe, ≈ 2 x 10^8 cpm per μg, 10^7 cpm/mL, for 18 h at 45°C), the membrane was washed twice in 2X SSPE with 0.1% SDS at 48°C for 30 min each, twice at the same temperature and the same time in 1X SSPE with 0.1% SDS and twice at 65°C for 10 min in 0.1X SSPE. The membrane was exposed to Kodak X-Omat AR film at -70°C for 6 days between intensifying screens (Dupont

Cronex).

Hybridizations with less stringency were also performed with high molecular weight flounder testis DNA (provided by Dr. Pliny Hayes and Dr. Peter Davies, Queen's University). Restriction enzyme digestions, agarose gel electrophoresis and alkali transfer of DNA onto the Hybond-N (Amersham) nylon membranes were done as previously described. The *Bam* HI fragment of the pWFMTC69 cDNA clone (Fig. 9 and 11) was purified on NuSieve (FMC Bioproducts) agarose gel and used as a probe for hybridization. This cDNA fragment contains the complete 3' untranslated region and the MT coding region, and was labelled by Nick Translation with [α ^{32}P]-dATP using a BRL Nick Translation Kit (Gibco/BRL Canada). The labelled probes ($\approx$ 2 x 10^8 cpm/ μg) were purified by a spin-column of Sephadex G-50 (Maniatis *et al.*, 1982), heat denatured and were used at concentration of 3.7 x 10^6 cpm/ mL for hybridization.

After pre-hybridization (5X SSPE, 10x Denhardt's, 7% SDS, 20 mM sodium phosphate pH 7.2, 100 μg/mL denatured calf thymus DNA at 68°C for 4 to 6 h), hybridization was performed in a solution of the same content plus the ^{32}p-labelled probes at 68°C for 22 h. Post-hybridization washes were as follows: (1) twice in 2X SSC at room temperature for 15 min each; (2) twice in 2X SSC, 0.1% SDS at 68°C for 15 min each; and (3) twice in 0.5X SSC, 0.1% SDS at 68°C for 10 min each. The membranes were exposed to Kodak X-Omat AR

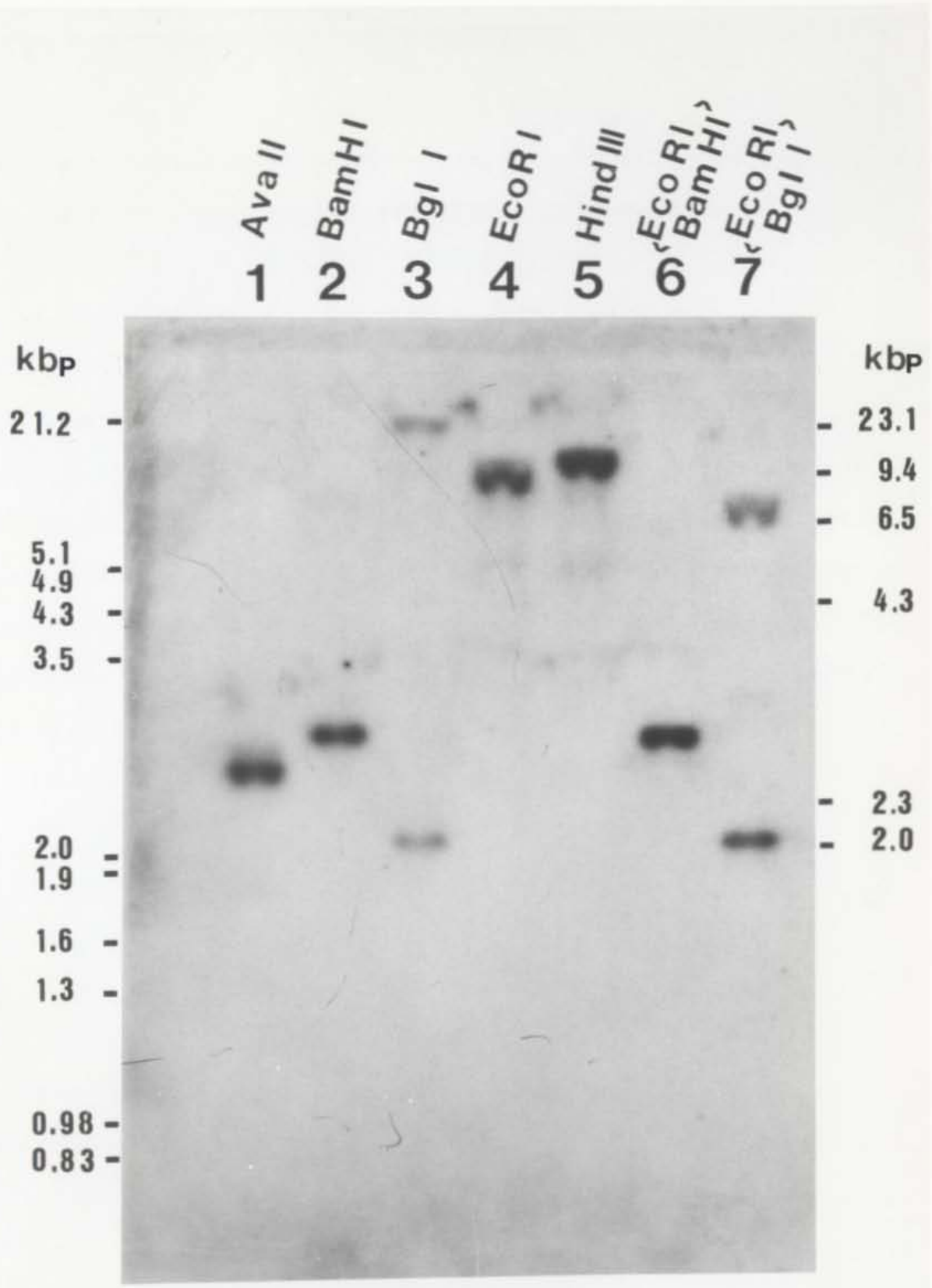
film at -70°C for 3 or 6 days between intensifying screens (Dupont Cronex).

5.3. RESULTS AND DISCUSSION

Figure 16 shows the autoradiogram of a Southern blot analysis of flounder genomic DNA probed with MT cRNA. The hybridization and washing conditions were stringent (0.1X SSPE at 65°C), therefore the hybridization signals should be very specific. Cleavage of the flounder DNA with *Ava* II, *Bam* HI, *Eco* RI or *Hind* III each resulted in a single band on the Southern blot (Fig. 16). Of these four enzymes, *Ava* II, *Eco* RI and *Hind* III are known not to cut the flounder MT cDNA, and thus the single band pattern on the blot suggests that the MT gene is present as a single copy in the winter flounder genome. *Bam* HI does have a restriction site on the flounder MT cDNA very close to the 5'- end (Fig. 9). The second *Bam* HI fragment is probably not seen on the blot because the length of the overlap with the MT cRNA probe is too short for efficient hybridization. There is a single restriction site for *Bgl* I in the middle of the MT cDNA (Fig. 9) which accounts for two *Bgl* I fragments (22 kbp and 2 kbp) being detected on the blot. Double digestion with *Eco* RI and *Bgl* I yielded a smaller fragment of around 6.8 kbp and the 2 kbp *Bgl* I fragment. Digestion with both *Eco* RI and *Bam* HI showed that the *Bam* HI fragment was located

Figure 16. Southern blot analysis of flounder genomic DNA probed with a MT cRNA (pSpTMTC4).

Molecular size markers (kbp) are *Hind* III digested and *Eco* RI digested Lambda DNA standards visualized after EtBr staining of the gel before alkali transfer. The enzymes used for digestions are indicated. After hybridization (probed with ^{32}P-labelled MT cRNA probe) and washing, the membrane was exposed to Kodak X-Omat AR film at -70°C for six days between two intensifying screens (Dupont Cornex).

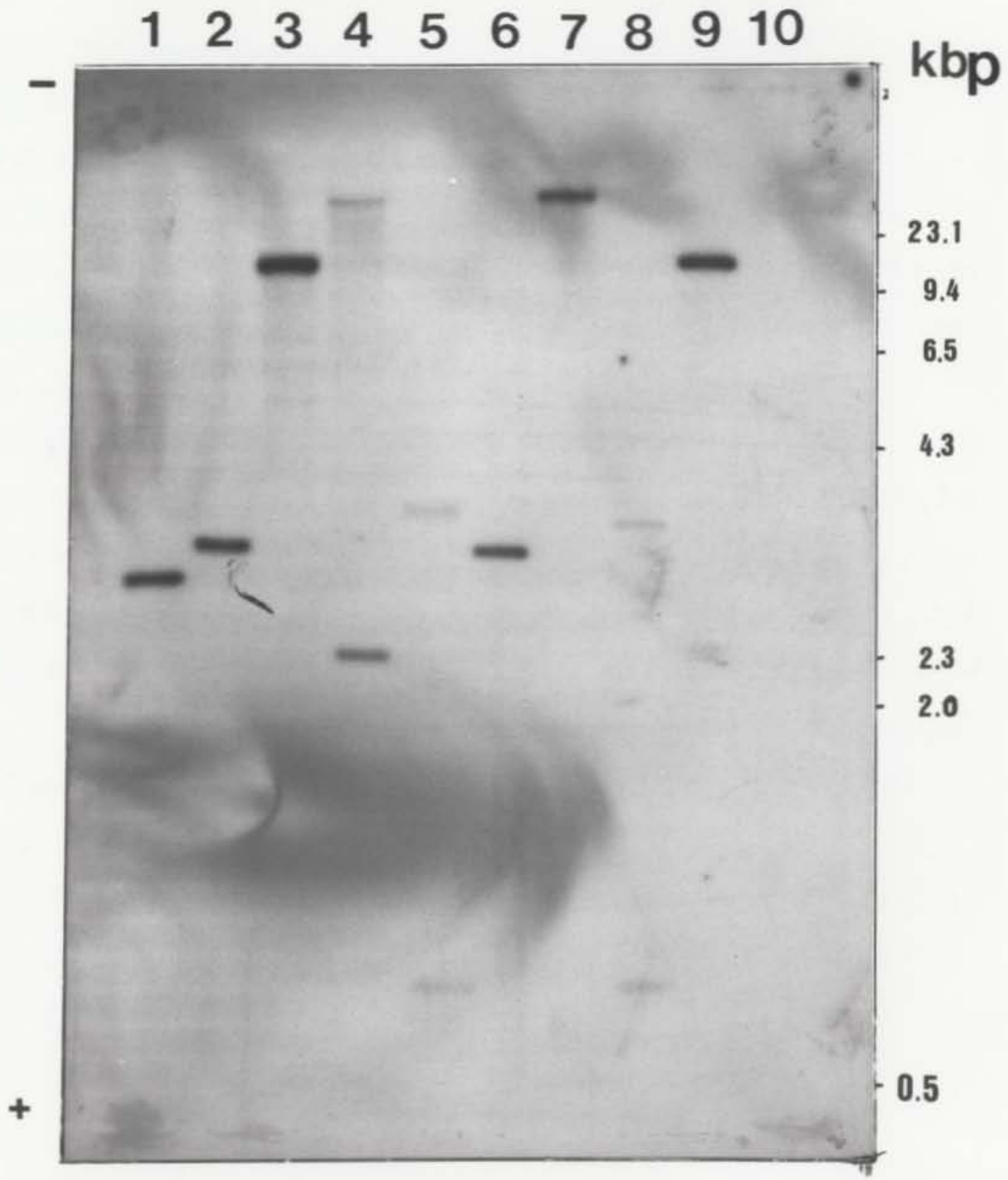

Ava II
BamHI
Bgl I
EcoR I
Hind III
EcoR I, BamHI
EcoR I, Bgl I
1
2
3
4
5
6
7
kbp
21.2
5.1
4.9
4.3
3.5
2.0
1.9
1.6
1.3
0.98
0.83
kbp
23.1
9.4
6.5
4.3
2.3
2.0

within the *Eco* RI fragment.

Similar results were obtained from a blot hybridized and washed under reduced stringency (aqueous hybridization and final washes in 0.5X SSC at 68°C). Figure 17 shows the autoradiogram of a Southern blot analysis of flounder genomic DNA probed with the nick-translated *Bam* HI fragment of pWFMTC69 which contains the entire coding region of flounder MT (Fig. 9). A similar pattern with single or double bands was observed for the flounder DNA purified from two different individuals. As restriction enzymes that did not cut the cDNA sequence (*Ava* II, *Hind* III, *Eco* RI) gave a single band pattern, and enzymes recognizing a digestion site within the coding sequence (*Bgl* I and *Pst* I) gave two bands on the Southern blot, it is very likely that only a single MT gene exists in the winter flounder genome. Enzymes (*Bgl* I and *Pst* I) that cut within the flounder MT gene generate two fragments with weaker signals than those produced by the intact gene. The two different sizes of the larger *Pst* I fragments (3.3 kbp and 3.5 kbp) observed in Figure 17 probably indicate a restriction fragment length polymorphism in the two different individuals examined. Restriction fragment length polymorphisms of antifreeze protein gene have also been observed in different individuals of flounder collected from Conception Bay, Newfoundland (Hayes *et al.*, 1991).

Figure 17. Southern blot analysis of flounder genomic DNA hybridized with a nick-translated MT cDNA probe.

Testis DNAs (5 μg) were purified from two different winter flounder, blotted onto nylon membrane for hybridization and the autoradiogram is shown here. Lanes 1 to 5 contained DNA samples from the same individual, the enzymes used for digestion are: Lane (1) *Ava* II, (2) *Bam* HI, (3) *Eco* RI, (4) *Bgl* I, (5) *Pst* I. Lanes 6 to 9 contained DNA samples from another individual, the enzymes used for digestion are: Lanes (6) *Bam* HI, (7) *Hind* III, (8) *Pst* I, and (9) *Eco* RI. Molecular size markers (kbp) are *Hind* III digested Lambda DNA standards (lane 10) visualized after EtBr staining of the gel before alkali transfer. After hybridization (probed with the nick-translated *Bam* HI fragment of pWFMTC69 MT cDNA) and a final wash in 0.5X SSC, 0.1% SDS at 68°C, the blot was exposed to Kodak X-Omat AR film at -70°C for three days between two intensifying screens (Dupont Cornex).


1 2 3 4 5 6 7 8 9 10
kbp
−
+
23.1
9.4
6.5
4.3
2.3
2.0
0.5

To date the only MT gene isolated from fish is the MT-B gene from rainbow trout (Zafarullah *et al.*, 1988). More recently, the nucleotide sequence of the trout MT-A promoter region has also been reported (Murphy *et al.*, 1990). While these trout MT genes appear to have several features in common with mammalian MT genes there are important differences. The rainbow trout genes have an AT-rich promoter compared with the highly GC-rich mammalian promoters and they lack binding sites for the transcription factor Sp1. These trout MT genes contain only one pair of MREs (metal regulatory element) proximal to their transcriptional start site (Zafarullah *et al.*, 1988; Murphy *et al.*, 1990).

In the future, it may be desirable to isolate the flounder MT gene using the cloned flounder MT cDNA as a probe. One important application of a cloned flounder MT gene is for transgenic fish experiments which require an inducible fish gene promoter. Isolation and characterization of the MT gene would also facilitate study of the mechanisms involved in MT expression in the flounder. How heavy metal ions activate MT gene transcription, or how they control the MT mRNA levels, are not understood yet. Experiments done on mammalian cells and trout cells suggest that the levels of MT mRNA and MTs are related to the ability of the metal ions to induce the MT mRNA synthesis at the level of MT gene transcription (Durnam and Palmiter,

1981, 1984; Hamer, 1986; Zafarullah *et al.*, 1988, 1989, 1990). Transfection experiments using fusion genes containing a MT gene promoter linked to a reporter gene would provide some insight in the regulation of flounder MT gene transcription in different types of cells under various conditions.

CHAPTER SIX

THESIS CONCLUSION

In the introduction to this thesis it was concluded that in order to gain a better understanding of the role that MT plays in metal metabolism in fish we must know more about MT gene regulation in fish. The winter flounder was chosen as the model marine fish to carry out this research because of the considerable body of literature available on its biology, physiology and biochemistry.

The complete amino acid sequence of the winter flounder MT was deduced from the cDNA sequence. It consists of 60 amino acids of which 20 are cysteine residues. Overall, the flounder MT sequence shows an approximate 85% identity with rainbow trout MT sequences and 50-62 % identity with chicken, pigeon and mammalian MT sequences.

Southern blot analyses of genomic DNA suggested that the MT gene is present as a single copy in the winter flounder genome. This result is consistent with the fact that only one MT was found in the liver cytosol and only one MT mRNA sequence was found in the MT cDNAs.

The basal MT mRNA levels in winter flounder tissues can be increased by the parenteral administration of heavy metal chlorides: Cd^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+}, and Hg^{2+}. This would suggest that the transcription of MT genes in fish can be induced by heavy metal ions.

Injections of the synthetic glucocorticoid, dexamethasone, did not induce increased MT mRNA levels in winter flounder tissues, suggesting the possibility that the

flounder MT gene lacks the *cis*-acting glucocorticoid regulatory elements present in mammalian MT systems.

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