Structure and Expression of Mammalian Metallothioneins

by

Adele Frances Holloway, B.Sc. Hons

Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

University of Tasmania (November, 1996)

Dept of Biochemistry

STATEMENTS

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and where duly acknowledged in the thesis, and to the best of my knowledge and belief contains no material previously published or written by another person except where due acknowledgement is made in the text of the thesis

SUMMARY

Metallothioneins (MTs) are metal binding proteins which have been implicated in a variety of physiological roles, including heavy metal detoxification, metal homeostasis and free radical scavenging. In both the human and the sheep there are multiple MT isoforms which may provide functional diversity. In both species differential regulation and expression patterns of MT isoforms have been reported. The work presented here investigates the expression and gene structure of MTs in these two mammalian species; firstly investigating MT in the developing sheep brain, and secondly studying a novel human MT isogene, *MT1L*. The underlying theme is to correlate the structure and expression profiles of specific MT isoforms with their proposed physiological functions.

Since the discovery of a novel MT in humans, MT-III, which is brain specific, much interest has centred on the expression and role of all MTs in the brain. In this thesis, the sheep was used as a model system in which to study the neural expression of MT at the RNA and protein level. MT-I and -II expression was found to be correlated with the development of glial cells, and limited solely to glial cells including foetal oligodendrocytes, which were previously not suspected to be involved in MT physiology. Distinctive shifts in the regional and cell-type expression of MT were observed during neural development, and these are discussed in terms of the functional role of MT.

The expression of MT-III, the brain specific isoform, is of current interest. This thesis reports the cloning of the gene encoding this isoform from a sheep brain cDNA library. Although homologous to MT-III isoforms from other species, the predicted sheep MT-III protein sequence contains several unique features, including the deletion of a highly conserved 3 amino acid sequence, and thus may have altered metal binding properties. Sheep MT-III mRNA was shown to follow the same regional and developmental expression patterns as MT-I and -II mRNA. This unique MT-III isoform may be useful to resolve pending questions about the role of MT-III in the brain, and the apparent neurotrophic actions of this isoform.

This work also investigates a novel human MT isogene, *MT1L*, which contains a TGA ("stop") codon midway through the transcribed region. By analogy to

previously characterised human MT genes, this codon interrupts the putative coding region of *MT1L*, suggesting the isogene is either not functional, expresses a truncated protein or potentially, produces a novel selenoprotein. In previous work, the author has shown that the gene has biological activity since it can confer resistance to cadmium to a transfected cell line. In this thesis, *MT1L* was shown to produce a full length mRNA transcript when cloned into expression vectors, and furthermore expression of *MT1L* mRNA was detected in a range of endocrine tissues and lymphocytes by northern blotting and RT-PCR. These findings confirm that *MT1L* has the potential to contribute to the repertoire of MT expression in the human.

ACKNOWLEDGMENTS

I wish to thank my supervisor Dr. Adrian West for his patience, unerring guidance and support, and for his encouragement and friendship over the past few years.

I'd like to thank Dr. Fiona Stennard for her friendship and support and for teaching me so much about molecular biology.

Thanks to Dr Inn Chuah, Dr Kate Dziegielewska, Dr. James Vickers, Dr. Jenny Penshow and Dr. Lee Weller for their ready advice and guidance. Thanks also to the members of the MBU for their support and friendship; particularly to Ros Thomson, Janine Miller, Jenny Penshow and Graeme McCormack for making the MBU a lab in which it was a pleasure to work.

Finally, a special thank you to Dale, my family and friends for their support and encouragement.

PUBLICATIONS

The following are publications arising from this thesis, to date:

Stennard, F.A., Holloway, A.F., Hamilton, J. and West, A.K. (1994) Chatacterisation of six additional human metallothionein genes. *Biochim. Biophys. Acta* **1218**, 357-365.

Holloway, A.F., Stennard, F.A., Dziegielewska, K.M., Weller, L. and West, A.K. Localisation and expression of metallothionein immunoreactivity in the developing sheep brain. *International Journal of Developmental Neuroscience in press.*

ABBREVIATIONS

bp	base pairs
BSA	bovine serum albumin
cDNA	complementary DNA
CuZnSOD	copper/zinc superoxidedismutase
DEPC	diethyl pyrocarbonate
DMF	N,N-dimethylformamide
DNA	deoxynucleic acid
DTT	dithiothreitol
EDTA	ethylene diamine tetra-acetic acid
FBS	foetal bovine serum
Gal-C	galactocerebroside
GFAP	glial fibrillary acidic protein
GSH	glutathione
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
IPTG	isopropyl-β-D-thiogalactopyranose
kb	kilobase pairs
LPS	lipopolysaccharide
mRNA	messenger ribonucleic acid
MOPS	3-[N-morpholino]propane-sulfonic acid
MT	metallothionein
O.D.	optical density
PBS	phosphate buffered saline
PCNA	proliferative cell nuclear antigen
PEG	polyethylene glycol
pfu	plaque forming unit
PVP	Polyvinylpyrrolidone
RNA	ribonucleic acid
RNAase	ribonuclease
RT-PCR	reverse transcriptase polymerase chain reaction
ROS	reactive oxygen species
SDS	sodium dodecyl sulphate
TEMED	N,N,N',N',-tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

NOMENCLATURE

MT nomenclature is not yet standardised despite recommendations by the Committee on the Nomenclature of Metallothionein appointed at the General Discussion Session of the Second International Meeting on Metallothionein and Other Low Molecular weight Metal-binding Proteins (see Fowler *et al.*, 1987).

This thesis discusses primarily the genes and cognate proteins from mouse, sheep and human and these will be distinguished when necessary by the prefixes m, s and h, respectively. The various isometallothioneins are referred to as MT-I, MT-II, MT-III and MT-IV.

Both the human and the sheep have complex isometallothionein families and therefore when specific subforms are referred to they are designated using the nomenclature established in the literature documenting these gene families. Therefore, the sheep MT subforms are designated in the style established by Peterson *et al.* (1988), i.e. the genes and cognate proteins are designated as MT-Ia, MT-Ib etc. The human isogenes are designated following human gene nomenclature guidelines in the style established by Karin *et al.* (1984a) and West *et al.* (1990), i.e. *hMT1A*, *hMT1B* etc and the cognate proteins as hMT1A, hMT1B etc.

TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION

1.1 Discovery

A metallothionein (MT) protein was first discovered in 1957 in equine kidney as a consequence of the search for components responsible for the natural accumulation of cadmium in mammalian tissues (Margoshes and Vallee, 1957). These proteins were subsequently found to bind both zinc and copper also (Pulido *et al.*, 1966; Kägi and Vallee, 1960).

1.2 Occurrence

MTs or MT-like proteins have been found throughout the animal kingdom, in plants, eukaryotic microorganisms and prokaryotes (Table 1.1, reviewed in Hamer, 1986). MTs are cytoplasmic proteins (e.g. Cherian *et al.*, 1981; Banerjee *et al.*, 1982). However, nuclear localisation of MT is well documented (e.g. Banerjee *et al.*, 1982) and polymeric copper containing MT has been shown to accumulate in lysosomes in the neonatal human liver also (Riordan and Richards, 1980).

1.3 Definition

MTs have been traditionally defined according to their structural features. They are characteristically low molecular weight proteins having a high content of heavy metals bound in thiolate clusters (Otvos and Armitage, 1980). The proteins have a high content of cysteine residues (22-33 mole %) and lack aromatic amino acids (Kägi *et al.*, 1974). MTs have been defined as polypeptides which resemble many of the properties of equine MT (Fowler *et al.*, 1987). There have been 3 classes of MT defined (Fowler *et al.*, 1987). Class I are polypeptides containing cysteine residues in positions closely related to equine MT and includes all mammalian MTs. Class II are polypeptides with the cysteine residues in positions only distantly related to equine MT and includes yeast MTs. Class III MTs are atypical, metal thiolate polypeptides including the phytoMTs and will not be discussed here further. This review will mainly focus on class I mammalian MTs.

human	frog
monkey	sea urchin
horse	crab
cow	trout
sheep	carp
pig	oyster
rabbit	mussel
hamster	wheat germ
rat	tobacco
mouse	tomato
seal	*Drosophila melanogaster*
chicken	*Caenorhabditis elegans*
pigeon	*Saccharomyces cerevisia*
duck	*Neurospora crassa*
dolphin	cyanobacteria

Table 1.1 The occurrence of MT and MT-like proteins.
This table lists some of the wide range of species which have be demonstrated to express MT or MT-like proteins. A more extensive list is presented in Hamer (1986).

1.4 Protein structure

Mammalian MTs can bind 7 equivalents of bivalent metal ions, e.g. cadmium and zinc (Kojima *et al.*, 1976), or 11-12 equivalents of univalent metal ions, e.g. copper (Nielson and Winge, 1984), exclusively through thiolate bonds to the cysteine residues. The binding is in a tetrahedral coordination for cadmium or zinc (Vašák *et al.*, 1981; Furey *et al.*, 1986) and a trigonal geometry for copper (Nielson *et al.*, 1985). The tertiary structure of the MT protein is dependent on metal binding, as the thionein exists as a random coil (Vašák *et al.*, 1980). The cysteine residues are distributed throughout the protein chain as cys-cys, cys-x-cys and cys-x-y-cys sequences, where x and y are any other amino acid (Kojima *et al.*, 1976). All cysteine residues occur in reduced form, coordinated to metals through mercaptide bonds, the native proteins containing no disulphide bonds (Bühler and Kägi, 1974; Kägi *et al.*, 1974). The proteins contain two distinct metal-thiolate clusters corresponding to two protein domains as demonstrated in Figure 1.1. Cluster A contains 11 cysteines, 4 zinc or cadmium ions, or 5-6 copper (CuI) ions and is located in the carboxyl-terminal α-domain. Cluster B consists of 9 cysteine residues, 3 cadmium or zinc ions or 6 copper (CuI) ions and is located in the amino-terminal β-domain (Winge and Miklossy, 1982; Boulanger *et al.*, 1983, Nielson and Winge, 1984). The α- and β-domains can be cleaved with subtisilin (Winge and Miklossy, 1982) and these domains are able to bind metals in the same stoichiometries as are found in the intact protein (Nielson and Winge, 1985). The three metals most commonly found bound to MTs are cadmium, copper and zinc, although MTs have been shown to bind at least 18 different metals (Nielson *et al.*, 1985). The metal composition of the protein is dependent of the species, tissue, stage of development and also the exposure of the organism to metals (reviewed in Vallee, 1979). Copper has the greatest binding affinity for MT, and the binding affinities for the most commonly bound metals are in the order copper>cadmium>zinc (see Nielson *et al.*, 1985). The α- and β- domains have differing stabilities and reactivities which are dependent on the metal species bound. For example cadmium and zinc can exchange within seconds in the β-domain, but within minutes in the α- domain (reviewed in Kägi and Kojima, 1987).

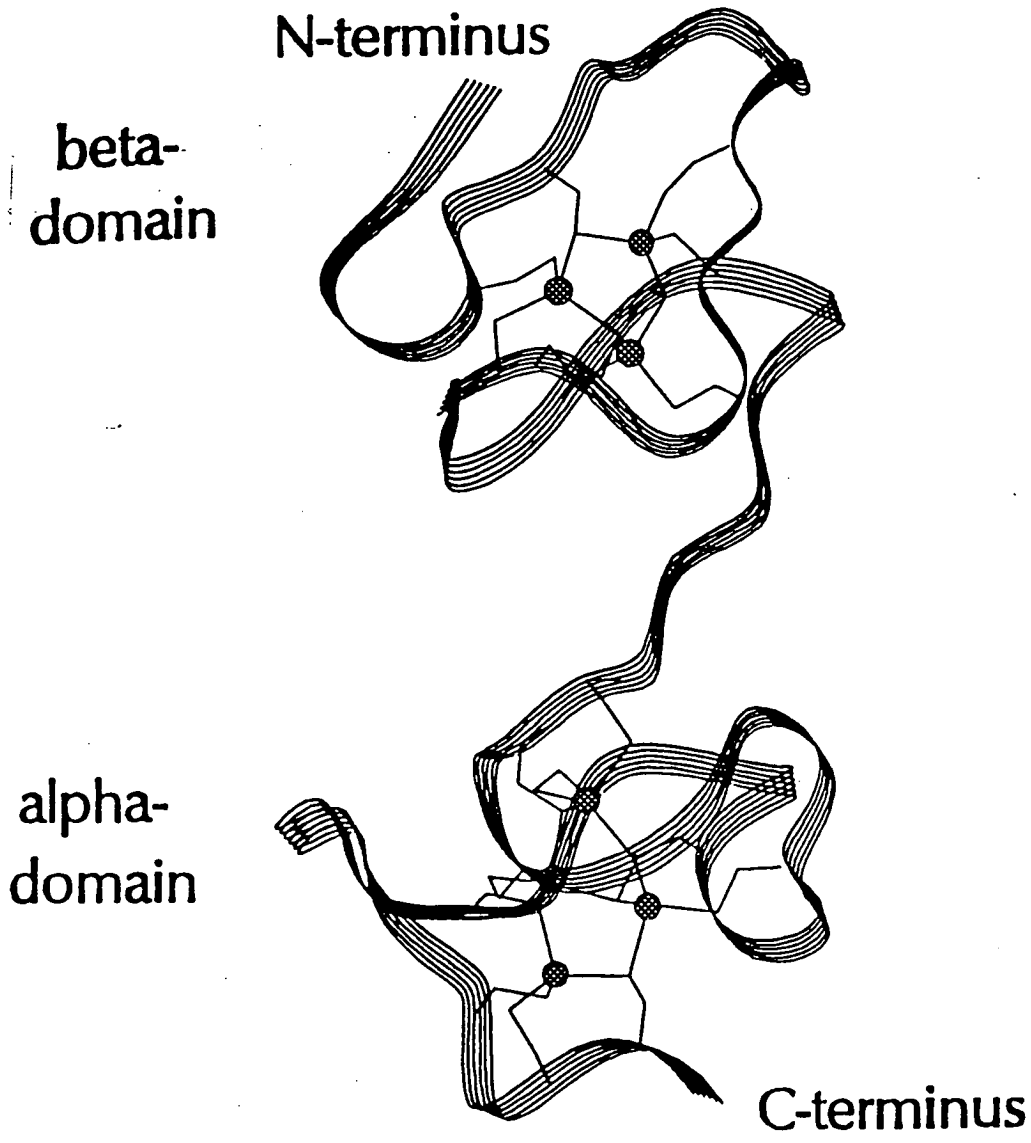


Figure 1.1 Metallothionein protein structure
A spatial structure model of rat MT-II derived from coordinates of its crystal structure is shown. It depicts a ribbon representation of the main chain fold with enclosed metal ion / cysteine (M/Cys) clusters (indicated by filled circles and thin lines, respectively). The alpha-domain enfolds the M_4Cys_{11} cluster and the beta-domain enfolds the M_3Cys_9 cluster (taken from Kägi, 1993).

1.5 Isoforms

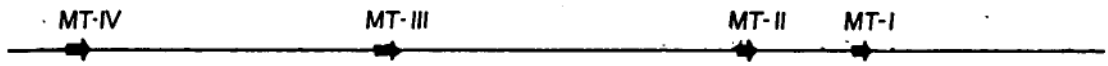
Mammalian MTs can be classified into 4 different subforms, which differ in their charge, size or amino acid composition. While two of the isoforms, MT-I and MT-II where characterised in many species relatively soon after the discovery of MTs (see Kägi and Kojima, 1987), the other two isoforms, MT-III and MT-IV were discovered only recently (Uchida *et al.*, 1991; Palmiter *et al.*, 1992; Quaife *et al.*, 1994). The genes which encode the various MT isoforms tend to be clustered on a single chromosome, the most well defined of the mammalian MT gene families being in the mouse. As shown in Figure 1.2A the 4 mouse MT isoforms (mMT-I, mMT-II, mMT-III and mMT-IV) are encoded by four genes clustered on chromosome 8.

1.5.1 MT-I and -II isoforms

In all mammalian species investigated so far, there are at least two functional MT genes which encode two electrophoretically distinguishable isoforms, designated MT-I and MT-II. In many instances there are multiple MT-I isoforms and a single MT-II isoform encoded by multigene families. For example in humans there are 7 functional MT-I genes; *hMT1A* (Richards *et al.*, 1984), *hMT1B* (Heguy *et al.*, 1986), *hMT1E* (Schmidt *et al.*, 1985), *hMT1F* (Schmidt *et al.*, 1985), *hMT1G* (Foster *et al.*, 1988), *hMT1H* (Stennard *et al.*, 1994) and *hMT1X* (Stennard *et al.*, 1994) and a single functional MT-II gene; *hMT2A* (Karin and Richards, 1982). In sheep there are 3 functional MT-I genes; sMT-Ia, sMT-Ib and sMT-Ic and a single functional MT-II gene; sMT-II (Peterson *et al.*, 1988). In contrast, in mouse there is only a single functional MT-I gene; mMT-I (Durnam *et al.*, 1980) and a single functional MT-II gene; mMT-II (Searle *et al.*, 1984). Characteristically, the MT-II isoform contains an acidic amino acid in position 10 or 11, instead of the neutral amino acid found in MT-I isoforms, as shown in Figure 1.2B.

MT-I and -II isoforms are the most extensively studied of the mammalian MTs and their expression within an organism appears to be widespread (see Hamer, 1986). Highest levels of MT-I and -II isoforms are found in parenchymatous tissues, e.g., in the mouse, highest levels of MT-I and -II are induced by metals in the liver, kidney, intestine and pancreas (Durnam and Palmiter, 1981). Expression of MT-I and -II isoforms have been shown to be induced by an array of factors including metals,

A



B

mouse MT-I
MDPN-CSCSTGGSCTCTSSCACKNCKCTSCKKSCCSCCPVGCSKCAQGCVCKG------AADKCTCCA

mouse MT-II
MDPN-CSCAS**D**GSCSCAGACKCKQCKCTSCKKSCCSCCPVGCSKCAQGCVCKG------AADKCTCCA

mouse MT-III
MDPE**T**CPCPTGGSCTCSDKCKCKGCKCTNCKKSCCSCCPAGCEKCAKDCVCKG**EEGAKA**EAEKCSCCQ

mouse MT-IV
MDPG**E**CTCMSGGICICGDNCKCTTCSCKTCRKSCCPCCPPGCAKCARGCICKG------GSDKCSCCP

Figure 1.2 The mouse metallothionein isoforms
The organisation of the functional metallothionein locus on chromosome 8 is shown (A). Adapted from Palmiter *et al.*, 1993. Arrows indicate the transcriptional orientations of the individual genes. The amino acid sequences of the 4 mouse isoforms encoded by these genes are shown (B). Amino acid differences which characterise the MT isoforms are indicated (bold, underlined).

hormones and xenobiotics. In the mouse, MT-I and -II genes have been demonstrated to be induced coordinately by metals, glucocorticoids and lipopolysaccharide (LPS, Searle *et al.*, 1984; Yagle and Palmiter, 1985), whereas in other species like the human (for example, Heguy *et al.*, 1986; Jahroudi *et al.*, 1990) and the sheep (Peterson and Mercer, 1988; Pitt *et al.*, 1992) differential regulation of MT-I and -II genes has been documented.

1.5.2 MT-III isoform

MT-III was first purified and characterised as a 68 amino acid MT-like protein from the human brain (Uchida *et al.*, 1991). Molecular cloning of the full length cDNA revealed remarkable homology to known MTs, including conservation of the number and position of cysteine residues, the conservation of lysine residues juxtaposed to cysteine residues, the absence of aromatic amino acids or histidine and the binding of 7 bivalent metal atoms per molecule (Tsuji *et al.*, 1992). Human MT-III however contains two unique insertions of 1 and 6 amino acids in the amino- and carboxyl-terminal regions respectively, as depicted in Figure 1.2B. Unlike previously characterised MTs, MT-III expression is largely restricted to the nervous system (Tsuji *et al.*, 1992).

The gene encoding the mouse homologue of human MT-III predicted a similar protein, containing inserts in the same positions and of the same size although the amino acid sequence of the carboxyl-terminal insert is not conserved. Mouse MT-III expression is also largely restricted to neural tissues (Palmiter *et al.*, 1992). Highly homologous rat, bovine and equine MT-III proteins have now been identified (Kobayashi *et al.*, 1993; Poutney *et al.*, 1994).

Although expression of MT-III isoforms appears to be mainly restricted to neural tissue (Tsuji *et al.*, 1992; Palmiter *et al.*, 1992; Kobayashi *et al.*, 1993), very low levels have been detected in the pancreas of the mouse (Erickson *et al.*, 1995). Masters *et al.* (1994b) showed that levels of MT-III mRNA in various regions of the mouse brain are approximately equivalent to levels of MT-I mRNA. However, unlike MT-I and -II isoforms, studies have failed to demonstrate upregulation of MT-III expression by metals or glucocorticoids (Palmiter *et al.*, 1992, Masters *et al.*, 1994b).

1.5.3 MT-IV isoform

The gene encoding a further isoform, MT-IV, was discovered in both human and mouse (Quaife *et al.*, 1994). The predicted protein has a single glutamine amino acid insertion at position 5 with respect to MT-I and -II isoforms, as shown in Figure 1.2B. This insertion is in the same place as the N-terminal insertion in MT-III isoforms. MT-IV is mainly expressed in differentiating cells of stratified squamous epithelia, and furthermore appears restricted to the suprabasal cells in the stratum spinosum of the epithelia (Quaife *et al.*, 1994).

As outlined above, MT-III and MT-IV isoforms display restricted expression patterns, while MT-I and -II isoforms are expressed more widely within an organism. However, in the mouse, all of the known MT isoforms, mMT-I, -II, -III and -IV, have been localised in the maternal deciduum during embryonic development. All the MT genes were shown to be co-expressed in at least some cells by *in situ* hybridisation (Liang *et al.*, 1996).

1.6 Gene structure

The functional mammalian MT genes are characterised by a tripartite structure consisting of 3 exons separated by 2 introns (Glanville *et al.*, 1981). Whilst the exons are highly conserved, the 5' and 3' untranslated regions and the introns show little homology and vary in size (Hamer, 1986). In all cases the intron/exon boundaries conform to the GT...AC splicing rule (Breathnach and Chambon, 1981).

1.7 Regulation

The synthesis of MTs is induced by a wide variety of factors including heavy metals, glucocorticoids, cytokines, growth factors and hormones as outlined in Table 1.2. The regulation of MT expression occurs largely at the level of transcription initiation, although small effects on MT mRNA stability have been suggested in some cases (Mayo and Palmiter, 1981). Studies investigating MT induction by zinc and copper, however, have suggested that MT gene expression is also modulated to some degree by factors at the post-transcriptional level (Sadhu and Gedamu, 1989; Vasconcelos *et al.*, 1996). The increase in MT protein synthesis in response to

Metals	**Cytotoxic agents**
Cd, Zn, Cu, Hg, Au, Ag, Co, Ni, Bi	butyrate
	retinoate
Hormones and cytokines	phorbol esters
glucocorticoids	endotoxin
progesterone	streptozotocin
estrogen	2-propanol
glucagon	ethanol
catecholamines	formaldehyde
interleukin-1	alkylating agents
interferon	chloroform
	carbon tetrachloride
Pathophysiological conditions	
starvation	
infection	
inflammation	
physical stress	
X-irradiation	
high oxygen tension	

Table 1.2 Physiological factors and experimental conditions which have been demonstrated to result in induction of metallothionein synthesis in cultured cells or *in vivo*. This table lists some of the wide range of factors which are inducers of metallothionein synthesis (adapted from Kagi and Schaffer, 1988)

inducers is accomplished through interactions between cis-acting elements (DNA sequence motifs) and trans-acting elements (cellular factors) (Karin *et al.*, 1987). MT gene expression is also regulated at more complex levels by changes in gene structure, such as amplification (Beach and Palmiter, 1981; Crawford *et al.*, 1985) and methylation (Compere and Palmiter, 1981).

Functional analysis has revealed metal-induced transcription of MTs to be mediated by metal-responsive elements (MREs), multiple copy imperfect repeats (Carter *et al.*, 1984; Stuart *et al.*, 1984), since shown to contain an essential core heptanucleotide motif TGCA/GCXC flanked by less conserved GC rich sequences (Culotta and Hamer, 1989). Individual MREs vary in their metal-activated transcriptional responses (Stuart *et al.*, 1985).

Genomic footprinting experiments suggest that a positively acting transcription factor binds to the MREs, since factors were only found to bind to these sequences after induction by metals (Andersen *et al.*, 1987; Mueller *et al.*, 1988). However, several different factors have in fact been reported to bind to MREs (Sequin and Prevost, 1988; Imbert *et al.*, 1989; Andersen *et al.*, 1990; Koizumi *et al.*, 1992). One of these factors, MTF-1, appears to be a constitutively active transcription factor capable of interacting with MREs (Radtke *et al.*, 1993). Palmiter (1994) demonstrated that a zinc-sensitive inhibitor, termed MT transcription inhibitor (MTI) was able to interact with MTF-1 and proposed a model in which the inhibitor and MTF-1 dissociate in the presence of zinc allowing MTF-1 to interact with the MREs of the MT promoter (see Figure 1.3). This model is supported by data which suggests that *de novo* synthesis is not required for activation of MT genes by metals (Karin *et al.*, 1980). However, it is still not known whether the zinc interacts directly or indirectly with the inhibitor.

A number of different trans-acting factors are now thought to be involved in the control of MT transcription. Most of these are general transcription factors, however the putative metal regulatory transcription factors thought to mediate induction of MTs by heavy metals appear to be MT-gene-specific (Culotta and Hamer, 1989). All MT genes investigated thus far have MREs present in their promoter region, and such elements have not been detected in promoters of other genes. The presence of other regulatory elements along with MREs within the 5'

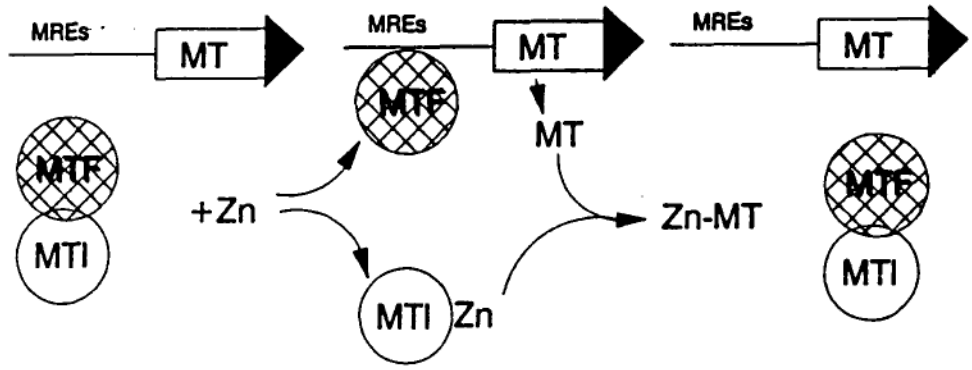


Figure 1.3 Model of MT gene regulation by zinc
The model depicted above proposes that the transcription factor MTF-I is complexed with metallothionein transcription inhibitor (MTI). This complex dissociates in the prescence of zinc allowing MTF-1 to interact with MREs of the MT promoter, activating MT transcription. The MT protein produced binds free zinc and the MTF-1/MTI complex reforms (taken from Palmiter, 1994)

flanking promoter region raises the possibility that protein-protein interactions may be an important component of regulatory mechanisms for MT genes (Thiele, 1992).

MT promoters contain binding sites for general transcription factors involved in the maintenance of basal level expression including the GC box sequence (GGGCGG) for the DNA binding protein Sp1 (Kadonaga *et al.*, 1986), which appears important for the general function of many promoters. This is often found to overlap promoter elements implicated in the control of basal level expression termed basal level elements (BLEs). These element may have an important role in determining the efficiency of transcription (Karin *et al.*, 1984).

Some MT promoters also contain recognition sequences for the activator protein AP-1 (Activator protein 1) (Lee *et al.*, 1987), and this is involved in the induction of the human gene *hMT2A* by 12-O-tetradecanoyl-13-phorbol acetate (TPA) (Angel *et al.*, 1986). The induction response involved is thought to utilize protein kinase C (PKC). AP-2 (Imagawa *et al.*, 1987) and AP-4 (Culotta and Hamer, 1989) binding sites have also been detected.

A glucocorticoid-responsive element (GRE) has been identified in the promoter of *hMT2A* and implicated as the major regulatory factor in induction of MTs by glucocorticoids (Karin *et al.*, 1987). The sequence motif in *hMT2A* determined to be responsible for induction of this gene by glucocorticoids is GGTACACTGTGTCCT (Karin *et al.*, 1984b), which is homologous to GREs identified in other promoters.

A region of the mouse MT-I promoter has been implicated in the induction of this MT by hydrogen peroxide. This region contains a composite element which consists of a major late transcription factor (MLTF) binding site overlapping an antioxidant response element (ARE) and/or MRE (Dalton *et al.*, 1994). The ARE which has the core sequence (GTGACnnnGC) can be bound by a factor(s) which is suggested to be activated by reactive oxygen species (ROS) (Rushmore *et al.*, 1991; Schroeder and Cousins, 1990) although the mechanism involved is unknown.

Although most cells exhibit MT expression and respond to a variety of inducers, there are a few cell lines such as Chinese hamster ovary (CHO) cells, which do not express MT even though the genes are present (see Grady *et al.*, 1987). Experiments have been conducted using the potent hypomethylating agent 5-

azacytidine and these studies have implicated DNA methylation as a regulatory mechanism in such cell-type specific expression of MT (Heguy *et al.*, 1986; Jahroudi *et al*, 1990). An inverse relationship between MT expression and the extent of methylation of cytosine residues has been postulated, and the methylation status of the 5' region appears particularly important. The mechanisms involved are unknown, but interference with the binding of RNA polymerase or regulatory factors, changes to the nucleosome phasing or alterations to higher order chromatin packaging have been suggested (Hamer, 1986). Methylation may also be an important regulating factor then, in the developmental control of tissue-specific MT gene expression (Palmiter, 1987).

There may also be domain regulatory elements which regulate genes within the MT locus. Palmiter *et al.* (1993) may in fact have detected a locus control region flanking the mouse MT-I and -II genes, which is responsible for the coordinate expression of these MTs. The MT-III and -IV genes, which are regulated and expressed differently, appear to be outside this locus control region. MT-III isoforms have very restricted expression and in fact the mouse MT-III promoter has been found to contain a tripartite repeat (25 X CTG) which has repressive activity on MT-III. This repeat also had repressive activity when joined to other promoters, in three different non-neural cell types (Imagawa *et al.*, 1995). A similar repeat has not been found in the human MT-III promoter however.

1.8 Physiological roles

1.8.1 Heavy metal detoxification

Several lines of evidence demonstrate that MTs are able to act in the detoxification of cadmium and other heavy metals. Such a role is suggested since MTs are transcriptionally activated by the heavy metals they bind (Durnam and Palmiter, 1981). Various studies have demonstrated the induction of MTs by heavy metals in both animal models (e.g. Shaik and Lucas, 1970; Nordberg *et al.*, 1971) and in cultured cell systems (e.g. Karin *et al.*, 1981). Cell lines which fail to produce MT due to methylation of the genes are sensitive to cadmium toxicity (Compere and Palmiter, 1981). Conversely, many cell lines which demonstrate resistance to cadmium toxicity, have been found to overexpress MT due to gene amplification

(Beach and Palmiter, 1981; Mayo and Palmiter, 1982; Gick and McCarty, 1982; Crawford *et al.*, 1985). Studies have also shown that transfection of MT genes on self replicating vectors causing overproduction of MT confers the transfected cells with cadmium resistance (Schmidt *et al.*, 1985; Karin *et al*, 1983). Furthermore, Morton *et al.* (1992) found that NIH/3T3 cells with increased MT levels due to transfection of the mouse MT-I gene, were at least 10 times more resistant to cadmium, at least in part due to a 4-fold reduced uptake of the metal into the cells.

In the whole animal model the situation is more complicated, however various studies have demonstrated that low dose treatment with a heavy metal resulting in induction of MTs can confer protection against the toxicity of subsequent higher dose heavy metal treatments (e.g. Leber and Miya, 1976). Furthermore, transgenic mice with non-functional MT-I and -II genes are extremely sensitive to cadmium poisoning (Michalska and Choo, 1993; Masters *et al.*, 1994a), while transgenic mice overexpressing MT-I are more resistant to cadmium toxicity (Liu *et al.*, 1995). However, there is some suggestion that excessive accumulation of cadmium-MT may be a major cause of kidney damage seen in acute cadmium poisoning (Nordberg *et al.*, 1975, Dorian *et al.*, 1992).

Much evidence then suggests that MT has a role in the detoxification of heavy metals as outlined above, however most researchers believe that this is not the primary physiological role of MTs (Karin, 1985, Webb, 1987). It is likely that this perceived role of MTs is adventitious, reflecting the similarity of cadmium and zinc. MT-I/II$^{-/-}$ transgenic mice which can not produce MT-I and -II proteins were crossed with mice which have disrupted copper effluxers due to mutation of the copper effluxing ATPase, producing mutant embryos which died due to copper toxicity (Kelly and Palmiter, 1996). This study demonstrated that MT provides a second line of defence against copper toxicity. Zinc transporters play an important role in homeostasis of this ion in cells (Palmiter and Findley, 1995) and it is likely that MTs provide a second line of defence against zinc toxicity also.

1.8.2 Metal homeostasis and metabolism

Roles for MT in hepatic storage, intestinal absorption, renal excretion and transport of copper and zinc have been suggested. Many of the characteristics of MT

suggest that they could be involved in such cellular metabolism. For example, MTs are usually found as copper and/or zinc-containing proteins, they have a conserved structure and are transcriptionally regulated by hormones and cytokines. Furthermore, high levels of MT are found in organs such as liver, kidney, intestine and pancreas which play important roles in homeostasis of these metals.

The transfer of metals from MT to apo enzymes has been demonstrated *in vitro* (Udom and Brady, 1980). For example, MT has been shown to donate zinc to carbonic anhydrase (Li *et al.*, 1980), and copper to ceruloplasmin (Schechinger *et al.*, 1986). Furthermore, copper MT not only donates copper to bovine dopamine β-monooxygenase resulting in activation of the enzyme, but also extracts copper from the enzyme resulting in inhibition of its enzymatic activity (Markossian *et al.*, 1988).

Expression of MT in many tissues is a direct reflection of dietary zinc intake (Blalock *et al.*, 1988; Huber and Cousins, 1988; Cousins and Lee-Ambrose, 1992) and there is substantial evidence to suggest that MT is involved in the regulation of absorption of essential metals in the intestine. Zinc administration induces MT in the intestine (Cherian, 1977; Hall *et al* 1979) and high levels of the protein correspond to reduced zinc absorption (Richards and Cousins, 1975). This inverse relationship between MT and zinc absorption has been well documented (Hempe and Cousins, 1992; Hoadley *et al.*, 1988) and has led to the suggestion that MT may function as an intracellular buffer limiting zinc absorption. Interestingly, MT induction and zinc absorption appear responsive to zinc but not other MT inducers such as dexamethasone and interleukin-1 (Hempe *et al.*, 1991). Tissue specific induction of MT appears also to be associated with the redistribution of zinc in the body (Cousins and Leinart, 1988; Huber and Cousins, 1988). Also, MT turnover is regulated by intracellular zinc levels, i.e. when zinc levels are low, MT is degraded (Karin *et al.*, 1981).

There is also evidence that metal containing MT can be secreted since it has been detected by radioimmunoassay in extracellular fluid such as urine, plasma and bile (reviewed in Bremner *et al.*, 1987). This has led to speculation that MT may be involved in transport of copper and zinc from one organ to another, or the excretion of these ions from the body.

1.8.3 Development and differentiation

A role for MT in development and differentiation has been suggested for a number of reasons. Firstly, high endogenous levels of MT have been reported in foetal and neonatal tissues (Bremner *et al.*, 1977; Bremner *et al.*, 1984; Panemangalore *et al.*, 1983; Elmes *et al.*, 1991) and MT appears to be subject to programmed regulation during both foetal and perinatal development (Nartey *et al.*, 1987a; Andrews *et al.*, 1984; Bakka and Webb, 1981; Klein *et al.*, 1991). Secondly, elevated levels of MT are seen in regenerating tissues such as the liver after partial hepatectomy (Ohtake *et al.*, 1979; Tohyama *et al.*, 1993) and the kidney after uninephrectomy (Zalups *et al.*, 1995). High levels of MT are often seen in actively proliferating and differentiating tissues, such as the basal layer of the epithelium of the cornea (Nishimura *et al.*, 1991). MT is also detected in hair follicles and the basal layer of hyperplastic epidermis, indicating it may be associated with the proliferation of keratinocytes (Karasawa *et al.*, 1991). Thirdly, the expression of MT in many tumour cells (Nartey *et al.*, 1987b; Bahnson *et al.*, 1991; Chauvin *et al.*, 1992) and particularly its association with the proliferative edge of tumourous tissue (Cherian, 1994) also suggest that these proteins are associated with cellular proliferation.

A study has found links between expression of MT and the cell cycle (Nagle and Vallee, 1995) and there is a suggestion that the protein is translocated to the nucleus during S-phase, where it may provide metals for nuclear factors or enzymes involved in the cell cycle (Tsujikawa *et al.*, 1991; Tohyama *et al.*, 1993). However, in *in vitro* studies, Imbra and Karin (1987), found no association between MT synthesis and the cell cycle. Furthermore, mouse embryo development *in vitro* was unaffected by microinjection of anti-MT antibodies (Ibáñez *et al.*, 1995), although Peters *et al.* (1995) demonstrated that MT antisense oligonucleotides inhibited the frequency of blastocyst formation in mouse preimplantation embryos. Finally, transgenic mice lacking MT-I and -II isoforms develop normally suggesting that MT can not play a critical role in development (Michalska and Choo, 1993; Masters *et al.*, 1994a).

1.8.4 Free radical scavenging

Several lines of study have led to the suggestion that MT may function in the defence against oxidative stress. Firstly, MT is induced in cells (Fornace *et al.*, 1988)

and tissues (Bauman *et al.*, 1991) by oxidative stress. Secondly, spin trapping experiments have shown that MT is able to scavenge hydroxyl radicals at least *in vitro* (Thornalley and Vašák, 1985; Renan and Dowman, 1989), and thirdly MT displays a protective effect against hydroxyl radical induced DNA damage *in vitro* that is concentration dependent (Abel and Ruiter, 1989).

Several studies have investigated the effect of MT on the lethality of free radicals. Overexpression of MT by induction with heavy metals and cytokines has been reported to result in resistance to oxidative stress caused by hydrogen peroxide (Mello-Filho *et al.*, 1988), organic peroxides (Ochi., 1988), hyperoxia (Hart *et al.*, 1990) and carbon tetrachloride (Schroeder and Cousins., 1990). However heavy metals and cytokines induce factors other than MT. For example, cadmium induces both MT and glutathione (GSH), a known free radical scavenger (Seagrave *et al.*, 1983), and in fact induction of MT by zinc which does not alter GSH concentrations failed to confer protection from free radicals in one study (Chubatsu *et al.*, 1992). This finding is supported by gene transfection studies in which overexpression of MT by transfection of the gene failed to confer resistance to ionizing radiation or bleomycin (Lohrer and Robson, 1989; Kaina *et al.*, 1990).

However, hydroxyl radicals may produce single strand scissions which are not necessarily lethal (Ward *et al.*, 1985), but result in mutagenic base damages (Aruoma *et al.*, 1989). Cells overexpressing MT by gene transfection have in fact been shown to have decreased hydrogen peroxide-produced DNA strand scissions, and depletion of MT by the antisense mRNA approach resulted in increased scissions (Chubatsu and Meneghini, 1993). These experiments suggest that MT may have antimutagenic properties and this line of reasoning is supported by studies which have demonstrated that cells overexpressing MT have resistance to alkylating agents (Kaina *et al.*, 1990). This has led to the suggestion that MT participates as a cofactor or regulatory element in repair or tolerance of toxic alkylation lesions.

Further studies which support the notion of the involvement of MT in free radical protection include its ability to confer resistance to the cytotoxic effects of monochromatic 365 nm UVA radiation, which is known to generate reactive oxygen species (ROS) intracellularly (Dudek *et al.*, 1993), the correlation of increased MT expression with decreased side effects of anti-cancer agents that produce oxidative

stress including cisplatin and adriamycin (Basu and Lazo, 1990) and also its ability to protect against carbon tetrachloride induced hepatotoxicity, following induction by zinc pretreatment (Cagen and Klaassen, 1979). It has also been suggested that MT may reduce lipid peroxidation possibly by releasing zinc to stabilize membranes (Thomas *et al.*, 1986).

Yeast and monkey MT proteins have also been shown to substitute for copper/zinc superoxide dismutase (CuZnSOD) in yeast. It was suggested therefore that MTs play a direct role in the defence against oxidative stress (Tamai *et al.*, 1993), although this activity was shown to be a property of only copper-containing MT. Interestingly, recent data has shown that CuZnSOD can play a role in copper buffering which is distinct from its role as a free radical scavenger (Culotta *et al.*, 1995). This raises the possibility that in the previous study MT is substituting for the copper homeostasis rather than free radical scavenging function of CuZnSOD.

Finally, the production of MT-I/I$^{I-/-}$ mice which fail to produce MT-I and -II isoforms has provided a useful tool for examining the function of MT isoforms (Michalska and Choo, 1993, Masters *et al.*, 1994a). These mice were shown to have increased sensitivity to both tertbutylhydroperoxide and paraquat, indicating that basal MT may be involved in the regulation of the redox state of the cell (Lazo *et al.*, 1995).

1.8.5 MT and the acute phase response

MTs are induced by many chemical and physical stresses, and the effect is seen most prominently in the liver. MTs are induced early in the acute phase response, and their induction by various cytokines including α-interferon (Friedman and Stark, 1985), tumour necrosis factor (TNF) (De *et al.*, 1990; Sato *et al.*, 1992), interleukin-1 (IL-1) (Cousins and Leinart., 1988) and interleukin-6 (IL-6) (Schroeder and Cousins, 1990) has been observed. *In vitro* studies on rat hepatocytes have also demonstrated induction of MT by interleukin-11 (IL-11), leukaemia inhibitory factor (LIF) and activated macrophages (Coyle *et al.*, 1995).

MTs then, have been suggested to play an important role in the acute phase response. Low plasma zinc levels have been observed during infection or inflammation corresponding to induction of MT in the liver and sequestration of zinc

in this tissue (Cousins, 1985; Bremner and Beattie, 1990). However, the induction of MT during infection is not mediated primarily by zinc, since MT induction in the liver precedes the accumulation of zinc in this tissue, in animals treated with endotoxin (Durnam *et al.*, 1984). Similarly glucocorticoids are not the primary mediator since MT is induced by inflammatory agents in adrenalectomized rats (Sobocinski *et al.*, 1981). Min *et al.* (1992) showed that MT induction by inflammation is in fact mediated by cytokines and evidence suggests that along with metal and glucocorticoid regulatory elements, MT genes contain DNA elements responsive to inflammatory stress (first suggested by, Durnam *et al.*, 1984). IL-6 has been implicated as a primary mediator in the induction of MT by inflammatory agents (Rofe *et al.*, 1992), and is capable of inducing MT synergistically with zinc and glucocorticoids (Schroeder and Cousins, 1990; Coyle *et al.*, 1993a; Coyle *et al*; 1993b). Thus a complex system involving many factors regulates MT expression in inflammation (reviewed in Coyle *et al.*, 1993c) and it seems likely that the induction of MT in the liver has the role of sequestering zinc in this tissue where it is required for the synthesis of DNA and proteins, including the acute phase proteins. In fact studies of MT-I/II$^{-/-}$ transgenic mice which fail to express MT-I and -II isoforms have demonstrated that MT is essential for the accumulation of zinc in the liver in endotoxin-induced inflammation (Philcox *et al.*, 1995).

It has also been suggested that MTs have an antioxidative role during the acute phase response, protecting tissues from injury by oxidative stress, since induction of MTs by oxidative stress appears partly to be mediated by cytokines (Sato *et al.*, 1995).

1.8.6 MT and anticancer drugs

A major factor in the failure of cancer chemotherapy appears to be resistance to anticancer drugs and MT has been implicated in this resistance. There are presently however conflicting results regarding the role of MT in anti-cancer drug resistance. MT induction by cadmium has been shown to result in resistance to cisplatin (Bakka *et al.*, 1981) and chlorambucil (Endresen *et al* 1983) and MT overexpression has been correlated to resistance to electrophilic anticancer drugs (Andrews PA *et al.*, 1987;

Kelley *et al.*, 1988). Non-metal inducers of MT have also been found to result in cisplatin resistance (Kondo *et al.*, 1994; Basu and Lazo, 1991).

In further studies overexpression of MT by transfection of cells with the human gene *hMT2A* correlated with resistance to cisplatin, chlorambucil and melphalon , but not the functionally unrelated 5-fluorouracil and vincristine (Kelley *et al.*, 1988). Resistance was also seen to alkylating agents such as mitomycin C, N-methyl-N-nitrosourea and cisplatin but not γ-irradiation or bleomycin (Kaina *et al.*, 1990). Other transfection studies however, found no cisplatin resistance (Morton *et al.*, 1993; Schilder *et al.*, 1990). It has been suggested that sensitivity to DNA alkylating agents following MT gene transfer may depend in part on the phenotype of cells (Lohrer and Robson, 1989; Lohrer *et al.*, 1990; Robson *et al.*, 1992) and examination of cisplatin resistant cells has revealed that although some have increased MT levels (Kelley *et al.*, 1988; Yang *et al.*, 1994; Kasahara *et al.*, 1991; Eichholtz-Wirth *et al.*, 1993) others do not (Andrews PA *et al.*, 1987; Kelley *et al.*, 1988; Schilder *et al.*, 1990; Shellard *et al.*, 1993). Several studies have also demonstrated the protective effects of induced MT on cisplatin *in vivo*, particularly in the mouse (Naganuma *et al.*, 1985; Satoh *et al.*, 1993a,b; Naganuma *et al.*, 1987; Satoh *et al.*, 1988).

Tumour samples have also been examined to investigate the link between alkylating agent resistance and MT (Nartey *et al.*, 1987b; Chin *et al*, 1993; Bahnson *et al.*, 1991; Haile-Meskel *et al.*, 1993). However no direct correlation between MT levels and response to therapy, age, stage, histology or tumour cell differentiation state has yet been found (Bahnson *et al.*, 1991; Wood *et al.*, 1993; Murphy *et al.*, 1991). In fact, Haerslev *et al.* (1995) concluded that immunohistochemically detectable MT was not prognostically significant in primary breast carcinomas.

Many of the anticancer drugs cause oxidative injury to cells, and also illicit an inflammatory response resulting in production of cytokines, IL-1, IL-6 and TNF. The involvement of MT in resistance to anticancer drugs is most likely related to the role of these proteins in the acute phase response and/or free radical scavenging.

1.8.7 Growth inhibitory function

MT-III was initially isolated as a growth inhibitory factor that is deficient in Alzheimer's disease (AD) (Uchida *et al.*, 1991), although a separate study did not find this down regulation to be statistically significant (Erickson *et al.*, 1994). MT-III inhibits the neurotrophic activity of AD brain extracts on neonatal rat cortical neurons (Uchida *et al*, 1991, Tsuji *et al.*, 1992). It is however only inhibitory in the presence of brain extract (either control or AD). In fact in the absence of brain extract MT-III (and MT-I) stimulate neuron survival (Erickson *et al.*, 1994). The inhibitory activity unique to MT-III is due to its N-terminal β-domain (Uchida and Ihara, 1995) and is more precisely dependent on the 2 prolyl residues in this domain which are only found in the MT-III isoform (Sewell *et al.*, 1995).

In transgenic mice overexpressing human MT-III, the expression profile of the human MT-III mimics that of the endogenous mouse MT-III. Zinc but not other metal concentrations are increased 9 fold, which is entirely accounted for by binding to the increased MT-III protein. However, despite the putative growth inhibitory activity of MT-III *in vitro*, the transgenic mice display no overtly deleterious effects in the development or function of the nervous system (Erickson *et al.*, 1995). The question remains, then, as to whether the growth inhibitory activity demonstrated for MT-III *in vitro* is physiologically relevant.

1.9 Expression of MT *in vivo*

MT expression has been detected in most organs and tissues examined. Highest levels of MT mRNA and endogenous protein are detected in parenchymatous tissues such as the liver, kidney, intestine and pancreas (Kägi and Kojima, 1987). Shaikh and Nolan (1987), reported MT levels in control rat of 7 μg/g in liver and 67 μg/g in kidney, as measured by radioimmunoassay. However, the actual levels of MT in a tissue vary widely depending on a range of factors including age, developmental status, dietary factors and exposure to metals. For example, measurement of hepatic MT levels by a cadmium binding assay during postnatal development of the rat demonstrated that MT levels are about 40 times greater immediately after birth than 30 days later (Cherian *et al.*, 1987). Notably MT has been detected in tissues which are rich in zinc ions such as Paneth cells of the small intestine (Danielson *et al.*, 1982;

Nishimura *et al.*, 1989) and the dorsolateral lobes of the prostate (Umeyama *et al.*, 1987; Nishimura *et al.*, 1990).

MT has been generally thought of as an ubiquitous protein however the use of immunohistochemical and *in situ* hybridisation methods, and the examination of endogenous rather than induced levels of MT have shown that its expression within an organ is not necessarily ubiquitous, but can be restricted to certain cell types and areas of the tissue. For example in the mammalian brain several studies have demonstrated the expression of MT-I and -II isoforms in glial but not neuronal cells by immunohistochemistry (e.g. Nishimura *et al.*, 1992; Blaauwgeers *et al.*, 1993). Furthermore, immunocytochemical studies have demonstrated that MT protein levels may vary markedly in individual cells within a tissue. This is evident both in various human organs (e.g. Nartey *et al.*, 1987a,b; Cherian, 1994) and in experimental animals (e.g. Nishimura *et al.*, 1991; Karasawa *et al.*, 1991; Elmes *et al.*, 1991; Danielson *et al.*, 1982).

MT mRNA has been detected using northern analysis and *in situ* hybridisation. These techniques are able to differentiate between mRNA for the various MT isoforms (e.g. Pitt *et al.*, 1992; Andrews *et al.*, 1991) and have demonstrated differential expression and regulation patterns. Similarly, various methods have been used to detect MT protein including immunohistochemistry, radioimmunoassay, an ELISA assay, and a silver saturation method. However, because of the similarity of the MT isoforms, specialized techniques are generally required to distinguish between them at the protein level. Many commonly used antibodies against MT cross react with both MT-I and -II isoforms (e.g. Jasani and Elmes, 1991), although isoform specific antibodies have been produced (e.g. Mehra and Bremner, 1983). Techniques such as capillary electrophoresis and high performance liquid chromatography are used most successfully to separate the various MT isoforms (Hunziker and Kägi, 1985; Richards and Beattie, 1993; Beattie *et al.*, 1993).

1.9.1 Nuclear localisation

Distinct differences in the cellular localisation of endogenous MT as detected by immunohistochemistry have been reported in developing tissues. MT was traditionally thought of as a cytoplasmic protein but its localisation in the cell nucleus

is now well documented. Although predominantly cytoplasmic localisation is observed in adult liver, in the foetal human liver, and foetal and neonatal rat liver MT is also localised to nuclei (Panemangalore *et al.*, 1983; Templeton *et al.*, 1985; Nartey *et al.*, 1987a; Andrews GK *et al.*, 1987; Elmes *et al.*, 1991). Nuclear localisation of MT in hepatocytes therefore appears to decrease with age. Both cytoplasmic and nuclear localisation have also been detected in studies of various tumours (e.g. Nartey *et al.*, 1987b; Schmid *et al.*, 1994; Suzuki *et al.*, 1991).

Tsujikawa *et al.*, (1991) investigated the subcellular localisation of MT in primary cultured adult rat hepatocytes stimulated by epidermal growth factor and insulin and found that MT localised in the cytoplasm of hepatocytes during G1 phase was translocated into the nucleus during early S-phase of the cell cycle. A further study using DNA flow cytometry demonstrated MT in the nucleus of diploid and aneuploid adenocarcenoma cells during S-phase but not G1-phase (Haile-Meskel *et al.*, 1993). Tohyama *et al.* (1993) also demonstrated that although MT is localised predominantly in the cytoplasm of normal adult rat liver, nuclear localisation was present in the regenerating liver after partial hepatectomy. The significance of MT in the nucleus is not yet known although one proposal is that it may be linked to the interactions of metal ions such as zinc with nuclear factors and enzymes involved in the cell cycle (Cherian, 1993). Chubatsu and Meneghini (1993) showed MT expression predominantly in the nucleus of V79 Chinese hamster lung fibroblasts both before and after chronic cadmium exposure. They proposed a link between the presence of MT in the nucleus and the ability of MT to protect DNA against free radical damage.

1.10 Expression in the brain

Various studies have documented the expression of MT in the mammalian brain. Expression of the MT-I and -II isoforms, as well as the brain specific isoform MT-III, have been recorded in the brain. Several studies have demonstrated that the relative levels of MT-III mRNA in regions of the brain closely follow the expression patterns of MT-I and -II mRNA (Masters *et al.*, 1994b; F.A.Stennard, Ph.D. Thesis). Furthermore, MT-I and -MT-III mRNA levels in the brain appear to be approximately

equal (Masters *et al.*, 1994b). However, there is currently debate as to whether MT-I and -II isoforms are expressed in the same cell type as the MT-III isoform.

1.10.1 MT-I and -II isoforms

MT-I and -II isoform expression in the brain has been studied in various species. In the mouse expression has been localised in glial cells, ependymal cells, arachnoid and pia mater (Nishimura *et al.*, 1992). Similarly in the rat brain expression has been detected in astrocytes throughout the brain (Young *et al.*, 1991; Young, 1994), ependymal cells, pia mater and arachnoid as well as the choroid plexus and blood vessels (Nakajima *et al.*, 1989; Nishimura *et al.*, 1992). In the monkey expression was reported in astrocytes, ependymal cells and pia mater (Suzuki *et al.*, 1992a) and in the human, various studies have localised MT-I and -II mainly to astrocytes (Nakajima *et al.*, 1991; Suzuki *et al.*, 1992b; Blaauwgeers *et al.*, 1993; 1994).

1.10.2 MT-III isoform

MT-III expression appears to be restricted to neural tissue (Tsuji *et al.*, 1992) however there is considerable debate regarding its cell-type expression. In the human brain expression of the protein detected with an antibody to a synthetic peptide has been reported to be restricted to actrocytes, primarily in the grey matter (Uchida *et al.*, 1991). In the mouse however, neural but not glial expression of MT-III mRNA was detected *in vivo*, by *in situ* hybridisation (Masters *et al.*, 1994b; Choudhuri *et al.*, 1995). Furthermore, transgenic mice generated by fusion of the MT-III promoter to the *E. coli* lac z gene demonstrated β-galactosidase activity localised to neurons (Masters *et al.*, 1994b). Zheng *et al.* (1995) however, found MT-III mRNA to colocalise with MT-I mRNA in the glial cells on the Purkinje layer of the cerebellum, and Masters *et al.* (1994b) reported MT-III mRNA in the choroid plexus indicating that the expression is not exclusively neuronal. In the rat brain also, *in situ* hybridisation revealed MT-III mRNA mainly in the neurons of the cortex, but both neuronal and non neuronal cells increased MT-III expression after ablation (Yuguchi *et al.*, 1995a). In a separate study, MT-III was colocalised to facial motorneurons and mainly neurons of the cortex (Yuguchi *et al.*, 1995b). Masters *et al.* 1994

showed that mouse astrocytes were capable of expressing MT-III after several days in culture, while Kobayashi *et al.* (1993) detected MT-III in rat astrocyte cultures, but not cultured neurons , fibroblasts or microglia.

1.11 MT-I and -II null mice

Although studies have demonstrated the ability of MTs to fulfil several roles, the primary physiological function of MT remains unresolved. Recently, transgenic mice have been employed to answer questions regarding MT and have proved a useful tool. Transgenic mice with disrupted MT-I and -II genes (MT-I/II$^{-/-}$ mice) where found to be apparently physiologically normal and fertile (Michalska and Choo, 1992; Masters *et al.*, 1994a), apart from increased sensitivity to cadmium. However, embryonic fibroblasts made from these mice were shown to have increased sensitivity to oxidative stress (Lazo *et al.*, 1995) and increased sensitivity to anticancer drugs (Kondo *et al.*, 1995). These mice have also been used to demonstrate the importance of MT in the sequestering of zinc in the liver during inflammation (Philcox *et al.*, 1995). Finally a study in which these mice were crossed with mice with disrupted copper effluxers demonstrated that MT is essential in providing a second line of defence against copper toxicity (Kelly and Palmiter, 1996). Thus, studies using MT-I/II$^{-/-}$ transgenic mice have demonstrated that although MTs are not necessary for normal development and function, they may provide essential back up systems, and may be required in situations of physiological stress.

1.12 Concluding Remarks

Despite the large number of studies investigating MTs over the past 30 years many features of MT remain enigmatic, not least being their physiological role and the requirement for the expression of multiple isoforms in mammals. Although many studies have investigated MT expression in artificially induced states, recent studies suggest that much can be learnt about this protein by examining its expression and regulation in the basal state and under physiological conditions, e.g. during developmental stages or during conditions of inflammation. Investigation of MT expression under such conditions may provide insight into the factors which are most important in regulating the expression of these proteins *in vivo*, and may lead to a

better understanding of their function. Similarly, the requirement for multiple isoforms to be expressed in mammals remains puzzling. These multiple isoforms may enable rapid responses to inducers, may provide a mechanism for tissue specific expression or may provide diversity of function. Examination of the individual MT genes, the features which set them apart, and the manner in which they are differentially regulated and expressed may provide insight into their requirement and their contribution to the physiology of MTs.

This thesis addresses the unresolved points outlined above. Although MT is known to be present in the mammalian brain, its expression during the complex process of neural development is uninvestigated. Therefore, this work will examine in detail the expression of MT during the development of the sheep brain, with particular emphasis on its localisation to specific regions and cell types. Furthermore, it is not known whether an MT-III isoform exists in this species, and thus whether it contributes to the repertoire of brain MT expression. Consequently, the existence of sheep MT-III will be investigated and if found, its expression profile compared to MT-I and -II isoforms. Finally, the reason for the expression of multiple isoforms of MT in all mammalian species remains. One approach to resolving the significance of multiple isoform expression is to determine whether each isoform has specific structural or regulatory properties. In this context a novel human gene, *MT1L* will be investigated to determine whether it is expressed in human tissues and therefore contributes to the repertoire of MT expression in the human.

CHAPTER 2: MATERIALS AND METHODS

2.1 Media and Buffers

All general chemicals and reagents were of molecular biology or analytical grade. Suppliers of specialised materials, enzymes or kits are listed. All buffers were prepared with double distilled water (dd water) purified using the Milli-Q Ultrapure Water System (Millipore) or glass distilled water, as specified.

Alkaline agarose 2 X Loading buffer

200 µl glycerol

750 µl dd water

46 µl saturated bromophenol blue

5 µl 5M NaOH

10 X Alkaline Buffer

3 ml 5 M NaOH

2 ml 0.5 M EDTA

45 ml distilled water

Alkaline agarose gel

Agarose (0.8 g) was dissolved in 72 ml distilled water, cooled to 55°C, then 8 ml of 10 X alkaline buffer added. A thin layer of gel was poured, and once set, loaded and electrophoresed at 100 mA.

Antibiotics

Antibiotics were added to media and agar at the following concentrations when required:

Ampicillin (Boehringer-Mannheim), added to 50 µg/ml

Tetracycline (Sigma), added to 12.5 µg/ml

Kanamycin (Boehringer-Mannheim), added to 50 µg/ml

Bouin's fixative

75 ml saturated aqueous picric acid

25 ml 40% formaldehyde

5 ml glacial acetic acid

Buffered glycerol

50 ml 0.5 M $NaHCO_3$, pH 8.3

0.6 ml 0.5 M Na_2CO_3, pH 11.5

100 ml glycerol

DNA Hybridisation Buffer

6 g HEPES (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) dissolved in

250 ml dd water

5 ml 10% SDS

1 g Ficoll

1 g BSA (bovine serum albumin)

1 g PVP (Polyvinylpyrrolidone)

1 ml 0.5 M EDTA

75 ml 20 X SSC

dd water to 500 ml

denatured salmon sperm DNA, added to 0.5 mg/ml before use

5 X DNA loading buffer

25% sucrose

6.25 mM EDTA

1.25% SDS

0.25% bromophenol blue

Hybridisation Buffer (50% formamide, for cDNA probes)

50% formamide

5 X SSC

0.5% milk powder

1% SDS

10% dextran sulphate

50 mM phosphate buffer

denatured salmon sperm DNA, added to 0.5 mg/ml before use

Hybridisation Buffer (40 % formamide, for in situ hybridisation)

As above, with 40% formamide and omission of dextran sulphate

Hybridisation Buffer (20% formamide, for oligonucleotide probes)

20% formamide

5 X SSC

50 mM phophate buffer, pH 6.8

1 mM sodium pyrophosphate

0.1% BSA (Bovine serum albumin)

0.1% PVP (Polyvinylpyrrolidone)

0.1% Ficoll

2% SDS

denatured salmon sperm DNA, added to 0.5 mg/ml before use

LB broth

10 g NaCl

10 g tryptone

5 g yeast extract,

distilled water to 1L, pH 7.0

LB agar

20 g agar in 1L LB broth

LB top agar

0.7% w/v agarose in LB broth

10 X MOPS Buffer

200 mM 3-[N-morpholino]propane-sulphonic acid (MOPS)

50 mM sodium acetate

10 mM EDTA

Adjusted to pH 6.5-7.0

NZY broth

5 g NaCl

2 g $MgSO_4.7H_20$

5 g yeast extract

10 g NZ amine (casein hydrolysate)

distilled water to 1 L, pH 7.5

NZY agar

15 g agar in 1 L NZY broth

NZY top agar

0.7% w/v agarose in NZY broth

Paraformaldehyde fixative

4% w/v paraformaldehyde dissolved in 500 ml distilled water at 60°C, cleared by addition of several drops of 10 M NaOH.

500 ml 0.2 M phosphate buffer

10 X PBS

80 g NaCl

13 g Na_2HPO_4

2 g NaH_2PO_4

in 1 L distilled water, pH 7.2

0.2 M Phosphate buffer

3.59 g $NaH_2PO_4.2H_2O$

10.93 g Na_2HPO_4, anhydrous

dissolved in 500 ml distilled water, pH 7.25

5 X RNA loading buffer

50% glycerol

1 mM EDTA, pH8.0

0.25% bromophenol blue

0.25% xylene cyanol FF

SM Buffer

5.8 g NaCl

2.0 g $MgSO_4.7H_2O$

50 ml 1 M Tris.HCl, pH 7.5

5.0 ml 2% (w/v) gelatin

dd water to 1 L

Solution D

250 g guanidinium thiocyanate in 293 ml distilled water

17.6 ml 0.75 M sodium citrate, pH 7.0

26.4 ml 10% sarcosyl

heated to 65°C and filter sterilized

20 X SSC Buffer

175.3 g NaCl

88.2 g sodium citrate

distilled water to 1 L, pH 7.0

1 X TAE Buffer

40 mM Tris.acetate

1 mM EDTA, pH 8.0

10 X TBE Buffer

121 g Tris.base

61.7 g boric acid

7.44 g EDTA, pH 8.3

TBS

6 g Tris.base

8.8 g NaCl

in 1 L distilled water, pH 7.6

TE

10 mM Tris.HCl, pH 7.4

1mM EDTA

TES

0.1 M NaCl in TE

2.2 Cloning techniques

Unless otherwise specified the cloning techniques used were as outlined in Sambrook *et al.* (1989).

2.2.1 Preparation of insert and vector DNA

Plasmid DNA was cleaved with restriction endonucleases (Pharmacia), using 1 unit of endonuclease per μg of DNA in reaction buffer supplemented with 0.1 mg/ml BSA. The DNA was electrophoresed through 0.75% agarose/1 X TBE, visualised by ethidium bromide staining, and the required DNA fragments excised from the gel. A hole was made in a 0.5 ml eppendorf tube with a 19 gauge needle and glass wool was placed in the bottom. The 0.5 ml eppendorf tube was placed inside a 1.5 ml eppendorf tube. The DNA was removed from the agarose by centrifugation at 7000 g for 3 min through the glass wool and collected in the 1.5 ml eppendorf tube. The DNA was then recovered from the elute by ethanol precipitation. DNA fragments

amplified by PCR were electrophoresed through 1.4% agarose/1 X TBE and purified similarly.

Vector DNA was digested with appropriate restriction endonucleases, and the linearized vector treated with calf-intestinal alkaline phosphatase (Pharmacia) to remove the 5'-phosphate groups and prevent recircularisation of the vector. The DNA was incubated for 30 min at 37°C with 1 unit of alkaline phosphatase. The reaction was phenol/chloroform extracted and the vector DNA recovered by ethanol precipitation.

2.2.2 Bluntending of DNA ends

DNA fragments isolated for subcloning into vectors, and linearized vectors were bluntended when necessary using mung bean nuclease (Promega) or *Pfu* DNA polymerase (Stratagene). DNA was incubated for 1 hr with 4.5 units of mung bean nuclease at 37°C, phenol/chloroform extracted and the DNA recovered by ethanol precipitation. Alternatively, DNA was incubated with 2.5 units of *Pfu* DNA polymerase for 30 min at 72°C in reaction buffer containing 1 mM dNTPs (Promega), and the DNA used directly in ligation reactions.

2.2.3 Ligation of insert DNA into vectors

Insert DNA (typically at 3 molar excess) was ligated into linearized vector DNA (50 ng) using T4 DNA ligase (New England Biolabs). Reactions were incubated for 16 to 24 hr at 16°C with excess ligase (50 to 500 units).

2.2.4 Preparation and transformation of competent cells

L-broth (100 ml) was innoculated with 1 ml of overnight bacterial culture, and incubated with shaking at 37°C until an $O.D._{650\ nm}$ of 0.4-0.5 was reached for MC1061 bacterial cells, or $O.D._{590\ nm}$ of 0.5-0.6 was reached for JM101 bacterial cells. The cells were centrifuged at 7000 g for 5 min at 2°C, the cell pellet resuspended in 20 ml of ice cold 50 mM $CaCl_2$ and the cell suspension incubated on ice for 30 min. The cells were pelleted by centrifugation at 7000 g for 5 min, and resuspended in 3.33 mls ice cold 50 mM $CaCl_2$.

Competent cells (200 µl) were incubated with DNA (half of a ligation reaction or typically 2 ng of plasmid DNA) for 40 min on ice. The cells were heat shocked by incubation at 42°C for 90 secs. Transformations (1/10 th and 9/10 th of the cells) were plated onto L-agar plates containing the appropriate antibiotics, and incubated for 16 hr at 37°C

2.2.5 Detection of recombinant clones

The method used to transfer bacterial colonies to nitrocellulose filters was based on that of Grunstein and Hogness (1975). Nitrocellulose filters (0.45 µm, Schleicher and Schuell) marked with ink were lowered onto bacterial colonies on agar plates for 2 min and the markings transferred to the bottom of the plates. The filters were placed on blotting paper soaked in 0.5 M NaOH/0.5 M NaCl for 5 min to lyse the bacteria and fix the DNA, transferred to blotting paper soaked in 2 M NaCl/0.5 M Tris.HCl, pH 7.0 for 10 min, then rinsed in 2 X SSC. The filters were baked under vacuum for 2 hr at 80°C.

The filters were prehybridised in DNA hybridisation buffer for 1 hr, then hybridised in the same buffer with the addition of a radioactive probe for 16 hr at 55°C to 65°C depending on the specificity of the probe. The filters were washed in 0.2 X SSC, 1% SDS at temperatures up to 55°C to remove background radioactivity. The filters were exposed to autoradiography film (Hyperfilm-MP, Amersham) and the developed autoradiograph aligned against the filters and agar plates to select recombinant clones.

2.2.6 Isolation of plasmid DNA by alkaline lysis

L-broth (100 ml) containing the appropriate antibiotics was innoculated with plasmid containing bacteria and incubated at 37°C overnight. The cultures were centrifuged for 15 min at 7000 g and the pellet suspended in 4 ml of lysis buffer 1 (50 mM glucose, 25 mM Tris.HCl, 10 mM EDTA, 20 mg/ml lysozyme [Boehringer-Mannheim]). After a 5 min incubation at room temperature, 8 ml of buffer 2 (0.2 M NaOH, 1% SDS) was added and incubated on ice for 10 min, followed by 6 ml of buffer 3 (5 M K-acetate, pH 4.8) and a further 10 min incubation on ice. After centrifugation at 17000 g for 20 min, the supernatant was removed and

phenol/chloroform extracted. The DNA was precipitated by addition of 0.6 volumes of isopropanol. The DNA was pelleted by centrifugation at 10000 g for 30 min, washed in 70% ethanol, and dissolved in 0.5 ml TE. The DNA was treated with RNAase (100 μg/ml, Boehringer-Mannheim) for 30 min at 37°C, then pronase (100 μg/ml, Boehringer-Mannheim) for 30 min at 37°C, phenol/chloroform extracted and ethanol precipitated at -20°C overnight. The DNA was pelleted, washed with 70% ethanol, dried and dissolved in 200 μl of TE.

2.2.7 Caesium chloride purification of DNA

DNA was isolated by alkaline lysis from 500 ml cultures, and dissolved in 2.4 ml TE, then purified with caesium chloride as follows. To the DNA solution was added 0.6 ml of 2.5 M NaCl/50% PEG (polyethylene glycol). This was mixed by inversion and incubated on ice for 2 hr. The DNA was pelleted by centrifugation at 3000 g for 1.5 min, and dissolved in 5 ml TES. Caesium chloride (8 g) and ethidium bromide (0.6 ml, of 10 mg/ml) was added to the DNA and incubated on ice for 30 min. The solution was centrifuged at12000 g for 30 min to pellet the PEG precipitate. The DNA solution was decanted, and 1.5 ml TES added. The solution was centrifuged at 100000 g for 40 hr at 18°C. The lower DNA band was removed with a syringe and extracted 4 times with n-butanol equilibrated against TE to remove the ethidium bromide. The DNA was dialysed against TE buffer.

2.3 Genomic DNA and RNA isolation and analysis

Unless otherwise specified the DNA and RNA analysis techniques used were as outlined in Sambrook *et al.* (1989).

2.3.1 Isolation of genomic DNA from frozen tissue

Frozen tissue (up to 1 g) was ground under liquid nitrogen, then homogenized in 10 ml of ice cold N.K.M buffer (150 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$). The nuclei were pelleted by centrifugation at 600 g for 5 min. The nuclei were suspended in 1 ml of N.K.M buffer and added dropwise to 5 ml of P.K.B. buffer (10 mM NaCl, 10 mM Tris.HCl, pH 8, 10 mM EDTA, 0.5% SDS) containing 100 μl of 20 mg/ml proteinase K (Boehringer-Mannheim). This was mixed gently and incubated at 37°C for 16 hr. The DNA solution was phenol/chloroform extracted twice, chloroform

extracted and the DNA was ethanol precipitated. The DNA was spooled, washed in 70%, then 100% ethanol, dried and dissolved in 0.5 ml TE. RNAase (Boehringer-Mannheim) was added to a final concentration of 100 μg/ml and incubated for 1 hr at 37°C. The DNA was recovered by phenol/chloroform extraction and ethanol precipitation. The DNA was then dialysed in TE and quantitiated by measuring the optical density at 260nm (1 O.D.= 50 μg/ml).

2.3.2 Southern analysis of genomic DNA

DNA (15 μg) was digested with appropriate restriction endonucleases (Pharmacia) and electrophoresed through a 1% agarose/1 X TBE gel at 25 volts, for 24 hr. The DNA was visualised by ethidium bromide staining and transferred to a nylon membrane (Zeta-Probe GT, BioRad) by alkaline blotting (0.4 M NaOH) for 16 hr. The membrane was rinsed in 2 X SSC, then baked for 30 min at 80°C.

The membrane was sandwiched between hardened filter paper (Whatman, #54), sealed in a plastic bag in hybridisation buffer (50% formamide for cDNA probes or 20% formamide for oligonucleotide probes) and prehybridised for 1 hr at 37°C. A radioactive probe was added to the same buffer and incubated for 16 to 24 hr at 37°C to 42°C, depending on the specificity of the probe.

The membrane was washed in SSC/SDS buffers generally to a stringency of 37°C in 0.2 X SSC, 1% SDS, although temperatures up to 65°C were used to remove background radioactivity. The membrane was sealed in plastic wrap, and exposed to autoradiography film (Hyperfilm MP, Amersham) in a cassette containing intensifying screens at -80°C.

2.3.3 Isolation of total RNA from frozen tissue

RNA was isolated by the method of Chomczynski and Sacchi (1987) with some modifications. Frozen tissue (0.5 g) was ground under liquid nitrogen and homogenized in 10 ml of guanidinium buffer (Solution D). To this was added, 1 ml of 2 M sodium acetate pH 4.5, 10 ml TE-saturated phenol, 2 ml chloroform, and 40 μl isoamyl alcohol, with mixing after each addition. After centrifugation (15000 g, 10 min) the aqueous layer was collected and the RNA precipitated by addition of an equal volume of isopropanol, and incubation for 16 hr at -20°C. The RNA was

recovered by centrifugation (10000 g, 15 min), washed with 70% ethanol, and dried. The pellet was dissolved in either 0.5% SDS or in DEPC-treated water if to be used for reverse-transcription. The RNA was quantitated by measuring the optical density at 260 nm (1 O.D.= 40 μg/ml). In some instances "mock extractions" were carried out in parallel with RNA isolations from cells or tissues. In these instances the isolation procedure was carried out as above but no tissue or cells were added to the Solution D. These extractions were used to check for contamination in the isolation materials.

2.3.4 Isolation of total RNA from cultured cells

RNA was isolated as above with some modification. RNA was isolated from adherent cells by removing the media, washing the cells with PBS, and adding 5 ml of Solution D directly to the cells. Cells in suspension were pelleted (1000g, 5 min), washed with PBS, and then solubilized in 5 ml of Solution D. Extractions were then carried out as above, except that all quantities were halved.

2.3.5 Northern analysis of RNA

RNA (typically 50 μg) was denatured by incubation at 65°C for 15 min in 16% formaldehyde and 45% deionised formamide. Once cool, RNA loading buffer and ethidium bromide (0.04 mg/ml final concentration) were added and the RNA electrophoresed through a 1.2% agarose denaturing gel (2.2 M formaldehyde in 1 X MOPS buffer). RNA was transferred to a nylon membrane (Zeta-Probe GT, BioRad) by alkaline blotting (0.05 M NaOH) for 6 to 8 hr. The membrane was washed in 2 X SSC then baked for 30 min at 80°C.

The membrane was sandwiched between pieces of hardened filter paper (Whatman, #54) and sealed into a plastic bag with hybridisation buffer and a radiolabelled probe. For cDNA probes a 50% formamide buffer was used, while for oligonucleotide probes a 20% formamide buffer was used. The membrane was hybridised for 16 to 24 hr at 37°C to 42°C depending on the specificity of the probe. The membrane was washed in SSC/SDS buffers generally to a stringency of 37°C in 0.2 X SSC, 1%SDS, although temperatures up to 65°C were used to remove background radioactivity.

The membrane was sealed in plastic film (Gladwrap) and exposed to autoradiographic film (Hyperfilm MP, Amersham) in a cassette with intensifying screens (Amersham), at -80°C. The autoradiographic film was pre-flashed using the Sensitize™ Pre-flash unit (Amersham) before exposure to the membrane.

2.3.6 Removing probes from nylon membranes

Radioactive probes were stripped from nylon membranes (Zeta-Probe GT, Bio Rad) by incubation in 0.5% SDS, 0.1 X SSC for 2 X 20 min at 95°C. Membranes were rinsed in 0.2 X SSC and stored between sheets of blotting paper.

2.3.7 cDNA probes

- general MT probe:

A 279 bp human *MT2A* RT-PCR transcript produced by RT-PCR of HeLa cell RNA and cloned into pSP64 (as described in F.A.Stennard, Ph.D. Thesis).

- human *MT1B* gene-specific probe:

The *Hin*dIII/*Bam*HI 5' flanking region of the human *MT1B* gene (as described in A.K.West, Ph.D. Thesis).

- human β-actin probe

Approximately full length cDNA for human fibroblast cytoplasmic β-actin cloned into the Okayama-Berg cDNA cloning expression vector (Ponte *et al.*, 1984).

- Glial fibrillary acidic protein (GFAP) probe

Mouse GFAP cDNA cloned into pBR322 (Lewis *et al.*, 1984).

2.3.8 Radiolabelling of cDNA probes

Plasmids containing the required cDNA insert were digested with restriction endonucleases (Pharmacia). The released cDNA insert was purified as outlined in section 2.2.1. The DNA (25-50 ng) was denatured by boiling for 5 min and labelled with α-^{32}PdCTP (Bresatec) in the following reaction using the ^{T7}QuickPrime Kit (Pharmacia):

34 µl denatured DNA

10 µl reagent mix

5 µl α-^{32}PdCTP (3000 Ci/mmol)

1 µl T7 DNA Polymerase

The reaction was incubated for 30 min at 37°C, and the unincorporated label removed by running the reaction through a G50 Sephadex column. The labelled cDNA was denatured by boiling for 5 min before addition to the hybridisation buffer. Hybridisation of cDNA probes was in hybridisation buffer containing 50% formamide.

2.3.9 Oligonucleotide probes

The following oligonucleotides were used in northern analysis or *in situ* hybridisation. The oligonucleotides hybridise to the 3' or 5' flanking regions of the respective genes, and are designed such that there is no crossreactivity with the other human MT genes. The oligonucleotides were prepared by the Bresatec or Auspep companies.

Gene	**Oligonucleotide**
human *MT2A* (20mer)	5'-ATC CAG GTT TGT GGA AGT CG-3'
human *MT1A* (20mer)	5'-ATG GGT CAG GGT TGT ATG GA-3'
human *MT1E* (20mer)	5'-CAA AGG GGA TGC TGG AGC TC-3'
human *MT1F* (20mer)	5'-GAG AGA CTG GAC TTT CCA AG-3'
human *MT1G* (21mer)	5'-GGT CAC TCT ATT TGT ACT TGG-3'
human *MT1H* (24mer)	5'-CGT GTC ATT CTG TTT TCA TCT GAC-3'
human *MT1L* (18mer)	5'-AAA TGA CGG GAG AGG CGA-3'
human *MT1X* (24mer)	5'-GCT CTA TTT ACA TCT GAG AGC ACA-3'
human β-actin (21mer)	5'-CAC ACT TCA TGA TGG AGT TGA-3'
sheep MT-III (18mer)	5'-TTT CAC ACG GGA CAG TGG-3'
conserved MT-I/II (19mer)	5'-GCA GCC CTG GGC ACA CTT G-3'

2.3.10 Radiolabelling of oligonucleotide probes

Oligonucleotides were radiolabelled under the following conditions using reagents in a 5'-terminal kinasing kit (Bresatec) and γ-^{32}PATP (Bresatec):

2 µl 10 X TM buffer

2 µl 100 mM DTT

100-500 ng oligonucleotide

10 µl γ-^{32}PATP (4000 Ci/mmol)

sterile water to 19 µl

1 µl T4 polynucleotide kinase

The reaction was incubated for 20 min at 37°C, then the kinase was deactivated by heating to 65°C for 5 min. Unincorporated label was removed by loading the sample onto a G25 Sephadex column and centrifuging it at 800 g for 5 min. The eluted labelled oligonucleotide was boiled for 5 min before addition to hybridisation buffer. Hybridisation of oligonucleotide probes was in 20% formamide hybridisation buffer.

2.4 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Unless otherwise specified the techniques used were as outlined in Sambrook *et al.* (1989) with some modification:

2.4.1 Reverse Transcription

Total RNA (1 µg) was reverse transcribed under the following conditions, using reagents supplied by Promega:

4 µl 5 X First Strand Buffer (250 mM Tris.HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$)

2 µl DTT (1 mM stock, 100 µM final concentration)

4 µl dNTP (2.5 mM stock, 0.5 mM final concentration of each)

1 µl oligonucleotide (random hexamers or oligo $d(T)_{16}$, 10 µg/ml final concentration)

1µg RNA

1 µl RNasin

1 µl M-MLV Reverse Transcriptase (200 units)

DEPC-treated dd water to a final volume of 20 µl

The reaction was incubated at room temperature for 10 min, then 1 hr at 37°C. The reverse transcriptase was deactivated at 99°C for 5 min.

2.4.2 Polymerase Chain Reaction

DNA or cDNA was amplified by polymerase chain reaction under the following conditions:

2.5 µl 10 X PCR buffer (100 mM Tris.HCl, 15 mM $MgCl_2$, 500 mM KCl, pH 8.3)

2.5 µl dNTPs (2.5 mM stock, 250 µM final concentration, Promega)

1 µl forward primer (10 µM stock, 0.04 µM final concentration)

1 µl reverse primer (10 µM stock, 0.04 µM final concentration)

0.5 µl Taq (2.5 Units, Boehringer-Mannheim)

10 ng cDNA

dd water to a final volume of 25 µl

PCR was performed in a DNA thermocycler (Perkin Elmer Cetus), using the following cycle sequence:

95°C, 2 min

95°C, 1 min⎫

60°C, 1 min⎬ (35 cycles)

72°C, 1 min⎭

72°C, 7 min

In some circumstances an annealing temperature other than 60°C was used, and in these cases the annealing temperature is specifically indicated where appropriate in the text.

2.4.3 PCR primers

The following PCR primers were designed to the 5' and 3' flanking regions of the human MT genes, such that they do not amplify other MT genes. Those PCR primer pairs taken from published reports are referenced appropriately. Primers were obtained from Bresatec or Auspep.

Primer	**Primer sequence**
• human *MT2A* primers (F.A.Stennard, Ph.D. Thesis)	
hMT2A sense (20mer)	5'-GCA ACC TGT CCC GAC TCT AG-3'
hMT2A antisense (20mer)	5'-ATC CAG GTT TGT GGA AGT CG-3'
• human *MT1H* primers (F.A.Stennard, Ph.D. Thesis)	
hMT1H sense (20mer)	5'-CGT GTT CCA CTG CCT CTT CT-3'
hMT1H antisense (24mer)	5'-CGT GTC ATT CTG TTT TCA TCT GAC-3'

- human *MT1L* primers

hMT1L sense (18mer) 5'-TGC CCT CTC CCG TCA TTT-3'

hMT1L antisense (16mer) 5'-AGC AGG GCT GTC CCC A-3'

- human *MT1X* primers (F.A.Stennard, Ph.D. Thesis)

hMT1X sense (20mer) 5'-AAC TCC TGC TTC TCC TTG CC-3'

hMT1X antisense (24mer) 5'-GCT CTA TTT ACA TCT GAG AGC ACA-3'

- human MT-III primers (F.A.Stennard, Ph.D Thesis)

human MT-III sense (19mer) 5'-CGA CAT GGA CCC TGA GAC C-3'

human MT-III antisense (17mer) 5'-GCC TCA GCT GCC TCT CC-3'

- human actin primers (F.A.Stennard, Ph.D. Thesis)

human actin sense (20mer) 5'-CAT CCT CAC CCT GAA GTA CC-3'

human actin antisense (21mer) 5'-CAC ACT TCA TGA TGG AGT TGA-3

- mouse MT-III primers

mouse MT-III sense (20mer) 5'-TGG ATA TGG ACC CTG AGA CC-3'

mouse MT-III antisense #1 (16mer) 5'-CCT TGG CCC CCT CTT C-3'

mouse MT-III antisense #2 (19mer) 5'-GTT GTG CCC CAC CAG GGA C-3'

* the antisense #1 primer is designed to the 3'flanking region of the MT-III gene, the antisense #2 primer is designed to the third exon of the MT-III gene.

- MT-III degenerate primers (Palmiter *et al.*, 1992)

MT-III degenerate sense (11mer) 5'-ATG GA(CT) CC(AGCT) GA(AG) AC-3'

MT-III degenerate antisense (10mer) 5'-CA(CT) TT(CT) TC(AGCT) GC(CT) TC-3'

2.5 DNA sequencing

2.5.1 Sanger dideoxy sequencing

The Sanger dideoxy sequencing method used was as outlined in Sambrook *et al.* (1989). Double stranded fragments cloned into pUC19 were purified using Magic Minipreps (Promega) and sequenced using Sequenase version 2 (USB). The pUC

forward or reverse primers were annealed to the template to act as a priming site. Fragments incorporating ^{35}SdATP (Bresatec) were electrophoresed through polyacrylamide gradient gels and detected by autoradiography.

2.5.2 Automated sequencing

The ABI PRISM™ Dye Primer Cycle Sequencing Reaction Kit (Perkin Elmer Cetus) and ABI PRISM™ Dye Terminator Cycle Sequencing Reaction Kit (Perkin Elmer Cetus) were used for DNA sequencing. Double stranded plasmids in pUC19 or pBK-CMV were purified using the QIAGEN Plasmid mini kit (QIAGEN) and the Cycle Sequencing Reaction Kits were used according to the manufacturers instructions.

PCR products were purified for sequencing as outlined in section 2.2.1, and the ABI PRISM™ Dye Terminator Cycle Sequencing Reaction Kit used according to the manufacturers instructions. Primers specific for the PCR product including those outlined in section 2.4.3 were used. An ABI Prism 377 DNA Sequencer (Perkin Elmer Cetus) was used for automated sequencing.

2.6 Immunocytochemistry and *In situ* hybridisation

2.6.1 Fixation of Tissues

Tissues were fixed in Bouin's fixative for immunocytochemistry and in paraformaldehyde fixative for *in situ* hybridisation.

Immediately after dissection tissues were immersed in Bouin's fixative for 9-16 hr depending on the size of the tissue. Tissues were dehydrated through graded alcohols, cleared in chloroform and embedded in paraffin wax (MP 56°C). Sections were cut 5 μm thick and floated onto poly-L-lysine (Sigma) treated slides or silanized slides (DAKO).

Immediately after dissection tissues were immersed in paraformaldehyde fixative for 12-24 hr depending on the size of the tissue. Tissues were rinsed in 0.1 M phosphate buffer for 3 X 10 min, rinsed briefly in distilled water and dehydrated through graded alcohols. Tissues were cleared in chloroform and embedded in paraffin wax (MP 56°C). Sections were cut 5 μm thick and floated onto silanized slides (DAKO).

2.6.2 Immunocytochemical staining

The enzyme-labelled streptavidin technique was used for immunocytochemical staining (Giorno, 1984).

Sections were dewaxed in xylene (2 X 10 min) and rehydrated in graded alcohols (absolute ethanol, 5 min; 95% ethanol, 5 min). Endogenous peroxidase activity was removed by incubating the sections in 0.3% H_2O_2 in methanol for 30 min. Sections were further rehydrated (95% ethanol, 5 min; 70% ethanol, 5 min), then washed in phosphate buffered saline (PBS) containing 0.01% v/v nonidet P-40 (PBS-NiP) for 3 X 10 min. Sections were incubated in blocker solution; 10% gelatin solution (Amersham) in PBS-NiP for 30 min in a humidified box.

Primary antiserum, diluted in blocker solution was applied to the sections for 16 hr at 4°C in a humidified box. After washing in PBS-NiP for 3 X 10 min the sections were incubated in the blocker solution for 20 min. Secondary antibody, diluted in blocker solution was applied to the sections for 1 hr at 37°C. After washing and blocking the sections again, they were incubated for 1 hr at 37°C with peroxidase-conjugated streptavidin diluted in blocker solution. The sections were again washed in PBS-NiP for 3 X 10 min, then in PBS for 5 min and Tris buffered saline (TBS) for a further 5 min. Peroxidase activity was visualised with 0.02% 3,3'-diaminobenzidine (Sigma) and 0.003% H_2O_2 TBS. The sections were dehydrated in graded alcohols, cleared in xylene and mounted in D.P.X. (Koch-Light Laboratories, Ltd)

2.6.3 Fluorescent immunocytochemical staining

Tissue sections were double-labelled with 1: anti-MT and anti-GFAP, 2: anti-MT and anti-S100 and 3: anti-MT and anti-Gal-C antibodies. Immunoreactivity was visualised with fluorescent secondary antibodies. The antibody staining was visualised with either Fluoroscein anti-mouse IgG or with anti-rabbit IgG - Texas Red Avidin D. The same method as above was used for fluorescent labelling but with several modifications as follows. The sections were incubated with the secondary antibodies (fluoroscein anti-mouse IgG or anti-rabbit IgG) for 2 hr at 37°C and the Texas Red Avidin D for 1 hr at 37°C. After incubation with Texas Red the sections were washed

in PBS-NiP for 3 X 10 min, PBS for 5 min, mounted in buffered glycerol and photographed.

2.6.4 Controls for immunocytochemistry

Control sections for immunocytochemistry were prepared by the procedures outlined above but with the omission of one of the antibodies. Control sections were prepared omitting all primary antibodies, all secondary antibodies and also the peroxidase conjugated streptavidin and Texas Red Avidin.

Control sections were also prepared using anti-MT antibody which had been preadsorbed with horse kidney MT-I and -II protein (Sigma). An aliquot of anti-MT antibody was incubated with a 10:1 ratio of MT protein for 1 hr at 37°C, then 16 hr at 4°C. Before use the antibody was centrifuged at 13000 g for 15 min.

2.6.5 Antibodies and dilutions

The following antibodies were used in immunocytochemistry at the specified dilutions.

Immunocytochemistry		
Primary antibody	**Secondary antibody**	**Tertiary antibody**
Monoclonal anti-MT (E9, Accurate Chemical and Scientific Corporation), 1:50	Biotinylated anti-mouse IgG (Biogenex), 1:50	Peroxidase conjugated streptavidin (Biogenex), 1:100
Monoclonal anti-GFAP (Boehringer Mannheim), 1:4	Biotinylated anti-mouse IgG (Biogenex), 1:50	Peroxidase conjugated streptavidin (Biogenex), 1:100
Rabbit anti-S100 (DAKO), 1:50	Biotinylated anti-rabbit IgG (Biogenex), 1:50	Peroxidase conjugated streptavidin (Biogenex), 1:100
Monoclonal anti-PCNA (Zymed), 1:200	Biotinylated anti-mouse IgG (Biogenex), 1:50	Peroxidase conjugated streptavidin (Biogenex), 1:100
Fluorescent Immunocytochemistry		
Primary antibody	**Secondary antibody**	**Tertiary antibody**
Monoclonal anti-MT (E9, Accurate Chemical and Scientific Corporation), 1:25	Fluoroscein anti-mouse IgG (Vector), 1:200	-
Rabbit anti-GFAP (DAKO), 1:50	Biotinylated anti-rabbit IgG (Vector), 1:200	Texas Red Avidin D (Vector), 1:200
Rabbit anti-S100 (DAKO), 1:50	Biotinylated anti-rabbit IgG (Vector), 1:200	Texas Red Avidin D (Vector), 1:200
Rabbit anti-Gal-C (Sigma), 1:25	Biotinylated anti-rabbit IgG (Vector), 1:200	Texas Red Avidin D (Vector), 1:200

2.6.6 In situ hybridisation

In situ hybridisation was by the method described by Penschow and Coghlan (1994). Sections were dewaxed in xylene, rehydrated through alcohols and rinsed in distilled water. Sections were transferred to TE buffer (50 mM Tris.HCl, pH 7.5, 5 mM EDTA). An aliquot of 625 µl of frozen pronase E stock (40 mg/ml) which had been predigested for 4 hr at 37°C was added to 200 ml of prewarmed TE buffer. The sections were incubated in this buffer for 10 min at 37°C. The tissue sections were rinsed in 0.1 M phosphate buffer and post fixed in 4% paraformaldehyde for 10 min at room temperature. The slides were rinsed in 4 X SSC and transferred to prehybridisation buffer. Radiolabelled oligonucleotide probes were added to tissue sections at a concentration of 300 ng/ml in 40% formamide hybridisation buffer. Sections were covered with coverslips and groups of slides were covered with plastic film (Gladwrap) and hybridised in a humid chamber for 16 to 24 hr at 37°C for general probes and 42°C for specific probes. After hybridisation sections were washed briefly in 2 X SSC, 1 X SSC, followed by a 1 hr wash in 1 X SSC at 40°C and a 30 min wash in 1 X SSC at 60°C. Sections were washed in ethanol, dried, and exposed to autoradiography film (Hyperfilm MP, Amersham) and then to liquid emulsion (Amersham) according to the manufacturer's directions.

2.7 Tissue Culture

2.7.1 Media and Reagents

All media and reagents were of tissue culture grade:

- Dulbecco's modification of Eagle's Medium (DMEM, Commonwealth Serum Laboratories)
- Solution PS (Penicillin G, 5000 U/ml, streptomycin sulphate, 5000 µg/ml, Commonwealth Serum Laboratories)
- 200 mM glutamine solution (2.92%), (Commonwealth Serum Laboratories)
- RPMI-1640 medium (Sigma)
- Foetal bovine serum (FBS, Commonwealth Serum Laboratories)
- 10 X Hanks' balanced salts solution (modified) (10 X HBS, ICN)
- Dulbecco's phosphate buffered salts (modified, without calcium and magnesium) (PBS, ICN)

- calcium chloride (Sigma)
- HEPES (N-2-Hydroxyethyl piperazine-N-2-ethane sulphonic acid) (BDH)
- Trypsin-Versene (Commonwealth Serum Laboratories)
- All tissue culture flasks and equipment were from Falcon

2.7.2 Maintenance of cultured cell lines

Unless specified tissue culture techniques were as oulined by Adams (1980).

COS cells, HeLa cells and embryonic fibroblasts were maintained in Dulbecco's modification of Eagle's Medium (DMEM, CSL) supplemented with 10% FBS, 2% penicillin/streptomycin solution (solution PS, CSL) and 2% glutamine solution (200 mM glutamine solution, 2.92%, CSL). Cells were subcultured by removing the adherent cells from the bottom of the flask using trypsin versene. Cells were maintained in an atmosphere of 5% CO_2 at 37°C.

Lymphocyte cell lines (K562 and CEM) were maintained in RPMI-1640 medium (Sigma), supplemented with 5% FBS, 2% penicillin/streptomycin solution (PS solution, CSL) and 2% glutamine solution (200mM glutamine solution, CSL). Cells were maintained in an atmosphere of 5% CO_2 at 37°C.

2.7.3 Calcium phosphate mediated transfections

The calcium phosphate mediated transfection protocol was as described by Keown *et al.*, (1990). Adherent cells were grown to 50-80% confluency in 25 cm^2 tissue culture flasks. The media was replaced with 3.5 ml of fresh media 4 hours before transfection. Caesium chloride purified plasmid DNA (20 μg) was precipitated, washed with 70% ethanol and dissolved in sterile TE (220 μl). A 30 μl aliquot of 2 M $CaCl_2$ in 10 mM HEPES was added to the DNA solution and mixed well. The calcium/DNA solution was added slowly to 250 μl of 2 X HBS, 40 mM HEPES, pH 6.95-7.05, with vigorous bubbling, and incubated for 30 min at room temperature. This solution was added to the cells and incubated overnight at 37°C in 5% CO_2 overnight. After incubation, the cells were washed twice with DMEM and the media replaced.

2.7.4 Preparation of embryonic fibroblasts

Embryonic mouse fibroblasts were prepared by the method of Harvey *et al.* (1993), with some modification. Pregnant mice were sacrificed by carbon dioxide asphyxiation, and the E14 embryos removed into a sterile petri dish. In a laminar flow hood the embryos were placed in a clean petri dish and rinsed in PBS. The head, tail and liver of the embryos were removed and the remaining embryonic tissue (from 3-4 embryos) was homogenised by forcing the tissue through a 19 gauge needle on a syringe with 0.1 ml DMEM. A further 5 ml of DMEM was added to the homogenised tissue and centrifuged for 5 min at 1000 g. The DMEM was removed, the cells resuspended in 10 ml of fresh DMEM and recentrifuged. The cell pellet was resuspended in 5 ml of fresh DMEM (supplemented with FBS, glutamine and penicillin/streptomycin solution) and incubated for 6 hr in an atmosphere of 5% CO_2 at 37°C in a 25 cm^2 tissue culture flask. The medium was aspirated to remove clumps of tissues and cells which had not adhered to the bottom of the flask, and replaced with fresh medium.

2.7.5 Radiolabelling cells

Flasks of cells (25 cm^2) were radiolabelled with ^{35}S-L-cysteine (1188Ci/mmol, 10.09 mCi/ml; ICN). Media was removed from the cells and replaced with 1 ml of fresh media which is enough to cover the bottom of the tissue culture flask. ^{35}S-L-cysteine (200 μCi) was added to the cells and incubated for 16 hours in 5% CO_2 at 37°C. The media was removed from the cells, the cells were washed in PBS and harvested.

2.8 Protein isolation and gel electrophoresis

2.8.1 Isolation of protein from cultured cells

Cells were scraped from the bottom of tissue culture flasks in 1 ml PBS with a rubber policeman, centrifuged for 1 min at 13000 g and resuspended in 25 μl of protein isolation buffer (10 mM Tris.HCl, pH 7.4 / 0.15 M NaCl). Cells were lysed by freeze/thawing which involved submerging the cells alternately in a dry ice/ethanol bath and 37°C water bath. The cell membranes were removed by centrifugation for 5

min at 13000 g. The supernatant was heat denatured at 95°C for 2 min and the heat unstable proteins removed by centrifugation for 5 min at 13000 g.

2.8.2 Carboxymethylation of metallothionein protein

Carboxymethylation of proteins was by the method of Koizumi and Kimura, (1982). To 25 µl of protein solution was added 12.5 µl of Tris.HCl carboxymethylation buffer (0.2 M Tris.HCl, pH 8.8, 8% SDS, 50% glycerol) and 5 µl of 0.2 M dithiothreitol (Boehringer-Mannheim). This was boiled for 5 min and after cooling, 7.5 µl of 1 M recrystalized iodoacetic acid, pH 8 was added to the solution and heated to 50°C for 15 min. To the protein solution, 5 µl of 0.1% bromophenol blue was added before electrophoresis on a 15% polyacrylamide protein gel with a 4.5% stacking gel.

2.8.3 SDS polyacrylamide gel electrophoresis

SDSPAGE of proteins was carried out as outlined in Sambrook *et al.* (1989). A 15% polyacrylamide gel was prepared containing 11.25 ml of lower gel buffer (1.5 M Tris, 0.4% SDS, pH 8.8), 22.5 ml of acrylamide stock (30% acrylamide, 0.8% bisacrylamide) and 11.25 ml of distilled water. This was degased, 150 µl of 10% ammonium persulphate and 30 µl of TEMED added, the gel poured and allowed to set overnight. A 4.5% stacking gel, containing 5 ml stacking gel buffer (0.5 M Tris, 0.4% SDS, pH 6.8), 3 ml of acrylamide stock and 12 ml of distilled water was degased, 80 µl of 10% ammonium persulphate and 20 µl of TEMED added and poured above the 15% gel.

The gel was prerun in SDS running buffer (0.025 M Tris, 0.192 M glycine, 0.1% SDS) for 1 hr before the radioactive samples and protein markers were added and electrophoresed for 6 hr at 125 volts. Lanes containing the protein markers were cut off the gel, stained with coomassie blue R-250 (0.05% in 50% methanol, 10% acetic acid) and destained in 7% acetic acid/5% methanol. Lanes containing the radioactive protein samples were dried in a gel drier (Model 583, BioRad) for 2 hr at 60°C then 4 hr at 80°C, and exposed to autoradiography film (Hyperfilm-MP, Amersham).

2.8.4 β-galactosidase assays

Assays were as outlined in Sambrook *et al.* (1989) with some modification. Cell extract (30 µl) was incubated with 1 µl of Mg solution (0.1 M $MgCl_2$; 4.5 M β-mercaptoethanol), 22 µl of 1 X ONPG solution (4 mg/ml 2-nitrophenyl-β-D-galactopyranoside) and 47 µl of 0.1 M $NaHPO_4$. Reactions were incubated for 30 min at 37°C and stopped by addition of 167 ml of 1 M Na_2CO_3. Absorbances were measured at 420 nm using mock transfected cell extracts as a standard. Values for β-galactosidase activity were determined using a standard curve.

2.9 cDNA library construction

Poly(A)$^+$ mRNA was purified from total RNA using the Dynabeads mRNA Purification Kit (Dynal). Poly(A)$^+$ mRNA was then used in the construction of a cDNA library using the ZAP Express™ cDNA synthesis kit from Stratagene.

2.9.1 Preparation of poly(A)$^+$ mRNA

The Dynabeads mRNA Purification Kit (Dynal) was used to purify poly(A)$^+$ mRNA from total RNA according to the manufacturer's directions. Briefly, 100 µl of RNA (75 µg) was heated to 65°C for 2 min, then added to 1.0 mg of oligo$(dT)_{25}$ Dynabeads suspended in 2 X binding buffer (20 mM Tris.HCl, pH 7.5, 1.0 M LiCl, 2 mM EDTA). The RNA was hybridized to the beads for 5 min at room temperature, placed in the magnetic particle concentrater (Dynal MPC®-E1) for 30 sec and the supernatant removed. The beads were washed twice with 200 µl of washing buffer (10 mM Tris.HCl, pH 7.5, 0.15 M LiCl, 1 mM EDTA). The poly(A)$^+$ mRNA was eluted in 20 µl of elution solution (2 mM EDTA, pH 7.5) by heating at 65°C for 2 min, then removing the solution from the beads immediately. The integrity of the poly(A)$^+$ mRNA was checked by electrophoresis of a sample through 1% agarose/1 X TBE and visualisation by ethidium bromide staining.

2.9.2 cDNA synthesis and addition of EcoRI adaptors

All reagents were supplied in the ZAP Express™ cDNA synthesis kit from Stratagene unless otherwise specified.

First strand synthesis was performed under the following conditions:

5.0 µl 10 X first strand buffer

3.0 µl first strand methyl nucleotide mixture

2.0 µl linker-primer (1.4 µg/µl)

1.0 µl RNase Block Ribonuclease Inhibitor (40 U/µl)

37.5 µl poly(A)$^+$ RNA (3 µg) isolated as outlined above.

The template and primer were allowed to anneal for 10 min at room temperature, then 1.5 µl MMLV-RT (50 U/µl) added. A sample (5 µl) of the reaction was added to 0.5 µl of α-^{32}PdATP (3000 Ci/mmol, Bresatec). Both the reaction and radioactive sample were incubated at 37°C for 1 hr. A control first strand synthesis was set up as above using 5 µl of test poly(A)$^+$ RNA (0.2 µg/µl) provided with the kit. After incubation the radioactive sample was stored at -20°C for later analysis.

Second strand synthesis was performed under the following conditions:

45.0 µl first strand synthesis reaction

20.0 µl 10 X second strand buffer

6.0 µl second strand nucleotide mixture

88.9 µl sterile distilled water

2.0 µl α-^{32}PdATP (3000 Ci/mmol, Bresatec)

2.0 µl RNase H (1.5 U/µl)

11.1 µl DNA polymerase I (9.0 U/µl)

The reaction was incubated at 16°C for 2.5 hr. The cDNA termini was bluntended by adding 23 µl blunting dNTP mix and 2.0 µl cloned *Pfu* DNA polymerase (2.5 U/µl) to the second strand synthesis reaction. The reaction was incubated at 72°C for 30 min. After incubation the reaction was phenol/chloroform extracted, chloroform extracted then the cDNA precipitated overnight at -20°C by the addition of 20 µl 3 M sodium acetate and 400 µl 100% ethanol.

The cDNA was pelleted by centrifugation at 13000g for 60 min at 4°C. The radioactive supernatant was discarded and the pellet was washed with 500 µl of 70% ethanol. The pellet was lypholized until dry, then resuspended in 9.0 µl of *Eco*R I adapters and incubated at 4°C for 30 min. A sample (1.0 µl) of the second strand synthesis reaction was removed at this stage and loaded onto an alkaline agarose gel with the radioactive sample of first strand synthesis reaction. After electrophoresis the gel was dried and autoradiographed.

*Eco*RI adaptors were ligated to the cDNA by adding the following components to the cDNA dissolved in *Eco*RI adaptor solution:

1.0 µl 10 X ligase buffer

1.0 µl 10 mM rATP

1.0 µl T4 DNA ligase (4 U/µl)

The reaction was incubated overnight at 8°C, then the ligase was heat inactivated by incubation at 70°C for 30 min.

The *Eco*RI ends were kinased by addition of the following components to the above reaction:

1.0 µl 10 X ligase buffer

2.0 µl 10 mM rATP

6.0 µl sterile water

1.0 µl T4 polynucleotide kinase (10 U/µl)

The reaction was incubated for 30 min at 37°C, then heat inactivated by incubation for 30 min at 70°C.

*Xho*I digestion of the cDNA was performed by addition of 28.0 µl *Xho*I buffer supplement and 3.0 µl *Xho*I (40 U/µl) to the reaction above. The reaction was incubated for 1.5 hr at 37°C.

2.9.3 Size fractionation of cDNA

All reagents other than those required for electrophoresis were supplied with the ZAP Express™ cDNA synthesis kit from Stratagene.

Size fractionation of the cDNA was performed using a Sephacryl S-500 spin column. The spin column was prepared by pipetting the sephacryl into a 1 ml syringe with a cotton plug inserted in the bottom. The column was spun at 400 g for 2 min, refilled and spun again. The column was washed with 2 X 300 µl of 1 X STE. The cDNA was pipetted into the top of the column and centrifuged at 400 g for 2 min, and the first fraction was collected. More fractions were collected by adding 60 µl 1 X STE to the top of the column and centrifuging to collect the eluent. The size of the cDNA in each fraction was determined by running 10% of each fraction on a 5% (29:1) nondenaturing acrylamide gel. The cDNA fractions were phenol/chloroform extracted, chloroform extracted and ethanol precipitated by addition of 2 X volume of

100% ethanol. The cDNA was precipitated overnight at -20°C. The cDNA was pelleted by centrifugation at 13000 g for 1 hr at 4°C, washed with 200 µl of 70% ethanol and dried under vacuum. The cDNA pellets (fractions 1 to 5 combined) were resuspended in 10.5 µl sterile water.

2.9.4 Cloning of cDNA into the ZAP™ Express vector

All reagents were supplied with the ZAP Express™ cDNA synthesis kit from Stratagene. Ligation of the cDNA into the ZAP Express vector arms was performed under the following conditions:

2.5 µl resuspended cDNA (~100 ng, as determined by the cDNA ethidium bromide plate assay, section 2.10.1)

0.5 µl 10 X ligase buffer

0.5 µl 10 mM rATP (pH 7.5)

1.0 µl ZAP Express vector (1 µg/µl)

0.5 µl T4 DNA ligase (4 U/µl)

The reaction was incubated overnight at 12°C. A test ligation was set up as follows:

1.0 µl ZAP Express vector (1.0 µg/µl)

1.6 µl test insert (0.4 µg)

0.5 µl 10 X ligase buffer

0.5 µl 10 mM rATP (pH 7.5)

0.9 µl sterile water

0.5 µl T4 DNA ligase (4 U/µl)

2.9.5 Packaging a library

The cDNA library was packaged using the ZAP Express™ cDNA Gigapack® II Gold Cloning kit. Packaging of the cDNA library was carried out by adding 2.5 µl of ligation reaction to a tube (10 µl) of freeze-thaw extract (supplied in the ZAP Express™ cDNA Gigapack® II Gold Cloning kit) and placing it on ice. A sample (15 µl) of sonic extract (supplied in the ZAP Express™ cDNA Gigapack® II Gold Cloning kit) was added immediately with gentle mixing and the reaction incubated at 22°C for 2 hr. To this was added 500 µl of SM buffer and 20 µl of chloroform. After

brief centrifugation to sediment the debris, the supernatant was transferred to a new tube and stored at 4°C. As a positive control, 1 µl of λcI857 *Sam*7 wild-type lambda control DNA (0.2 µg, supplied in the ZAP Express™ cDNA Gigapack® II Gold Cloning kit) was packaged.

Plating of the library was carried out by adding 200 µl of XL1-Blue MRF' cells (supplied with the ZAP Express™ cDNA Gigapack® II Gold Cloning kit) at $O.D._{600}$ of 0.5 (see section 2.10.2) to 1 µl of the final packaged reaction and 1 µl of a 1/10 dilution. The phage were allowed to attach to the cells by incubation at 37°C for 15 min. The following components were added to the phage:

3 ml NZY top agar (48°C)

15 µl 0.5 M IPTG

50 µl X-gal (250 mg/ml in DMF)

This was plated immediately onto NZY agar plates and incubated overnight at 37°C. The packaged wildtype lambda control DNA was plated out as for the packaged library however VCS257 host cells were used instead of XL1-Blue MRF' cells

2.9.6 Amplification of the ZAP Express library

The primary library was amplified by mixing aliquots of library suspension containing up to 50000 plaque-forming bacteria with 600 µl of XL1-Blue MRF' cells at $O.D._{600}$ of 0.5 (see section 2.10.2), and incubating at 37°C for 15 min. Melted NZY top agar (6.5 ml) was added and the mixture poured onto fresh 150 mm NZY agar plates. The plates were incubated for 6-8 hr, so that the plaques were visible but less than 1 mm in size. The plates were overlayed with 10 ml of SM buffer and stored at 4°C overnight. The bacteriophage suspension was recovered from each plate and pooled. The plates were rinsed with 2 ml SM buffer, chloroform was added to the pooled suspension at 5% final concentration and incubated at room temperature for 15 min. The cell debris was removed by centrifugation at 500 g for 10 min, and 0.3 % final concentration of chloroform was added to the supernatant. Aliquots were stored at 4°C and in 7% DMSO at -80°C.

2.9.7 Screening the cDNA library

The cDNA library was titred to determine its concentration and plated onto 150 mm NZY agar plates at 15000 pfu/plate, by mixing an aliquot of the library with 600 µl of XL1-Blue MRF' cells at OD_{600} of 0.5 (see section 2.10.2). The phage were allowed to attach to the cells by incubation at 37°C for 15 min. NZY top agar (6.5 ml) was added and the mixture poured onto NZY agar plates. The plates were incubated for 8 hr at 37°C, then stored at 4°C overnight. The phage was transferred to a nitrocellulose membrane (0.45 µm, Schleicher and Schuell) by placing the membrane on the agar plate for 2 min, and for 4 min for a duplicate membrane. The orientation of the membrane was marked by pushing a needle with ink through the membrane and into the agar. The nitrocellulose was submerged in denaturing solution (1.5 M NaCl/0.5 M NaOH) for 2 min, then neutrilized in 1.5 M NaCl/0.5 M Tris.HCl, pH 8.0 for 5 min. The membrane was rinsed in 2 X SSC/0.2 M Tris.HCl, pH 7.5 for 30 sec, then baked under vacuum for 2 hr at 80°C. The membranes were prehybridised for 2 hr at 55°C in DNA hybridisation buffer then the radioactive cDNA probe added and the membranes hybridised overnight at 55°C. Membranes were washed in SDS/SSC to a stringency of 0.2 X SSC, 1% SDS at room temperature and exposed to autoradiography film (Hyperfilm MP, Amersham).

Positive plaques were identified by orienting the membranes with the developed autoradiography film and the original agar plates. Positive plaques selected from the agar plates were removed in a plug of agar with a pipette tip. The agar plug was added to 1 ml of SM buffer and 20 µl of chloroform. Aliquots (1 µl and and 1 µl of a 1/10 dilution) were plated onto NZY agar as before and incubated at 37°C overnight. Plaques were lifted onto nitrocellulose as before and hybridized with the radioactive probe. Subsequent screenings were performed until a positive plaque was isolated to purity.

2.9.8 Single-clone excision

The single-clone excision protocol was used to excise the pBK-CMV phagemid vector from the ZAP Express vector, using the ExAssist helper phage (Stratagene). Single-clone excision was performed on phage from a purified plaque

which had been stored in 0.5 ml SM buffer/20 µl chloroform at 4°C for 24 hours. The protocol involved combining the following:

200 µl XL1-Blue MRF' cells at OD_{600} 1.0 (see Preparation of host cells, section 2.10.2)

250 µl phage stock (containing > 1 X 10^5 phage particles)

1 µl ExAssist helper phage (>1 X 10^6 pfu/µl)

This was incubated at 37°C for 15 min, 3 ml NZY broth added and the incubation continued with shaking at 37°C for 3 hr. The culture was heated at 70°C for 20 min then centrifuged at 1000 g for 15 min. The supernatant was stored at 4°C. The phagemids were plated at two concentrations by combining the following

200 µl freshly grown XLOLR cells at OD_{600} 1.0 (see Preparation of host cells, section 2.10.2)

100 µl phage supernatant or 10 µl phage supernatant

This was incubated at 37°C for 15 min, 300 µl NZY broth added and the incubation continued for 45 min at 37°C. Aliquots (200 µl) were plated on LB-kanamycin plates and incubated overnight at 37°C.

2.10 Miscellaneous Procedures

2.10.1 Quantitation of cDNA-ethidium bromide plate assay

100 ml of 0.8% (w/v) agarose in TAE buffer was prepared. Ethidium bromide (10 µl of 10 mg/ml stock) was added to the cooled agarose, and poured into petri dishes. The plates were dried at 37°C, then spotted with 0.5 µl aliquots of DNA standard solutions (200 to 10 ng/µl solutions). A 0.5 µl aliquot of the sample cDNA was immediately spotted onto the plate adjacent to the standards. The samples were allowed to absorb for 15 min, the plate inverted and photographed under UV light and the concentration of the sample cDNA estimated in comparison to the standards.

2.10.2 Preparation of host bacteria

Bacterial glycerol stocks were streaked onto appropriate agar plates and incubated overnight at 37°C. An isolated colony was innoculated into 50 ml of appropriate media, and grown overnight at 30°C with shaking at 200 rpm to an OD_{600} of less than 1. The bacteria was pelleted by centrifugation at 500 g for 10 min. The

cells were gently resuspended in half the volume of sterile 10 mM $MgSO_4$, and stored at 4°C to be used within 48 hr. The cells were diluted to an OD_{600} of 0.5 in sterile 10 mM $MgSO_4$ and used immediately. For the single-clone excision protocol the cells were prepared as above, however they were used at an OD_{600} of 1.0.

The following table demonstrates the various host strains used in the preparation of the cDNA library and the conditions under which they were grown:

Host strain	Agar plates	Medium for glycerol culture	Medium for bacterial cultures
XL1-Blue MRF'	LB-tetracycline	LB-tetracycline	LB + 0.2% maltose-10mM $MgSO_4$
XLOLR	LB-tetracycline	LB-tetracycline	LB
VCS257	LB	LB	LB + 0.2% maltose-10mM $MgSO_4$

2.10.3 Preparation of TE-saturated phenol

Phenol was melted at 65°C, then 40% v/v TE buffer added, and shaken vigorously for 5 min. The phases were allowed to separate overnight at 4°C, the aqueous layer removed and repeated with 25% v/v TE buffer. After the phases had separated, most of the aqueous layer was removed and 8-hydroxyquinoline added to a final concentration of 0.1%.

2.10.4 Preparation of sephadex columns

Sephadex G50 or G25 was added to sterile distilled water, washed several times then equilibrated in TES. The slurry was autoclaved for 10 min and stored at 4°C. A G50 sephadex column was prepared by filling a 0.5 cm diameter glass column with sephadex to 1 cm in length. A G25 spin column was prepared by filling a 1 ml syringe with sephadex slurry and centrifuging in at 800 g for 5 min.

CHAPTER 3: EXPRESSION OF MT-I AND -II ISOFORMS IN THE DEVELOPING SHEEP BRAIN.

3.1 Introduction

3.1.1 Metallothionein in the brain

In 1983 a low molecular weight zinc-binding and zinc-inducible protein was detected in rat brain (Itoh *et al.*, 1983), and later determined to be a MT-like protein (Ebadi, 1986a). Since then MT has been identified in the brain of various species (e.g. Paliwal and Ebadi, 1989). More recently, the cellular localisation of MT in the brain has been examined by immunohistochemistry. In young rat brain, MT has been localised to ependyma, pia mater, arachnoid and also cells of the choroid plexus (Nishimura *et al.*, 1992), while in adult rat MT was detected in ependymal cells, choroid plexus as well as blood vessel (Nakajima *et al.*, 1989), and also in astrocytes throughout the brain (Young *et al.*, 1991; Young, 1994). Similarly, in the mouse, MT has been localised to ependymal cells, glia, arachnoid and pia mater (Nishimura *et al.*, 1992).

MT expression has also been examined in monkeys and humans. In the monkey brain, MT was detected in pia mater, ependymal cells and astrocytes (Suzuki *et al.*, 1992a). In the human, several studies have localised MT mainly in astrocytes (Nakajima *et al.*, 1991; Suzuki *et al.*, 1992b). The expression was found to be in a subset of astrocytes mainly in the grey matter, and mutually exclusive to GFAP (glial fibrillary acid protein) immunoreactive astrocytes which were found mainly in the white matter. Some cells at the junction between white and grey matter, however, were both MT and GFAP immunoreactive (Blaauwgeers *et al.*, 1993; 1994).

Studies examining MT expression during development have shown that MT levels in the brain increase dramatically after birth (Ebadi., 1986b), in contrast to the liver which has higher levels of expression during foetal development (e.g. Paynter *et al.*, 1990). However, no detailed studies have investigated the localisation of MT in the foetal brain. Suzuki *et al.* (1994) examined MT expression in human brain of several foetal ages. In the human brain, MT immunoreactivity was not detected at 21 weeks gestation, but was detected in astrocytes in grey and white matter at 35 weeks and 40 weeks gestation. In infant brain MT was also expressed in the pia mater and

adventitia of blood vessels. In the adult brain MT was detected in the pia mater, ependymal cells, astrocytes and adventitia of blood vessels. Other than this brief study, the localisation of MT in the developing brain has not been described.

Although the expression of MT in tissues such as the liver during development has been investigated in detail (Andersen *et al.*, 1983; Andrews *et al.*, 1984; Mercer and Grimes, 1986; Lehman-McKeeman *et al.*, 1988; Paynter *et al.*, 1990), less is known about MT regulation in the brain, and the factors important in its regulation in this tissue are still largely uninvestigated. Systemic factors are often excluded from the brain by the blood-brain barrier. For example, MT in the brain is induced by intracerebroventricular, but not peripheral administration of zinc (Ebadi, 1986a; Paliwal *et al.*, 1990). MT in the brain is also induced by stress (Hidalgo *et al.*, 1990; 1991), dexamethasone and corticosterone (Gasull *et al.*, 1994). Brain MT has also been shown to be induced by lipopolysaccharide (LPS) in both mouse (Searle *et al.*, 1984) and rat (Itano *et al.*, 1991).

3.1.2 Sheep metallothioneins

In the sheep, metallothioneins are encoded by a multigene family (Peterson *et al.*, 1988) and like in humans there are multiple MT-I isoforms (MT-Ia, MT-Ib and MT-Ic) and a single MT-II isoform (MT-II). The sheep MT genes have been demonstrated to be differentially regulated (Peterson and Mercer, 1988) as have the human MT-I and -II genes (e.g. Heguy *et al.*, 1986). In cultured fibroblasts only MT-II is responsive to dexamethasone treatment, while all genes are responsive to metals, although to differing degrees (Peterson and Mercer, 1988). In contrast, the mouse MT-I and -II isoforms have been shown to be coordinately regulated (e.g. Searle *et al.*, 1984). The sheep MT genes however have remained largely uninvestigated *in vivo*, although a study has documented expression of MT mRNA in the foetal lung (E120-132) in a study of late gestational lambs (Pitt *et al.*, 1992).

3.1.3 Development of the brain.

An outline of mammalian neurodevelopment is presented here, highlighting particular aspects which put in context the results presented in this chapter. Further

information can be found in the following texts: Arey, 1965; Larsen, 1993; Sadler, 1990; Jacobson, 1991.

The central nervous system develops from an indentation in the neural plate, which originates from ectoderm of the embryo. The indentation along the midline of the plate forms the neural groove. Fusion of the edges of this groove forms the neural tube which gives rise to the brain and spinal chord (as depicted in Figure 3.1). The brain consists of 3 general classes of cells: neurons, glia and ependymal cells, mainly originating from the neuroepithelial layer (as depicted in Figure 3.2). The ratio of neurons to neuroglia in the brain is in the range of 1:10-50. The neuroepithelial cells in the ventricular zones of the brain cycle through mitosis, the postmitotic cells moving outwards forming the subventricular and intermediate zones. The intermediate zone consists of differentiated neuroblasts and glioblasts. The remaining cells in the neuroepithelial layer differentiate into ependymal cells. Glial cells consist of several subclasses; astrocytes, oligodendrocytes, radial glial cells and microglia. Astrocytes develop from the gliablasts, whereas microglial cells develop from mesenchymal cells. The origin of oligodendrocytes is debatable, although evidence suggests that they originate from the same precursors as some astrocytes. Neuroblasts develop into neurons migrating into the intermediate zone where they settle and mature, losing their ability to divide. As more neuroblasts are formed they migrate through the settled neurons forming subsequent layers, and thus there is progressive expansion of the walls of the brain (as depicted in Figure 3.3). In this way the brain is said to develop from the "inside out". It is thought that the gliablasts and neuroblasts form at the same time, but the differentiation of glia is delayed and continues for some time after all the neurons are formed. Therefore migration of neurons is detected first, while glia migrate later in a second wave. The migration of neurons is thought to be guided by long thin cells, termed radial glial cells which stretch from the ventricular zone out to the marginal (outer) zone. Radial glial cells are only present in the foetal brain, and it is thought that some astroctyes develop from transformation of radial glial cells.

At first the outer marginal zone consists only of processes of the neuroepithelial cells, but gradually becomes invaded by processes of the post-mitotic cells in the intermediate zone, and later mature glial cells. Astrocytes near the surface

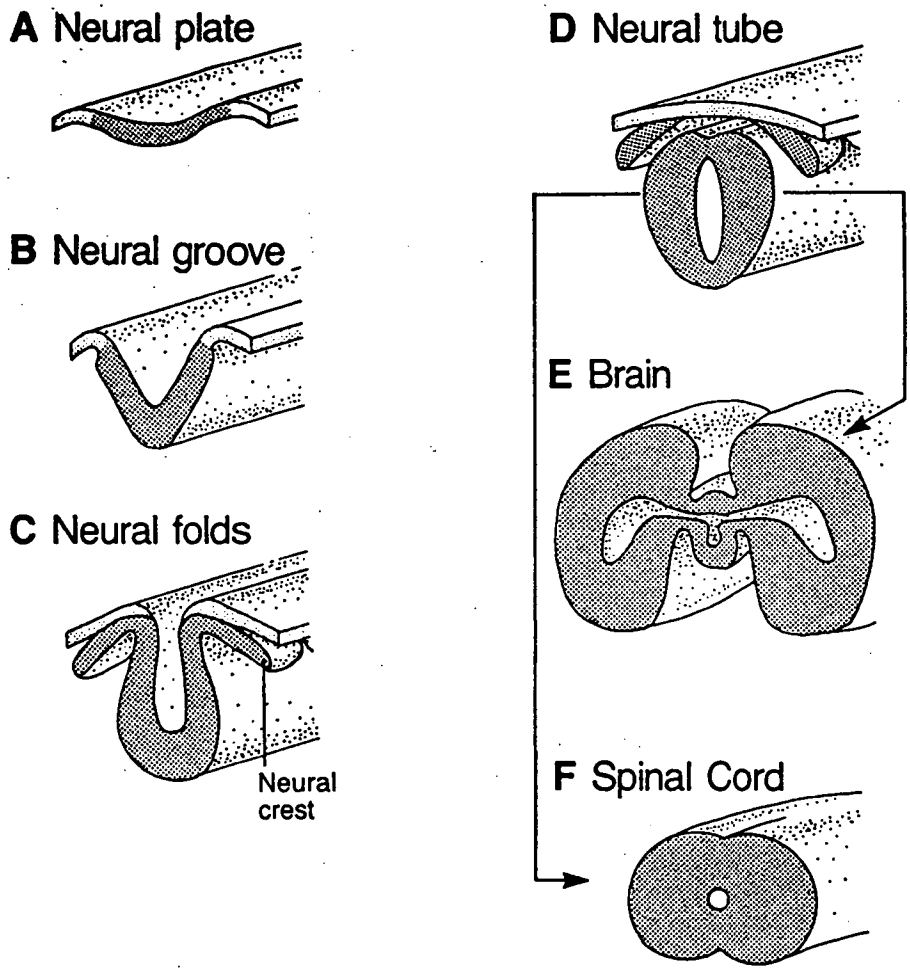


Figure 3.1 Diagrammatic representation of the development of the mammalian brain and spinal chord. Taken from: Introduction to Histology (D.H.Cormack).

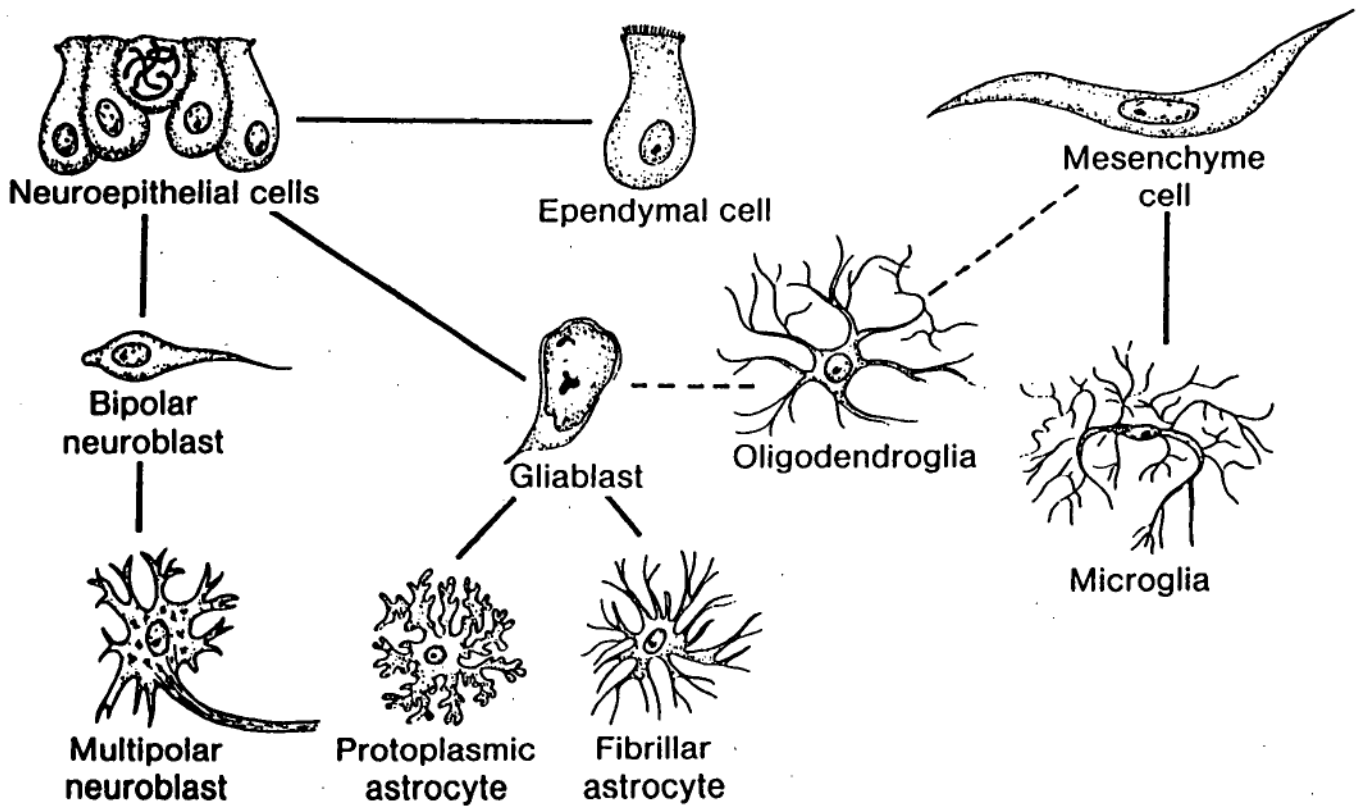


Figure 3.2 Diagrammatic representation of the cell types found in the brain and their origins
The neuroblasts, astrocytes and ependymal cells are shown to originate from the neuroepithelial cells. The origin of oligodendroglia remains unclear. Taken from: Langman's Medical Embryology, 6th edition (J.W.Sadler).

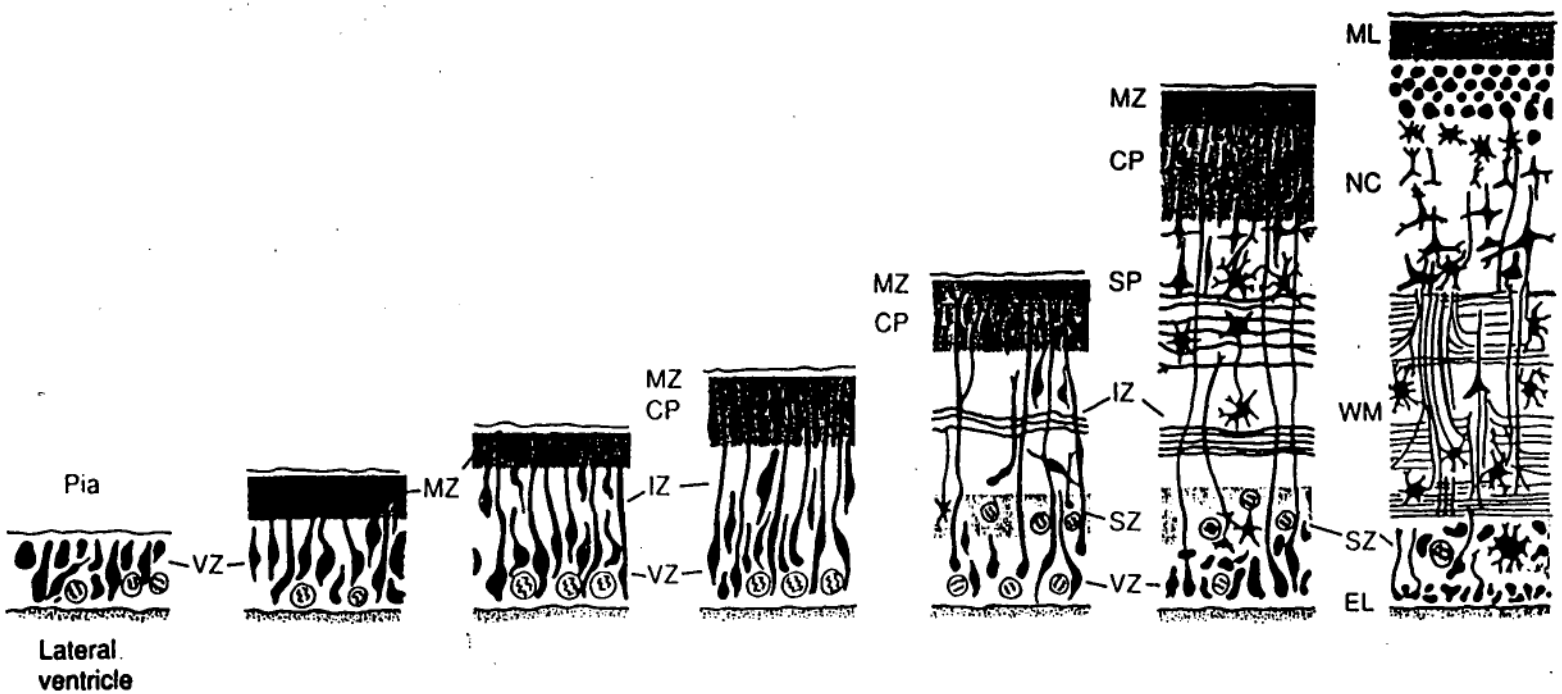


Figure 3.3 Representation of the differentiation of the cerebral neocortex
This diagram illustrates the expansion of the cerebrum from a single zone of cells to a multilayered structure containing differentiated neural cells. Immature cells are depicted moving outwards from the proliferative ventricular zone causing expansion of the walls of the brain. VZ, ventricular zone; MZ, marginal zone; IZ, intermediate zone; CP, cortical plate; SZ, subventricular zone; SP, subplate; EL, ependymal layer; WM, white matter; NC, neocortex; ML, molecular layer. Taken from: Human Embryology (W.J.Larsen).

of the brain usually have one or more processes extending to the pial surface of the brain where they expand to form 'endfeet' forming the glia limitans. Other astrocytes have endfeet associated with blood vessel walls. Astrocytes are classified phenotypically as protoplasmic (predominantly in the white matter) or fibrillar (predominantly in the grey matter). Oligodendrocytes found in both the white and grey matter provide the myelin sheath of neurons and are therefore often found associated with neurons.

Glial fibrillary acidic protein (GFAP), S100 and galactocerebroside (Gal-C) are frequently used as cell type markers in the brain. GFAP is a 51 kd protein of 10 nm intermediate filaments of astrocytes, and is an astrocyte specific marker. S100 proteins are a class of calcium binding proteins. They are dimers consisting of 2 subunits, and are markers of ependymal cells and astrocytes. There is some evidence that S100 is also expressed in oligodendrocytes (Janeczko, 1994). Gal-C is the major galactosphingolipid of myelin. It is expressed in cell membranes of oligodendrocytes in the central nervous system, and in Schwann cells in peripheral nerves.

The aim of this work was to examine the cell-type expression of MT-I and -II isoforms in the developing mammalian brain; to determine where and when MT is first expressed in the brain and whether the regional and cellular expression patterns change as the brain develops. Also, to determine whether the expression profiles of MT during development correlate with known changes in the physiology of brain development. The overall aim of these studies was to determine whether the expression profile of MT protein at the cellular level could provide insight into the function of MT in the brain in light of the roles played by the various cell-types in the adult brain and during development.

3.2 Results

Northern blotting and immunocytochemistry were used to examine the expression profile of MT-I and -II mRNA and protein in the developing sheep brain. Regional expression patterns of MT protein were compared with the astrocyte marker GFAP in the developing brain. Double-labeling fluorescent immunocytochemistry

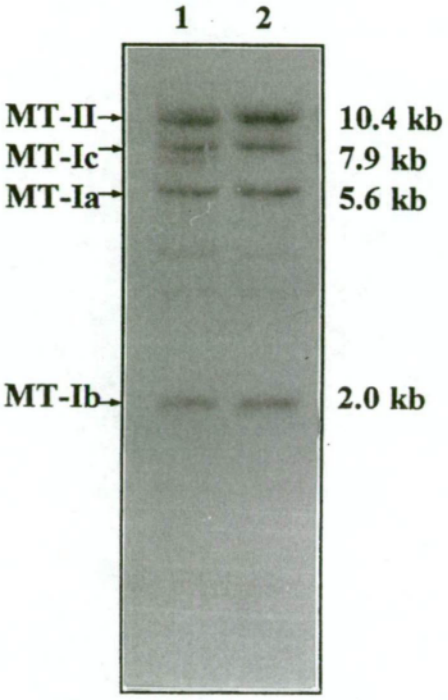
with anti-MT antibodies and anti-GFAP, anti-S100 or anti-Gal-C antibodies was used to confirm cell type expression of MT.

3.2.1 Selection of foetal ages.

The cortical plate of the neocortex develops in the sheep brain between E30 and E35, and by E40 all regions of the neocortex have a cortical plate of 1-2 cell layers. By E80, most of the layers of the cortex are present, and all the adult layers are present by about E120. The period of gestation is 147 days. Foetal samples were obtained from the following ages: For northern blotting ages E40, E58, E64, E72, E86 and E115 were used and for immunocytochemistry ages E34, E40, E58, E73, E80 and E116 were used. Samples represent the pre-cortical plate stage (E34; n=2), the cortical plate proper stage (E40, E58, E64, E72, E73; n=5), the late-sub-plate stage (E80, E86; n=3) and the "mature foetal" stage in which all of the adult layers are present (E115, E116; n=4), as discussed in Dziegielewska *et al.* (1993).

3.2.2 Expression of MT-I and -II mRNA in the foetal and adult sheep brain.

Southern blot analysis (section 2.3) of sheep genomic DNA was used to determine whether a human *MT2A* RT-PCR transcript hybridised to all sheep MT-I and -II genes, and was therefore a suitable probe to examine MT mRNA expression in the developing sheep brain. Genomic DNA from 2 sheep was digested with the restriction endonuclease, *Eco*RI, electrophoresed through 1.4% agarose and transferred to a nylon membrane by alkaline blotting. The membrane was hybridised with a radiolabelled probe for 16 hr at 37°C in 50% formamide hybridisation buffer, and washed to a stringency of 0.2 X SSC, 1% SDS at 37°C. The probe used was a 279 bp human *MT2A* RT-PCR transcript produced by RT-PCR of HeLa cell RNA using the following primers: 5'-GCAACCTGTCCCGACTCTAG-3' (sense) and 5'-ATCCAGGTTTGTGGAAGTCG-3' (antisense), and cloned into pSP64 (as described in F.A.Stennard, Ph.D. Thesis). Southern blot analysis revealed 4 bands previously attributed to sheep MT-I and -II genes (Peterson *et al.*, 1988) confirming that the



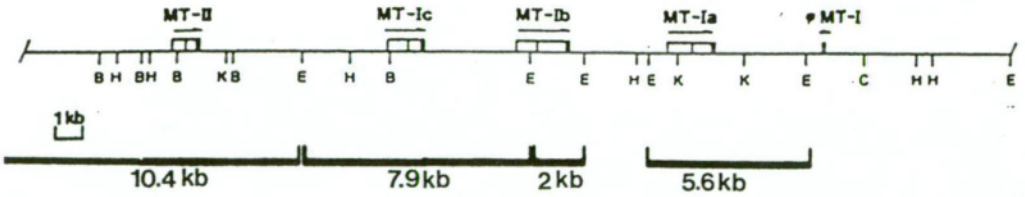


Figure 3.4 Southern blot analysis of the sheep genome with a human MT probe
A: Genomic DNA from 2 sheep (lanes 1 and 2) was digested with *Eco*RI and subjected to Southern blot analysis with a human *MT2A* RT-PCR transcript as a probe. The genes which hybridised to the probe and the sizes of the restriction fragments containing them are indicated.
B: A restriction map of the sheep MT locus demonstrates the restriction fragments which hybridised to the human *MT2A* RT-PCR transcript and the sheep MT genes which they represent. Restriction sites for the following restriction endonucleases are indicated: E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; C, *Cla*I; K, *Kpn*I. Modified from Peterson *et al.*, 1988.

probe used cross reacted with all known sheep MT genes (MT-Ia, -Ib, -Ic and -II), as depicted in Figure 3.4. Some other fainter bands were observed on the autoradiograph. It is possible they represent additional MT isogenes, or processed pseudogenes outside of the mapped locus. However, no other MT genes have been reported in the sheep, and cross hybridisation with these bands was significantly less than with the known genes, and in fact these bands were undetectable on lesser exposures of the blot.

RNA was isolated from the brains and livers of foetal and adult sheep and analysed by northern blotting (section 2.3) for the presence of metallothionein mRNA using the human *MT2A* RT-PCR transcript as a probe, under the conditions described above. As shown in Figure 3.5, high MT mRNA levels were observed at E115 and in the adult brain, but a very low signal was also detected at E72 and E86 which was more obvious following overexposure of the autoradiogram (not shown). In contrast, MT mRNA was observed in the liver at all foetal ages and in adults, and was most intense in the E40-E72 foetuses.

For comparison the levels of GFAP mRNA were examined using a mouse GFAP cDNA, isolated from a clone supplied by N.J.Cowan (Lewis *et al.*, 1984). This probe was hybridised at 37°C in 50% formamide hybridisation buffer for 16 hr and washed to a stringency of 0.2 X SSC, 1% SDS at 37°C. GFAP mRNA was present in the foetal sheep brain from E58. Like MT, highest levels were observed in the E115 and in the adult brain (Figure 3.5). As expected GFAP mRNA was not detected in the liver at any age. The northern blot was also hybridised to a human β-actin cDNA, which is often used to demonstrate that equal amounts of RNA are present in each lane (Figure 3.5). This probe was hybridised for 16 hr at 37°C in 50% formamide hybridisation buffer and washed to a stringency of 0.2 X SSC, 1% SDS at 37°C. However, β-actin can be subject to developmental regulation (e.g. Lazarini *et al.*, 1991) and therefore Figure 3.5 also shows the ethidium-bromide stained gel used for northern blotting, to demonstrate that equal levels of RNA were loaded in all tracks.

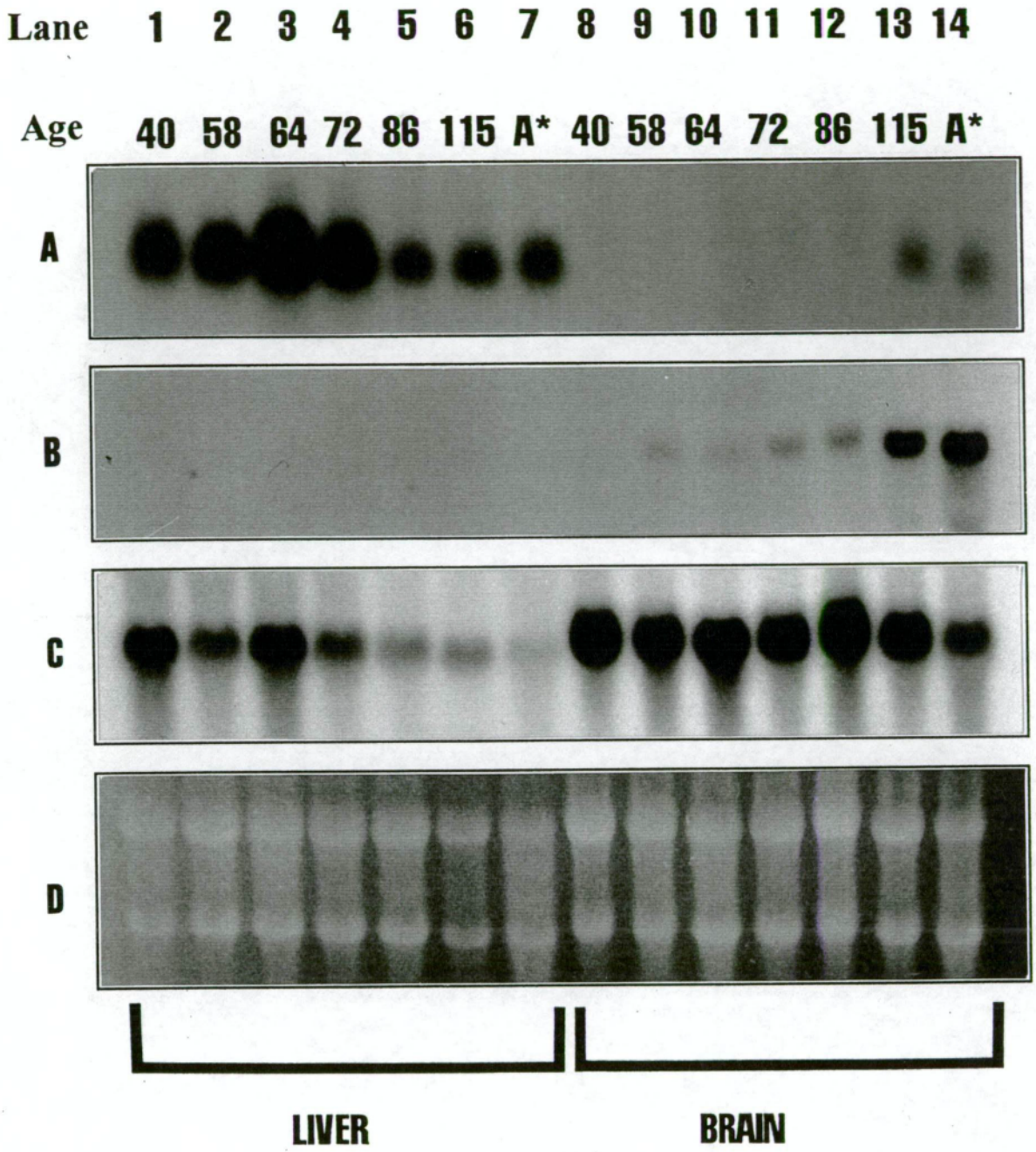


Figure 3.5 Expression of MT and GFAP mRNA in the brain and liver of the foetal and adult sheep

Total RNA was extracted from foetal (E40-E115) and adult (A*) tissues. After electrophoresis and northern blotting, the RNA was hybridised to MT (A), GFAP (B) and β-actin (C) radiolabelled probes. A photograph of the ethidium bromide stained gel is shown to demonstrate that approximately equal amounts of RNA were loaded in each lane (D).

3.2.3 Expression of MT-I and -II protein in the foetal and adult sheep brain.

An anti-MT antibody was used to examine the expression of MT-I and -II proteins in the developing sheep brain. The anti-MT antibody used was a monoclonal antibody raised against horse MT, and has been shown to cross react with both MT-I and -II isoforms (Jasani and Elmes, 1991). Cross reactivity with MT-III and MT-IV was not examined since these isoforms have not been identified in the sheep, and in other species their expression in the foetal brain is very low (Kobayashi *et al.*, 1993; Quaife *et al.*, 1994). However, it has been shown that this antibody does not cross react with mouse MT-III and MT-IV protein (West and Skabo, personal communication). The specificity of the immunostaining was confirmed by processing slides with the omission of primary antibody, secondary antibody or the peroxidase-conjugated streptavidin. In addition, antibody preadsorbed with a mixture of horse kidney MT-I and MT-II (section 2.6.4) was used in some experiments. In each case, no specific immunoreactivity was observed (Figure 3.6). Immunocytochemical staining with an anti-GFAP antibody was examined on similar sections in order to compare MT and GFAP regional expression patterns.

Pre-cortical plate and cortical plate proper stage (E34-E73)

The first positive but weak immunostaining against MT was observed amongst the population of proliferating cells in the ventricular zone of the diencephalon at E73 (data not shown). No staining of other cell types or in other regions of the brain were observed. At earlier ages, from E34 to E58, there was no MT immunostaining on sections of whole foetal brains, either of cellular or non-cellular regions. Similarly, no positive staining was observed with the astrocyte marker, GFAP.

Late sub-plate stage (E80)

In the E80 foetal brain MT staining was observed in radial glial cells, ventricular zone cells and astrocytes, as depicted in Figure 3.7. Little staining of astrocytes was observed in the cerebral cortex, with the exception of the molecular

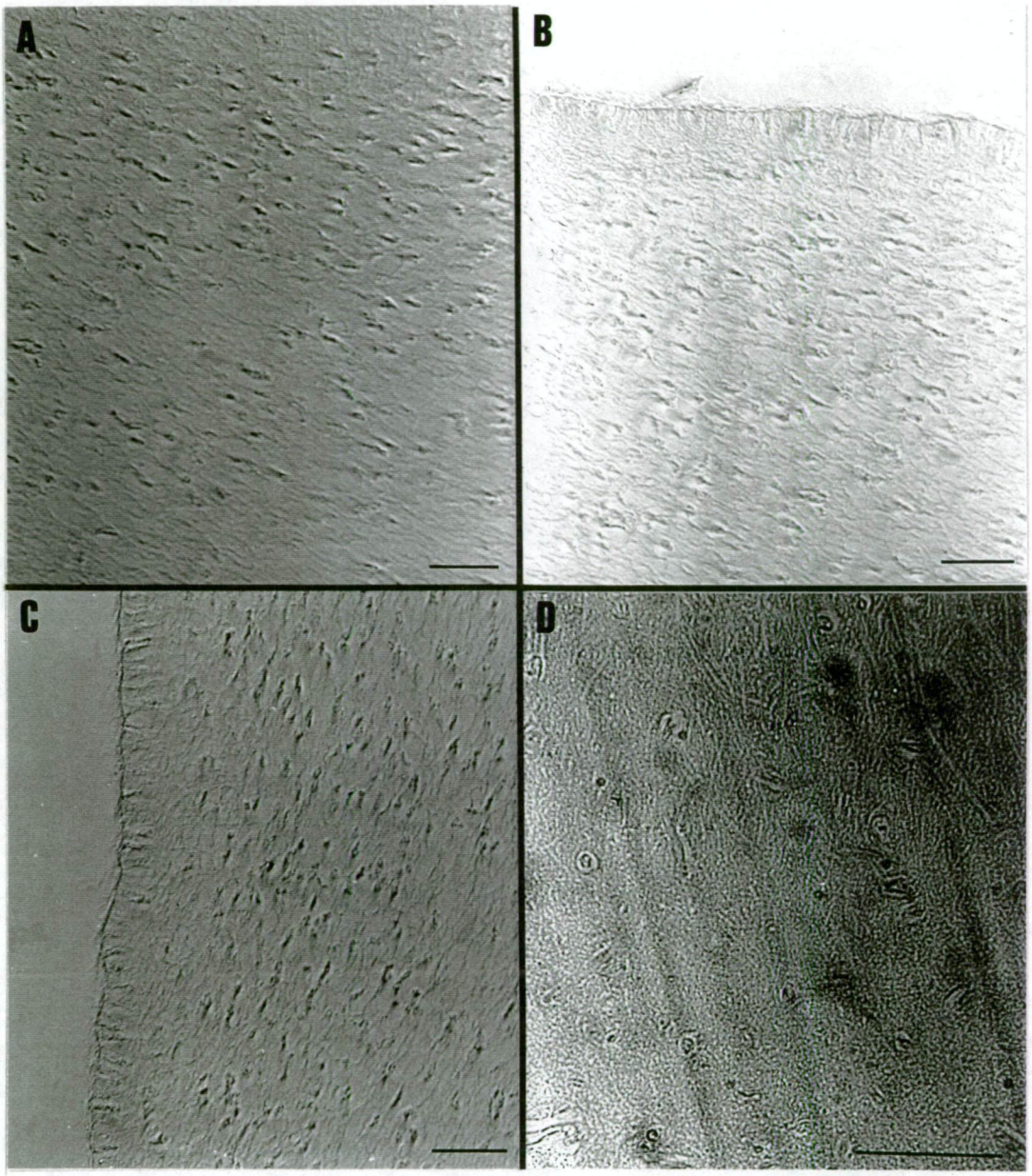
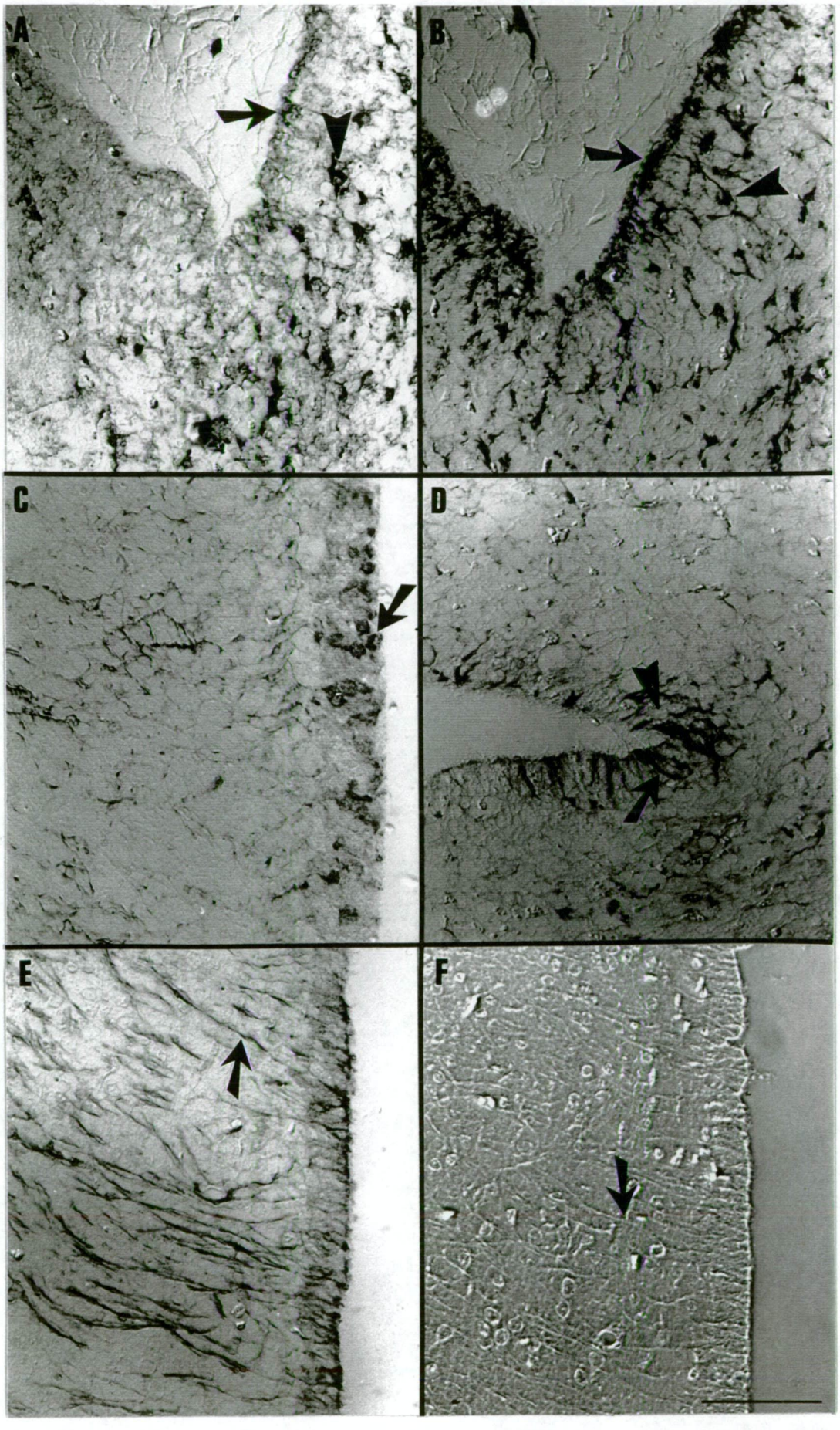


Figure 3.6: Controls for immunocytochemistry in the sheep brain.
Sections were processed for immunocytochemistry as outlined in materials and methods. Panels A and B show lack of specific staining resulting from the omission of primary antibody (A) and secondary antibody (B), respectively. Panel C demonstrates the lack of specific staining resulting from omission of the peroxidase-conjugated streptavidin. Panel A shows an area of grey matter in the cerebral cortex of the E116 foetal sheep brain. Panels B and C show areas of the molecular layer of the cortex of the E116 foetal sheep brain. Panel D demonstrates the lack of specific immunoreactivity for MT in the adult sheep brain cortex when the anti-MT antibody was preadsorbed with horse kidney MT-I and -II. Panels A to D, bars=50 μm.

Figure 3.7 MT and GFAP immunoreactivity in the E80 foetal sheep brain
Sections were stained with anti-MT (A,C,E) or anti-GFAP (B,D,F) antibodies. Panels A and B show glial cells (arrowheads) and the glial limiting membrane (arrows) of the molecular layer immunostained for MT (A) and GFAP (B), respectively. Panels C and D show the proliferative ventricular zone immunostained for MT and GFAP, respectively. Progenitor cells in the ventricular zone are immunoreactive for MT (arrow in C), but not GFAP (arrowhead in D), while radial glial cells are immunoreactive for GFAP (arrow in D). Panels E and F show radial glial cells extending from the ventricular zone at the base of the diencephalon which are MT immunoreactive (arrow in E) but not GFAP immunoreactive (arrow in F). Bar =50 μm (Panels A to F).


A
B
C
D
E
F

layer where glial cells (arrowhead) and also astrocyte end-feet (arrow) of the glial limiting membrane were MT positive (Figure 3.7A). GFAP staining was also observed in the molecular layer in astrocytes (arrowhead) and end-feet (arrow) of the glial limiting membrane (Figure 3.7B). MT staining of the glial limiting membrane was less complete and weaker than GFAP. Some immunoreactivity (both MT and GFAP) was also observed in astrocytic fibres in this region.

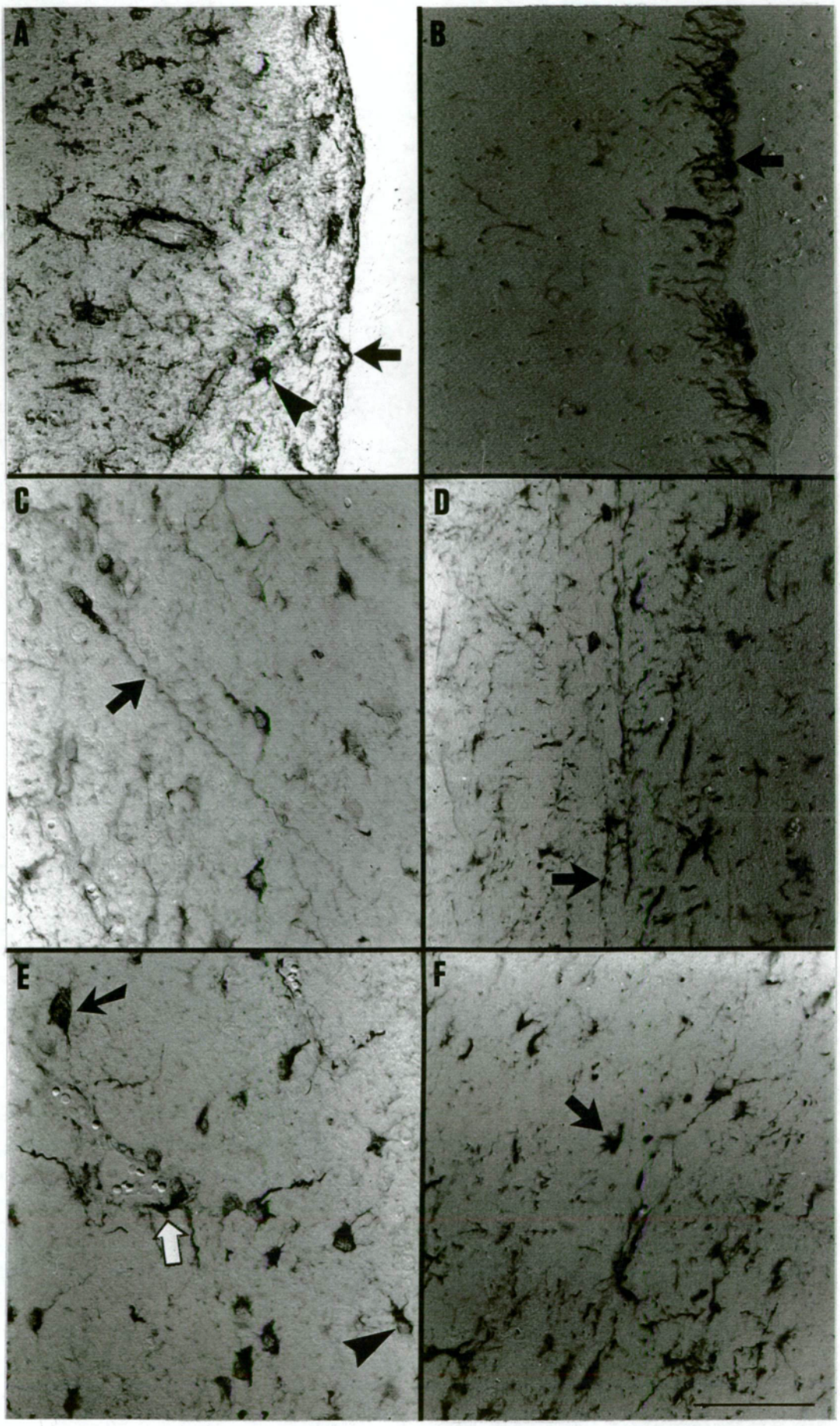
Some progenitor cells in the ventricular zones were intensely MT stained (see arrow, Figure 3.7C). GFAP staining was also present in the ventricular zones however in radial glial cells (e.g. arrow) rather than progenitor cells (e.g. arrowhead) confined to the proliferative ventricular zone (Figure 3.7D). MT positive cells having the morphological appearance of radial glial cells (arrow) which extend out from the ventricular zone were observed at the base of the diencephalon (Figure 3.7E). GFAP immunoreactivity was not observed in this region (Figure 3.7F), although radial glial cells which were GFAP positive were found in other areas, as indicated above (e.g. Figure 3.7D). Overall, the GFAP staining was similar to that of MT, but not identical.

Mature foetal stage (E116)

In the E116 foetal sheep brain MT staining was observed in oligodendrocytes, astrocytes, fibre tracts, and astrocyte end feet in the molecular layer, as depicted in Figure 3.8. The limiting glial membrane, consisting of astrocyte end-feet was particularly intensely stained (see arrow, Figure 3.8A). Some morphologically distinct MT positive astrocytes were also observed in this region (see arrowhead, Figure 3.8A). The limiting glial membrane was also intensely stained with GFAP (see arrow, Figure 3.8B), although the staining pattern was not identical to that of MT. The considerable punctate staining observed in the molecular layer is interpreted as being due to cross sections of processes from MT positive astrocytes.

Compact astrocytic fibre tracts (arrow, Figure 3.8C) were observed in the white matter of the cortex which were MT positive. GFAP positive fibre tracts were also seen in this region (see arrow, Figure 3.8D). MT positive cells which

Figure 3.8 MT and GFAP immunoreactivity in the E116 foetal sheep brain
Sections were stained with anti-MT (A,C,E) or anti-GFAP (B,D,F) antibodies. Panels A and B show the glial limiting membrane (arrows) of the molecular layer immunostained for MT (A) and GFAP (B), respectively. MT positive cells resembling astrocytes are also seen in the molecular layer (arrowhead in A). Astrocytic fibre tracts in the white matter of the cerebrum are both MT (Panel C, arrow) and GFAP (Panel D, arrow) immunoreactive. Panel E shows MT immunoreactive cells morphologically resembling astrocytes (arrow) and oligodendrocytes (arrowhead). Glial cells associated with blood vessels are also indicated (white arrow). Panel F shows GFAP immunoreactive astrocytes (arrow) in the white matter of the cerebrum. Bar=50 μm (Panels A to F).


A
B
C
D
E
F

morphologically resembled astrocytes were observed in the white matter of the cerebrum (see arrow Figure 3.8E). Smaller cells, morphologically resembling oligodendrocytes were also observed to be MT immunoreactive (see arrowhead, Figure 3.8E). GFAP positive astrocytes were also observed throughout the cerebrum (Figure 3.8F). MT positive cells were often found associated with blood vessels throughout the cortex (e.g. white arrow, Figure 3.8E).

Adult

In the adult sheep cortex MT immunoreactivity was observed in astrocytes (in the white matter of the cerebral cortex and outer molecular layer), in glial processes throughout the grey matter and in astrocyte end feet in the limiting glial membrane, as depicted in Figure 3.9. This figure depicts MT and GFAP expression in the adult brain, as detected by fluorescent immunocytochemistry. The same monoclonal antibodies were used as in the foetal brain, however the MT and GFAP immunoreactivities were visualised with the fluorescent secondary antibodies, FITC and Texas Red, respectively (as detailed in section 2.6.3).

In the molecular layer MT and GFAP staining of astrocytes and astrocyte end-feet (arrow) was similar although not identical (see Figure 3.9A,B). MT staining was confined to a narrower region of the molecular layer than GFAP. GFAP staining throughout the white matter was considerably more widespread than that for MT, although many MT immunoreactive astrocytes where present. There was widespread MT immunoreactivity throughout the grey matter probably due to the dense network of glial cell processes (Figure 3.9C,D), while GFAP staining was weaker in this region than in the white matter. A MT+/GFAP+ astrocyte in the grey matter is indicated (arrows, Figure 3.9C,D). No cells resembling oligodendrocytes were immunoreactive for MT in the adult brain.

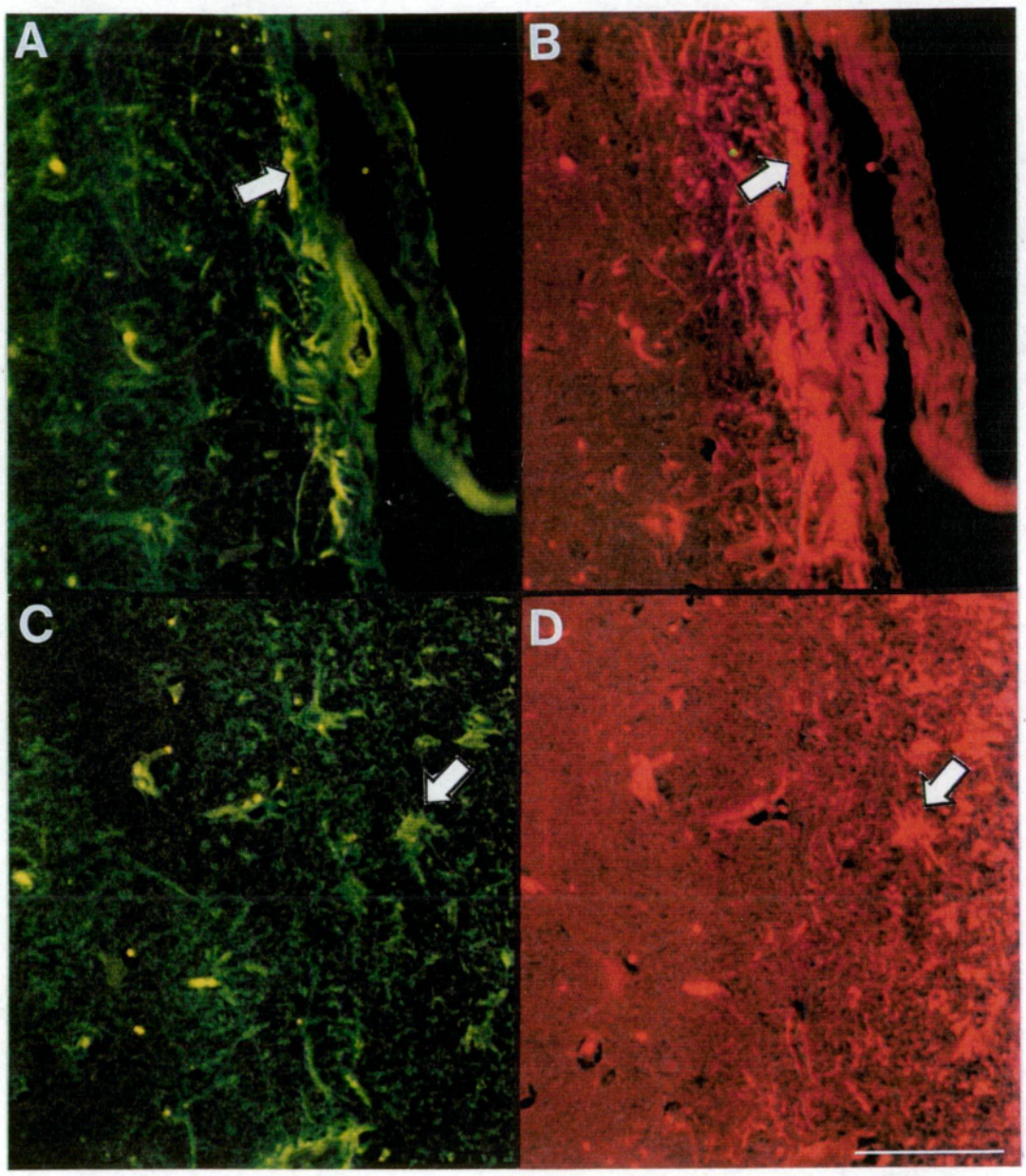


Figure 3.9: MT and GFAP immunoreactivity in the adult sheep brain.
Sections were stained with anti-MT (A,C) or anti-GFAP (B,D) antibodies. Panels A and B show the glial limiting membrane (arrows) of an area of the molecular layer of the cerebral cortex immunostained for MT (A) and GFAP (B), respectively. Panels C and D show an area of grey matter of the cerebral cortex. An MT+/GFAP+ astrocyte is indicated (arrows). Bar=50 μm (Panels A to D).

In summary, MT immunostaining was first observed in the foetal sheep brain at E73 at a similar time to when MT mRNA was initially observed by northern blotting (see Figure 3.5). MT immunoreactivity was detected in radial glial cells, astrocytes and oligodendrocytes, as outlined in Table 1. MT immunoreactivity was consistently observed in the molecular layer of the cerebral cortex, ventricular zones of the telencephalon and diencephalon, as well as throughout the white and grey matter of the cortex in the "mature" brain (E116 and adult). Shifts were observed in regional localisation and cell-type expression during development, for example, MT immunoreactivity was observed in oligodendrocytes in the foetal but not the adult brain. No MT immunoreactivity was observed in neurons at any stage of brain development.

3.2.4 Expression of MT and cellular markers in the E116 foetal sheep brain.

Double-labeling immunocytochemistry (section 2.6.3) was performed with anti-MT antisera and antisera to the cellular markers; GFAP, S100 and Gal-C to confirm correlations made earlier between MT immunoreactivity and cell type, based on cell morphology. MT was visualised using as a secondary antibody, anti-mouse IgG conjugated to FITC while the cellular markers GFAP, S100 and Gal-C were visualised using as a secondary antibody, anti-rabbit IgG conjugated to Texas Red. The specificity of the secondary antibodies was verified by processing sections as normal but either the anti-GFAP antibody (Figure 3.10A,B) or the anti-MT antibody (Figure 3.10C,D) was omitted. GFAP is a marker of astrocytes, S100 is a marker of astrocytes, ependymal cells and possibly oligodendrocytes, and Gal-C is a marker of oligodendrocytes (see section 3.1.3: Development of the brain)

Comparison of the MT staining pattern with that of GFAP, particularly in the E116 foetal brain (Figures 3.11 and 3.12), showed that the regional distribution of these proteins was similar but their expression patterns were not identical. For example, MT+/GFAP+ cells (i.e. astrocytes, e.g. arrows) were observed in the white matter of the cortex, but were in close proximity to MT+/GFAP- cells (e.g.

Age	Cell type	MT	GFAP
E73	•progenitor cells	+	-
E80	•radial glial cells	+	+
	•progenitor cells	+	-
	•astrocytes	+	+
	•astrocyte end feet	+	+
E116	•astrocytes	+	+
	•astrocyte end feet	+	+
	•oligodendrocytes	+	-
	•astrocytic fibre tracts	+	+
Adult	•astrocytes	+	+
	•astrocyte end feet	+	+

Table 3.1 Cellular localisation of MT and GFAP immunoreactivity in foetal and adult sheep brain

MT and GFAP proteins were localised by immunocytochemistry in foetal and adult sheep brain. This table outlines the cell types in which unambiguous MT and GFAP immunoreactivity was detected, from E73 when staining was first observed to adult.

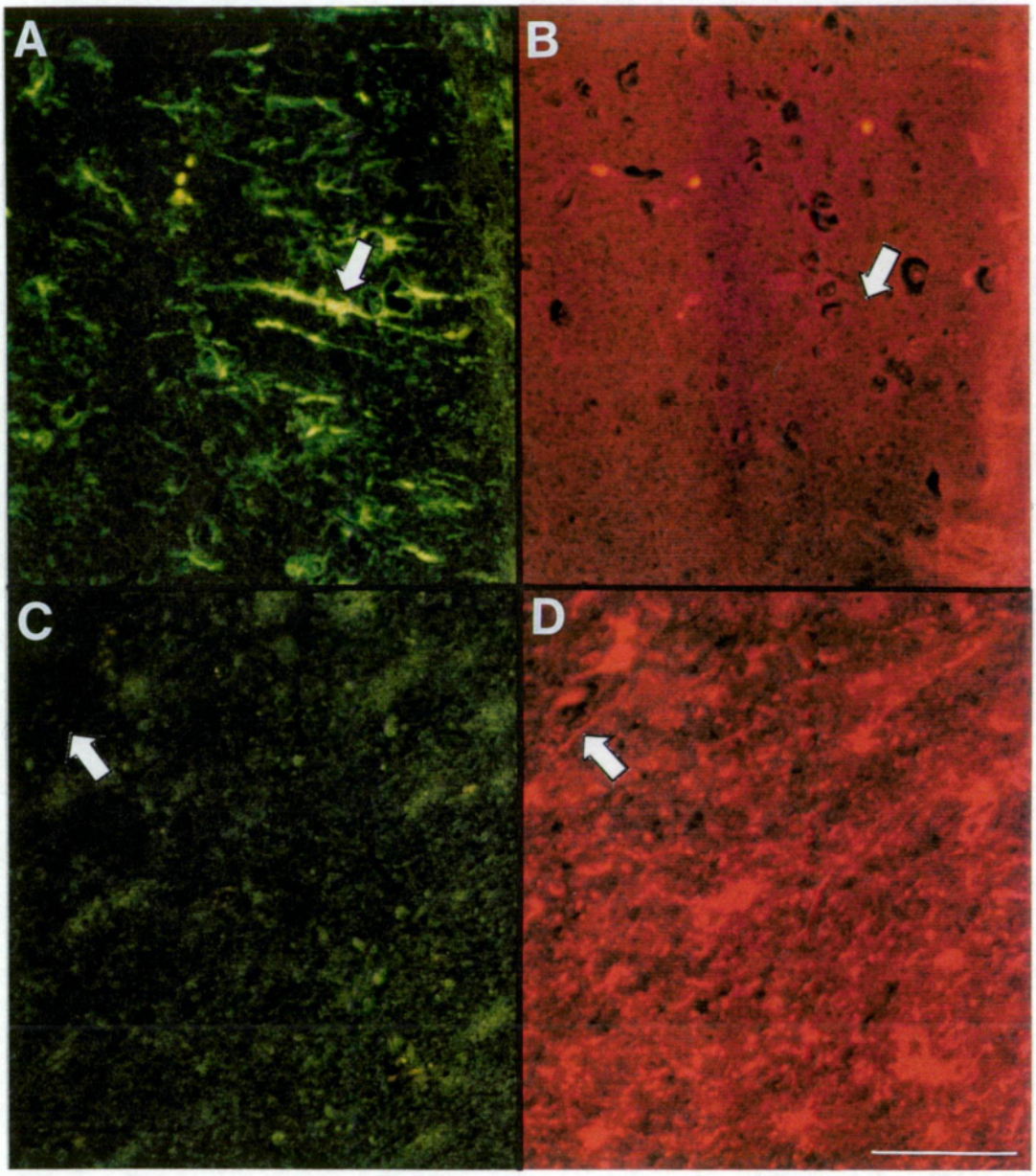
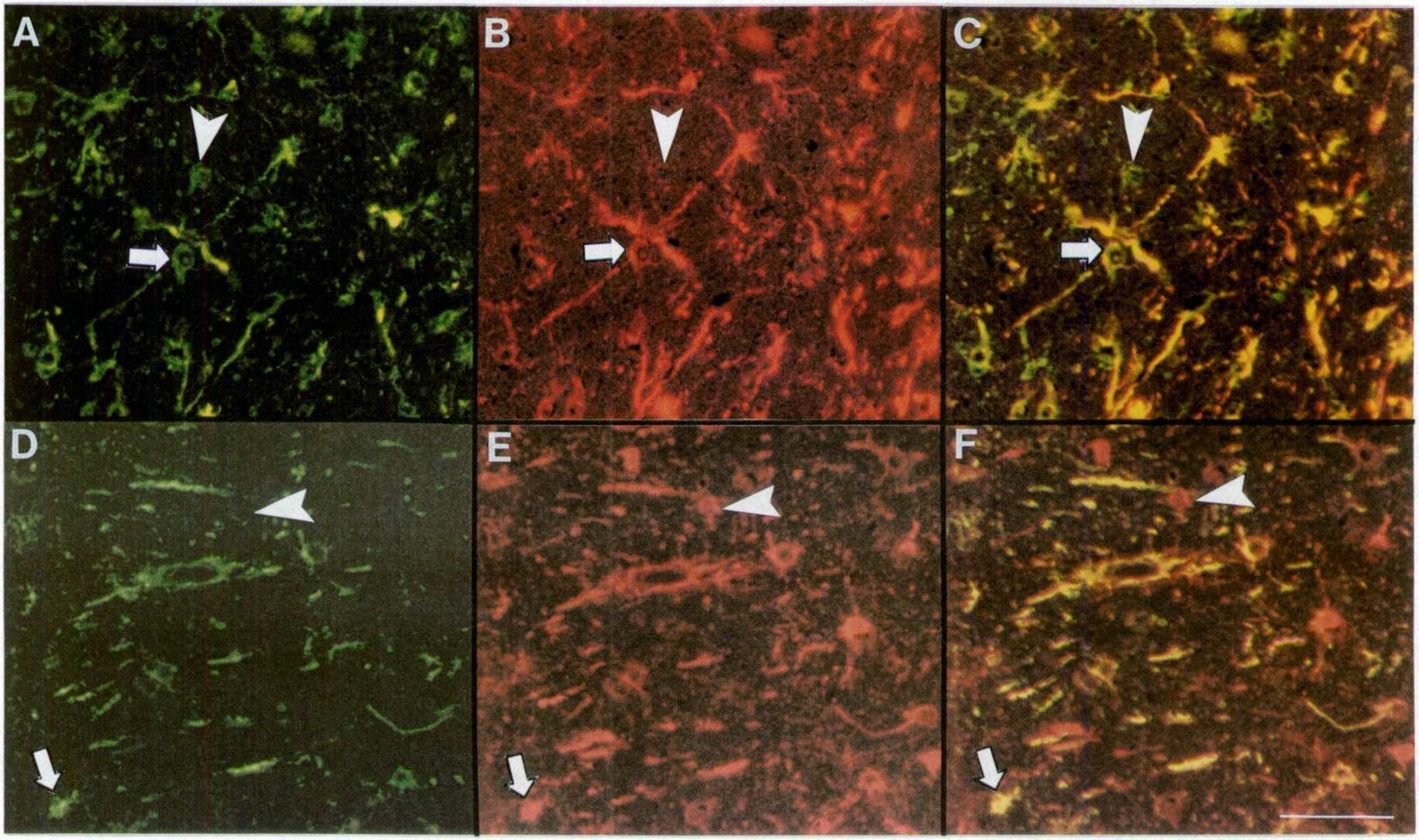


Figure 3.10: Controls for fluorescent immunocytochemistry in the E116 foetal sheep brain.
Single sections were processed with anti-MT and anti-GFAP antibodies, and visualised by conjugation to FITC and Texas Red, respectively. Panels A and B show a section processed with anti-MT but not anti-GFAP antibodies. MT immunoreactivity is visualised with FITC (eg arrow in A) but there is no cross reactivity with Texas Red (eg arrow in B). Panels C and D show a section processed with anti-GFAP but not anti-MT antibodies. GFAP immunoreactivity is visualised with Texas Red (eg arrow in C) but there is no cross reactivity with FITC (eg arrow in D). Bar=50 μm (Panels A to D).

Figure 3.11 MT and GFAP fluorescent double-labelling in the E116 foetal sheep brain
Panels A and B show immunostaining of a single section from the white matter of the cerebrum with anti-MT (A) and anti-GFAP (B) antibodies, respectively. Panel C is a double exposure photograph showing both MT (green) and GFAP (red) immunoreactivity. Cells expressing both proteins are therefore stained yellow. MT+/GFAP+ astrocytes (see arrows) can be seen in close proximity to MT+/GFAP- cells resembling oligodendrocytes (see arrowheads). Panels D and E show immunostaining of a single section of cortex immunostained with anti-MT (D) and anti-GFAP (E) antibodies. Panel F is a double exposure photograph showing both MT (green) and GFAP (red) immunoreactivity. Both MT+/GFAP+ astrocytes (see arrows) and MT-/GFAP+ astrocytes (see arrowheads) are shown. Bar=50 μm (Panels A to F).


A
B
C
D
E
F

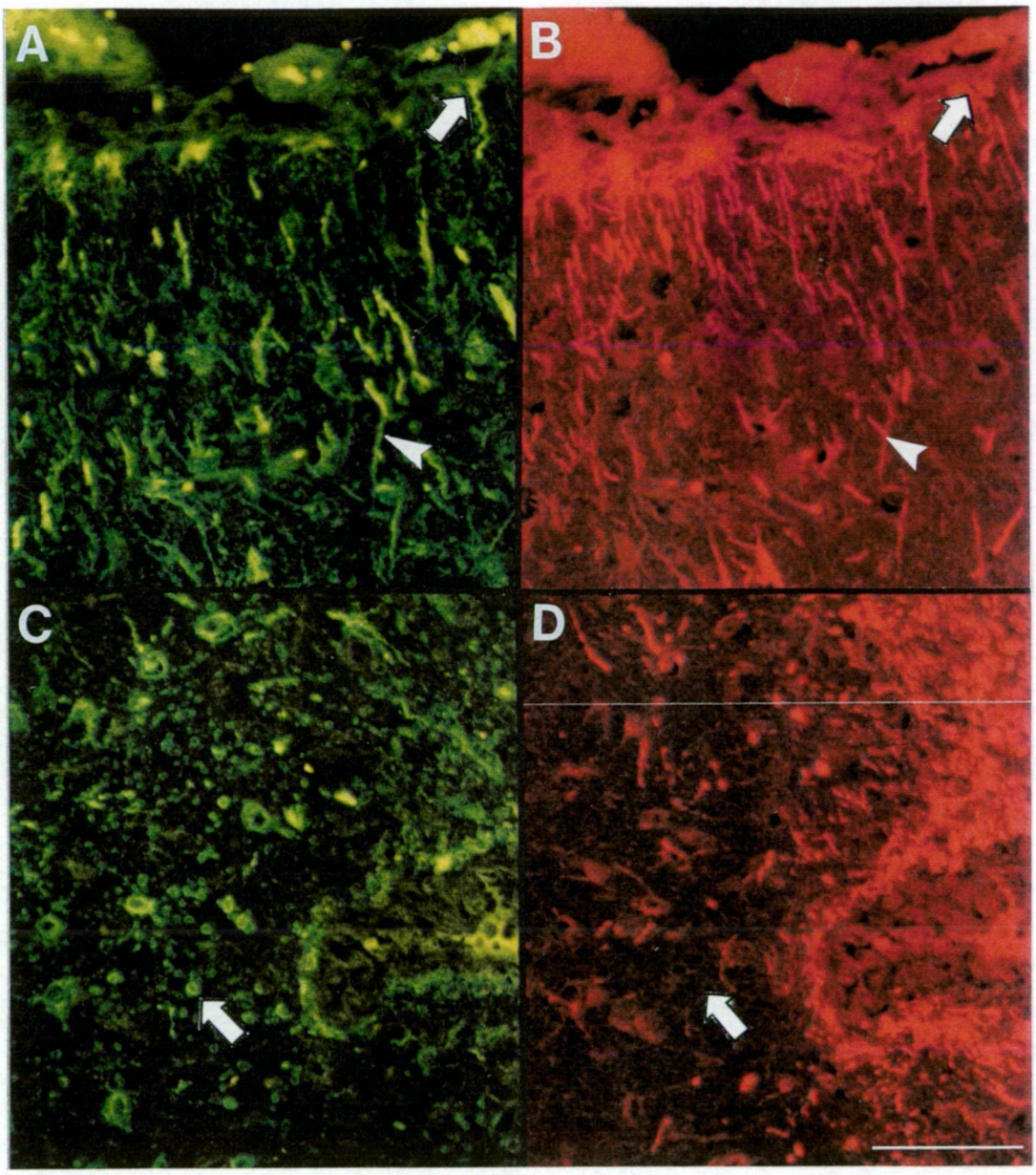


Figure 3.12: MT and GFAP fluorescent double-labelling in the E116 foetal sheep brain.

Panels A and B show a section of the molecular layer of the cerebral cortex double-stained with anti-MT (A) and anti-GFAP (B) antibodies.The glial limiting membrane is immunoreactive for both MT and GFAP (arrows), as are many glial processes (arrowheads). Panels C and D show a section of the grey matter of the diencephalon double-stained with anti-MT (C) and anti-GFAP (D) antibodies. Small circular profiles morphologically resembling oligodendrocyte processes encircling axons are MT+/GFAP- (eg arrows). Bar=50 μm (Panels A to D).

arrowheads) which morphologically resembled oligodendrocytes (see Figures 3.11A,B,C). Furthermore, not all astrocytes within a region expressed MT. As demonstrated in Figures 3.11D,E,F, GFAP+/MT- (e.g. arrowhead) as well as GFAP+/MT+ (e.g. arrow) cells were evident by fluorescent double labeling.

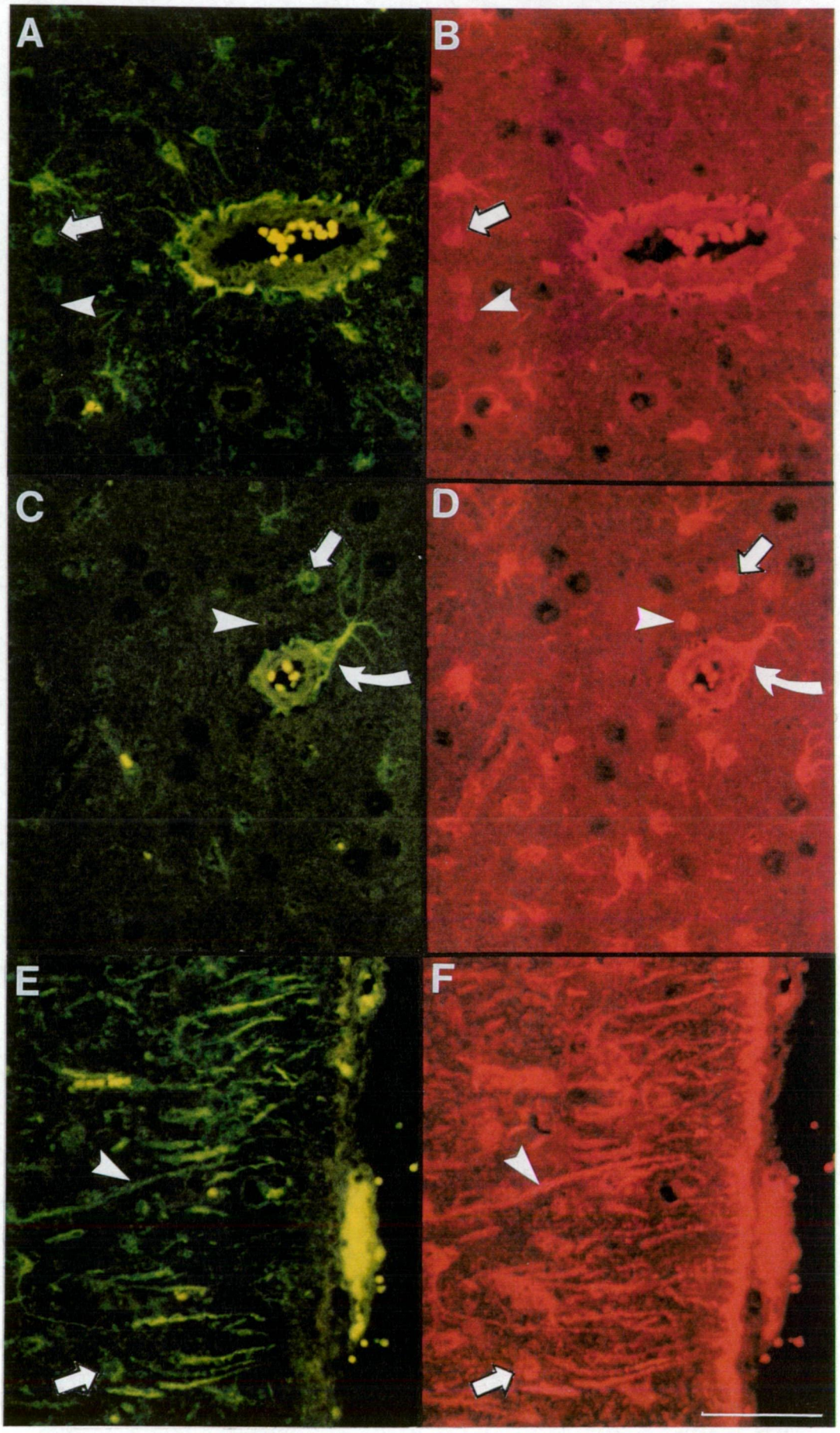
In the molecular layer both MT and GFAP staining was observed although again the staining was not identical. The staining of the glial limiting membrane with MT, appeared to be more limited than with GFAP. Astrocytic processes both within the glial limiting membrane (see arrows, Figure 3.12A and B) and in more internal regions of the molecular layer (see arrowheads, Figure 3.12A and B) were observed to be MT+/GFAP+. In areas characteristic of grey matter in the diencephalon, MT staining, which gave rise to small circular profiles surrounding non-staining cores was observed. These structures did not exhibit GFAP immunoreactivity, as demonstrated by double labeling immunocytochemistry (see arrows, Figure 3.12C and D, respectively) and it is proposed that they represent MT positive processes of oligodendrocytes encircling axons.

Double labeled sections then, illustrated cells which were MT+/GFAP-, MT+/GFAP+ and MT-/GFAP+ indicating that there is no simple correlation between the expression of these two proteins.

Staining with anti-S100 antibodies did not correlate well with the MT immunoreactivity. The S100 staining was much more widespread than MT and therefore did not help to clarify the identity of MT positive cells, although MT+/S100+ cells were observed (see Figure 3.13), and in fact MT staining appeared to be a subset of the S100+ cells. MT+/S100+ cells were observed, (see arrows, Figures 3.13A,B and C,D), in close proximity to morphologically similar cells which were MT-/S100+ (see arrowheads, Figures 3.13A,B and C,D). A MT+/S100+ glial cell with processes encircling a blood vessel is indicated (curved arrows, Figure 3.13C,D). There appear to be no cells which are MT+/S100-. Similarly, in the molecular layer, the S100 staining is more widespread than MT, and all MT+ cells/processes appear to be S100+ (e.g. arrows/arrowheads in Figures 3.13E,F).

Figure 3.13 MT and S100 fluorescent double-labelling in the E116 foetal sheep brain

Sections were stained with anti-MT (A,C,E) and anti-S100 (B,D,F) antibodies. Panels A and B shows immunostaining of a single section containing a major blood vessel in the white matter of the cerebral cortex with MT and S100, respectively. MT+/S100+ glial cells (e.g. arrows) are in close proximity to MT-/S100+ glial cells (e.g. arrowheads). Panels C and D also show MT and S100 immunoreactivity, respectively in a region of the white matter of the cortex. A MT+/S100+ glial cell (arrow) and MT-/S100+ glial cell (arrowhead) are indicated. A MT+/S100+ glial cell with processes encircling a blood vessel is indicated (curved arrow). Panels E and F show MT and S100 immunoreactivity respectively, in the molecular layer of the cortex. MT+/S100+ processes (e.g. arrowhead) and cell bodies (e.g. arrow) are indicated. Bar=50 mm (Panels A to F).


A
B
C
D
E
F

Some MT+ cells had been assessed to be oligodendrocytes by their morphology and their failure to express GFAP. Double-labeling immunocytochemistry with anti-Gal-C and anti-MT antisera confirmed that some but not all oligodendrocytes are MT immunoreactive (see Figure 3.14). Both MT+/Gal-C+ (e.g. arrowhead) and MT-/Gal-C+ (e.g. arrow) cells were observed. The small circular profiles previously proposed to be processes of oligodendrocytes encircling axons were observed to be Gal-C+ (arrow).

The correlation between MT and PCNA expression was examined in the E116 foetal brain. PCNA is a 36 kDa protein which functions as a cofactor for DNA polymerase δ in S-phase and also in DNA synthesis associated with DNA repair. It is often employed as a marker of proliferation (Hall *et al.*, 1990). The expression of MT did not necessarily correlate with PCNA in the foetal sheep brain as demonstrated dramatically in Figure 3.15, which depicts expression of MT (A), GFAP (C), S100 (D) and also PCNA (B) in areas surrounding fissures of the brain. Although MT, GFAP and S100 are all expressed in cells which form the glial limiting membrane of the sulcus (arrows, Figure 3.15A, C and D, respectively), PCNA is not expressed in these cells (see arrowhead, Figure 3.15B), but in cells further inward (arrow, Figure 3.15B). Note that PCNA expression is exclusively nuclear (see arrow, Figure 3.15B), whereas MT expression was detected in both the nucleus (see arrow, Figure 3.15A) and cytoplasm (see arrowhead, Figure 3.15A).

3.3 Discussion

3.3.1 Localisation of MT-I and -II in the developing sheep brain

Northern analysis and immunocytochemistry were used to investigate the expression and distribution of MT-I and -II isoforms in the foetal and adult sheep brain. Both approaches indicate that MT expression begins at a timepoint around E70 in the foetal brain since MT mRNA and protein were first detected at E72 and E73, respectively (see Figure 3.5). MT expression in the brain increased during further foetal development and immunoreactivity appeared most prominent in the E116 foetal

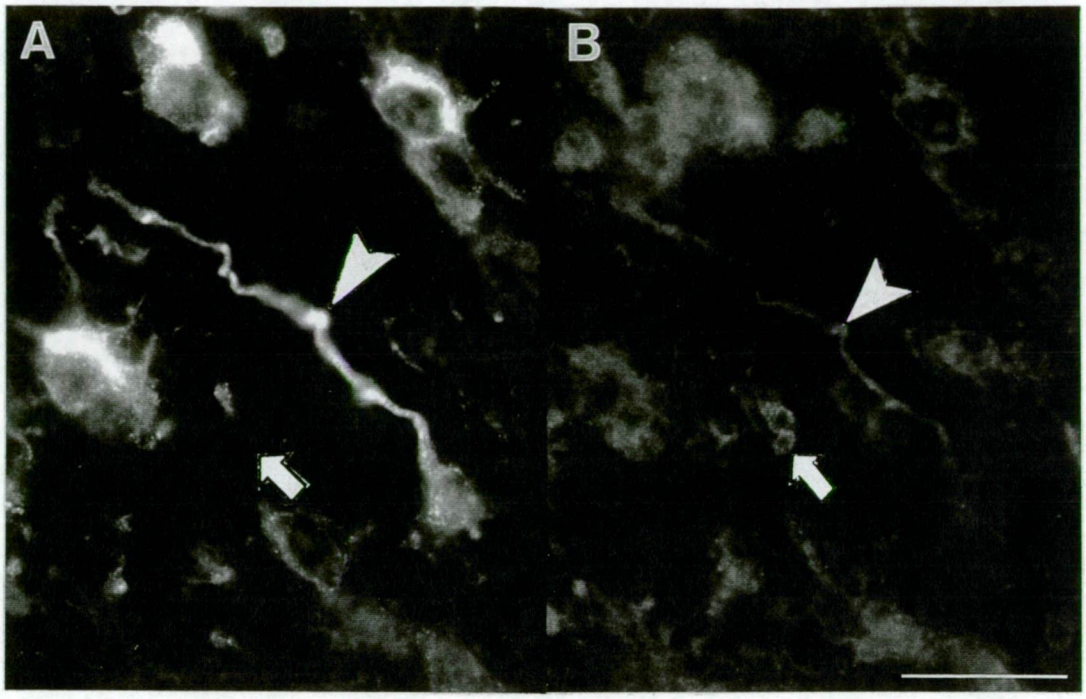


Figure 3.14 MT and Gal-C fluorescent double-labelling in the E116 foetal sheep brain

Sections were stained with anti-MT (A) and anti-Gal-C (B) antibodies. The immunostaining is of a single section from the white matter of the cortex with MT and Gal-C, respectively. A MT+/Gal-C+ oligodendrocyte is indicated (arrowhead). A MT-/Gal-C+ process of an oligodendrocyte is indicated (arrow). These small circular profiles are processes of oligodendrocytes encircling axons. Bar=20 μm.

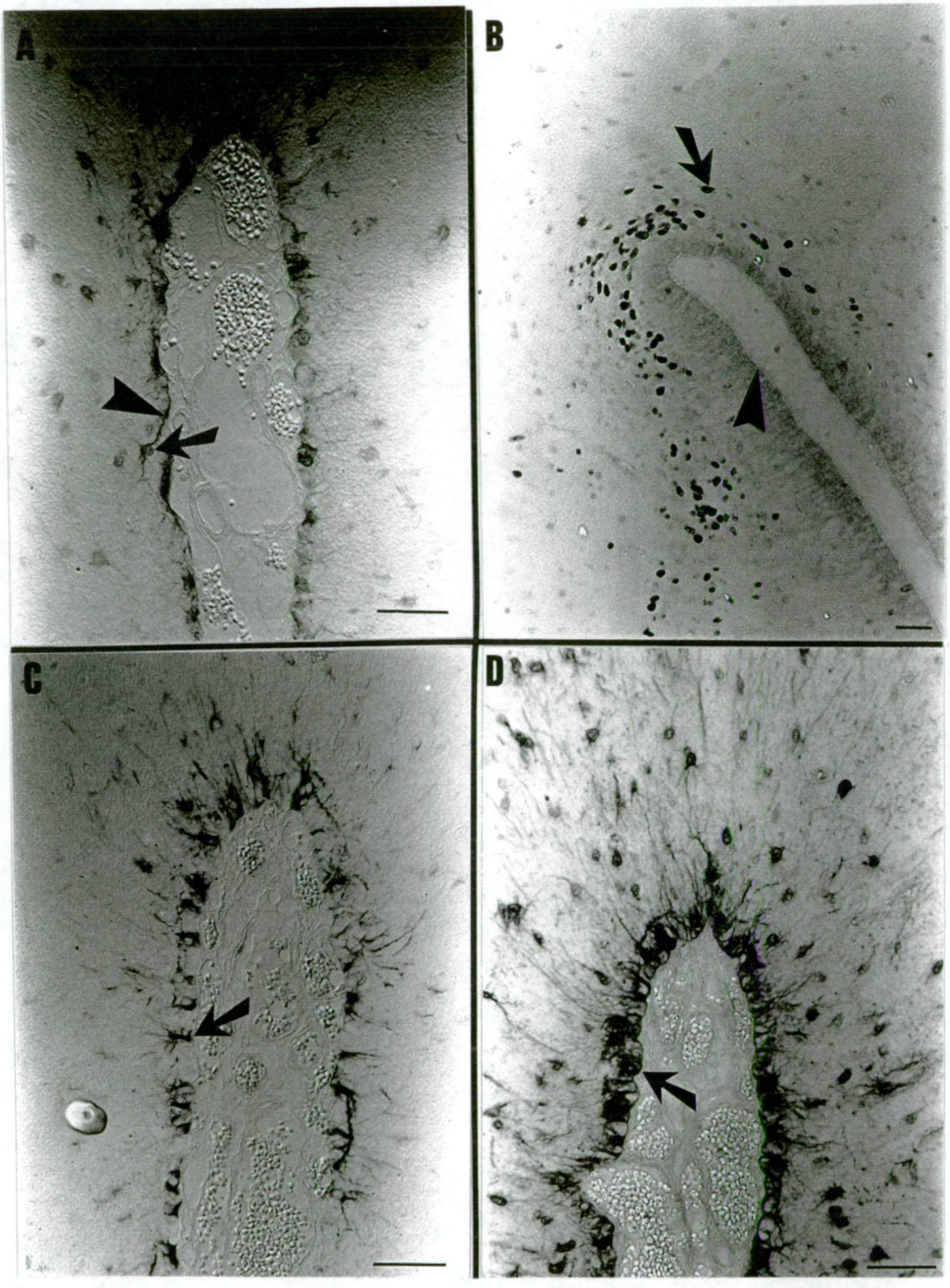


Figure 3.15 Immunocytochemical staining of fissures of the E116 foetal sheep brain.

Panels A to D show immunostaining in the area surrounding fissures of the brain. Panel A shows MT immunoreactivity in glial cells with processes lining the sulcus and forming the glial limiting membrane. The MT immunoreactivity is in both the nucleus (arrow) and the cytoplasm/processes (arrowhead). Panel B shows PCNA immunoreactivity in the area surrounding a sulcus of the brain. The immunoreactivity is exclusively nuclear (eg arrow). The cells which form the glial limiting membrane are not PCNA immunoreactive (arrowhead). Panel C.shows GFAP immunoreactivity in cells forming the glial limiting membrane of a sulcus in the foetal brain (eg arrow). Panel D shows S100 immunoreactivity in the area surrounding a sulcus of the brain. Glial cells which form the limiting membrane are S100 immunoreactive (eg arrow). Panels A to D, bars=50 μm.

brain. Immunostaining appeared relatively less prominent in the adult brain, and the high MT mRNA levels possibly reflect the relatively low, but constant staining of fibres and glial processes throughout the entire adult cortex. Alternatively, not all the mRNA detected by northern blotting may have been translated into protein. Although regulation of MT genes is primarily at the level of transcription (see Palmiter, 1987), modulation of MT gene expression at the post-transcriptional level has been documented (Sadhu and Gedamu, 1989; Vasconcelos *et al.*, 1996).

In the development of the foetal brain, migration of neuroblasts precedes that of most glial cells; for example, in the sheep brain neuroblasts appear before E20 and their migration continues up to at least E65. The cortical layers contain only neuroblasts and their derivatives (apart from radiating fibres and rare epithelial cells) up to E58. Glial cells appear in increasing numbers after this (Åstrom, 1967). It is apparent then from this study, that the appearance of MT immunoreactivity in the foetal sheep brain coincides with the development of glial cells and that MT-I and -II isoforms are present in neither neuroblasts nor developed neurons. Similarly, in studies in other species it has been demonstrated that MT-I and -II isoforms are not expressed in neurons (e.g. Nishimura *et al.*, 1989; 1992; Blaauwgeers *et al.*, 1993)

It is clear that MT expression undergoes shifts in both regional and cellular localisation during the development of the sheep brain. For example, MT immunoreactivity was confined mainly to the ventricular zone and molecular layer in the E73-E80 foetal brain, but expression was considerably broader in the E116 and adult brain. In the E73 brain immunoreactive cells were in the still proliferating ventricular zone, but in the E80 and E116 brain radial glial cells and morphologically distinct astrocytes and oligodendrocytes exhibited MT immunoreactivity. These identifications were confirmed by double labeling experiments with anti-GFAP and anti-Gal-C antisera, and are significant because oligodendrocytes have not been previously recorded as expressing MT. Interestingly, no trace of MT immunoreactivity was observed in the adult oligodendrocytes suggesting that this is a phenomenon restricted to the foetal brain. Further work is necessary to determine if

oligodendrocytes in the adult brain are able to express MT after induction with, for example, endotoxin. It has been shown in the rat optic nerve that oligodendrocytes and type-2 astrocytes develop from the same progenitor cells (Raff, 1989) and it is therefore possible that the MT positive progenitor cells observed in the ventricular zone give rise to MT positive astrocytes and oligodendrocytes. Interestingly, in a previous study low-level ovine GFAP immunoreactivity was detected as early as E60 in radial glial cells (Hewicker-Trautwein and Trautwein, 1993), which may explain the early detection of GFAP mRNA in this study (Figure 3.5).

We were able to show in the E116 and adult brain that MT and GFAP expression in astrocytes were not directly correlated, but there were overlaps in their expression profiles. Morphologically distinct astrocytes, which express all visible permutations of MT and GFAP, could be identified in single fields. This is of particular interest because previous studies in, for example, the adult human brain have shown the expression of these two proteins to be in different subsets of glial cells (Blaauwgeers *et al.*, 1993). Furthermore, in contrast to the situation in the human brain where MT and GFAP are expressed in regionally separated sets of astrocytes (Blaauwgeers *et al.*, 1993), in the sheep MT and GFAP were expressed in the same regions. In the sheep, MT immunoreactivity was observed in both white and grey matter regions of the E116 and adult brains whilst in the adult human, MT immunoreactivity is associated mainly with the grey matter (Blaauwgeers *et al.*, 1993). Interestingly, Suzuki *et al.* (1994) showed that in the foetal and infant human brain astrocytes in both the grey matter and white matter were positive for MT. Clearly, in both the human and the sheep, there are changes in the expression profiles of MT during development.

It has been suggested previously that MT may be associated with cell growth and proliferation (Nishimura *et al.*, 1992; Wlostowski *et al.*, 1993) This is supported by our study in that MT expression is detected in the proliferative ventricular zone in the E73 sheep brain and also in subsequent ages. However, we also noted high levels of expression in the molecular layer and the glial limiting membrane, regions not

associated with rapidly proliferating cells. Furthermore, in the E116 sheep brain it is apparent from the lack of correlation between MT and PCNA immunoreactivity that although MT is often expressed in proliferative regions, it is not intrinsic to dividing cells. A plausible explanation for the distribution observed is that MT expression is confined to a subset of glial cells and its expression is seen as they are formed in the ventricular zone, and then later in the subcortical layer and the molecular layer as these cells migrate outwards. MT may then be one of the earliest known markers of this class of cell.

3.3.2 The role of MT in the brain

MT has been most extensively studied in organs other than the brain, such as the liver and kidney, and much previous work has emphasised the ability of systemic factors such as blood-borne heavy metals, glucocorticoid hormones and interleukins 1 and 6 to regulate expression of MT genes (Kägi and Kojima, 1987). The regional and cellular distribution of MT protein in the developing sheep brain suggests, however, that localised factors may be more important in the regulation of MT expression in neural cells under normal conditions. It is intriguing to observe adjacent cells with identical patterns of GFAP or S100 staining and morphological appearance, but which display MT immunoreactivity ranging from very intense to undetectable. Since each of these cells is presumably exposed to the same blood supply, it appears likely that cell-specific factors, such as intracellular zinc levels, redox state or proliferation status, may be determining MT expression in the brain under basal conditions. It is possible that at another timepoint, as the state of cells and the conditions of their microenvironment change, different populations of glial cells would be seen to express MT. It is well documented that under conditions of stress, MT levels increase, and it is most likely that in the brain this is due to an increase in the number of MT expressing cells, rather than simply an increase in MT levels in those cells already expressing the protein. This assertion could be tested by examining the expression of MT in the sheep brain before and after LPS treatment. Such a treatment would

possibly demonstrate the total population of cells which are capable of expressing MT protein when required.

Clearly, in the mammalian brain, MT-I and -II isoforms are expressed in glial cells but not neurons, which would suggest that the function of MT-I and -II isoforms in the brain is related to the role played or circumstances encountered by glia but not neurons in the brain. Glial cells are the largest population of cells in the brain, and it is becoming evident that astrocytes have a number of varied roles in the brain. These include maintenance of the blood brain barrier, transport of factors from the blood to neurons, involvement in neuron migration and neurite outgrowth, regulation of water ions and amino acids in the brain, transfer of proteins and nutrients to neurons and also a protective role in the brain (Jacobson, 1991).

Astrocytes have been suggested to play a role in the protection of neurons from free radicals (Juurlink, 1994; Makar *et al.*, 1994). *In vitro* studies have demonstrated that pure cultures of neurons are highly susceptible to anoxic conditions, however when co-cultured with astrocytes, the degeneration of neurons under anoxic conditions is dramatically reduced (Vibulsreth *et al.*, 1987). The manner in which astrocytes provide such protection to neurons is unclear. A role for MT in protection against free radicals has been suggested (Thornalley and Vašák, 1985; Abel and Ruiter, 1989; Chubatsu and Meneghini, 1993) and it is possible that MTs fulfill such a role in astrocytes. Not all astrocytes within a given area are likely to be subject to the same levels of oxidative stresses as the redox status of individual cells may differ depending on for example the physiological state of the cell, and this may explain the different intensities of MT staining seen in glial cells within a single region of the brain. Although the main role of oligodendrocytes is seen as providing the myelin sheath for neurons, evidence is surfacing that they have a much wider role in the brain also (Byravan *et al.*, 1994; Caroni and Schwab, 1989). The possibility exists that MT expressed in astrocytes and oligodendrocytes acts as a free radical scavenger in the developing sheep brain. However, although evidence suggests MT may be able to scavenge free radicals within a cell, MTs are not generally thought to be secreted,

so a mechanism by which MTs expressed in glial cells would be able to protect associated neurons from free radical damage is unknown.

The ability of MT proteins to protect against heavy metal toxicity has been well documented. The appearance of MT consistently in the glial limiting membrane and also consistently surrounding blood vessels supports a protective role for MT, and it may be an important component of the blood brain barrier. Neurons do not have contact directly with the blood supply and therefore a protein with such a protective role would not necessarily be required to be expressed in neurons. This may explain the failure of neurons to express MT-I and -II isoforms. A single astrocyte is often associated with blood vessels and neurons, and acts to transport some factors from the blood to neurons, while also preventing other factors from reaching neurons (Jacobson, 1991). MT protein expressed in astrocytes has been shown to protect against methylmercury cytotoxicity *in vitro* (Rising *et al.*, 1995) and the role of MT in astrocytes may be to scavenge heavy metals and prevent them reaching neurons in the brain. However this protective role would not necessarily explain the expression patterns observed for MT in which a single cell near a blood vessel may express MT, while a neighbouring cell may not.

Alternatively, MT has been proposed to have a role in zinc homeostasis and transport. High levels of zinc are found in the brain and it is essential for normal brain development. Zinc levels vary in different areas of the brain, as do MT levels, and although studies indicate that MT levels do not correlate completely with cytoplasmic zinc levels (Hidalgo *et al.*, 1991; Gasull *et al.*, 1994) it is possible that MT functions in astrocytes in the storage and transport of this metal. High levels of MT are in fact seen in zinc rich areas such as the pineal gland (F.A.Stennard, Ph.D. Thesis). A proportion of the zinc in the brain is found in vesicles concentrated in synaptic terminals of neurons (see Fredrickson, 1989). This zinc is released at glutamatergic synapses in response to depolarisation. It is possible that the role of MT-I and -II proteins in astrocytes is involved in the sequestration of this released zinc. This is possible since it is known that astrocytes are involved in scavenging excess potassium

ions released after excessive stimulation of neurons (see Jacobson, 1991). Under normal physiological conditions, MT may then be expressed only in those cells associated with glutamatergic synapses.

It is also conceivable that MT expressing cells are responding to paracrine secretion of agents like interleukin 1 or 6, known inducers of MT (Bauer *et al.*, 1993) since these are known to be produced by a number of cell types in the brain (Benveniste *et al.*, 1990; Lee *et al.*, 1993). Therefore, a productive line of future research will be to use available techniques to search for changes at the cellular level of agents such as zinc, interleukins or demonstrated free radical scavengers, and to correlate these with MT expression. Identification of the key factors which induce MT expression in neural cells may provide insight into the actual function of MT in the brain.

Finally, it is unclear why MT-I and -II isoforms are not expressed in neurons and further studies are necessary to determine this. It is possible that MT-I and -II genes are methylated in neurons, and there is certainly evidence of regulation of MT genes in certain cell types by methylation (e.g. Compere and Palmiter, 1981). Alternatively, it is possible that factors involved in the regulation of MT, such as those that interact with the metal regulatory elements (MREs) are not expressed in this cell type. Another possibility is that those factors which regulate MT -I and -II isoform expression in the brain, have no effect on neurons, i.e. neurons do not express receptors that interact with these factors, or else they are excluded from contact with neuronal cells by the surrounding glia.

The physiological basis for the exclusively glial expression of MT-I and -II in the brain is no doubt linked to the function of MT in this tissue. MT expression may not be required by neurons since they rely on glial cells for many protective mechanisms, or alternatively neurons may be unable to tolerate the accumulation of metals such as copper and zinc within the cell. Alternatively, there is some evidence, at least in the mouse, that the brain specific MT isoform, MT-III, is expressed in neurons (Masters *et al.*, 1994b) and it is possible that this isoform has a compensatory

role in neurons. It should be noted, however, that in the rat, MT-I protein has been detected in neurons both in culture and *in vivo* (Hidalgo *et al.*, 1994). However, the levels of MT-I protein in cultured neurons determined by RIA were 10 times lower than in glial cells.

The investigation of MT in the sheep brain has revealed shifts in both regional and cellular expression during development. Although MT appears to be expressed in many of the same cell types in various species there are often subtleties which distinguish expression of MT in different species. The sheep however, may prove to be a useful model to examine the factors which are most important in regulating MT expression in the brain, thus providing insight into the role of MT in this tissue.

CHAPTER 4: CLONING OF THE SHEEP MT-III CDNA

4.1 Introduction

A protein was isolated from human brain extract and termed growth inhibitory factor (GIF) because of its ability to inhibit neurite sprouting *in vitro* (Uchida *et al.*, 1991). GIF was originally isolated as a factor deficient in the Alzheimer's disease brain, although a further study failed to confirm this deficiency (Erickson *et al.*, 1994). The protein was a novel MT isoform, containing 2 structural insertions with respect to MT-I and -II isoforms, (Tsuji *et al.*, 1992), and thus termed MT-III. Genes encoding highly homologous MT-III isoforms have since been identified in mouse (Palmiter *et al.*, 1992) and rat (Kobayashi *et al.*, 1993), and MT-III proteins have been isolated from bovine and equine brain (Poutney *et al.*, 1994).

In all species examined, MT-III expression is restricted almost exclusively to neural tissue, although a very low level of MT-III mRNA has been detected in the pancreas and small intestine of the mouse (Erickson *et al.*, 1995), and also in the deciduum during early embryonic development (Liang *et al.*, 1996). However, the cell type expression of MT-III in the brain remains controversial. In the human brain, MT-III protein was detected using an antibody to a synthetic peptide, and its expression was found to be restricted to astrocytes (Uchida *et al.*, 1991). In a further study, both MT-III mRNA and protein were detected in astrocytes in layers 2-6 of grey and white matter of the human cerebrum, and were found to co-localise with MT-I (Nakajima and Suzuki, 1995). In contrast, in the mouse, MT-III mRNA expression was primarily detected in neurons (Choudhuri *et al.*, 1995), and Masters *et al.* (1994b) reported a correlation between MT-III expression and neurons that sequester zinc in terminal vesicles. However, the expression of MT-III in the mouse does not appear to be exclusively neuronal as MT-III mRNA was found to co-localise with MT-I in glial cells of the purkinje layer of the cerebellum (Zheng *et al.*, 1995), and was also detected in the choroid plexus (Masters *et al.*, 1994b). Interestingly, in transgenic mice in which the human MT-III promoter was fused to the *E.coli* lac Z gene, expression of β-galactosidase was detected in neurons (Masters *et al.*, 1994b). Furthermore, transgenic mice containing the human MT-III gene expressed a high

level of human MT-III mRNA in the brain in a pattern which generally reflected the neuronal expression of endogenous mouse MT-III mRNA (Erickson *et al.*, 1995).

MT-III mRNA expression has also been examined in neural cells in culture. MT-III mRNA was detected in rat astrocyte primary cultures, but not cultures enriched for neurons, fibroblasts or microglial cells (Kobayashi *et al.*, 1993). Similarly, Masters *et al.* (1994b) found that although MT-III mRNA was not expressed in mouse astrocytes *in vivo,* astrocytes maintained in culture began to express MT-III after 1 week, suggesting that MT-III expression can be induced by culture conditions. However, MT-III was not detected in 5 transformed human astrocyte cell lines (Masters *et al.*, 1994b).

Low levels of MT-III mRNA have been detected in the embryonic rat (E18), increasing dramatically postnatally (Kobayashi *et al.*, 1993). In the mouse, MT-III mRNA was detectable in the foetal head at E13.5, enriched in the foetal brain at E16.5 and reached maximum levels at day 13 after birth (Masters *et al.*, 1994b). However, significant levels of MT-III mRNA were not detected in the foetal human brain (Tsuji *et al.*, 1992). In the mouse, MT-III mRNA has also been detected in early development in cells of the deciduum, co-expressed with MT-I and -II mRNA (Liang *et al.*, 1996). The expression of MT-III in this instance appeared to be highly regulated, both temporally and spatially, and appeared to be co-regulated with other MT isoforms. In the various regions of the adult mouse brain MT-I mRNA and MT-III mRNA have also been shown to be expressed at approximately equal levels, except in the hippocampus in which MT-III mRNA was enriched (Masters *et al.*, 1994b), suggesting a degree of co-regulation of MT isoforms in the adult mouse brain.

However, MT-III regulation appears to differ from that of other MT genes in many respects. In the mouse MT-III is unaffected by cadmium chloride, dexamethasone, ethanol, kainic acid, lipopolysaccharide (LPS) or zinc chloride, while both LPS and zinc chloride increase MT-I mRNA levels in the brain (Zheng *et al.*, 1995; Palmiter *et al.*, 1992). Furthermore, in cultured mouse astrocytes the MT inducers phorbol 12-myristate 13-acetate, dibutyryl cAMP, dexamethasone and zinc sulphate failed to affect MT-III expression. However, removal of serum from

astrocytes in culture, resulted in growth arrest and increased MT-III mRNA expression (Masters *et al.*, 1994b).

Examination of the promoters of both the human and mouse MT-III genes revealed regulatory elements often found in MT promoters, including AP-2 like binding sites, Sp1 binding sites and also metal regulatory elements (MREs, Naruse *et al.*, 1994). Despite the presence of three MREs in the promoters of both human and mouse MT-III genes, metal regulation of neither MT-III gene has been demonstrated. Interestingly, the human MT-III gene also contains an element similar to the sequence detected in the gene which encodes glial fibrillary acidic protein (GFAP) thought to be responsible for the astrocyte-specific expression of GFAP, and it was proposed that this element may be responsible for the astrocyte-specific expression which has been documented for human MT-III (Naruse *et al.*, 1994). The mouse MT-III promoter has been shown to contain a CTG triplet repeat which is capable of acting as a repressor. However, binding proteins for this sequence have not been detected, and its activity in brain cells is yet to be examined (Imagawa *et al.*, 1995).

The growth inhibitory activity of human MT-III *in vitro* was first reported by Uchida *et al.* (1991), and has since been demonstrated for MT-III proteins from mouse (Erickson *et al.*, 1994), horse and cow (Poutney *et al.*, 1994). The growth inhibitory activity of the MT-III protein demonstrated *in vitro*, is entirely due to the N-terminal portion of the protein (Uchida and Ihara, 1995), and more particularly has been mapped to the CPCP sequence in the N-terminal domain, which is unique to the MT-III isoform (Sewell *et al.*, 1995). Several studies have demonstrated changes in MT-III expression *in vivo* after neural damage, leading to the suggestion of a growth inhibitory role for MT-III *in vivo* also. For example, MT-III appears to be upregulated in rat brain after stab wound injury. Both MT-III mRNA and protein levels increased 4 days after injury and the response was sustained for 28 days (Hozumi *et al.*, 1995). The increase in MT-III was suggested to be a result of upregulation of the gene due to the acute phase response after injury, and an indication that MT-III may be involved in repair after brain injury. Similarly, MT-III mRNA levels increased transiently in both neuronal and non-neuronal cells after ablation (Yuguchi *et al.*, 1995a). It was proposed that MT-III was involved in maintaining the neuronal network and controlling the outgrowth of injured neurites.

In a further study, MT-III expression was dramatically suppressed after transection of peripheral facial nerves, and this response lasted for 5 weeks (Yuguchi *et al.*, 1995b). In this instance it was suggested, that suppression of MT-III would reduce inhibition of neurite outgrowth which is necessary for regeneration of axons. In contrast, overexpression of human MT-III in transgenic mice did not result in any obvious deleterious effects in the development or physiology of the brain, raising questions as to whether MT-III does have a growth inhibitory role *in vivo*. These mice however accumulated increased levels of zinc (but not other metals) in the brain entirely accounted for by zinc bound to the human MT-III protein, raising the possibility that MT-III is involved in some aspect of zinc regulation (Erickson *et al.*, 1995).

The sheep MT gene family consists of 4 well characterised functional MT genes (MT-Ia, MT-Ib, MT-Ic and MT-II). However a MT-III isoform has not been documented in the sheep. The purpose of this study was to clone the MT-III cDNA from sheep, investigate its homology to MT-III genes from other species, and examine whether its expression patterns are consistent with those found in other species. Investigation of MT-III in a species with a complex MT locus (like humans), and in which MT isoforms have been well studied in the brain, may shed light on the function of MT-III *in vivo* and its relationship to other MT isoforms. Questions still remain as to whether the MT-III isoform does in fact have a function distinct from MT-I and -II isoforms and whether the growth inhibitory activity of MT-III demonstrated *in vitro* is physiologically relevant. Investigation of the cell type expression of MT-III in a further species will also be useful as studies to date on mouse, rat and human are contradictory and suggest that cell type expression of MT-III may in fact be species specific.

4.2 Results:

4.2.1. Cloning of mouse MT-III cDNA and its suitability as a sheep MT-III probe

Mouse MT-III cDNA (274 base pairs including the entire coding region) was amplified from mouse brain RNA by RT-PCR using mouse MT-III sense (5'-TGGATATGGACCCTGAGACC-3") and antisense (5'-CCTTGGCCCCCTCTTC-3') PCR primers. The RT-PCR protocol was as outlined in section 2.4, using an

annealing temperature of 60°C, and 35 cycles. The amplified product was blunt ended using *Pfu* DNA polymerase and ligated into the pUC-19 vector at the *Sma*I endonuclease restriction site. The RT-PCR product was sequenced by the dideoxy sequencing method (section 2.5.1) and confirmed to be identical to that reported previously for mouse MT-III (Palmiter *et al.*, 1992). The hybridisation of this RT-PCR product to sheep and mouse DNA and RNA was then examined, as outlined below.

Genomic DNA was isolated from mouse and sheep tissue, digested with the restriction endonucleases *Eco*RI and *Hin*dIII, electrophoresed through 1.4% agarose and transferred to a nylon membrane (ZetaProbe GT, BioRad) as outlined in section 2.3. The Southern blot was hybridised at 37°C in 50% formamide hybridisation buffer for 16 hr with the radiolabelled mouse MT-III RT-PCR probe, and washed to a stringency of 0.2 X SSC, 1% SDS at 37°C. Southern analysis of sheep and mouse DNA under these conditions demonstrated that the probe hybridised to mouse as well as sheep sequences (Figure 4.1). The probe hybridised to an *Eco*RI fragment of mouse DNA of 4.5 kb as expected, as well as an *Eco*RI fragment of sheep DNA of 4.4 kb, which did not correspond to any of the known sheep MT genes (Peterson *et al.*, 1988, see Chapter 3).

Total RNA was isolated from various tissues of both foetal (E72, E86, E116) and adult sheep. Northern analysis of the RNA (section 2.3) using the radiolabelled mouse MT-III RT-PCR product as a probe, under the conditions outlined above, demonstrated that the mouse MT-III probe hybridised to RNA from sheep brain (E116 and adult), but not RNA from other tissues (Figure 4.2). In contrast, the human *MT2A* RT-PCR product used as a general MT-I/II probe since it hybridises to all known sheep MT-I and MT-II isogenes (see Chapter 3), hybridised to RNA from sheep brain (E116 and adult) as well as liver (E72, E86, E116 and adult), kidney (E116 and adult), and weakly to RNA from lung (adult), and heart (adult).

4.2.2 Cloning sheep MT-III cDNA

Attempts were made to amplify sheep MT-III cDNA by low stringency RT-PCR using PCR primers designed to the coding regions of the human and the mouse MT-III gene sequences and low annealing temperatures. Degenerate MT-III primers

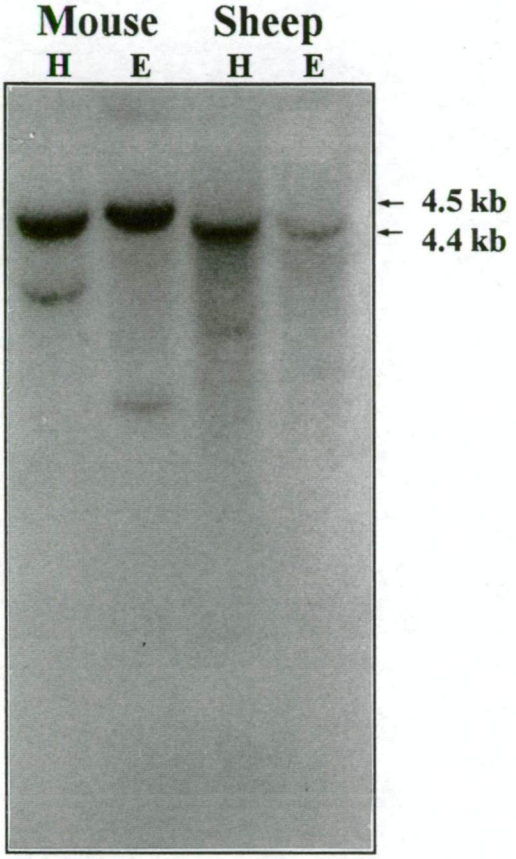


Figure 4.1 Analysis of mouse and sheep DNA with a mouse MT-III probe
Mouse and sheep genomic DNA was digested with the restriction endonucleases *Eco*RI (E) and *Hin*dIII (H), and subjected to Southern blot analysis with a mouse MT-III cDNA probe. The probe hybridised strongly to a 4.5 kb *Eco*RI fragment of mouse DNA and a 4.4 kb *Eco*RI fragment of sheep DNA as indicated.

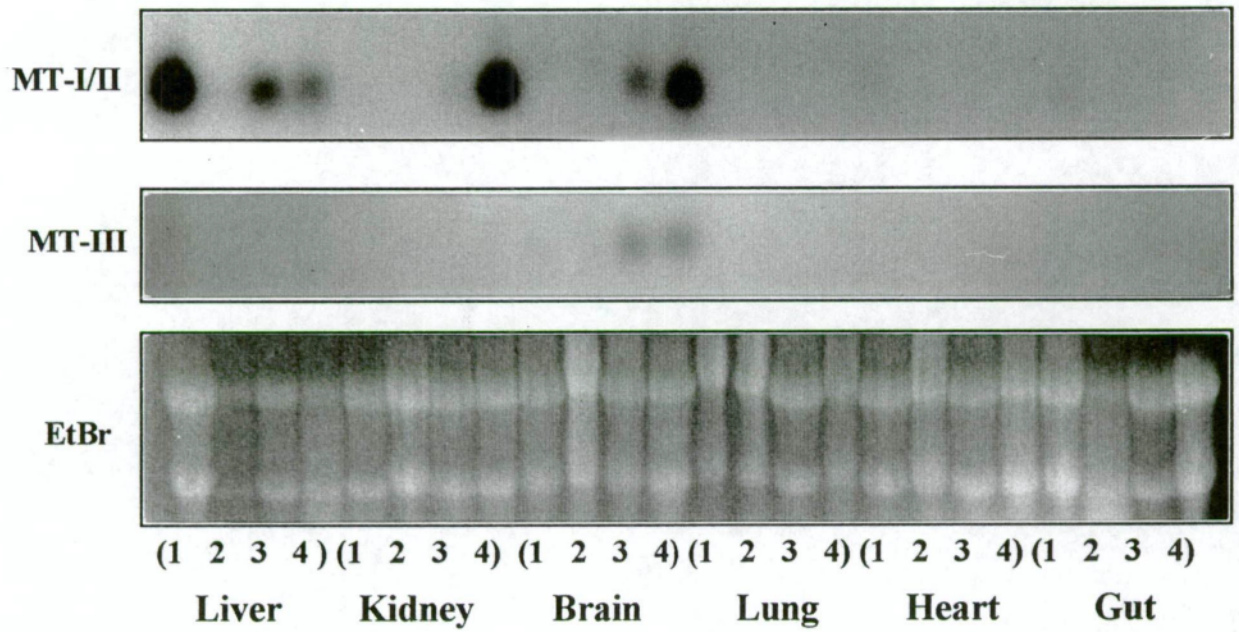


Figure 4.2 Expression of MT mRNA in foetal and adult sheep tissues
Total RNA was isolated from foetal and adult sheep tissues and after electrophoresis subjected to northern blotting. Tissues were from sheep of the following ages; 1: E72, 2: E80, 3: E116 and 4: Adult. The RNA was hybridised to a mouse MT-III cDNA probe (MT-III), and a human *MT2A* cDNA probe (MT-I/II) which hybridises to all sheep MT-I and -II genes. The ethidium bromide stained gel is shown to demonstrated that approximately equal amounts of RNA were loaded in each lane.

used to clone the mouse MT-III cDNA in a previous study (Palmiter *et al.*, 1992), were also used. A product of approximately 150 bp was amplified from sheep brain RNA but not liver RNA, using primers designed to the coding region of the mouse MT-III gene (sense: 5'-TGGATATGGACCCTGAGACC-3' and antisense: 5'-GTTGTGCCCCACCAGGGAC-3') at an annealing temperature of 32°C. This transcript was shown to hybridise to the mouse MT-III RT-PCR product probe under the conditions outlined above. However, due to the low stringency of the PCR many transcripts were amplified, and despite numerous attempts an MT-like product could not be cloned.

A sheep choroid plexus cDNA library in λgt10 (Tu *et al.*, 1989) was screened (see section 2.9.7) using the mouse MT-III RT-PCR product as a probe. Approximately 120,000 plaques were lifted onto nitrocellulose filters, hybridised with the radiolabelled probe at 55°C in DNA hybridisation buffer, and washed to a stringency of 0.2 X SSC, 1% SDS at room temperature. However, no positive plaques were detected from this library.

Therefore it was decided to construct a sheep cortex cDNA library. Total RNA was isolated from brain cortex of 2 adult sheep. Poly(A)$^+$ RNA was isolated from the total RNA using magnetic Dynabeads from Dynal, which have oligo(dT) residues covalently coupled to the surface (section 2.9.1). The poly(A)$^+$ mRNA (3 μg) was used to construct a cDNA library with the ZAP express vector system from Stratagene (section 2.9). First strand cDNA was synthesized with MMLV-RT (Figure 4.3A). Rnase H was used to nick the RNA and DNA polymerase was then used in synthesis of the second strand of the cDNA (Figure 4.3A). The prepared cDNA was size fractionated with Sephacryl and the first 5 fractions (Figure 4.3B) were combined and ligated into the ZAP Express vector. After packaging and amplification the sheep brain cortex cDNA library consisting of 52,000 recombinants and with a titre of 1.2 X 10^{10} pfu/ml was screened with the mouse MT-III RT-PCR product (section 2.9.7). Approximately 60,000 plaques were lifted onto nitrocellulose filters, hybridised with the radiolabelled mouse MT-III probe for 16 hr at 55°C in DNA hybridisation buffer, and washed to a stringency of 0.2 X SSC, 1% SDS at room temperature. Twelve plaques which hybridised to the mouse MT-III RT-PCR product were selected and purified after 3 rounds of screening. The required inserts

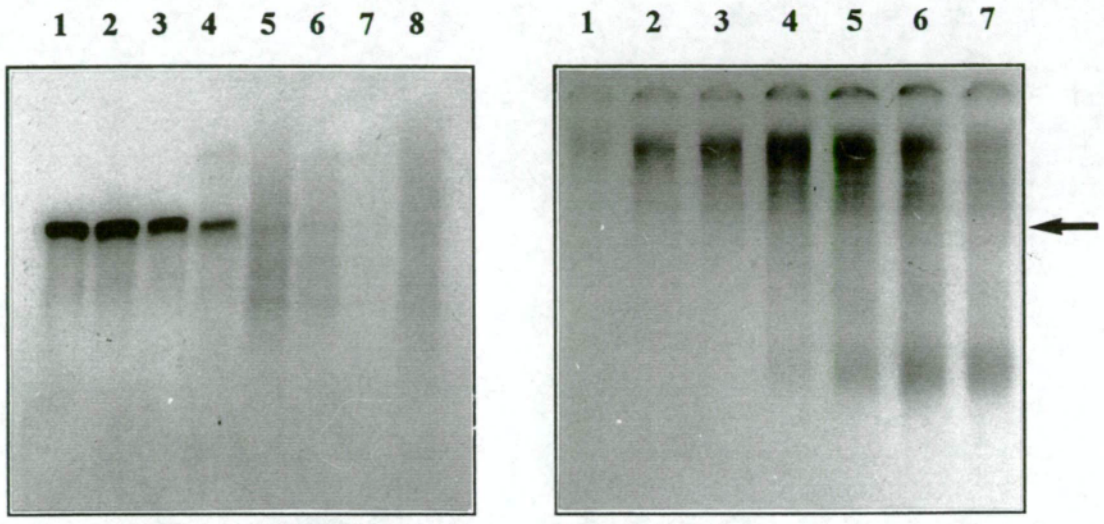


Figure 4.3 Construction of a sheep brain cDNA library
Poly(A)$^+$ RNA (3μg) was isolated from sheep brain cortex and used to construct a cDNA library using the Statagene ZAP Express vector system.

A: First and second strand synthesis of the cDNA was monitored by electrophoresis of an aliquot of the reactions through an alkaline agarose gel. The dried gel was exposed to autoradiography film. Test poly(A)+ RNA provided by Stratagene was used as a control of first and second strand synthesis. Lanes 1-3: test poly(A)$^+$ RNA, first strand synthesis (4%, 2% and 1% of reaction, respectively), Lane 4: test poly(A)$^+$ RNA, second strand synthesis (10% of reaction), Lanes 5-7: sheep brain cortex poly(A)$^+$ RNA, first strand synthesis (4%, 2%, and 1% of reaction, respectively), Lane 8: sheep brain cortex poly(A)$^+$ RNA, second strand synthesis (10% of reaction).

B: The cDNA was size fractionated through a S-500 sephadex spin column. Aliquots (10%) of the 7 collected fractions were electrophoresed through a 5% (29:1) nondenaturing acrylamide gel and exposed to autoradiography film. Inclusion of DNA markers on the gel allowed the size of the cDNA fractions to be determined, and a size of 500 bp is indicated (arrow).

were excised out of the phage in the kanamycin-resistant pBK-CMV phagemid vector (section 2.9.8). Clones were sequenced in both directions using the ABI PRISM™ Dye Primer Cycle Sequencing Ready Reaction Kit (section 2.5.2). Sequence analysis of the clones revealed 2 full length independent clones of a novel MT cDNA (Figure 4.4).

4.2.3 Analysis of a novel MT cDNA, sheep MT-III

The protein predicted by the novel sheep cDNA is a 65 amino acid MT-like protein containing 17 cysteine residues, appearing in conserved positions when compared with the known sheep MTs (Figure 4.5), and devoid of aromatic amino acids. Insertions of a single amino acid in the N terminal domain and a 6 amino acid insertion in the C terminal domain identify the protein as a MT-III isoform. The putative sheep MT-III protein is clearly homologous to the known MT-III proteins from human, mouse, rat, cow and horse (Figure 4.5) with 87% and 85% amino acid conservation to the bovine and equine proteins, respectively. In fact, the putative sheep MT-III amino acid sequence is identical to the MT-III protein isolated from bovine brain at 61 out of 68 positions. Slightly less conservation is seen with the human, mouse and rat protein with 84%, 79% and 75% amino acid identity, respectively. The most significant difference in the putative sheep MT-III protein is a 3 amino acid deletion of the amino acid sequence SCC, normally conserved at positions 33-35 of MT-III proteins. Furthermore, a serine residue at position 30 replaces a cysteine residue which is conserved in all other MT-III proteins. The 6 amino acid insertion in the C-terminal domain of the sheep MT-III protein is identical to that found in the corresponding bovine protein, although the single amino acid insertion at position 5 which is a threonine residue in all known MT-III proteins is an alanine residue in the putative sheep protein. The 3' untranslated region of the sheep MT-III cDNA has little similarity to those reported for the mouse and human (Palmiter *et al.*, 1992) which are themselves unrelated, although the position of the AATAAA polyadenylation site is relatively conserved.

The putative sheep MT-III protein is identical to all 4 known sheep MT-I and -II isoforms (Ia, Ib, Ic and II) at 32 positions, and contains unique residues at 18 positions (not including the 2 insertions and the 3 amino acid deletion). At the

```
        10          20          30          40          50          60
*   *                                                    Hae III
CTAGAAGCCG ATTCGCCTCT TCGGTGGTCG CTCCTCTCCG GCATGGACCC TGAGGCCTGC
                                                  MetAspPr oGluAlaCys

        70          80          90          100         110         120
                                                        C
CCCTGCCCTA CTGGCGGCTC CTGCACCTGC TCCGACTCCT GCAAGTGTGA GGGCTGCACA
ProCysProT hrGlyGlySe rCysThrCys SerAspSerC ysLysCysGl uGlyCysThr

       130         140         150         160         170         180
TGTGCCTCCA GCAAGAAGAG CTGCTGCCCC GCAGAGTGCG AGAAATGTGC CAAGGATTGT
CysAlaSerS erLysLysSe rCysCysPro AlaGluCysG luLysCysAl aLysAspCys

       190         200         210         220         230         240
                       Hae III
GTGTGTAAAG GTGGAGAGGG GGCCGAAGCT GAGGAGAAGA AGTGCGGCTG CTGCCAGTGA
ValCysLysG lyGlyGluGl yAlaGluAla GluGluLysL ysCysGlyCy sCysGlnEnd

       250         260         270         280         290         300
                                                       Hae III
GGACGTGCCC ACTGTCCCGT GTGAAACGTG TGTGAATAGT GCAGGGTGGC CCAGTGCCAC

       310         320         330         340         350         360
CTGCCCTGTG GTGAGGCCCA GCAGTGTGTC CCCTTCCCTG CAAGCCACTG GCAAGTGACA

       370         380         390         400         410         420
ATAAATCCTA TGAATGGCAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAA
```

Figure 4.4: Nucleotide sequence of sheep MT-III cDNA
The sequence of sheep MT-III cDNA was obtained from 2 independent clones from a sheep brain cortex cDNA library. The start of the 2 clones are indicated (asterisks). The polymorphism identified by RT-PCR is indicated in bold (at position 108). Recognition sites for the *Hae*III restriction endonuclease are indicated. Binding sites for PCR primers are indicated (double underlined). The polyadenylation site is indicated (bold).

A:

Sheep MT-Ia
MDPN-CSCPTGGSCSCAGSCTCKACRCPSCKKSCCSCCPVGCAKCAQGCVCKG------ASDKCSCCA

Sheep MT-Ib
MDPN-CSCPTSGSCSCAGSCTCKACRCPSCKKSCCSCCPVGCAKCAQGCVCKG------ASDKCSCCA

Sheep MT-Ic
MDPN-CSCSTGSSCSCAGSCTCKACRCPSCKKSCCSCCPVGCAKCAQGCICKG------ASDKCSCCA

Sheep MT-II
MDPN-CSCTAGESCTCAGSCKCKDCKCASCKKSCCSCCPVGCAKCAQGCVCKG------ASDKCSCCA

Sheep MT-III
MDPEACPCPTGGSCTCSDSCKCEGCTCASSKK---SCCPAECEKCAKDCVCKGGEGAEAEEKKCGCCQ
*** * *1½1**2* **2* * *2* ** **** * *** *½*** ** **

B:

Human MT-III
MDPETCPCPSGGSCTCADSCKCEGCKCTSCKKSCCSCCPAECEKCAKDCVCKGGEAAEAEAEKCSCCQ

Mouse MT-III
MDPETCPCPTGGSCTCSDKCKCKGCKCTNCKKSCCSCCPAGCEKCAKDCVCKGEEGAKAEAEKCSCCQ

Rat MT-III
MDPETCPCPTGGSCTCSDKCKCKGCKCTNCKKSCCSCCPAGCEKCAKNCVCKGEEGAK--AEKCSCCQ

Cow MT-III
MDPETCPCPTGGSCTCSDPCKCEGCTCASCKKSCCSCCPAECEKCAKDCVCKGGEGAEAEEKKCSCCQ

Horse MT-III
MDPETCPCPTGGSCTCSGECKCEGCKCTSCKKSCCSCCPAECEKCAKDCVCKGGEGAEAEAEKCSCCQ

Sheep MT-III
MDPEACPCPTGGSCTCSDSCKCEGCTCASSKK---SCCPAECEKCAKDCVCKGGEGAEAEEKKCGCCQ
----*----------*--***----------*---

Figure 4.5 Comparison of the putative sheep MT-III protein with known MT proteins

A: Comparison of the predicted sheep MT-III amino acid sequence with sheep MT-I and -II amino acid sequences (from Peterson *et al.*, 1988). Amino acids common to all proteins are indicated with asterisks, those in MT-III also found in MT-I (1) or MT-II (2) isoforms are indicated, and the amino acids in the MT-III sequence found in some MT-I and -II sequences are indicated (½).

B: Comparison of the predicted sheep MT-III amino acid sequence with MT-III proteins from human (Uchida *et al.*, 1991), rat (Kobayashi *et al.*, 1993), mouse (Palmiter *et al.*, 1992) and cow and horse (Poutney *et al.*, 1994). Substitutions unique to the sheep MT-III protein are indicated (asterisks).

remaining 8 positions it has identity to one or more of the known sheep MTs (see Figure 4.5). The sheep MT-III protein has 37 positions identical to MT-Ia and MT-II, 36 positions identical to MT-Ib and 34 positions identical to MT-Ic.

The sequence of the sheep MT-III cDNA was confirmed from 4 more individuals to verify the sequence. The following PCR primers were designed to the sheep MT-III cDNA in the 5' and 3' non-coding regions:

sheep MT-III sense: 5'-CGGTGGTCGCTCCTCT-3'

sheep MT-III antisense: 5'-TTTCACACGGGACAGTGG-3'.

The binding sites for the primers in the cDNA nucleotide sequence are indicated in Figure 4.4. RNA was isolated from 4 additional sheep brains and RT-PCR used to amplify the sheep cDNA. The RT-PCR protocol was as outlined in section 2.4, using an annealing temperature of 60°C and 35 cycles. Transcripts were electrophoresed through an agarose gel, purified through a spin column (section 2.2.1) and subjected to a second round of amplification. Purified transcripts were sequenced using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit and the sheep MT-III antisense oligonucleotide described above. Analysis of the 4 PCR products determined that they were identical to the sequences obtained from the cDNA library, except for a single polymorphism where the cysteine at position 22 was either encoded by a TGT or TGC codon (as indicated in Figure 4.4).

4.2.4 Expression of sheep MT-III mRNA

A 147 bp *Hae*III fragment of the sheep MT-III cDNA (bases 56 to 202, Figure 4.4) was subcloned into pUC-19 at the *Sma*I endonuclease restriction site, creating a partial clone of sheep MT-III cDNA minus the poly $(A)^+$ tail. The clone contains 77% of the coding region of the cDNA. This *Hae*III fragment of the sheep MT-III cDNA was used as a probe in Southern and northern analysis of sheep DNA and RNA, as outlined below.

Genomic DNA from 2 sheep was digested with the restriction endonuclease *Eco*RI and subjected to Southern analysis (section 2.3). The Southern blot was hybridised with the radiolabelled sheep MT-III cDNA probe described above for 16 hr at 42°C in 50% formamide hybridisation buffer, and washed to a stringency of 0.2 X SSC, 1% SDS at 37°C. Under these conditions the sheep MT-III cDNA probe

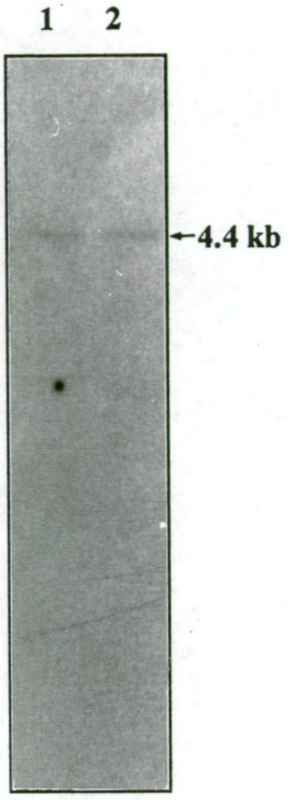


Figure 4.6 Southern blot analysis of sheep DNA with a sheep MT-III probe
Genomic DNA from 2 sheep (lanes 1 and 2) was digested with the restriction endonuclease *Eco*RI and subjected to Southern blot analysis with a sheep MT-III cDNA probe. The probe hybridised to a 4.4 kb *Eco*RI fragment of sheep DNA as indicated.

hybridised to a 4.4 kb *Eco*RI fragment of sheep DNA (Figure 4.6), confirming earlier results using the mouse MT-III RT-PCR product as a probe. The sheep MT-III cDNA probe did not hybridise to fragments of DNA containing sheep MT-I and -II genes, confirming that the probe was specific for the MT-III isogene (see Chapter 3).

The *Hae*III fragment of sheep MT-III cDNA was used as a probe in northern analysis (section 2.3) of RNA from sheep brain and liver of different developmental ages. RNA was isolated from liver and brain of foetal and adult sheep, electrophoresed through agarose, and transferred to a nylon membrane by alkaline blotting. The northern membrane was hybridised under the conditions outlined above with the radiolabelled *Hae*III fragment of sheep MT-III cDNA. Expression of MT-III mRNA was detected in the E116 foetal and adult brain but not at earlier time points (Figure 4.7) or in the foetal or adult liver. MT-I and -II isoforms were detected in the E116 and adult brain using as a probe the human *MT2A* RT-PCR product (i.e. MT-I/II general probe) which hybridises to these isoforms. Low levels were also detected in the E72 and E86 brain, as detailed previously (see Chapter 3).

Northern analysis was also used to examine the expression of sheep MT-III mRNA in different regions of the adult sheep brain (Figure 4.8). Using the *Hae*III fragment of sheep MT-III cDNA and the conditions outlined above, expression was detected in all regions examined with highest levels of expression detected in the pineal gland, relatively high levels in the basal ganglia and midbrain and lowest levels in the brain stem and thalamus. MT-III mRNA again was not detected in the adult liver. Expression of mRNA for MT-I and -II isoforms was also examined using the human *MT2A* RT-PCR product as a probe. The northern blot was hybridised at 37°C for 16 hr in 50% formamide hybridisation buffer, and washed to a stringency of 0.2 X SSC, 1% SDS at 37°C; conditions under which the probe hybridises to all MT-I and -II isoforms, as outlined in Chapter 3. Expression of MT-I and -II mRNA was similar to that of MT-III mRNA, with highest levels of expression detected in the pineal gland (Figure 4.8). Unlike MT-III, MT-I and -II isoforms were detected in the adult liver, as expected. Hybridisation of the northern blot with a human β-actin probe demonstrated that approximately equal amounts of RNA were loaded in each lane. Hybridisation was at 37°C in 50% formamide hybridisation buffer, and the blot was washed to a stringency of 0.2 X SSC, 1% SDS at 37°C.

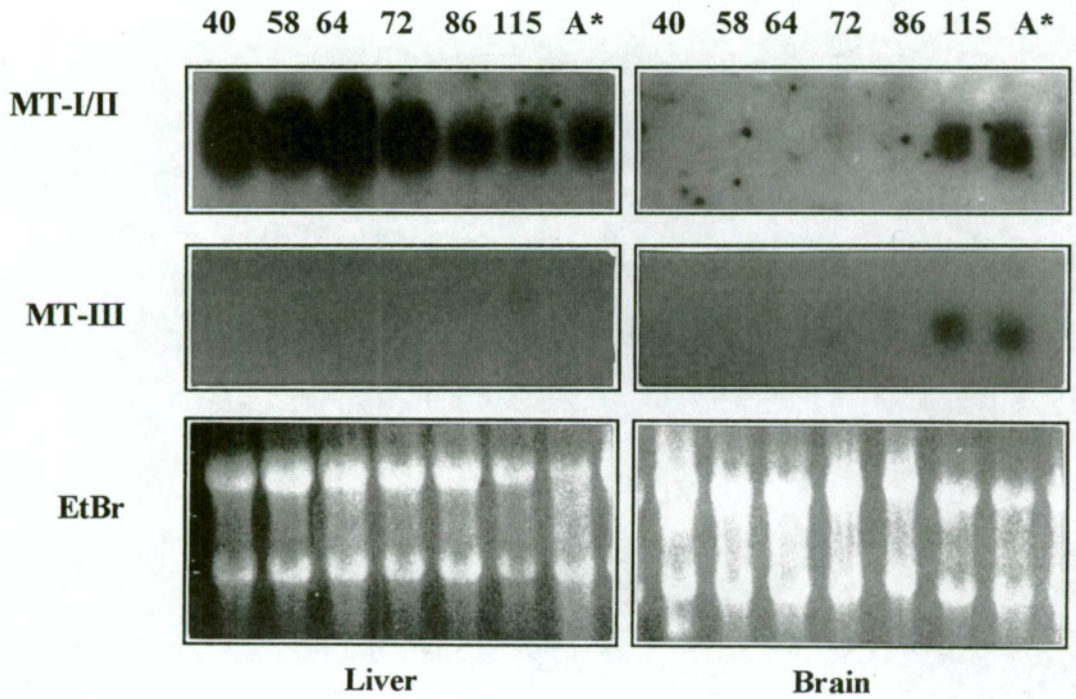


Figure 4.7 Expression of MT mRNA in the foetal sheep brain and liver
Total RNA was isolated from foetal (E40, E58, E64, E72, E86 and E115) and adult (A*) sheep brain and liver. After electrophoresis and northern blotting, the RNA was hybridised to a human *MT2A* RT-PCR product (MT-I/II) which hybridises to all sheep MT-I and -II genes and a sheep MT-III cDNA. The ethidium bromide stained gel is shown to demonstrate that approximately equal amounts of RNA were loaded in each lane.

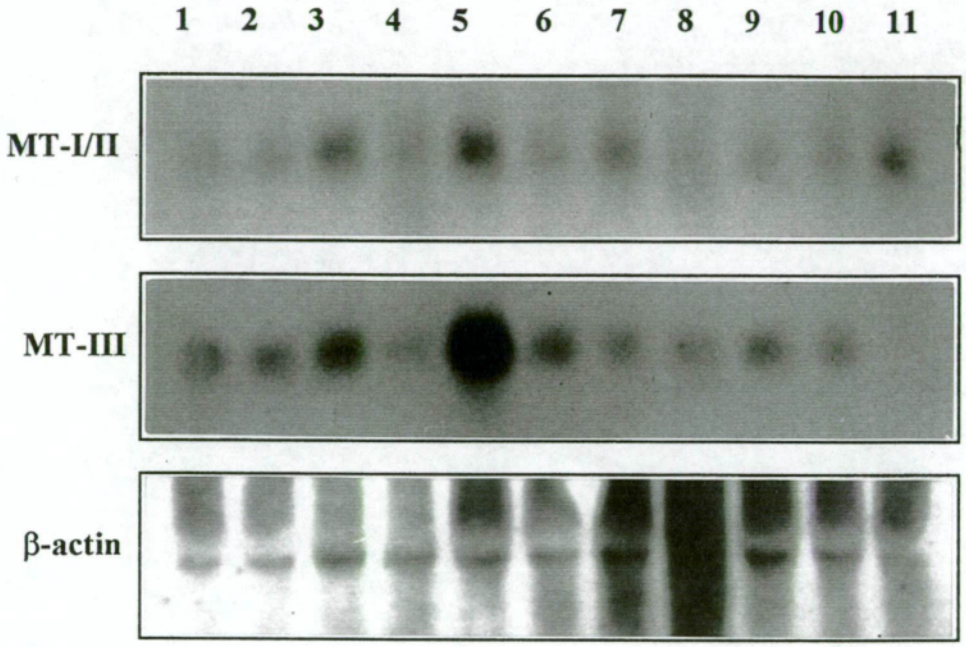


Figure 4.8 Regional expression of MT mRNA in the adult sheep brain
Total RNA was isolated from dissected regions of the adult sheep brain and from the adult liver. After electrophoresis and northern blotting, the RNA was hybridised to a human *MT2A* RT-PCR product (MT-I/II) which hybridises to all sheep MT-I and -II genes and a sheep MT-III cDNA. Hybridisation to a human β-actin cDNA demonstrated that approximately equal amounts of RNA were loaded in each lane. RNA was from the following tissues regions: 1: frontal cortex, 2: occipital cortex, 3: midbrain, 4: brain stem, 5: pineal gland, 6: basal ganglia, 7: hypothalamus, 8: thalamus, 9: hippocampus, 10: choroid plexus, 11: liver.

4.2.5. Cellular localisation of sheep MT-III

Preliminary studies were conducted to investigate whether the cellular localisation of MT-III mRNA could be determined by *in situ* hybridisation. Adult sheep brain was dissected into anatomical regions, fixed in paraformaldehyde, sections 5 μm thick cut and floated onto silanized slides. An oligonucleotide probe designed specifically to the sheep MT-III sequence, and a conserved oligonucleotide designed to MT-I and -II isoforms were radiolabelled with γ-^{32}PATP. The oligonucleotide sequences used were:

sheep MT-III antisense: 5'-TTTCACACGGGACAGTGG-3'

conserved MT-I/II antisense: 5'-GCAGCCCTGGGCACACTTG-3'

In situ hybridisation studies using these probes were conducted as outlined in section 2.6.6. Sections were hybridised for 16 hr with the radiolabelled oligonucleotide probes. A hybridisation temperature of 37°C was used for the conserved MT-I/II oligonucleotide and a hybridisation temperature of 42°C was used for the MT-III specific oligonucleotide. The sections were washed up to 60°C in 1 X SSC and exposed to autoradiography film. Figure 4.9 shows autoradiography of sheep brain cortex, basal ganglia and pineal gland as well as mouse kidney sections hybridised with the radiolabelled probes. These brain sections were chosen for preliminary studies as northern analysis demonstrated that these regions of the brain express widely varying levels of MT-III mRNA (see Figure 4.8). As demonstrated in Figure 4.9A, highest level of MT-III mRNA detected by *in situ* hybridisation were in the pineal gland, followed by the basal ganglia and the brain cortex. This is as expected from earlier northern analysis (Figure 4.8). However, although differential expression could be detected between sections taken from different regions of the brain, within a region there was little variation in the intensity of signal and at a microscopic level, the hybridisation signal could not be assigned to individual cells or a particular cell type. This is because the large amount of scatter from ^{32}P radionucleotides means that signal from an individual cell is observed over many of the surrounding cells. Figure 4.9B demonstrates that while MT-III mRNA was detected in pineal gland and basal ganglia, no signal was detected in the mouse kidney sections, as expected, and therefore the probe was specific for the MT-III isoform the

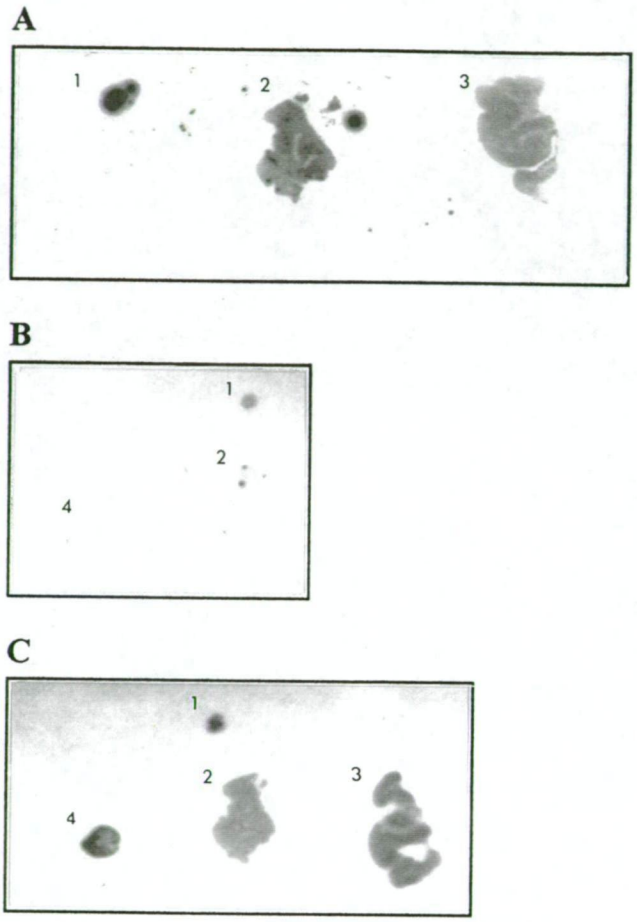


Figure 4.9 *In situ* hybridisation of MT in sheep brain regions
Tissue sections were hybridised *in situ* to radiolabelled oligonucleotide probes as described in the text. The probes used were a sheep MT-III oligonucleotide probe (A and B) and conserved MT-I/II oligonucleotide probe (C). The hybridised sections were exposed to autoradiography film (Hyperfilm -MP, Amersham). Tissue sections were from: 1 sheep pineal gland, 2 sheep basal ganglia, 3 sheep brain cortex, 4 mouse kidney.

probe was specific for the MT-III isoform. As demonstrated in Figure 4.9C the highest level of MT-I/II mRNA detected by *in situ* hybridisation was in the pineal gland, with lower levels in the basal ganglia and cortex as expected from previous northern analysis (Figure 4.8). Unlike the MT-III probe the conserved probe detected MT-I and -II mRNA in the mouse kidney, as expected. Again, the signal could not be assigned to any cell type at the microscopic level. However, the preliminary results presented here suggest that these oligonucleotides may be useful in examining the cellular localisation of MT-III in the sheep brain but more informative studies would necessitate using large areas of the sheep brain, containing various anatomical regions so that glial or neuronal rich areas of the brain can be correlated with the intensity of the MT-III signal. Also, a different radioisotope may be required which produces less scatter.

4.3 Discussion

4.3.1 Sheep MT-III structure and homology

A novel MT cDNA was cloned from a sheep brain cortex cDNA library. The cDNA encodes a putative sheep MT-III protein of 65 amino acids, containing 26% cysteine residues appearing in conserved positions throughout the protein chain. The predicted amino acid sequence is consistent with other MT proteins, containing no aromatic amino acids or histidine. The putative sheep MT-III protein shows a high degree of conservation to MT-III isoforms from other species, and in fact shares 87% and 85% identity to the bovine and equine MT-III proteins characterised by Poutney *et al.* (1994). Palmiter *et al.* (1992), suggested that the MT-I, -II and -III isoforms arose from the triplication of an ancestral MT gene. The mouse MT-III protein appears more similar to the mouse MT-I protein, while the human MT-III protein appears more similar to the human MT-II protein. At the protein level, sheep MT-III appears equally divergent from the MT-I and -II isoforms, having 37 amino acids identical to the sheep MT-Ia protein and 37 amino acids identical to the sheep MT-II protein.

The most striking feature of the putative sheep MT-III protein in comparison to any mammalian MT is a deletion of 3 amino acids (SCC) at positions 33-35. This is a deletion in the SCCSCC sequence that occurs directly after the 2 lysine residues

which form the bridge between the α- and β- domains of the protein. This sequence forms the start of the α-domain of the protein, and is conserved among all mammalian MT proteins sequences documented thus far (see Kägi, 1993). This deletion is likely to affect the structure of the protein and its metal binding properties and is unlikely to be an artifact since it was observed from a total of 5 individual sheep. In fact 3 conserved cysteine residues are missing from the sheep MT-III protein, those normally found at positions 34 and 35 which are deleted, as well as the cysteine residue usually conserved at position 30 which is a serine residue in sheep MT-III. The absence of 3 cysteine residues is likely to diminish the metal binding properties of the sheep MT-III protein, since cysteine residues are critical in metal binding.

The metal binding stoichiometries of MT-I and -II isoforms are well documented and appear to be identical for all MTs, as the metal complexing is dependent on the conserved cysteine residues alone, although other amino acids may contribute to the stability of the complex (see Kägi and Kojima, 1987). These conserved cysteine sulfhydryls can be classified as either terminal (liganded to one metal atom) or bridging (liganded to two metal atoms). Human and mouse MT-III proteins have been shown to exhibit the same metal binding stoichiometries with zinc, cadmium and copper as are documented for MT-I and -II isoforms (Sewell *et al.*, 1995), i.e. the MT-III proteins bind 7 mole equivalents of the divalent metals cadmium and zinc, or 12 copper ions. MT-III isoforms, like MT-I and -II isoforms, consist of 2 domains enfolding separate polymetallic clusters (Sewell *et al.*, 1995), i.e. MT-III proteins contain a Me_3Cys_9 cluster (β-domain containing 3 divalent metals via 3 bridging and 6 terminal cysteines) and a Me_4Cys_{11} cluster (α-domain containing 4 divalent metals via 5 bridging and 6 terminal cysteines). The zinc complexes of MT-III proteins also exhibit similar cluster reactivities as have been established for MT-I and -II isoforms (Sewell *et al.*, 1995). The putative sheep MT-III protein is missing one terminal cysteine residue from the β-domain and two cysteine residues (one bridging, one terminal) from the α-domain. These cysteine residues are normally involved in the binding of 3 of the 7 divalent metal ions in the MT-metal clusters (represented in Figures 4.10 and 4.11).

A number of studies have looked at the effect of mutations of the various conserved cysteine residues on the metal binding properties of MT mutants, and also

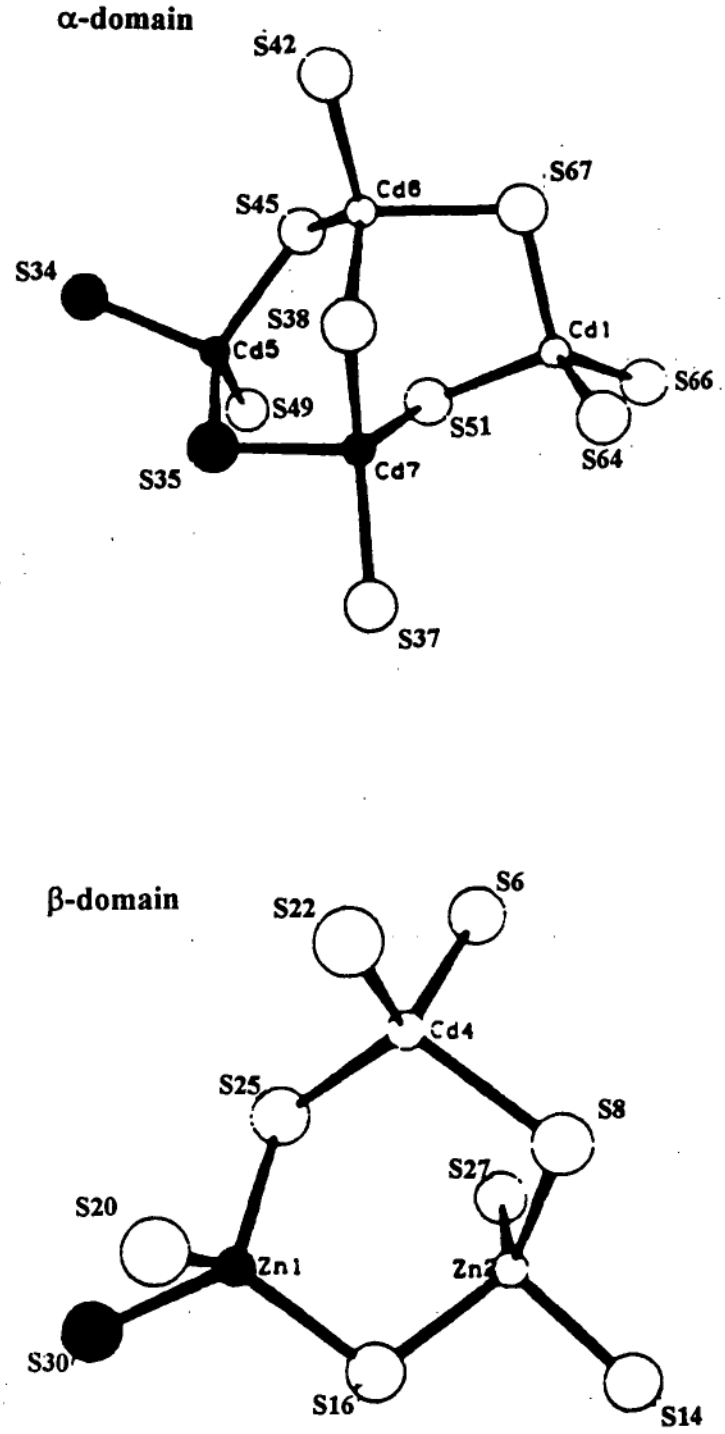


Figure4.10Disruptions in metal binding of the putative sheep MT-III protein. The metal thiolate clusters as determined for rat liver MT are shown. The β-domain cluster ($Metal_3cysteine_9$) and α-domain cluster ($Metal_4cysteine_{11}$) are shown. The sulfur atoms of the cysteine residues are represented as S_n where n indicates the position in the sheep MT-III amino acid sequence. Those cysteine residues either deleted or mutated in the sheep MT-III protein are indicated (black) and those metal atoms normally involved in binding to these residues are indicated (black). The numbering of the metals correspond to n.m.r spectral assignments. Adapted from Robbins *et al.*, (1991).

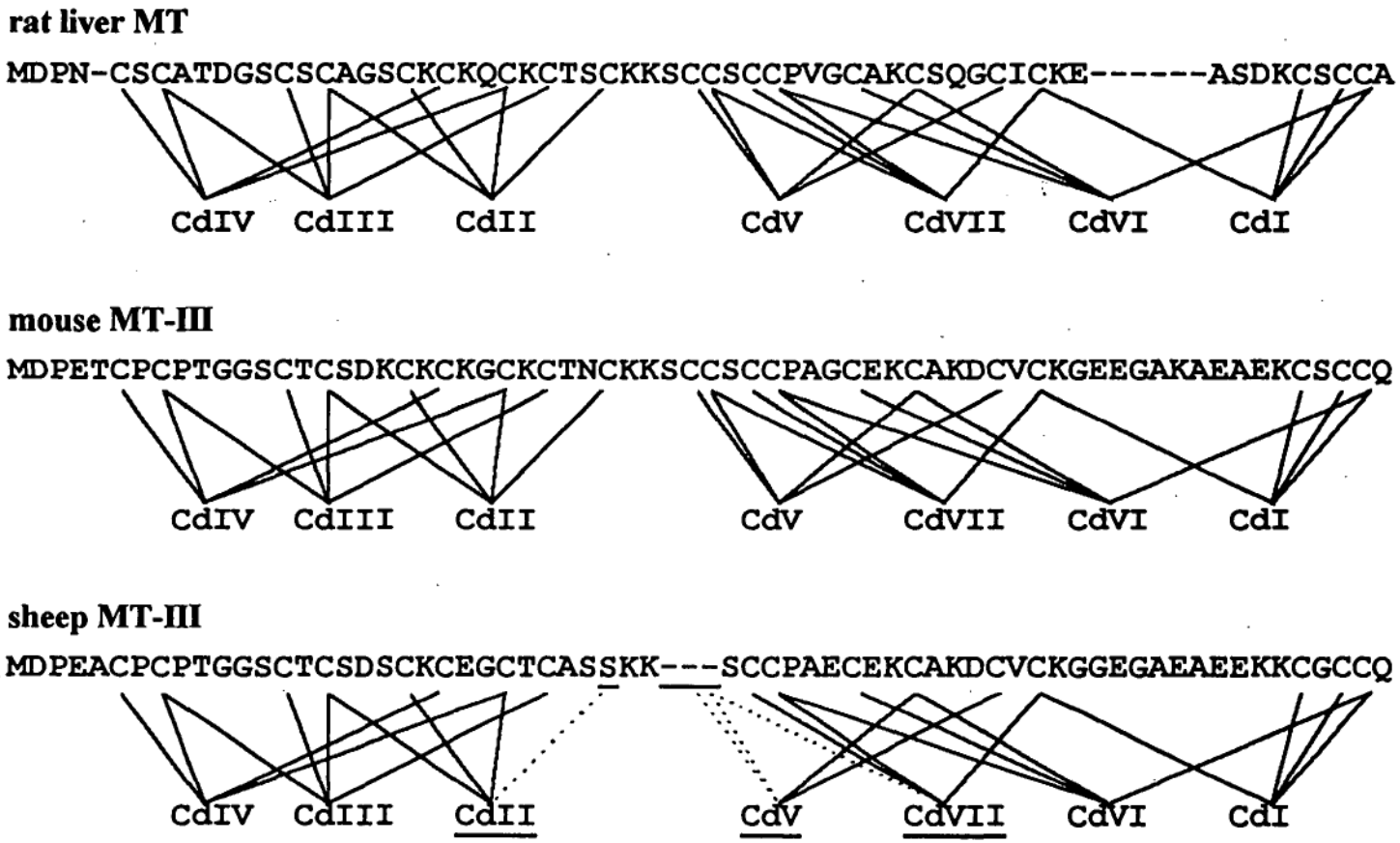


Figure 4.11 Cadmium-thiolate bonds which are disrupted in the putative sheep MT-III protein
Cadmium-cysteine connectivities of rat liver $^{113}Cd_7$-thionein established by 2-dimensional n.m.r spectroscopy are shown (adapted from Kagi and Schaffer, 1988). These connectivities are extrapolated to mouse MT-III, since identical conformations have been demonstrated for this isoform (Sewell *et al.*, 1995). The putative sheep MT-III amino acid sequence is shown, and those cadmium-cysteine ligands which are disrupted due to mutation or deletion of cysteine residues are indicated as dotted lines. Those cadmium atoms which are affected are underlined.

the ability of these mutants to protect against cadmium toxicity. In general, such MT mutants do not have major structural disruptions of the cadmium-thiolate binding geometry. For example, chinese hamster MT mutants C13Y, C50Y and C13,50Y (in which cysteine 13, cysteine 50 or both cysteine 13 and 50 are mutated to tyrosine residues) all retained a tetrahedral geometry of metal binding. They also retained the 2 independent domain structure characteristic of MT proteins, although the mutations created domains which tended to be folded less tightly. All three mutants bound 6 rather than 7 mole equivalents of cadmium (Cismowski and Huang, 1991). The C13Y mutant (with altered β-domain) was found to retain its ability to protect against cadmium toxicity, whereas the C50Y and C13,50Y mutants with altered α-domains did not. A second study determined the effect of mutation of terminal and bridging cysteine residues on detoxification capacity of the protein. While the mutation of all bridging cysteines examined resulted in decreased detoxification function, the mutation of some terminal cysteines resulted in decreased detoxification function while the mutation of other terminal cysteines was inconsequential (Chernaik and Huang, 1991).

Since the putative sheep MT-III protein retains the lysine bridge which links the 2 domains and is the only interaction between them, the structural alterations in the two domains may be considered separately. The β-domain of the sheep MT-III protein has a terminal cysteine residue mutated to a serine residue (C30S, position 30 corresponding to position 29 of MT-I and -II isoforms) as represented in Figures 4.10 and 4.11. Like the C13Y mutant with a mutated terminal cysteine residue in its β-domain, this domain of the sheep MT-III protein may still coordinate metals in a geometry similar to that of normal MT complexes (Cismowski and Huang, 1991). However, the analogous MT-II mutant C29S was found to have decreased ability to protect against cadmium toxicity (Chernaik and Huang, 1991).

The α-domain of the putative sheep MT-III protein is missing a terminal cysteine (C33) and a bridging cysteine residue (C34), as represented in Figure 4.10 and 4.11. The mutation of the analogous terminal cysteine residue in Chinese hamster MT incidentally, had no effect on the ability of the protein to confer protection against cadmium toxicity (Chernaik and Huang, 1991). In contrast, mutation of any bridging cysteine residue appears to result in decreased ability to protect against metal toxicity

(Chernaik and Huang, 1991). Therefore, the loss of the C34 bridging cysteine residue in the sheep MT-III protein would be expected to result in a decreased ability to protect against heavy metal toxicity. In a 'normal' mammalian MT protein the Me_3cys_9 β-domain has a cysteine to metal ratio of 3:1 whereas the Me_4cys_{11} α-domain has a cysteine to metal ratio of 2.75:1. It may be possible that the α-domain of the sheep MT-III protein which contains 9 rather than the usual 11 cysteine residues, may be able to form a β-domain-like conformation, binding 3 divalent metal atoms.

Studies of MT proteins with mutated cysteine residues like those outlined above, have demonstrated the plasticity of the metal-cysteine complexing, i.e. the ability of mutant MT proteins to bind a reduced number of metal atoms, but still in a structure resembling that found in the wildtype MT. Other studies have demonstrated the flexibility of the MT protein in accommodating divalent ions of vastly differing sizes, and the ability of MT proteins to accommodate univalent as well as divalent ions has been well documented (see Kägi and Kojima, 1987). There seems little doubt then that the putative sheep MT-III protein could bind metal ions, but the structure of the complex formed, the number of metals bound and how closely it resembles the mammalian MT-metal complexes documented to date remains to be determined. The studies outlined above have demonstrated that mutant MT proteins, although able to bind metals in a structure resembling that found in wildtype MT, may have impaired ability to protect against heavy metal toxicity. The ability of MT to protect against cadmium toxicity is often used to determine whether a MT protein is functional, however the role of MTs *in vivo* is still unclear and such an assay does not necessarily assess the ability of MT to function in the various other physiological roles proposed for MT, such as metal metabolism, homeostasis and transport, free radical scavenging or most importantly for MT-III isoforms, growth inhibition.

All MT-III proteins contain 2 insertions, a single amino acid insertion at position 5 in the N-terminal domain, and a larger insertion (usually of 6 amino acids) in the C terminal domain. In all MT-III isoforms examined to date, the single amino acid insertion at position 5 is a threonine residue, whereas the novel sheep MT-III protein contains an alanine residue at this position (see Figure 4.5B). However, the substitution of an alanine for a threonine residue would not be expected to significantly affect the structure of the protein as the amino acids are similar in size

and charge. Notably, no specific function or involvement directly in metal binding has been assigned to this single amino acid insertion, and it is interesting to note that rabbit MT-2a and MT-2c contain a single insertion of an alanine residue in their β-domain also, although the insertion is at position 9 (see Kägi, 1993). The 6 amino acid insertion in the C terminal domain of the MT-III proteins appears in the only extended loop of the MT-I and -II structure (Robbins *et al.*, 1991), and is a generally less conserved, although predominantly acidic, sequence. The insertion GEGAEA in the sheep MT-III protein is in fact identical to that identified in the bovine protein (see Figure 4.5B).

The actual positioning of the C-terminal insertion with respect to the MT-I or -II protein structure is debatable. This insertion found in the various MT-III proteins has been designated in the literature as occurring between residues 52 and 53 of the corresponding MT-I and -II isoforms for mouse and human (Palmiter *et al.*, 1992), between residues 52 and 53 for rat and human (Kobayashi *et al.*, 1993; Tsuji *et al.*, 1992) or between residues 51 and 52 for the bovine and equine MT-III proteins (Poutney *et al.*, 1994). MT-I and -II isoforms have conserved lysine residues at positions 51 and 56 which correspond to conserved lysine residues in the MT-III amino acid sequence at positions 52 and 63. The amino acid residues between these lysines are divergent between MT-I, -II proteins and MT-III proteins which has led to difficulties in assigning the exact position of the insertion in MT-III simply from the amino acid sequence of the proteins. In positioning the insertion found in the putative sheep MT-III protein I have followed Palmiter *et al.* (1992), in designating the insertion between residues 52 and 53 with respect to the sheep MT-I and -II protein (see Figure 4.5A).

Apart from those differences discussed above the only other amino acid in the sheep MT-III protein which is found to differ from those of the known MT-III isoforms is that the serine residue at position 65 in all other MT-III proteins is substituted by a glycine residue in the putative sheep MT-III. The serine residues throughout the MT protein chain are relatively conserved and although they are not directly involved in metal binding may have some effect on the stability of metal binding (see Kägi and Kojima, 1987).

Future work will involve confirming the predicted sheep MT-III structure at the protein level, either by purification of the protein and amino acid sequencing or by isolation of this isoform from sheep brain by chromatography and analysis by mass spectrometry to confirm its molecular weight.

4.3.2 Sheep MT-III expression *in vivo*

The sheep MT-III cDNA (and mouse MT-III RT-PCR product) hybridised to a single restriction fragment after Southern analysis of sheep genomic DNA, but not those previously attributed to the sheep MT-I and -II isoforms (Peterson *et al.*, 1988). Northern analysis with these MT-III probes indicated that sheep MT-III mRNA expression is restricted to the brain, in contrast to the more widespread expression of MT-I and -II isoforms. This is in keeping with the expression profiles seen in other species (Palmiter *et al.*, 1992; Tsuji *et al.*, 1992; Kobayashi *et al.*, 1993). MT-III mRNA was detected in the foetal (E116) and adult brain but not in the liver, kidney, gut, lung or heart of foetal or adult sheep (Figure 4.2). MT-III mRNA was not detected in the foetal liver at any time, or in the early foetal brain (before E116). It is interesting to note that the MT-III mRNA levels in the foetal E116 brain are approximately equal to those in the adult brain, whereas in other species a dramatic increase in MT-III mRNA has been reported after birth (e.g. Kobayashi *et al.*, 1993). However, it should be noted that the E116 foetal sheep brain is relatively mature, since cortical development relative to gestation period proceeds more quickly in sheep than in other mammals (Åstrom, 1967). Interestingly, MT-III was detected in the E116 foetal brain, at the same time as significant levels of MT-I and -II mRNA were detected suggesting similar controls may be involved in the regulation of MT-I, -II and -III isoforms during development.

Furthermore, the expression profile of MT-III mRNA in different regions of the adult sheep brain appeared to mimic to some degree that of MT-I and -II (Figure 4.8). For example, the highest level of MT-III mRNA was observed in the pineal gland, as was observed for MT-I and -II. Similarly, in the mouse (Masters *et al.*, 1994b) and in the human (see Figure 4.12), MT-III mRNA expression has been shown to have similar regional expression patterns to other MT isoforms. This seemingly concurrent expression of MT-III mRNA with MT-I and -II mRNA in the

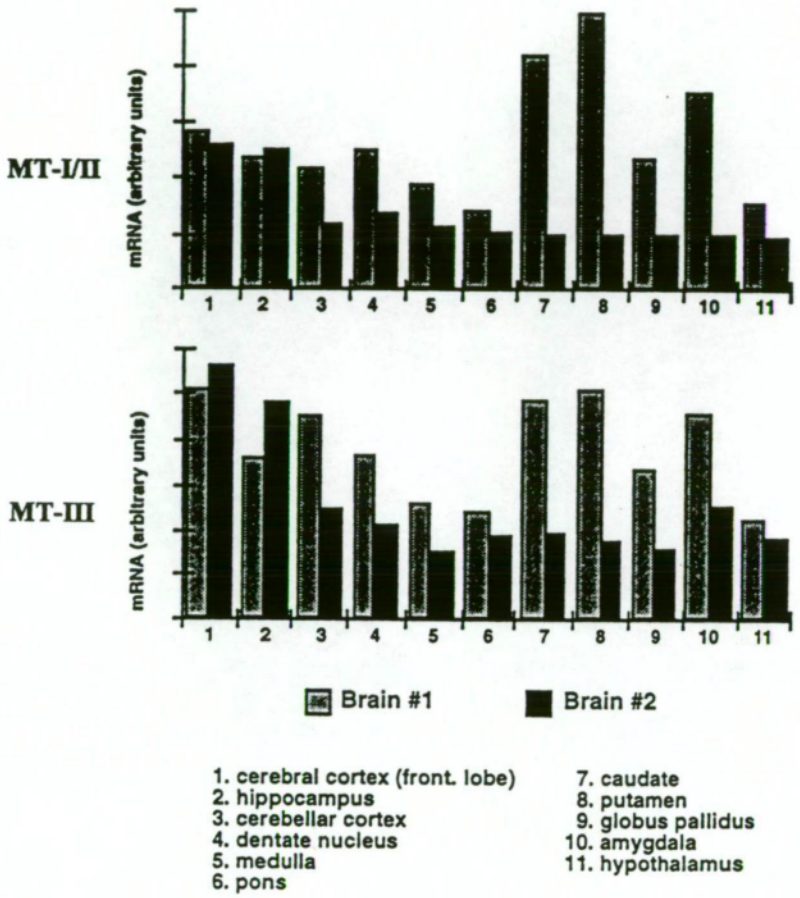


Figure 4.12 Regional expression of MT-I, -II and -III mRNA in the human brain
Graphical representation of the relative proportions of MT- I/II and MT-III mRNA in various regions of 2 human brains, as determined by northern analysis, is shown. Levels of the mRNA are arbitrary, and comparisons can not be made between different isoforms or between the 2 brains. Taken from F.A.Stennard, Ph.D thesis, 1995.

brain, at least under basal conditions, suggests that they may be subject to similar regulatory controls. However, studies have failed to show induction of MT-III by factors such as LPS and dexamethasone which are well documented regulators of MT-I and -II expression in the brain (Palmiter *et al.*, 1992). Another explanation may be that all MT isoforms are expressed in the same cell types, and the regional expression patterns reflect the relative proportion of MT expressing cells within each region of the brain. Similarly, the concurrent appearance of the MT isoforms during development may be due to the development/maturation of the MT expressing cells. The cell-type expression of MT-III is in fact controversial. MT-III mRNA and protein has been demonstrated to be co-expressed with other MT isoforms in glial cells in the human (e.g. Uchida *et al.*, 1991), but MT-III mRNA has been shown to be expressed in neurons in the mouse brain (Masters *et al.*, 1994b). This difference may be species specific, and investigation of cell type expression of the novel sheep MT-III isoform may shed light on this phenomenon. Preliminary *in situ* hybridisation experiments demonstrated varying intensities of hybridisation signal for MT-III in different regions of the sheep brain. However, the more elegant *in situ* hybridisation experiments have been in rat and mouse, where large regions of the brain have been investigated and comparison of the regional expression patterns has allowed MT-III expression to be correlated to glial rich or neuronal rich areas of the brain (e.g. Masters *et al.*, 1994b; Yuguchi *et al.*, 1995a). Such experiments in the sheep brain would require a large and detailed study and would require the perfusion of the sheep brain to successfully fix such large pieces of tissue. The preliminary experiments detailed in this study have however identified oligonucleotide probes which could be employed in such a study.

Antibodies have been made to both human and rat MT-III. Synthetic peptides covering the C-terminal inserts in the MT-III isoforms were used in both cases to generate antibodies (Uchida *et al.*, 1991; Hozumi *et al.*, 1995), however, Masters *et al.* (1994b), were unable to produce antisera to either these peptides or a maltose-binding protein-MT-III fusion protein. Due to the conservation of MTs difficulties have been encountered in generating isoform specific antibodies. Studies on the antibodies which cross react with MT-I and -II isoforms have revealed the antigenic region of the protein to be the first 7 amino acids (Winge and Garvey, 1983; Kay *et*

al., 1991). Since the sheep MT-III protein has a unique amino acid sequence in this region it may be an ideal candidate for the production of a specific MT-III antibody, and immunocytochemical techniques could then be employed to examine the cell type expression of sheep MT-III.

MT-III proteins are the only MTs for which a functional assay exists, in that they inhibit neurite sprouting *in vitro* (Uchida *et al.*, 1991; Erickson *et al.*, 1994; Poutney *et al.*, 1994). This, and its discovery as a factor deficient in Alzheimer's disease has led to MT-III being implicated as a growth inhibitory factor *in vivo*. Interestingly, despite the amino acid substitutions and deletions in the ovine MT-III protein, which may alter its metal binding properties, it retains the CPCP sequence in the N-terminal domain (positions 6-9) to which the growth inhibitory function of MT-III proteins has been attributed (Sewell *et al.*, 1995). The role of MT-III *in vivo* however, remains to be determined and the similarity of MT-I, -II and -III mRNA expression profiles raises the possibility that the function of MT-III may not be entirely dissimilar to that of MT-I and -II. This is supported by studies which demonstrate that transgenic mice overexpressing human MT-III protein are apparently physically and physiologically normal, apart from increased sequestration of zinc in the brain, which is completely accounted for by the overexpression of human MT-III (Erickson *et al.*, 1995). Much the same effect would be expected from overexpression of MT-I or -II isoforms. It is therefore possible that the *in vitro* assay in which MT-III exhibits growth inhibitory activity and is thus distinguishable from other MT isoforms may not be physiologically relevant, and may not represent the role of MT-III *in vivo*.

The structurally distinct sheep MT-III isoform may be very useful in understanding the function of MT-III *in vivo*, and its relationship to other MT isoforms. Analysis of its metal binding properties, its ability to provide protection against heavy metal toxicity, and its growth inhibitory activity, may provide insight into which of the properties of MT-III are physiologically relevant and whether it has a role in the brain distinct from MT-I and -II isoforms.

CHAPTER 5: EXPRESSION OF THE HUMAN *MT1L* GENE

5.1 Introduction

5.1.1 Human metallothionein genes

As is found in the sheep, humans have a large multigene MT family which encodes a single MT-II isoform and multiple MT-I isoforms. MT-II isoforms are distinguished from MT-I isoforms by the presence of an acidic amino acid at position 10 or 11 rather than a neutral amino acid in these positions. In the human all functional MT genes appear to be restricted to human chromosome 16 (Karin *et al.* 1984a) with all MT-I and -II genes linked in a cluster of 14 genes in a single locus, 16q13 as indicated in Figure 5.1. Genes encoding MT-III and MT-IV isoforms have also been identified in the human (Palmiter *et al.*, 1992; Quaife *et al.*, 1994) and these genes have been shown to be located in close proximity to the functional locus on chromosome 16 (Palmiter *et al.*, 1993), although linkage has not yet been demonstrated.

The human MT-I and -II genes have been characterised to varying degrees, and 8 of these genes (indicated in Figure 5.1) have been shown to be expressed at the protein or the mRNA level *in vivo*. A single MT-II isoform (*MT2A*) and multiple MT-I isoforms have been isolated from human liver (Hunziker and Kägi, 1987; Kägi, 1993). *MT2A* is the best characterised of the human MT genes and *MT2A* mRNA is expressed at high levels in most tissues (Richards *et al.*, 1984). *MT1A* is also expressed in most tissues at the RNA level, but at lower levels than *MT2A* (Richards *et al.*, 1984). *MT1F* and *MT1G* have also been investigated at the mRNA level, and appear to be expressed in a tissue specific manner (Varshney *et al.*, 1986; Jahroudi *et al.*, 1990). Similarly, expression of *MT1B* has been shown to be cell-type-specific (Heguy *et al.*, 1986) and studies using cell lines derived from human embryonic tissues suggest that the expression of *MT1E* and *MT1F* are developmentally regulated (Schmidt and Hamer, 1986). *MT1H* and *MT1X* mRNA have both been detected in HeLa cells after cadmium treatment (Stennard *et al.*, 1994) and their expression *in vivo* has also been demonstrated (F.A.Stennard, Ph.D. Thesis). Although the expression of multiple human isoforms has been demonstrated in the human liver by HPLC (Hunziker and Kägi, 1985; 1987), the technical difficulties associated with

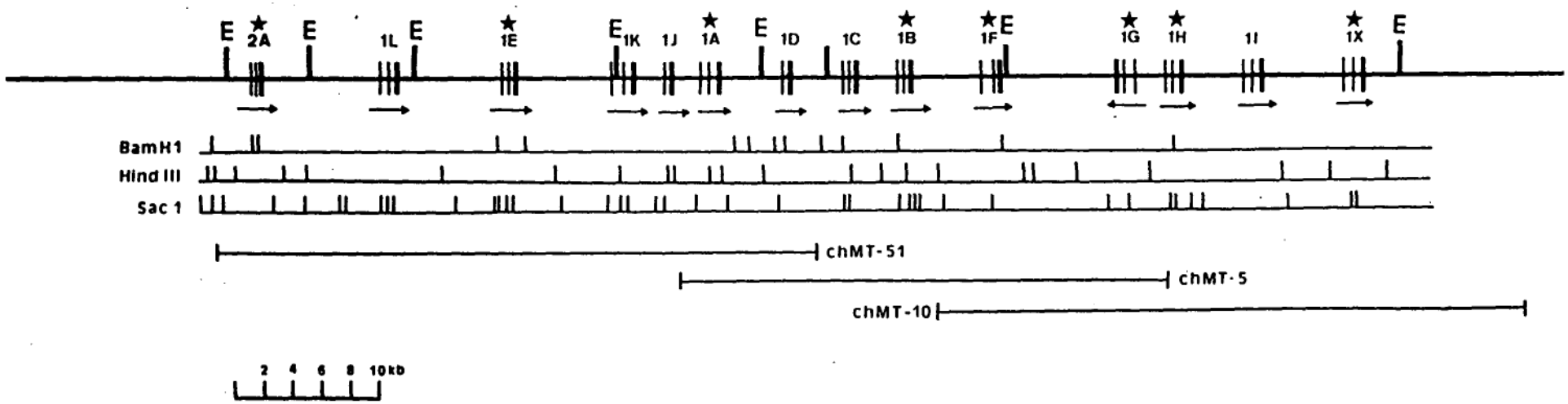


Figure 5.1 The MT-I/II functional locus on human chromosome 16

The location of the MT genes in the locus on chromosome 16 and their transcriptional orientations (arrow) are shown. Those genes which have been previously shown to be functional are indicated (asterix). Recognition sites for the restriction endonucleases *Bam*HI, *Hin*dIII, *Sac*I and *Eco*RI (E) are indicated. The cosmid clones chMT-51, chMT-5 and chMT-10 which encompass the entire functional MT-I/II locus are shown below.

investigating individual MT isoforms has meant that most of the human MT genes are only characterised at the RNA level.

The most recently discovered genes which encode the MT-III and MT-IV isoforms have restricted expression patterns *in vivo*. Human MT-III expression appears largely restricted to the brain (Tsuji *et al.*, 1992), while MT-IV expression appears to be restricted to squamous epithelial tissue, such as the nasal epithelia in the mouse (Quaife *et al.*, 1994).

The reason for multiple MT isogenes in the human is unclear. It is possible that their role is simply to allow the rapid accumulation of MT protein in cells, and certainly activation of multiple genes could theoretically result in the immediate expression of much larger amounts of protein than could be achieved by activation of a single gene. Such a system would be of value in situations of stress or physiological insults, conditions under which MT is often upregulated. However, the demonstration of differential expression patterns for various human MT isogenes has led to the suggestion that the multiple MT genes provide diversity of function. This is supported by studies which have demonstrated that the different isoforms can be induced by a variety of factors and to different extents. However, it remains to be seen whether the various MT isoforms have slightly different functions or whether they have identical roles, but in different cells or under different circumstances. Investigation of the individual MT genes, their expression in various tissues and their regulation may provide insight into the function of the various MT genes, and what part they play in the overall action of MTs *in vivo*.

5.1.2 The human metallothionein gene, *MT1L*

The sequence of the human MT gene *MT1L* has been obtained previously and is shown in Figure 5.2 (A.F.Holloway, B.Sc. Honours Thesis). The amino acid sequence encoded by *MT1L* is consistent with other human MT-I proteins (Figure 5.3), except for the presence of an in-frame TGA codon in exon 2 which is usually a signal for the termination of protein translation. Otherwise the MT1L amino acid sequence contains no atypical amino acids, and all of the 20 conserved cysteine residues are present apart from that replaced by the termination codon at position 26. However, the presence of a TGA ("stop") codon at position 26 of the predicted

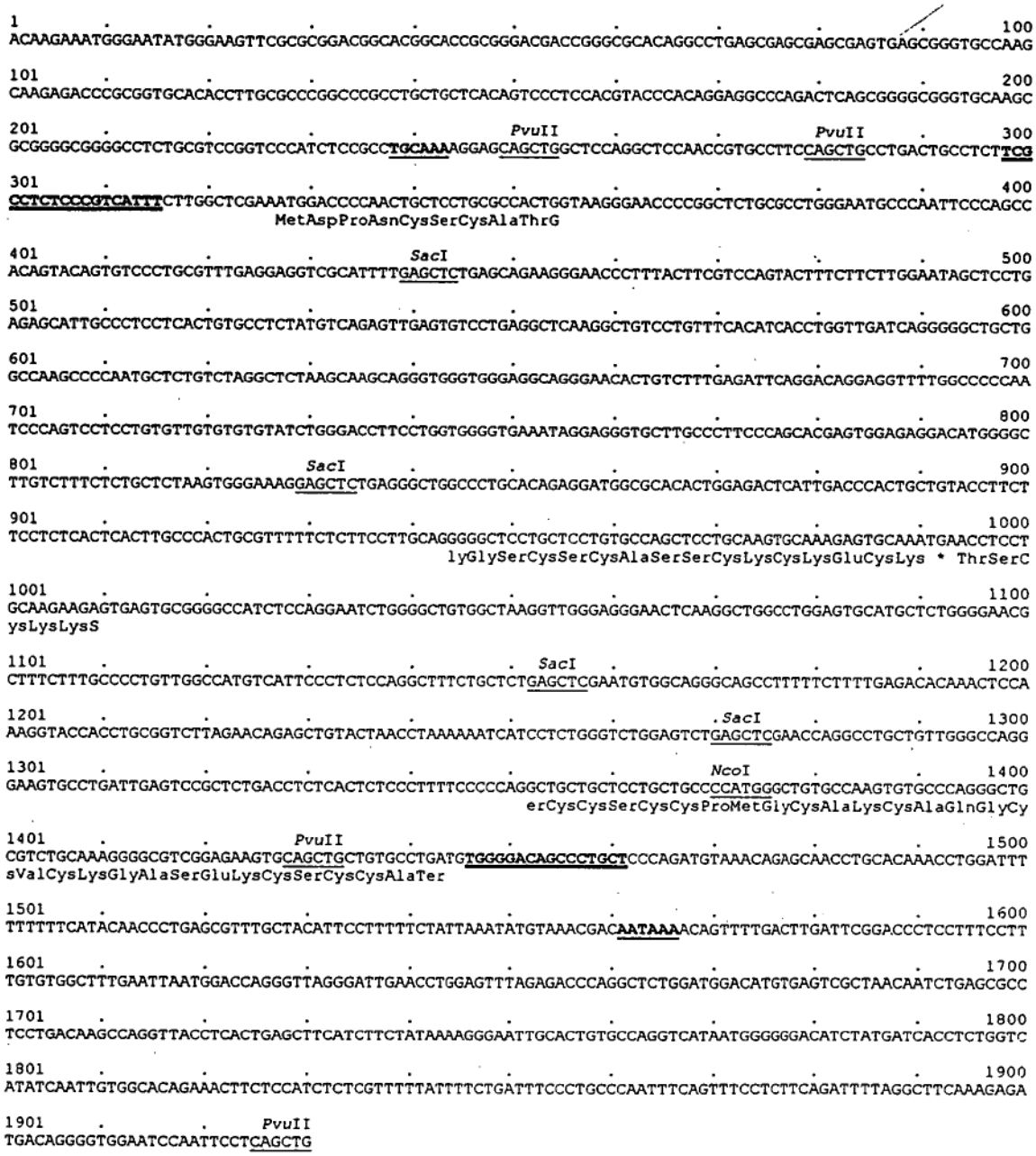


Figure 5.2 Nucleotide sequence of the human *MT1L* gene

The nucleotide sequence of the *MT1L* gene was determined by dideoxy sequencing (A.F.Holloway, B.Sc. Honours Thesis). The putative amino acid sequence is indicated below the coding regions. Recognition sites for the restriction endonucleases *Nco*I, *Sac*I and *Pvu*II are indicated (underlined). The putative transcription start site and polyadenylation site are indicated (bold, underlined). Binding sites for oligonucleotides designed to amplify the gene or cDNA by PCR are shown (bold, double underlined).

```
.....C.C.....C.C...C.C..C.C..C...CC.CC...C..C...C.C...........C.CC.     .
MDPN.CSCATGGSCSCASSCKCKECK*TSCKKSCCSCCPMGCAKCAQGCVCKG......ASEKCSCCA MT1L
MDPN.CSCATGGSCTCTGSCKCKECKCNSCKKSCCSCCPMSCAKCAQGCICKG......ASEKCSCCA MT1A
MDPN.CSCTTGGSCACAGSCKCKECKCTSCKKCCCSCCPVGCAKCAQGCVCKG......SSEKCRCCA MT1B
MDPN.CSCATGGSCTCAGSCKCKECKCTSCKKSCCSCCPVGCAKCAQGCVCKG......ASEKCSCCA MT1E
MDPN.CSCAAGVSCTCAGSCKCKECKCTSCKKSCCSCCPVGCSKCAQGCVCKG......ASEKCSCCD MT1F
MDPN.CSCAAGVSCTCASSCKCKECKCTSCKKSCCSCCPVGCAKCAQGCICKG......ASEKCSCCA MT1G
MDPN.CSCEAGGSCACCGSCKCKKCKCTSCKKSCCSCCPLGCAKCAEGCICKG......ASEKCSCCA MT1H
MDPN.CSCSPVGSCACAGSCKCKECKCTSCKKSCCSCCPVGCAKCAQGCICKG......TSDKCSCCA MT1X
MDPN.CSCAAGDSCTCAGSCKCKECKCTSCKKSCCSCCPVGCAKCAQGCICKG......ASDKCSCCA MT2A
MDPETCPCPSGGSCTCADSCKCEGCKCTSCKKSCCSCCPAECEKCAKDCVCKGEAAEAEGAEKCSCCQ  MT3
MDPRECVCMSGGICMCGDNCKCTTCNCKTCRKSCCPCCPPGCAKCARGCICKG......GSDKCSCCP  MT4
```

Figure 5.3 Comparison of the putative human MT1L protein sequence with known human MT proteins

The putative amino acid sequence encoded by the human *MT1L* gene is shown. For comparison the amino acid sequences of the other MT-I and -II proteins encoded by genes of the functional locus on chromosome 16 are shown. Amino acid sequences of the human MT-III and -IV proteins are also shown. The features which define the MT-II, MT-III and MT-IV isoforms are demonstrated (underlined) and those features unique to MT1L are indicated (underlined). The conserved cysteine residues (C) are shown above the sequences. Conventional single letter abbreviations are used for the amino acids and an asterix is used to indicate a TGA codon.

amino acid sequence makes the amino acid assignments which follow this position irrelevant and would initially suggest that the *MT1L* gene is non-functional, i.e. does not produce a protein. However, it is possible that a shortened, but still functional protein consisting of 25 instead of 62 amino acids is encoded by this gene. MT proteins consist of 2 domains and studies have shown that these are individually capable of binding heavy metals (Furey *et al.*, 1986). A shortened *MT1L* protein consisting of 25 amino acids is an almost complete β-domain and therefore may be functional in itself (see Figure 5.4).

Alternatively, in several cases a TGA codon has been shown to encode an unusual amino acid residue, selenocysteine, rather than a termination signal (reviewed in Böck *et al.*, 1991). This results in a functional selenoprotein being produced by the cotranslational insertion of a selenocysteine residue in the protein. All except one of the selenoproteins known are encoded by a gene with a single inframe TGA codon, like that of *MT1L*, resulting in one selenocysteine residue in the protein chain. The best characterised of the mammalian selenoproteins is glutathione peroxidase and the selenol moiety is essential for the redox properties of this protein (Forstrom *et al.*, 1978). It has been proposed that the selenol moiety imparts increased reactivity compared with the thiol of a normal cysteine residue (Stadtman, 1996).

In previous work by the author, *MT1L* (as well as the more highly characterised *MT2A*) was cloned into a eukaryotic expression vector, pSVL, and after transfection into the monkey kidney cell line, COS, were both shown to produce mRNA (A.F.Holloway, B.Sc. Honours Thesis). In addition the transfected genes conferred enhanced cadmium resistance (Figure 5.5), suggesting that *MT1L*, like *MT2A* encodes a functional MT protein.

The aim of this work was to determine whether *MT1L* is expressed at the mRNA level in human tissues and cell lines, and to determine whether the *MT1L* gene encodes a functional protein, either a 61 amino acid selenoprotein or a truncated 25 amino acid metallothionein protein.

5.2 Results

During the course of this work the entire sequence of the *MT1L* gene as shown in Figure 5.2, was rechecked, and the appearance of an inframe TGA codon in

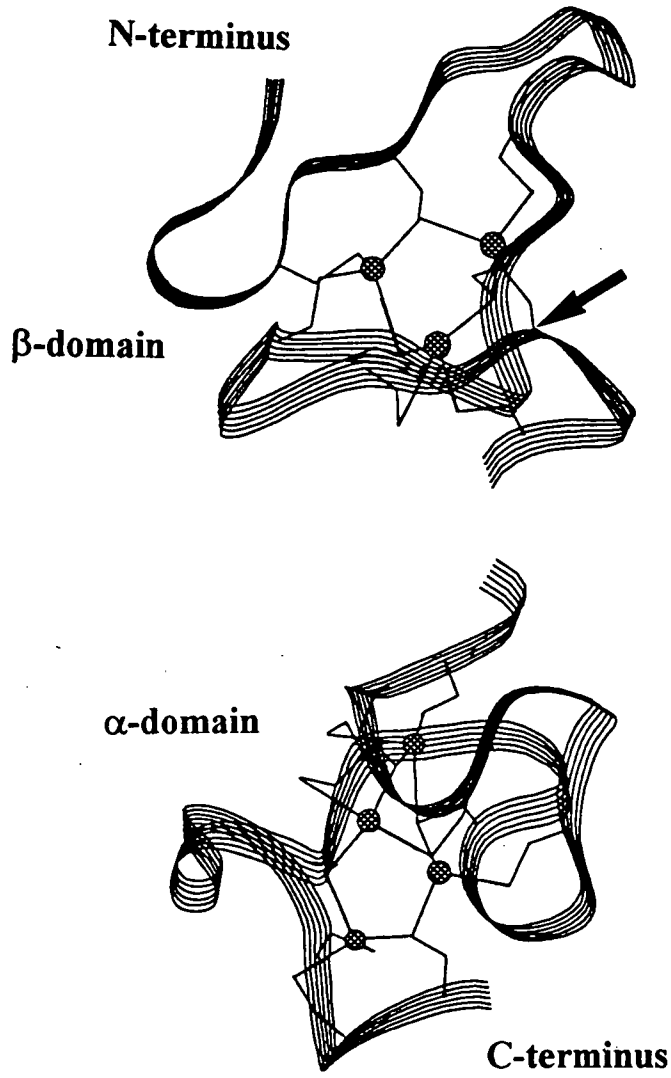


Figure 5.4 Models of the α- and β- domains of the MT protein
The separated α- and β-domains of an MT protein are shown. The model is a ribbon representation of the MT protein with the metal-cysteine clusters depicted as filled circles and thin lines. The α-domain consists of a 4 metal 11 cysteine cluster and the β-domain consists of a 3 metal 9 cysteine cluster (taken from Kägi, 1993). The position of cysteine residue 26 which is replaced with a TGA ("stop") codon in *MT1L* is indicated by an arrow. Note that this position is close to the end of the β-domain of the protein.

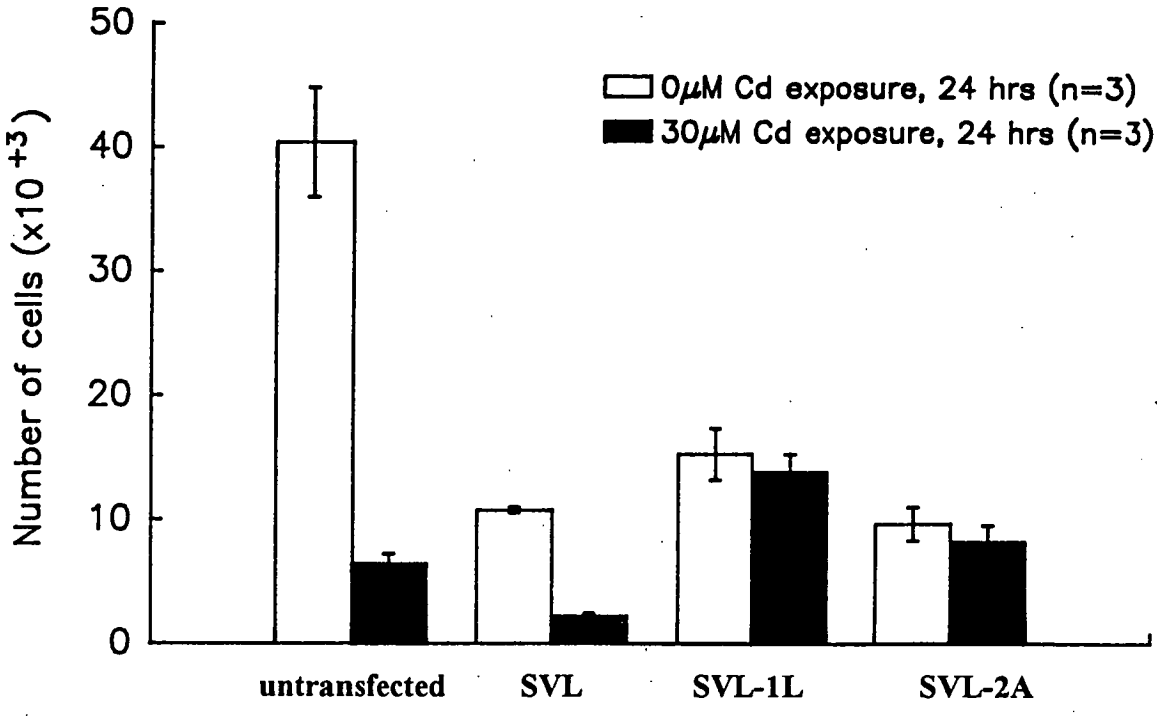


Figure 5.5 Ability of *MT1L* to protect against cadmium toxicity
The human *MT1L* gene and *MT2A* cDNA were cloned into the eukaryotic expression vector, pSVL to create the vectors SVL-1L and SVL-2A, respectively. COS cells were transfected with the vectors by Transfectam (Promega) mediated transfection and treated with 30 μM $CdCl_2$ for 24 hours. Untransfected cells and cells transfected with the pSVL vector alone represent negative controls.

exon 2 of this gene was reconfirmed from 2 separate clones, chMT-51 and λ39.1, derived from 2 unrelated individuals (see West *et al.*, 1990).

A series of experiments were conducted to determine whether *MT1L* was expressed in human tissues or in human cultured cell lines. RT-PCR and northern blotting were used to detect *MT1L* mRNA.

5.2.1 Detection of *MT1L* mRNA in human tissues by RT-PCR

5.2.1.1 Specificity of human *MT1L* PCR primers

PCR primers were designed to the 5' and 3' flanking regions of the *MT1L* gene. These regions are considerably less conserved than the coding regions and allowed primers to be designed which were specific for the *MT1L* gene. The location of the *MT1L* PCR primers within the gene sequence are indicated in Figure 5.2. These primers would be expected to amplify a gene fragment of 1164 bp or a cDNA fragment of 230 bp. In some instances primers specific for other MT genes, designed similarly to their 5' and 3' flanking regions were used for comparison. The following primer pairs were used to amplify DNA or cDNA by PCR:

MT1L	sense	5'-TCGCCTCTCCCGTCATTT-3'
	antisense	5'-AGCAGGGCTGTCCCCA-3'.
MT2A	sense	5'-GCAACCTGTCCCGACTCTAG-3'
	antisense	5'-ATCCAGGTTTGTGGAAGTCG-3'
MT1H	sense	5'-CGTGTTCCACTGCCTCTTCT-3'
	antisense	5'-CGTGTCATTCTGTTTTCATCTGAC-3'
MT1X	sense	5'-AACTCCTGCTTCTCCTTGCC-3'
	antisense	5'-GCTCTATTTACATCTGAGAGCACA-3'
β-actin	sense	5'-CATCCTCACCCTGAAGTACC-3'
	antisense	5'-CACACTTCATGATGGAGTTGA-3'

The RT-PCR protocol was as outlined in section 2.4 and an annealing temperature of 66°C was used in the PCR reaction. PCR products were electrophoresed through 1.4% agarose/1 X TBE, and visualised by ethidium bromide staining.

The specificity of the *MT1L* PCR primers was confirmed by PCR of cosmids chMT-51, chMT-5 and chMT-10 which contain all of the human MT-I and -II genes in the locus 16q13 (see Figure 5.6). This experiment demonstrated that the *MT1L* PCR primers were specific for the *MT1L* gene and were not able to amplify other MT-I or -II genes. As shown in Figure 5.6, addition of cosmid chMT-51 DNA (which contains the *MT1L* gene) to the PCR reaction resulted in amplification of a single DNA fragment. The size of this fragment (1164 bp) was as predicted for the amplification of the *MT1L* gene using these primers (see Figure 5.2). Amplification products were not detected when either cosmid chMT-5 or chMT-10 DNA (which do not contain the *MT1L* gene) were added to the PCR reaction, as demonstrated in Figure 5.6.

5.2.1.2 Expression of *MT1L* mRNA in human tissues

Total RNA was isolated from human autopsy samples (section 2.3.3) and subjected to RT-PCR using the various PCR primers described above at an annealing temperature of 66°C. RT-PCR of pancreas RNA using *MT1L* PCR primers amplified a fragment consistent with the expected size from *MT1L* cDNA of 230 bp (see Figure 5.7A). *MT1X* (247 bp), *MT2A* (279 bp), and *MT1H* (286 bp) products were also amplified from pancreas RNA using the appropriate primers. Primers designed to amplify the ubiquitous β-actin mRNA were used in each reaction as an internal control, and a product of 668 bp was amplified in all samples except when using the *MT2A* primers. This absence of a β-actin product was frequently observed when using *MT2A* primers and has been observed elsewhere (F.A.Stennard, Ph.D. Thesis) and is probably due to a number of factors. The β-actin product is larger than that of *MT2A* and the extension time required to form this product is likely to be longer. Since *MT2A* is also expressed at relatively high levels it is likely that reaction components are consumed in the amplification of the human *MT2A* cDNA at the expense of the β-actin product.

Using the *MT1L* PCR primers a PCR product of 230 bp was also amplified from thyroid, pineal gland and hypothalamus RNA by RT-PCR, but not from liver RNA (Figure 5.7B). As indicated in Table 5.1, *MT1L* cDNA was also not detected by RT-PCR in small intestine, large intestine, spleen, heart or thymus, although *MT2A*

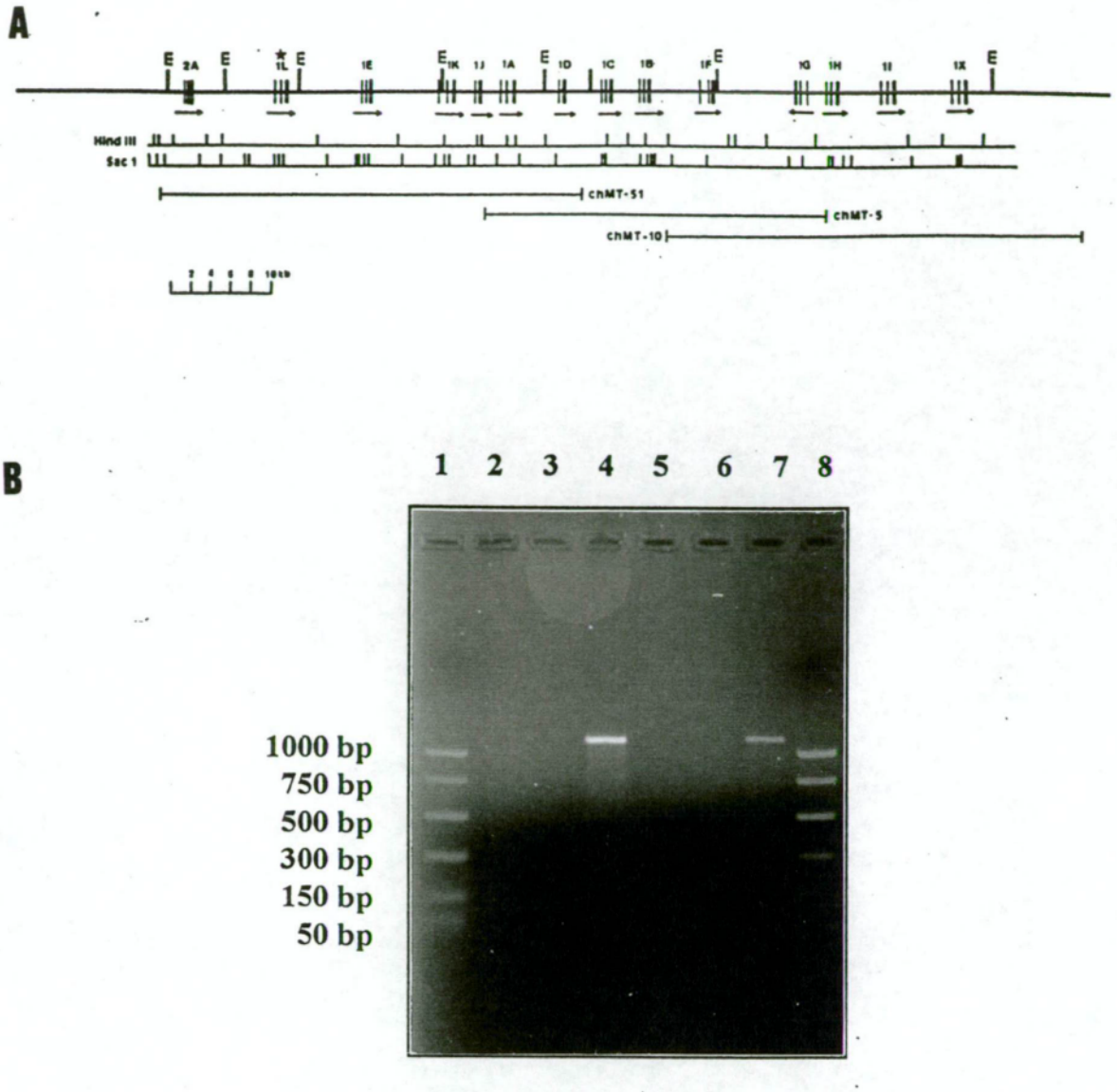


Figure 5.6 Specificity of *MT1L* PCR primers

The cosmid clones chMT-5, chMT-10 and chMT-51 which cover the entire functional MT-I and -II locus on human chromosome 16 were used to determine the specificity of *MT1L* PCR primers. The location of the human MT-I and -II genes in the functional locus and their transcriptional orientations (arrows) are shown (A). Restriction maps for the endonucleases *Hin*dIII, *Sac*I and *Eco*RI (E) are indicated. The *MT1L* gene is highlighted (asterisk). Regions encompassed by the cosmid genomic clones chMT-51, chMT-5 and chMT-10 are shown below the map (Adapted from West *et al.*, 1990). Cosmid DNA was subjected to PCR and PCR products were electrophoresed through 1.4% agarose and visualised by ethidium bromide staining (B).

1: Molecular weight markers (sizes as marked)
2: chMT-5, 1 ng
3: chMT-10, 1 ng
4: chMT-51, 1 ng
5: chMT-5, 0.1 ng
6: chMT-10, 0.1 ng
7: chMT-51, 0.1 ng
8: Molecular weight markers

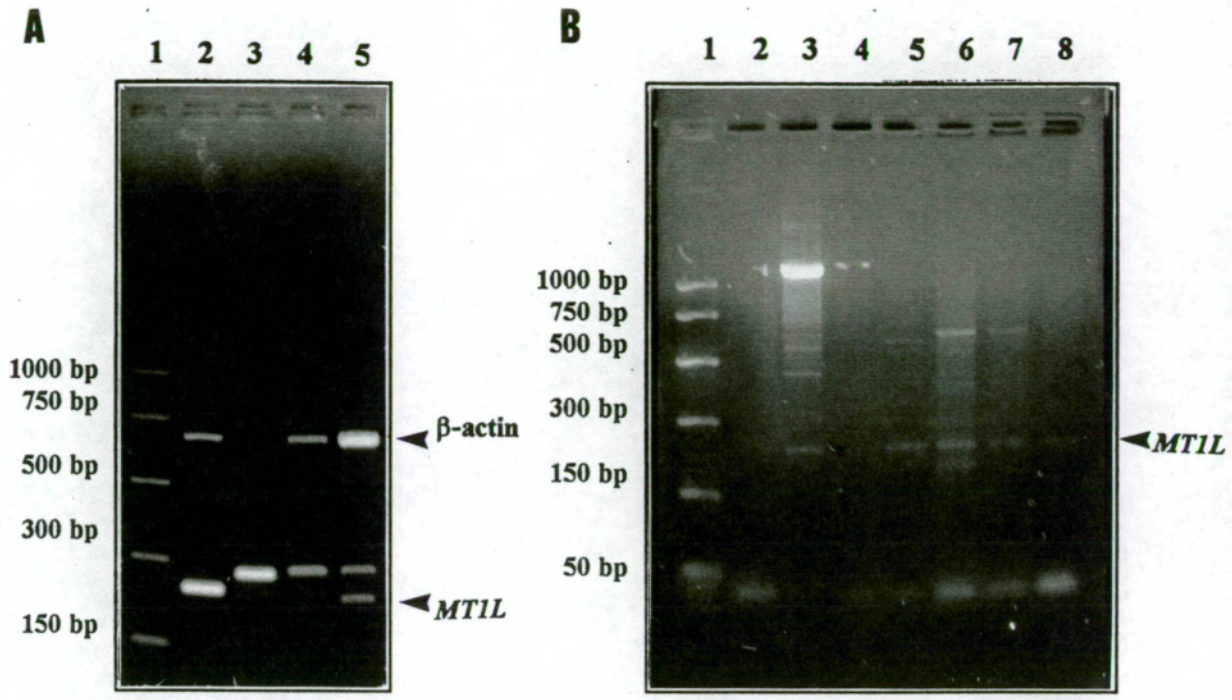


Figure 5.7 Detection of *MT1L* in human tissues by RT-PCR

A: RNA was isolated from human pancreas and subjected to RT-PCR using primers specific for human MT genes *MT1X*, *MT2A*, *MT1H* and *MT1L*. β-actin primers were also included in each reaction.

1: Molecular weight markers (sizes as marked)
2: Pancreas RNA, *MT1X* primers, β-actin primers
3: Pancreas RNA, *MT2A* primers, β-actin primers
4: Pancreas RNA, *MT1H* primers, β-actin primers
5: Pancreas RNA, *MT1L* primers, β-actin primers

MT1L (230 bp, lane 5) and β-actin (668 bp, all lanes) PCR products are indicated.

B: RNA was isolated from human tissue samples or COS cells transfected with the human *MT1L* gene, and subjected to RT-PCR using primers specific for the human *MT1L* gene.

1: Molecular weight markers (sizes as marked)
2: no RNA
3: RNA from COS cells transfected with the *MT1L* gene
4: Liver RNA
5: Thyroid RNA
6: Pancreas RNA
7: Pineal gland RNA
8: Hypothalamus RNA

The 230 bp *MT1L* PCR product is indicated.

	MT1L	*MT2A*
liver	-	+
small intestine	-	+
large intestine	-	+
pancreas	+	+
thyroid	+	+
spleen	-	+
heart	-	+
thymus	-	+
pineal gland	+	+
hypothalamus	+	+

Table 5.1 Amplification of *MT1L* and *MT2A* transcripts by RT-PCR
RNA was isolated from human tissues and subjected to RT-PCR using primers specific for the human *MT1L* and *MT2A* genes. The tissues in which products from the two genes were (+) and were not (-) amplified are indicated.

cDNA was detected in all of these tissues. All of these tissues were from a single individual (4 month old male, tissues obtained 48 hr *postmortem*).

When *MT1L* PCR primers were used to amplify cDNA a second amplification product, slightly larger or smaller than that expected was often observed. This occurred particularly when using cDNA from the pancreas (see Figure 5.7A). The nature of these products are unknown, but are presumed not to be from another MT gene as the primers had been proved to be specific for the *MT1L* gene. However, the 230 bp product obtained by RT-PCR using *MT1L* primers was confirmed to be amplification of *MT1L* cDNA by several methods as outlined below.

The *MT1L* gene and *MT2A* cDNA were previously cloned into the eukaryotic expression vector pSVL to create the constructs SVL-1L and SVL-2A (A.F.Holloway, B.Sc. Honours Thesis). The pSVL vector allows expression of cloned genes under the control of the SV40 promoter. RNA was isolated from COS cells, a monkey kidney cell line, transfected with these vectors: SVL, SVL-1L and SVL-2A (section 2.7.3). The RNA was subjected to RT-PCR using the *MT1L* PCR primers. A product of the same size as one of those amplified from human pancreas RNA (230 bp) was detected in cells transfected with the SVL-1L vector (*MT1L* gene), but not cells transfected with the SVL-2A vector (*MT2A* cDNA) or pSVL vector alone (Figure 5.8). β-actin PCR primers were included in all reactions as an internal control and a product of 668 bp was amplified in each instance. Figure 5.7B demonstrates that the products amplified by RT-PCR from thyroid, pineal gland and hypothalamus RNA using *MT1L* primers were also the same size as that amplified from RNA isolated from SVL-1L (*MT1L*) transfected COS cells.

As shown in Figure 5.2, *MT1L* contains a recognition site for the restriction endonuclease *Nco*I in the region encoding the third exon. This site should be present in the cognate PCR product from this gene. No other known human MT gene contains this *Nco*I site. Therefore, products of 230 bp amplified using *MT1L* primers from both the *MT1L* transfected COS cell RNA and human pancreas RNA were digested with the restriction endonuclease *Nco*I. Restriction fragments of 139 bp and 91 bp were produced as expected (Figure 5.9A). These DNA fragments (both before and after digestion with *Nco*I) were electrophoresed through 1.4% agarose/1 X TBE, transferred to a nylon membrane by alkaline blotting (section 2.3.2) and hybridised

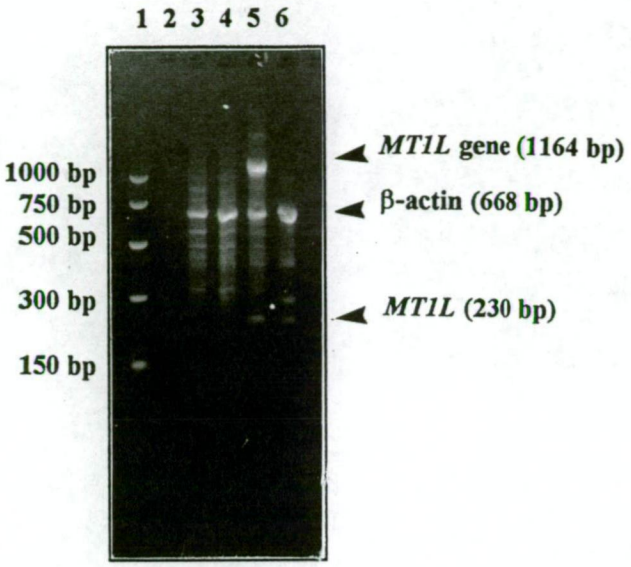


Figure 5.8 Detection of *MT1L* mRNA in transfected COS cells by RT-PCR
COS cells were transfected with the eukaryotic expression vector SVL, the human *MT2A* cDNA (SVL-2A) and the human *MT1L* gene (SVL-1L). RNA isolated from these cells was subjected to RT-PCR using primers specific for the *MT1L* gene and also the human β-actin gene.

1: Molecular weight markers (sizes as marked)
2: no RNA
3: RNA from SVL transfected COS cells
4: RNA from SVL-2A transfected COS cells
5: RNA from SVL-1L transfected COS cells
6: RNA from human pancreas

PCR products from amplification of the *MT1L* and β-actin mRNA are indicated (arrows). A product is also detected in lane 5 from amplification of the *MT1L* gene from contaminating SVL-1L vector DNA.

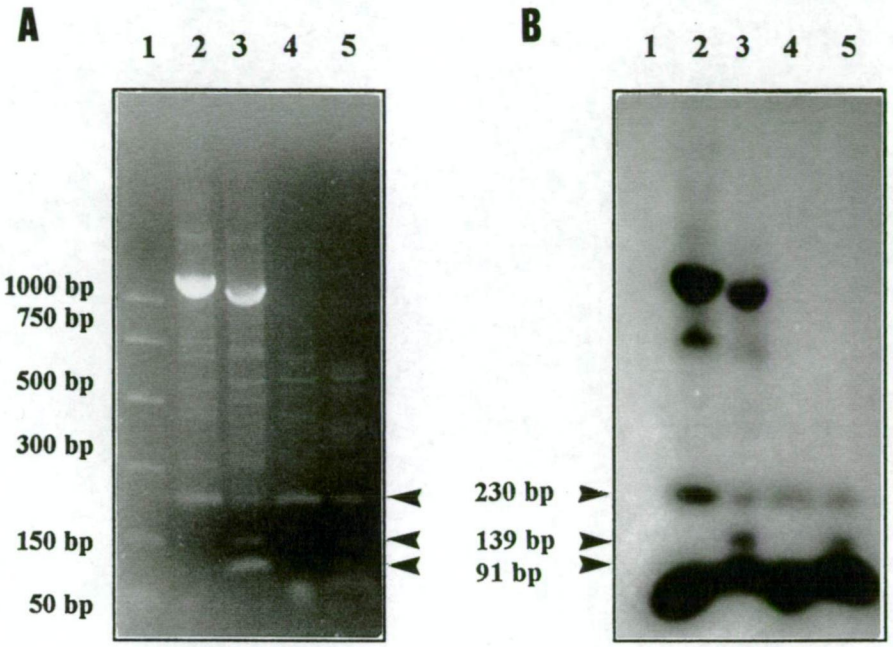


Figure 5.9 Confirmation of *MT1L* PCR product by restriction endonuclease digestion

RNA was isolated from human pancreas and from COS cells transfected with the human *MT1L* gene in the eukaryotic expression vector SVL. RNA was subjected to RT-PCR using primers specific for *MT1L*, restricted with *Nco*I and electrophoresed through a 1.4% agarose gel (A). The DNA fragments were transferred to nylon membrane by alkaline blotting and hybridised with a general MT-I/II radiolabelled probe, as described in the text. The resultant autoradiograph is shown (B). The 230 bp *MT1L* PCR product is indicated, as well as the 139 bp and 91 bp products of its restriction with *Nco*I.

1: Molecular weight markers (sizes as marked)
2: RNA from *MT1L* transfected COS cells
3: RNA from *MT1L* transfected COS cells, *Nco*I restricted
4: RNA from human pancreas
5: RNA from human pancreas, *Nco*I restricted.

with a general MT probe. The probe used in this analysis was a *MT2A* RT-PCR product which hybridises to all human MT-I and -II genes including *MT1L*, under the conditions discussed later in this chapter (see Figure 5.12, below). This probe hybridised to the 230 bp RT-PCR product amplified from the human pancreas RNA and SVL-1L transfected COS cell RNA (Figure 5.9B). It also hybridised to the 139 bp and 91 bp fragments produced by digestion of the products with the restriction endonuclease *Nco*I (Figure 5.9B). The *NcoI* digestion of the PCR products was incomplete and therefore hybridisation to the 230 bp product could still be detected after digestion. Hybridisation of the probe to the 91 bp fragment is partially masked by the signal from hybridisation to excess primers at the bottom of the blot. It should also be noted that although products of other sizes were often observed when using *MT1L* PCR primers, these products did not hybridise to the MT probe. However, RT-PCR of the RNA isolated from SVL-1L transfected COS cells resulted in amplification of a much larger fragment which hybridised to the MT probe (Figure 5.9, Lanes 2 and 3). This product (of 1164 bp) is due to amplification of the *MT1L* gene from contaminating vector DNA in the RNA preparation. This contamination occurred because of the replication of SV40-based vectors to a high copy number in COS cells.

The 230 bp PCR product obtained from amplification of human pancreas cDNA using *MT1L* PCR primers was excised from a 1.4% agarose gel and purified (section 2.2.1). The cDNA was blunt ended with mung bean nuclease and ligated into pUC19 (section 2.2). Sequence analysis of a purified clone confirmed that the RT-PCR product was *MT1L* cDNA (Figure 5.10A). The clone was incomplete due to the removal of nucleotides at either end of the transcript by mung bean nuclease which has exonuclease activity. However, the sequence obtained corresponded to the entire second exon and most of the third exon of the gene, and importantly included the TGA codon at position 26 which is unique to *MT1L*. The clone was sequenced in both directions and the TGA codon is clearly evident in both cases, as demonstrated in Figure 5.10B and C. This experiment confirmed that the mRNA detected in human tissues by RT-PCR was derived from the *MT1L* gene, and that the TGA codon was indeed present in the mRNA.

A

```
GGCTCCTGCTCCTGTGCCAGCTCCTGCAAGTGCAAAGAGT
GlySerCysSerCysAlaSerSerCysLysCysLysGluC

GCAAATGAACCTCCTGCAAGAAGAGCTGCTGCTCCTGCTG
ysLys***ThrSerCysLysLysSerCysCysSerCysCy

CCCCATGGGCTGTGCCAAGTGTGCCCAGGGCTGCGTCTGC
sProMetGlyCysAlaLysCysAlaGlnGlyCysValCys

AAAGGGGC
LysGlyAl
```

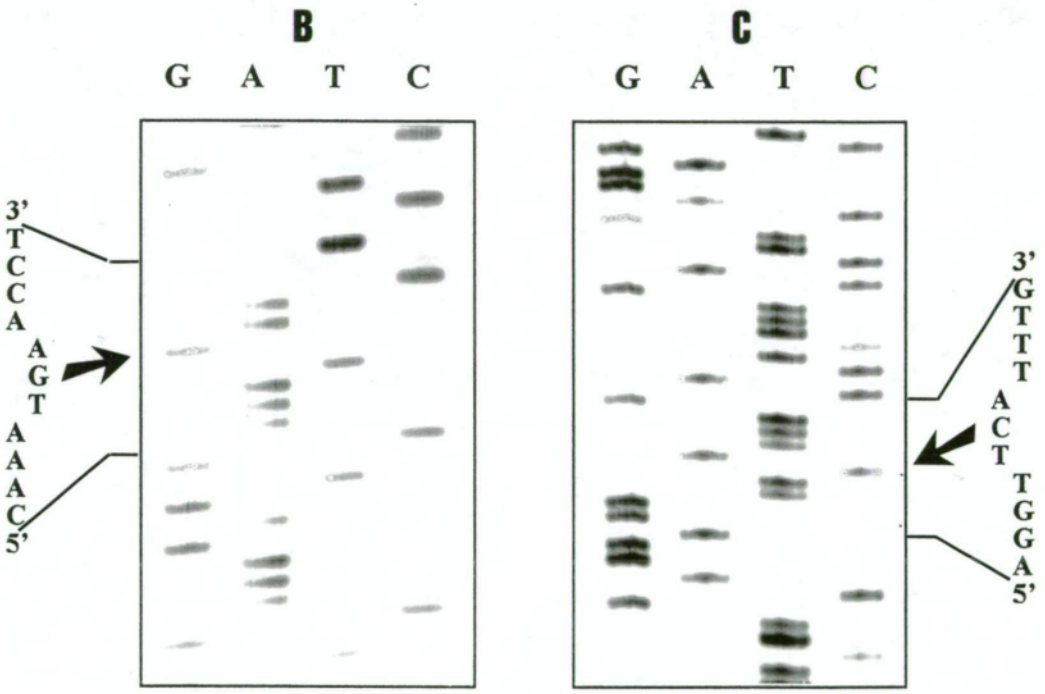


Figure 5.10 Sequence of a partial clone amplified from human pancreas
RNA was isolated from human pancreas and subjected to RT-PCR using primers specific for the human *MT1L* gene. The amplified transcript was blunt ended with mung bean nuclease, cloned into pUC19 and sequenced. Sequencing reactions were electrophoresed on a 6% acrylamide gel, the gel fixed and dried, and exposed to Hyperpaper (Amersham). The nucleotide sequence of the partial clone obtained (A) is identical to the *MT1L* gene, including an inframe TGA codon (***). The regions of the autoradiograph of the sequencing gel showing the TGA codon on both the sense (B) and antisense (C) strand are shown. Note that the TGA codon appears as the reverse compliment on the antisense strand ie TCA reading 5' to 3'.

5.2.1.3 Detection of *MT1L* mRNA in human cell lines by RT-PCR.

The expression of the human *MT1L* gene at the RNA level was examined in a primary culture of human lymphocytes and also white blood cell lines by RT-PCR.

RNA isolated from cultured human lymphocytes (prepared as outlined in Stennard *et al.*, 1995), untreated and treated with 10 μM $CdCl_2$ or 10 μM $ZnCl_2$ for 6 hr was supplied by F.A.Stennard. The RNA was subjected to RT-PCR using the *MT1L* PCR primers and a product consistent with the expected size from *MT1L* cDNA of 230 bp was amplified from cadmium and zinc treated samples (Figure 5.11). No product was detected using RNA from untreated lymphocytes.

RNA was isolated from CEM and K562 cell lines (two lymphoid cell lines) maintained in culture as described in section 2.7.2. These cells were either untreated or treated with 10 μM $CdCl_2$ for 16 hr. The RNA was subjected to RT-PCR using *MT1L* PCR primers and a product consistent with the expected size from *MT1L* cDNA of 230 bp was amplified from CEM cells (Figure 5.11). A product of the same size was amplified from RNA isolated from COS cells transfected with the SVL-1L vector, which contains the *MT1L* gene. This product (of 230 bp) was amplified from both the untreated and cadmium treated CEM cells however a product of this size was not amplified from K562 cells, even after cadmium treatment (see Figure 5.11).

5.2.2 Detection of *MT1L* mRNA in human thyroid by northern analysis

MT1L mRNA had been detected in human tissues by RT-PCR, however this technique is very sensitive and may detect transcripts at a level which is not necessarily physiologically relevant. Northern analysis is a less sensitive technique and detection of a message by this method is more likely to suggest physiological relevance. This technique can also be easily used to determine the relative amounts of a particular mRNA in different samples. The expression of *MT1L* mRNA was therefore examined in thyroid samples by northern blotting (5.2.2.2). To do this, the construction of gene-specific probes for *MT1L* and several other genes was required and is described below (5.2.2.1).

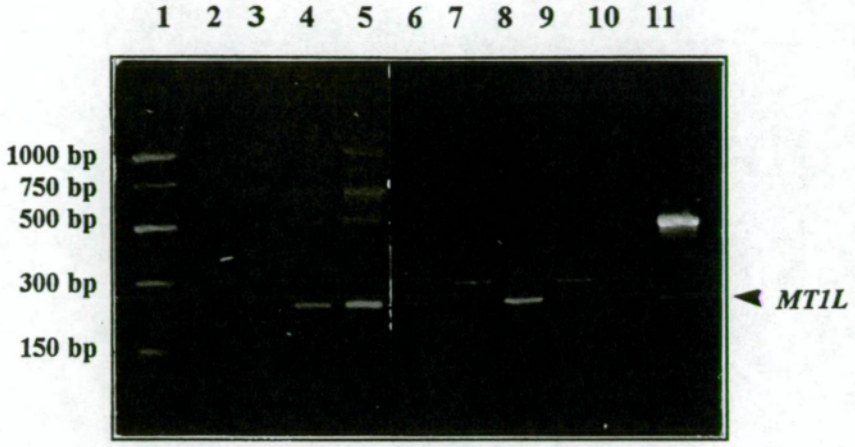


Figure 5.11 Detection of *MT1L* mRNA in human white blood cells by RT-PCR
RNA was isolated from primary cultures of human lymphocytes, CEM cells and K562 cells, and subjected to RT-PCR using primers specific for *MT1L*.

1: Molecular weight markers (sizes as marked)
2: no RNA
3: RNA from human lymphocytes, saline treated
4: RNA from human lymphocytes, 10 μM $CdCl_2$, 6 hr
5: RNA from human lymphocytes, 10 μM $ZnCl_2$, 6 hr
6: RNA from CEM cells
7: RNA from K562 cells
8: RNA from CEM cells, 10 μM $CdCl_2$, 16 hr
9: RNA from K562 cells, 10 μM $CdCl_2$, 16 hr
10: mock extraction (see section 2.3.3)
11: RNA from COS cells transfected with the *MT1L* gene

The 230 bp *MT1L* PCR product is indicated (arrow).

5.2.2.1 Specificity of a human *MT1L* oligonucleotide probe

An antisense oligonucleotide (5'-AAATGACGGGAGAGGCGA-3') was designed to the 5' flanking region of the human *MT1L* gene. The oligonucleotide was complementary to the *MT1L* sense primer used in RT-PCR. The specificity of this oligonucleotide was determined using a cosmid blot prepared by F.A.Stennard (see Stennard *et al.*, 1994) which contained the cosmid clones chMT-51, chMT-5 and chMT-10. These cosmids encompass the functional locus on chromosome 16q13 containing all MT-I and -II genes (see Figure 5.12A). These were digested with the restriction endonuclease *Hin*dIII, producing DNA fragments which contained only a single MT gene. This was with the exception of *MT1H* and *MT1I* which are contained on the same *Hin*dIII fragment and thus DNA from the cosmid CH-2 was digested with the restriction endonuclease *Sac*I to separate these genes. The digested DNA was electrophoresed on an agarose gel (Figure 5.12B) and transferred to nylon membranes by alkaline blotting. The specificity of the MT gene specific probes was determined by their hybridisation to these blots. The *MT1L* oligonucleotide was radiolabelled with γ^{32}PATP using T4 polynucleotide kinase (section 2.3.10) and hybridised to the cosmid blot at 42°C in 20% formamide hybridisation buffer, for 16 hr, then washed to a stringency of 0.2 X SSC, 1% SDS at 45°C. At this stringency the probe hybridised to only a single band on the cosmid blot, containing the human *MT1L* gene (Figure 5.12C).

The specificity of oligonucleotides designed to other MT genes were determined similarly. Oligonucleotides used as gene specific probes and the stringency at which they were washed are as follows:

MT1L	5'-AAATGACGGGAGAGGCGA-3'
	0.2 X SSC, 1% SDS at 45°C
MT2A	5'-ATCCAGGTTTGTGGAAGTCG-3'
	0.2 X SSC, 1% SDS at 45°C
MT1A	5'-ATGGGTCAGGGTTGTATGGA-3'
	0.2 X SSC, 1% SDS at 37°C
MT1E	5'-CAAAGGGGATGCTGGAGCTC-3'
	0.2 X SSC, 1% SDS at room temperature

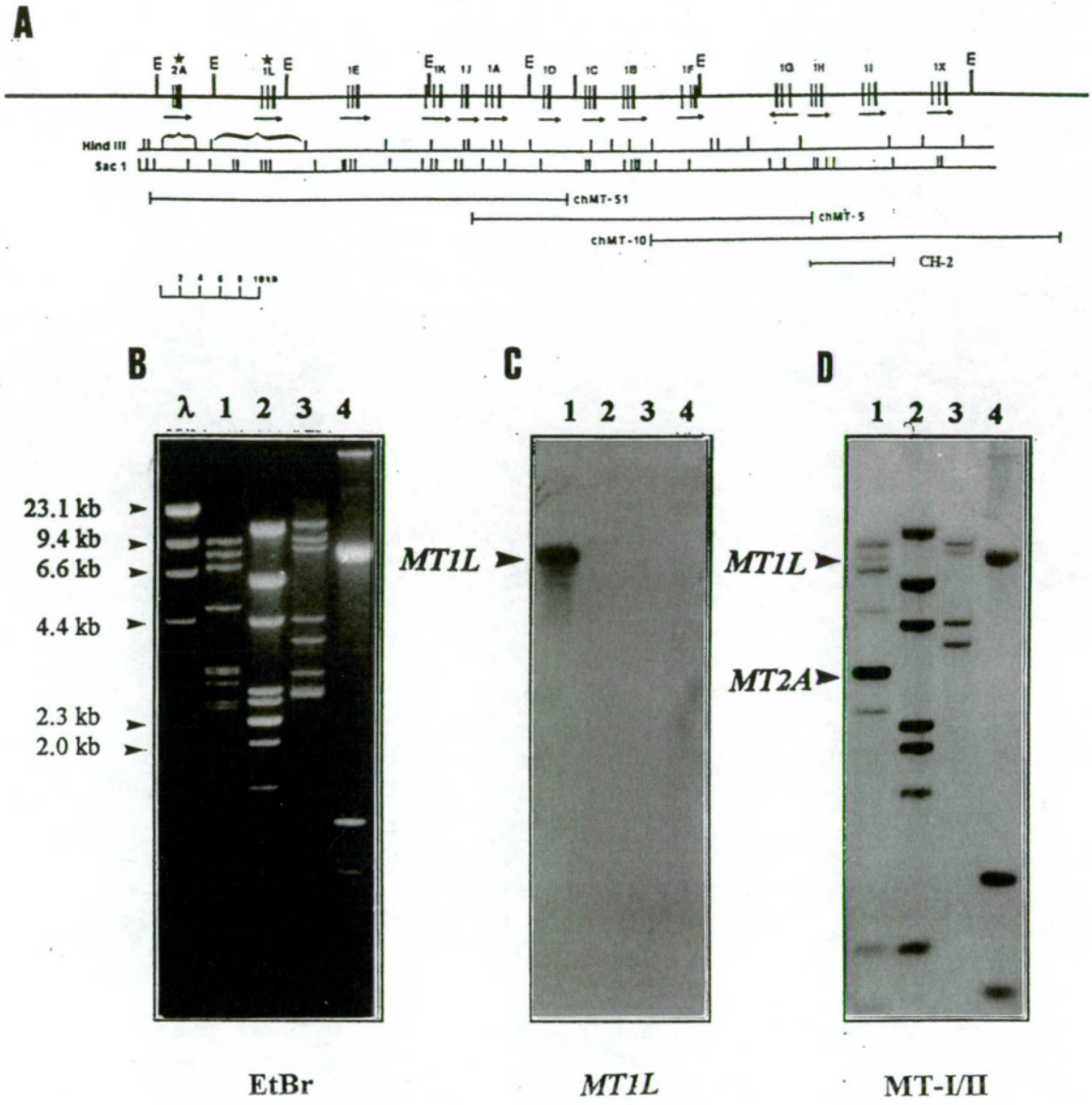


Figure 5.12 Specificity of MT probes

A cosmid blot encompassing the functional MT-I and -II locus on chromosome 16 was used to determine the specificity of MT probes. The location of the MT-I and -II genes in the functional locus and their transcriptional orientation (arrows) is shown (A). Restriction maps for the endonucleases *Hin*dIII, *Sac*I and *Eco*RI (E) are indicated. The *Hin*dIII restriction fragments representing the genes *MT1L* and *MT2A* are shown (bracket and asterisk). Regions encompassed by the cosmid genomic clones chMT-51, chMT-5, chMT-10 and CH-2 are indicated (adapted from West *et al.*, 1990).

Cosmid DNA was digested with restriction endonucleases, electrophoresed through an agarose gel and transferred to nylon membrane by alkaline blotting (Stennard *et al.*, 1994). The ethidium bromide stained gel is shown (B); λ: *Hin*dIII restricted λ markers (sizes indicated), 1: *Hin*dIII restricted chMT-51, 2: *Hin*dIII restricted chMT-5, 3: *Hin*dIII restricted chMT-10, 4: *Sac*I restricted CH-2. A *MT1L* oligonucleotide was used as a specific probe for this gene. It hybridises to only the *MT1L* gene (9.4 kb) as indicated (arrow, C). A human *MT2A* PCR product was used as a general probe as it hybridises to all MT-I and -II genes. Fragments representing *MT2A* (3.2 kb) and *MT1L* (9.4 kb) are indicated (arrows, D).

MT1F	5'-GAGAGACTGGACTTTCCAAG-3'
	0.2 X SSC, 1% SDS at room temperature
MT1G	5'-GGTCACTCTATTTGTACTTGG-3'
	0.2 X SSC, 1% SDS at 37°C
MT1H	5'-CGTGTCATTCTGTTTTCATCTGAC-3'
	0.2 X SSC, 1% SDS at room temperature
MT1X	5'-GCTCTATTTACATCTGAGAGCACA -3'
	0.2 X SSC, 1% SDS at room temperature
β-actin	5'-CACACTTCATGATGGAGTTGA-3'
	0.2 X SSC, 1% SDS at 37°C

MT1B expression was determined using a *Hin*dIII, *Bam*HI fragment from the 5' flanking region of the *MT1B* gene (A.K.West, Ph.D. Thesis). This was hybridised in 50% formamide hybridisation buffer at 42°C for 16 hr and washed to a stringency of 0.2 X SSC, 1% SDS at 65°C.

A 279 bp human *MT2A* RT-PCR product amplified by RT-PCR of HeLa cell RNA using the *MT2A* PCR primers described earlier was cloned into the plasmid pSP64 by F.A.Stennard. This product was used as a general MT-I/II probe. The RT-PCR product was radiolabelled with α-^{32}PdCTP (section 2.3.8) and hybridised to the cosmid blot at 37°C in 50% formamide hybridisation buffer, for 16 hr. The blot was washed to a stringency of 0.2 X SSC, 1% SDS at 37°C and under these conditions this probe hybridises to all MT genes in the 16q13 locus as indicated in Figure 5.12D. It is evident from Figure 5.12D that this probe hybridises considerably more strongly to the *MT2A* gene from which it is derived, than other MT genes including *MT1L*, under these conditions.

5.2.2.2 Expression of *MT1L* mRNA in the human thyroid

The expression of *MT1L* mRNA in human thyroid samples was determined by northern blotting using the *MT1L* oligonucleotide described above as a probe. For comparison, expression of the other MT-I and -II genes was examined using the appropriate gene specific probes.

RNA was isolated from 3 human thyroid samples and subjected to northern analysis (section 2.3) using the MT gene specific oligonucleotide and cDNA probes under the conditions indicated above. *MT1L* mRNA was detected in 2 of the 3 human thyroid samples examined (Figure 5.13). *MT2A* (3 of 3), *MT1H* (2 of 3), *MT1X* (2 of 3) and *MT1G* (1 of 3) mRNA were also detected in some of the thyroid samples, but *MT1A*, *MT1B*, *MT1E* and *MT1F* were not detected in any thyroid samples, even after long exposure of the autoradiographs. Hybridisation with the human β-actin oligonucleotide probe under the conditions described above indicated that equal amounts of RNA were present in all lanes.

5.2.3 Expression of *MT1L* under the control of the SV40 promoter

Studies were undertaken to investigate the expression of MT1L protein. Although *MT1L* mRNA had been detected in human tissues by both RT-PCR and northern analysis the question remained as to whether the *MT1L* gene encoded a shortened MT protein of 25 amino acids, a selenoprotein of 62 amino acids, or whether the mRNA was translated into protein at all. The ability of the *MT1L* gene to produce protein was investigated using the SVL vector, in which a cloned gene or cDNA is expressed under the control of the SV40 promoter.

Previously the *MT1L* gene and *MT2A* cDNA were cloned into the eukaryotic expression vector pSVL to create the constructs SVL-1L and SVL-2A, depicted in Figure 5.14 (A.F.Holloway, B.Sc. Honours Thesis). *MT1L* was shown to produce mRNA of the same size as that produced by *MT2A* after transfection into COS cells, a monkey kidney cell line, using the Transfectam reagent (Promega).

This experiment was repeated in the present study, but the SVL-1L, SVL-2A and pSVL vectors were transfected into COS cells, by calcium phosphate-mediated transfection (see section 2.7.3). RNA was isolated after 48 hours and subjected to northern analysis. Hybridisation with a human *MT2A* RT-PCR product which hybridises to all human MT-I and -II genes including *MT1L* and *MT2A* (as described earlier, see Figure 5.12) confirmed previous experiments, demonstrating that both *MT1L* and *MT2A* produce full length mRNA of approximately 350 bp (Figure 5.15). The considerably weaker signal observed from the *MT1L* transfected cells compared with the *MT2A* transfected cells most likely reflects the relative hybridisation strength

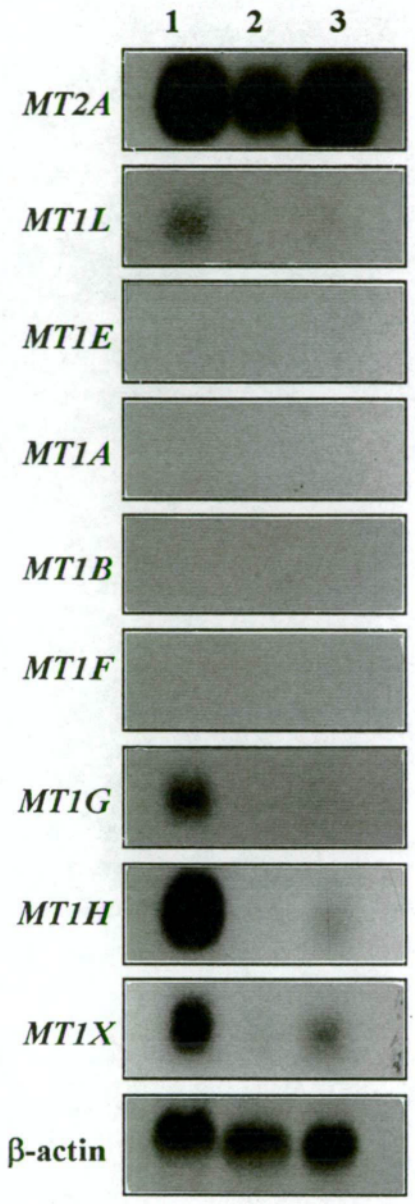
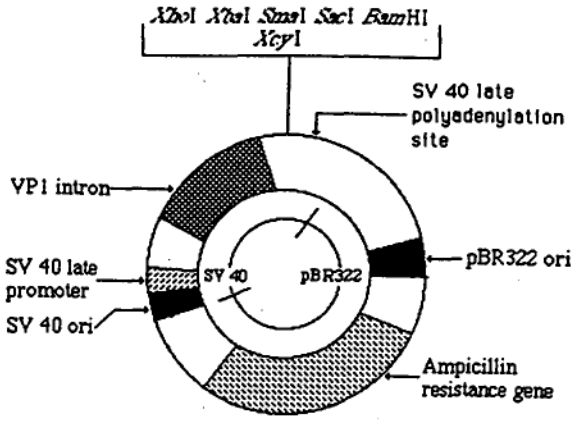
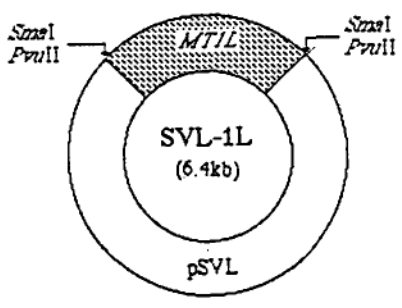


Figure 5.13 Expression of MT mRNA in the human thyroid
RNA was isolated from 3 human thyroids (lanes 1 to 3) and the expression of the various human MT-I and -II isogenes was examined by northern analysis. Radiolabelled oligonucleotide and cDNA probes specific for the human MT genes as specified in the text were used to examine the expression of the individual MT isogenes. A human β-actin probe was used to confirm that equal amounts of RNA were present in each lane.



pSVL



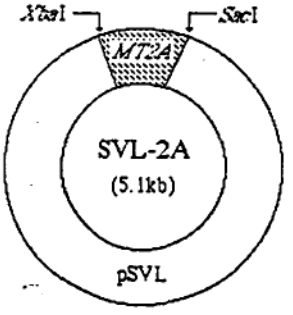


Figure 5.14 Diagrammatic representation of pSVL and the SVL-MT constructs
pSVL is a 4.8 kb SV40-derived eukaryotic expression vector containing SV40 and pBR322 elements as indicated. The polylinker containing multiple cloning sites is shown. The SVL-1L construct was created by the insertion of a 1.6 kb *Pvu*II fragment containing the *MT1L* gene into the SVL vector at the *Sma*I site as indicated. The SVL-2A construct was created by insertion of a 0.3 kb *MT2A* cDNA into the SVL vector at the *Xba*I/*Sac*I sites as indicated.

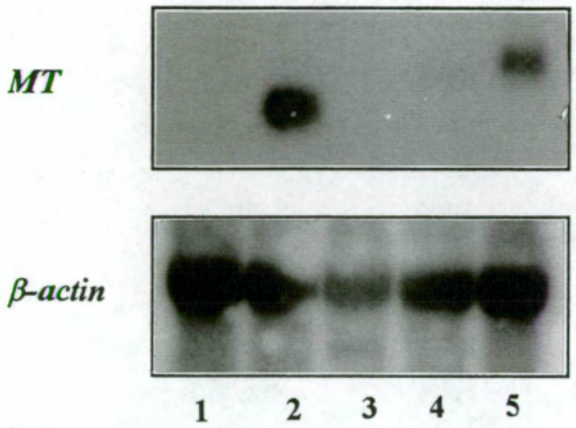


Figure 5.15 Expression of MT mRNA in transfected COS cells
COS cells were transfected with the eukaryotic expression vector SVL, the human *MT1L* gene (SVL-1L) and the human *MT2A* cDNA (SVL-2A). As a positive control COS cells were also treated with $CdCl_2$. RNA was isolated from the cells and analysed for MT mRNA by northern analysis using a general MT probe (*MT2A* PCR product). RNA was from COS cells treated as follows:

1: COS cells
2: COS cells, 10 μM $CdCl_2$, 16 hr
3: COS cells, SVL transfected
4: COS cells, SVL-1L transfected
5: COS cells, SVL-2A transfected

A β-actin probe was used to confirm that equal amounts of RNA were present in each lane.

of the probe to these 2 genes (see Figure 5.12D). Endogenous monkey MT mRNA induced by treatment of COS cells with 10 μM $CdCl_2$ for 24 hr was also detected with this probe as a slightly smaller transcript (Figure 5.15).

COS cells were then co-transfected with the same set of expression vectors (SVL, SVL-1L and SVL-2A) and a vector containing the β-galactosidase gene under the control of the RSV promoter (RSV-β-gal, as described in Stennard *et al.*, 1994). 24 hr after transfection these cells were incubated with ^{35}S-cysteine (200 μCi) for 16 hr. Protein was isolated from the cells, carboxymethylated and electrophoresed through a 15% polyacrylamide gel (as outlined in section 2.8). Autoradiography failed to detect an increased production of MT in the *MT1L* and *MT2A* transfected cells although the endogenous monkey MT was detectable, and a significant increase in MT levels was observed in COS cells which had been treated with 10 μM $CdCl_2$ for 24 hr (Figure 5.16). Assays for β-galactosidase (section 2.8.4), produced by the cotransfected RSV-β-gal vector determined that the transfections had been successful (data not shown).

A timecourse with cells transfected with the SVL-2A vector showed that the cells were viable and expressing *MT2A* mRNA up to 8 days after transfection (Figure 5.17), with maximum *MT2A* mRNA levels after 4-6 days. On the basis of this result the radiolabelling experiment outlined above and in Figure 5.16 was repeated but cells were incubated with ^{35}S-cysteine for 16 hr, 4 days after transfection. Again, no increases in MT protein were observed in the transfected cells, although endogenous monkey MT was detectable. There was a significant increases in the endogenous MT levels after treatment with cadmium (data not shown). As before β-galactosidase assays confirmed the vectors had successfully been transfected into the cells.

COS cells were again transfected with the expression vectors (SVL, SVL-1L and SVL-2A) but in the absence of ^{35}S-cysteine. Some COS cells were also treated with 10 μM cadmium chloride for 24 hr to induce endogenous MT. The cells were harvested 4 days after transfection and the isolated proteins heat treated to remove heat unstable proteins. RIA assays performed by Dr C. Tohyama failed to detect increased MT levels in the transfected COS cells, although an approximately 20 fold increase was detected in the cadmium treated COS cells (Table 5.2).

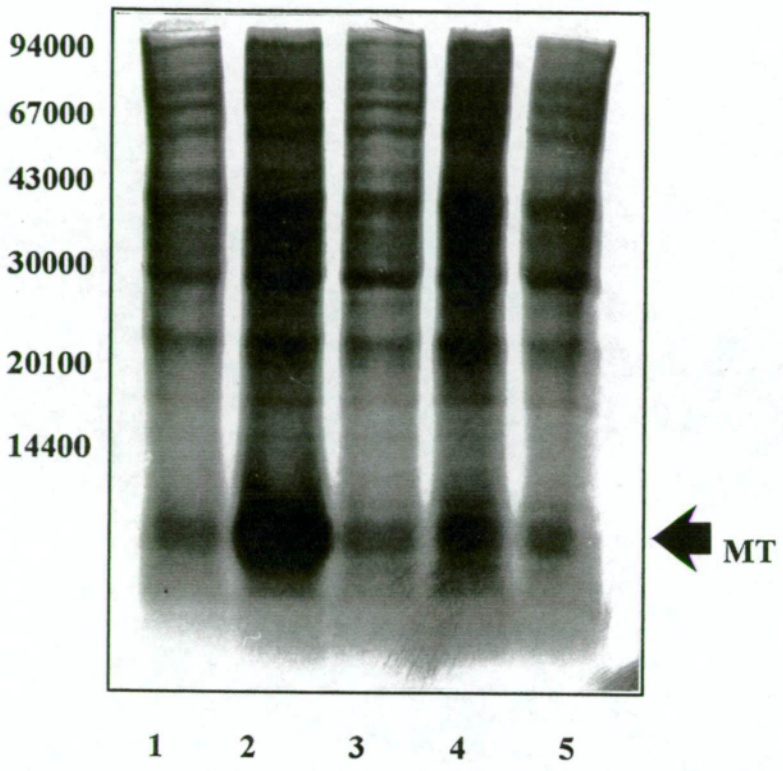


Figure 5.16 Expression of MT protein in transfected COS cells
COS cells were transfected with the eukaryotic expression vector SVL, the human *MT1L* gene (SVL-1L) and the human *MT2A* cDNA (SVL-2A). As a positive control COS cells were also treated with $CdCl_2$. Cells were incubated with ^{35}S-cysteine for 16 hr. Protein was isolated from the cells and subjected to SDSPAGE. The protein gel was dried and exposed to Hyperfilm (Amersham). Proteins were from COS cells treated as follows:

1: COS cells
2: COS cells, 10 μM $CdCl_2$, 16 hr
3: COS cells, SVL transfected
4: COS cells, SVL-1L transfected
5: COS cells, SVL-2A transfected.

The radiolabelled MT proteins are indicated (arrow). The position of protein molecular weight markers (Pharmacia) electrophoresed on the same gel are shown. The markers used were: phosphorylase b (94000), bovine serum albumin (67000), ovalbumin (43000), carbonic anhydrase (30000) soybean trypsin inhibitor (20100) and α-lactalbumin (14400).

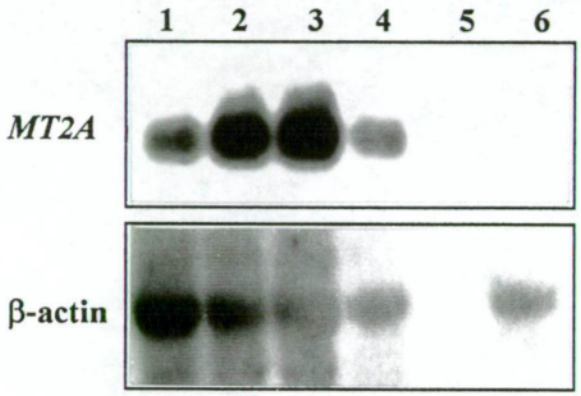


Figure 5.17 Timecourse for expression of *MT2A* mRNA in transfected COS cells
COS cells were transfected with the human *MT2A* cDNA cloned into the eukaryotic expression vector SVL. RNA was isolated from the cells and *MT2A* mRNA detected by northern analysis using a MT probe (*MT2A* PCR product). RNA was isolated from cells at the following timepoints after transfection: 2 days (lane 1), 4 days (lane 2), 6 days (lane 3), 8 days (lane 4), 10 days (lane 5). RNA was also isolated from untransfected COS cells (lane 6). A β-actin probe was used to demonstrate the relative levels of mRNA in each lane.

	MT (ng/ml)	total protein (μg/ml)	MT (μg/μg total protein)
COS cells	90.5	151	0.599
COS cells, SVL transfected	49.2	83.8	0.587
COS cells, SVL-1L transfected	51.5	124	0.415
COS cells, SVL-2A transfected	50.5	126	0.400
COS cells, 10 μM $CdCl_2$, 16 hrs	2160	167	12.93

Table 5.2 Detection of MT protein in transfected COS cells by radioimmunoassay

COS cells were transfected with the eukaryotic expression vector SVL, the human *MT1L* gene (SVL-1L) and the human *MT2A* cDNA (SVL-2A). As a positive control COS cells were also treated with $CdCl_2$. Protein was isolated from the cells, and the concentration of MT protein determined by radioimmunoassay by Dr C.Tohyama.

Attempts were then made to look at the expression of MT1L protein in a cell line incapable of producing endogenous MT protein, thus avoiding the problem of trying to detect the protein produced by the vectors as an increased signal above the background endogenous MT protein. Embryonic fibroblasts were prepared from E12-14 foetal mice (section 2.7.4). The mice used were transgenic MT-I/II$^{-/-}$ mice which fail to produce MT-I and -II proteins (Michalska and Choo, 1993). MT-I/II$^{-/-}$ fibroblasts were transfected with the expression vectors (SVL, SVL-1L and SVL-2A) by calcium phosphate-mediated transfection. The cells were harvested after 48 hr, RNA isolated and subjected to northern analysis with a general MT probe. The northern blot was hybridised with the *MT2A* RT-PCR probe in conditions described previously, under which the probe hybridises to all human MT-I and -II isogenes (see Figure 5.12). Although *MT1L* and *MT2A* mRNA was detected in COS cells transfected in parallel, significant levels of MT mRNA could not be detected in the transfected embryonic fibroblasts even after overexposure of the autoradiograph (see Figure 5.18). This may have been due to a combination of factors; firstly, transfection efficiencies are often reduced in primary cell lines and secondly, the SVL vectors are replicated in COS cells but not embryonic fibroblasts therefore reducing the amount of RNA produced by the vectors in the embryonic fibroblasts.

5.2.4 Expression of *MT1L* under the control of the CMV promoter

Attempts were made to observe protein produced by the *MT1L* gene using an alternate vector system, pBK-CMV (Stratagene). This vector contains the powerful cytomegalovirus (CMV) immediate early promoter and, unlike the SVL, vector does not rely on replication of the vector in host cells. The *MT1L* gene and *MT2A* cDNA were cloned into the pBK-CMV expression vector and transfected into embryonic mouse fibroblasts by calcium phosphate-mediated transfection.

The pBK-CMV vector contains promoters for both eukaryotic and prokaryotic expression (as depicted in Figure 5.19). The lac Z promoter (244bp, necessary for prokaryotic expression only) was removed from the vector by digestion with the restriction endonucleases *Nhe*I and *Xba*I. This digestion creates compatible ends allowing religation of the vector minus the lac Z promoter. The authenticity of

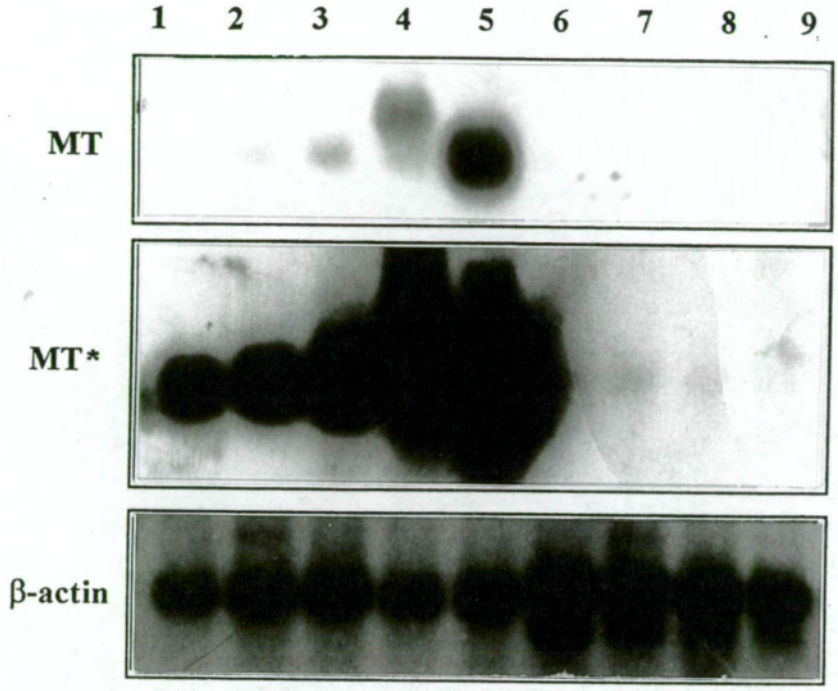


Figure 5.18 Expression of MT mRNA in transfected cells
COS cells and embryonic fibroblasts from MT-I/II$^{-/-}$ mice were transfected with the eukaryotic expression vector SVL, the human *MT1L* gene (SVL-1L) and the human *MT2A* cDNA (SVL-2A). As a positive control COS cells were also treated with $CdCl_2$. RNA was isolated from the cells and MT mRNA was detected by northern analysis using a general MT probe (*MT2A* PCR product) and the resultant autoradiograph is shown. Overexposure of the autoradiograph is also shown (MT*). RNA was from cells treated as follows:

1: COS cells
2: COS cells, SVL transfected
3: COS cells, SVL-1L transfected
4: COS cells, SVL-2A transfected
5: COS cells, 10 μM $CdCl_2$, 16 hr
6: MT-I/II$^{-/-}$ embryonic fibroblasts
7: MT-I/II$^{-/-}$ embryonic fibroblasts, SVL transfected
8: MT-I/II$^{-/-}$ embryonic fibroblasts, SVL-1L transfected
9: MT-I/II$^{-/-}$ embryonic fibroblasts, SVL-2A transfected

A β-actin probe was used to confirm that equal amounts of RNA were present in each lane.

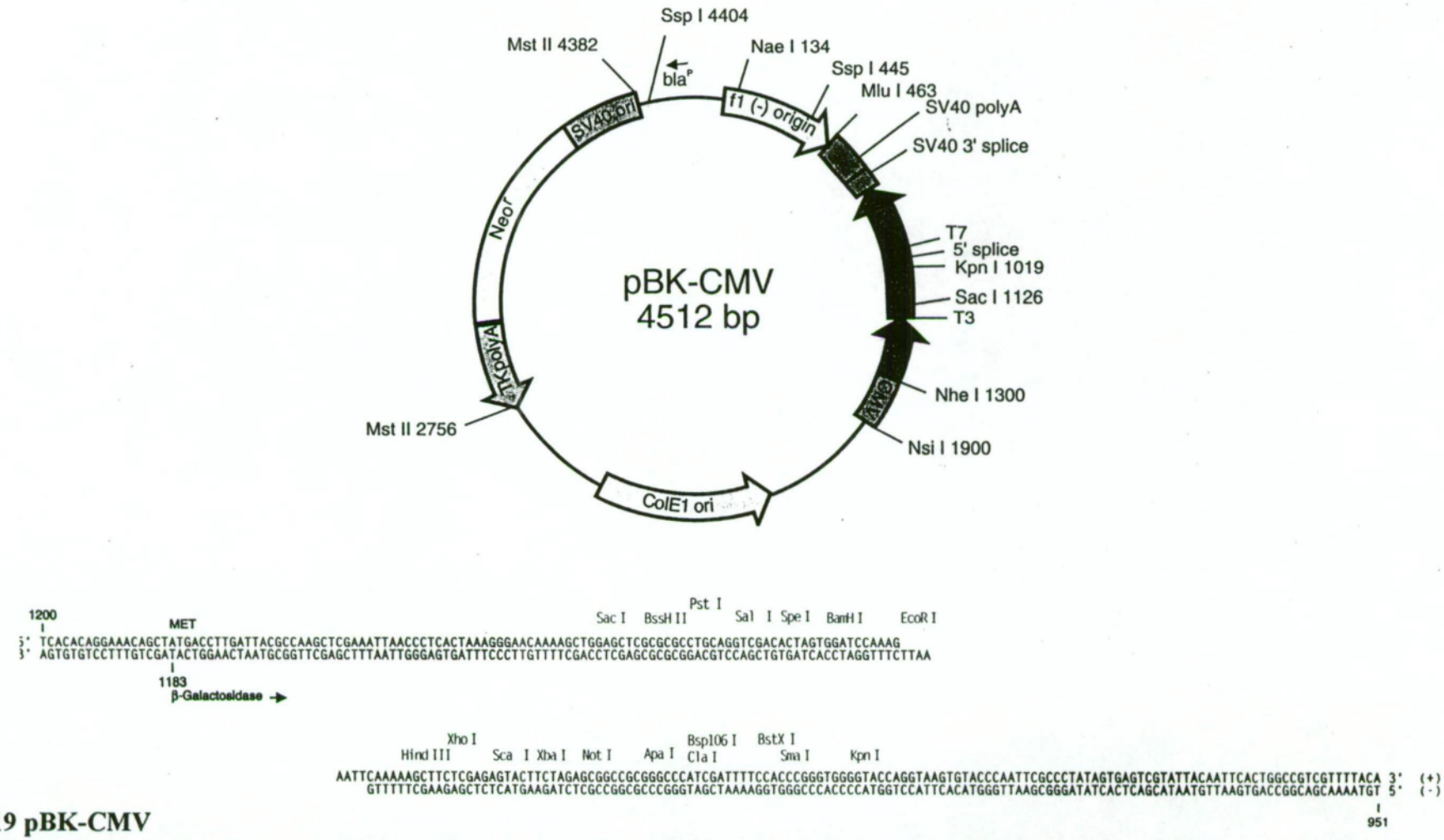


Figure 5.19 pBK-CMV

The pBK-CMV phagemid (from Stratagene) contains promoters for prokaryotic (LacZ) and eukaryotic (CMV) expression. It contains a multiple cloning site as shown below the phagemid map, and the neomycin/kanamycin resistance gene. The phagemid map is adapted from the 1994 Stratagene catalogue.

this vector was checked by sequencing clones using the ABI PRISM™ Dye Primer Cycle Sequencing Ready Reaction Kit (section 2.5.2).

The *MT1L* gene was removed from the pSVL-1L vector by digestion with the restriction endonucleases *Bam*HI and *Xba*I. The 1.6 kb fragment containing the *MT1L* gene was bluntended with *Pfu* DNA polymerase and ligated into pBK-CMV at the *Sma* I restriction endonuclease site to create the vector pBK-CMV-1L (as outlined in Figure 5.20). The *MT2A* cDNA was removed from the vector pSP64-2A by digestion with the restriction endonucleases *Xba*I and *Sac*I, bluntended with *Pfu* DNA polymerase and ligated into pBK-CMV to create the vector pBK-CMV-2A (as outlined in Figure 5.20). The authenticity of these constructs was confirmed by sequencing the regions where the gene or cDNA were ligated into the vector. The ABI PRISM™ Dye Terminator and Dye Primer Cycle Sequencing Ready Reaction Kits were used to sequence these clones.

Embryonic fibroblasts from MT-I/II$^{-/-}$ mice prepared as outlined in section 2.7.4, were transfected with the pBK-CMV-1L and -2A vectors by calcium phosphate-mediated transfections. The cells were harvested after 48 hr, RNA isolated and subjected to northern analysis with the *MT2A* RT-PCR clone which hybridises to all human MT-I and -II genes including *MT1L* and *MT2A* as a probe (as described earlier, see Figure 5.12). MT mRNA was detected in both pBK-CMV-1L and pBK-CMV-2A transfected cells, but not untransfected cells or cells transfected with vector alone (pBK-CMV). The pBK-CMV-1L vector produced mRNA of approximately 350 bp as expected while the pBK-CMV-2A vector produced a mRNA of approximately 600 bp (Figure 5.21). The differences in sizes of the mRNA produced by the 2 vectors is because the *MT2A* cDNA uses the polyadenylation site provided by the vector adding about 250 bp to this mRNA, whereas the *MT1L* gene includes its own polyadenylation site. A faint MT transcript was detected in the untransfected cells and cells transfected with vector alone because the targetted mutation to the MT-I gene in these transgenic mice disrupts the translation but not transcription of this gene and therefore endogenous MT mRNA is observed (Michalska and Choo, 1993). RNA was also isolated from HeLa cells treated with 10 μM $CdCl_2$ for 16 hrs and examined on the northern blot described above. Significantly more MT mRNA was detected in the HeLa cells than the MT transfected embryonic fibroblasts.

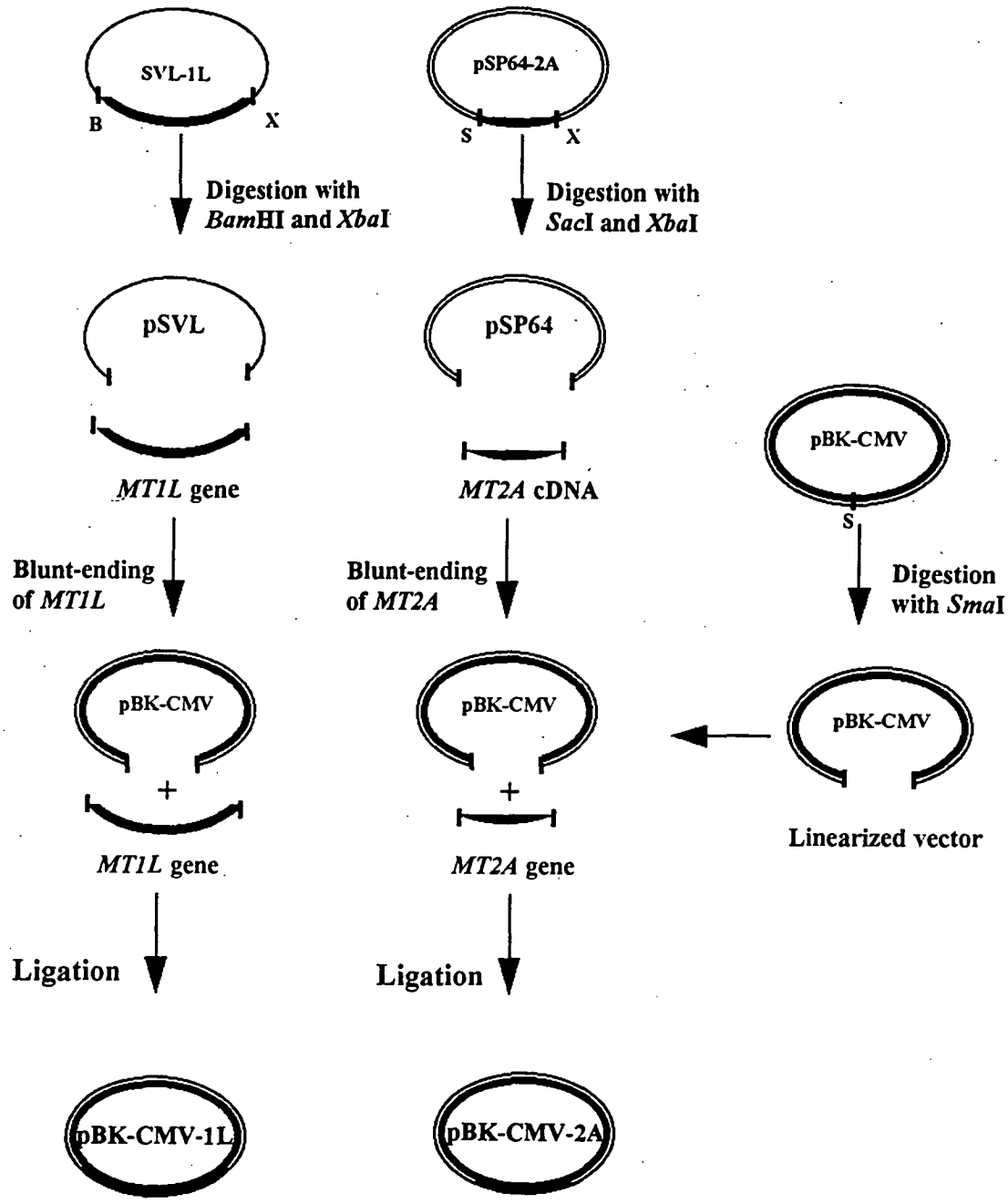


Figure 5.20 Construction of pBK-CMV-1L and pBK-CMV-2A vectors
The *MT1L* gene was removed from the SVL-1L vector and cloned into the pBK-CMV eukaryotic expression vector by the strategy outlined above to create pBK-CMV-1L. The *MT2A* cDNA was removed from the pSP64-2A vector and cloned into the pBK-CMV eukaryotic expression vector by the strategy outlined above to create pBK-CMV-2A. Constructs containing the gene or cDNA inserted in the required orientation were selected by digestion with a range of restriction endonucleases and confirmed by sequencing the constructs.

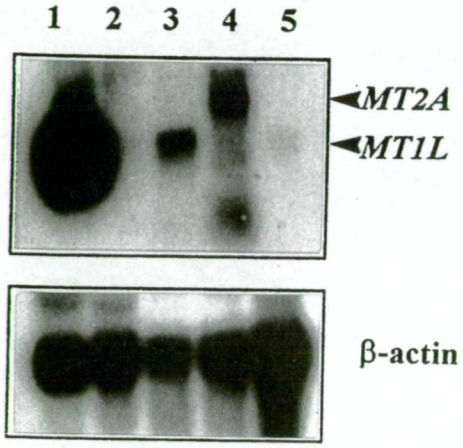


Figure 5.21 Expression of MT mRNA in transfected embryonic fibroblasts
Embryonic fibroblasts prepared from MT-I/II$^{-/-}$ mice were transfected with the eukaryotic expression vector pBK-CMV, the human *MT1L* gene (pBK-CMV-1L) and the human *MT2A* cDNA (pBK-CMV-2A). As a positive control HeLa cells were also treated with $CdCl_2$. RNA was isolated from the cells and analysed for MT mRNA by northern analysis using a general MT probe (*MT2A* PCR product). RNA was from cells treated as follows:

1: HeLa cells, 10 μM $CdCl_2$, 16 hr
2: MT-I/II$^{-/-}$ embryonic fibroblasts
3: MT-I/II$^{-/-}$ embryonic fibroblasts, pBK-CMV-1L transfected
4: MT-I/II$^{-/-}$ embryonic fibroblasts, pBK-CMV-2A transfected
5: MT-I/II$^{-/-}$ embryonic fibroblasts, pBK-CMV transfected

Arrows indicate the *MT2A* transcript and *MT1L* transcript. A β-actin probe was used to confirm that equal amounts of RNA were present in each lane.

However, since the pBK-CMV constructs were shown to be transfected into embryonic fibroblasts and MT mRNA could be detected in these cells, expression of MT1L protein was examined in this system. Embryonic MT-I/II$^{-/-}$ fibroblasts were transfected with the MT containing vectors, pBK-CMV-1L and pBK-CMV-2A by calcium phosphate-mediated transfection. 48 hr after transfection cells were radiolabelled with 200 μCi ^{35}S-cysteine for 16 hr. For comparison HeLa cells treated with 10 μM $CdCl_2$ were radiolabelled similarly. Protein was isolated from the cells, heat treated to remove heat unstable proteins, carboxymethylated and electrophoresed through a 15% polyacrylamide gel (as outlined in section 2.8). Autoradiography detected large amounts of the endogenous protein in cadmium treated HeLa cells, but failed to detect production of MT protein in the *MT1L* and *MT2A* transfected fibroblasts cells, i.e. a protein band could not be detected in the transfected cells that was absent in the untransfected cells (see Figure 5.22).

5.3 Discussion

Previously, a novel human MT gene, *MT1L*, from the functional locus on chromosome 16 was sequenced and found to contain an inframe TGA ("stop") codon midway through the coding region when compared to other MT genes. Earlier work had also indicated that this gene, when transfected into COS cells could confer resistance to cadmium, equivalent to the well characterised *MT2A* gene. Expression of the *MT1L* gene at the mRNA level was investigated in this study by RT-PCR and northern blotting. *MT1L* mRNA was detected in human pancreas, thyroid, pineal gland and hypothalamus by RT-PCR, using PCR primers specific for the human *MT1L* gene. The PCR product amplified from pancreatic tissue was confirmed to be *MT1L* cDNA by its size (230 bp), by comparison to products amplified from *MT1L* transfected COS cells, by its digestion with the restriction endonuclease *Nco*I, and its hybridisation to an MT probe. Finally, sequence analysis of a partial clone of the RT-PCR product confirmed it to be *MT1L* cDNA. The partial clone corresponded to the second exon and most of the third exon of the *MT1L* gene, and importantly included the unique inframe TGA codon in the second exon. Sequence analysis of this clone in both directions confirmed the presence of this TGA codon. Therefore, the *MT1L* gene was demonstrated to be expressed in some human tissues at the mRNA level.

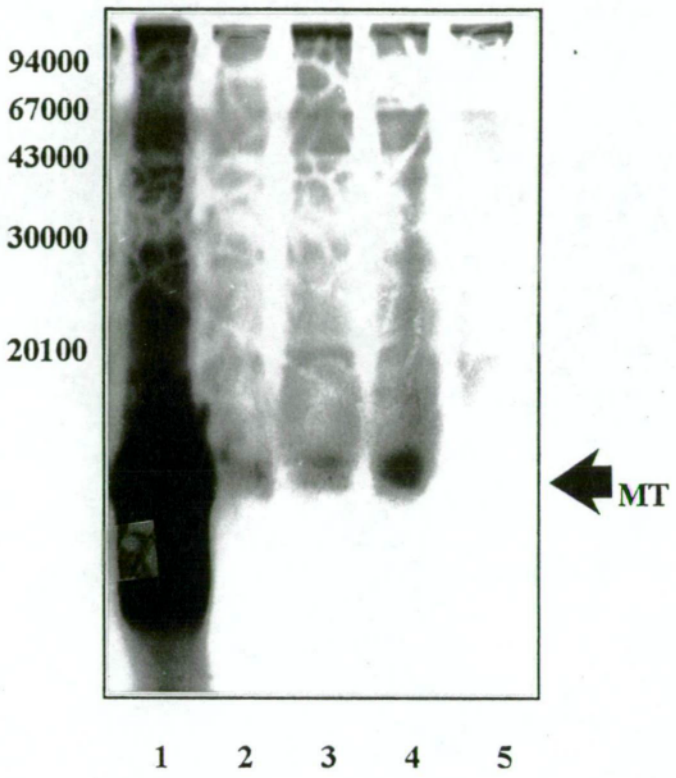


Figure 5.22 Expression of MT protein in transfected embryonic fibroblasts
Embryonic fibroblasts prepared from MT-I/II$^{-/-}$ mice were transfected with the eukaryotic expression vector pBK-CMV, the human *MT1L* gene (pBK-CMV-1L) and the human *MT2A* cDNA (pBK-CMV-2A). As a positive control HeLa cells were also treated with $CdCl_2$. Cells were incubated with ^{35}S-cysteine for 16 hr. Protein was isolated from the cells and subjected to SDSPAGE. The protein gel was dried and exposed to Hyperfilm (Amersham). Proteins were from cells treated as follows:

1: HeLa cells
2: MT-I/II$^{-/-}$ embryonic fibroblasts, pBK-CMV-2A transfected
3: MT-I/II$^{-/-}$ embryonic fibroblasts, pBK-CMV-1L transfected
4: MT-I/II$^{-/-}$ embryonic fibroblasts, pBK-CMV transfected
5: MT-I/II$^{-/-}$ embryonic fibroblasts

The radiolabelled MT proteins are indicated (arrow). The position of protein molecular weight markers (Pharmacia) electrophoresed on the same gel are shown. The markers used were: phosphorylase b (94000), bovine serum albumin (67000), ovalbumin (43000), carbonic anhydrase (30000), soybean trypsin inhibitor (20100) and α-lactalbumin (14400).

Furthermore, the existence of the TGA codon was thus confirmed in samples obtained from a third unrelated individual.

The human *MT2A* gene appears to be expressed at the RNA level in most tissues (Richards *et al.*, 1984), while differential expression patterns have been demonstrated for various MT-I isogenes (Varshney *et al.*, 1986; Jahroudi *et al.*, 1990; Heguy *et al.*, 1986). Similarly, the *MT1L* gene appears to be expressed in a tissue-specific manner, at least at the mRNA level. Interestingly, *MT1L* was detected by RT-PCR in those endocrine tissues indicated above, but was not detected in tissues such as the liver and intestine from the same individual. These latter tissues generally have high levels of MT expression (see Kagi and Kojima, 1987), and it is interesting that *MT1L* mRNA was not detected in the liver by RT-PCR.

MT1L mRNA was also detected by RT-PCR in cultured human lymphocytes after treatment with cadmium or zinc, but not in untreated human lymphocytes. This suggests that like other MT genes, *MT1L* may be inducible by the heavy metals cadmium and zinc. Furthermore, *MT1L* mRNA was detected in the human T-lymphoid cell line, CEM, by RT-PCR, but not in the human myeloid leukemia cell line, K562. This further demonstrates the differential expression of the human MT gene, *MT1L*.

MT1L mRNA was detected by northern analysis in 2 of 3 human thyroid samples using an oligonucleotide probe which was specific for the *MT1L* gene. Expression of other human MT genes (*MT1G*, *MT1H*, *MT1X* and *MT2A*) were also detected in the thyroid samples, and the thyroid sample in which *MT1L* mRNA was not detectable also contained lower levels of the other MT isoforms. Northern analysis is a considerably less sensitive technique than RT-PCR for detecting mRNA transcripts, and detection of *MT1L* by this technique is significant. It has been suggested that RT-PCR is sensitive enough to detect only a few copies of a mRNA transcript in a cell (Chelly *et al.*, 1989) and therefore can detect expression of a gene at levels which are not physiologically relevant. However, detection of *MT1L* in thyroid tissues by northern analysis suggests that this gene contributes significantly to the larger pool of MT expression in the thyroid gland. Although the expression levels of the different isogenes can not be directly compared by northern analysis, the fact that *MT1L* mRNA was detected by this method and mRNA from other MT genes

(*MT1A*, *MT1B*, *MT1E* and *MT1F*) which have been shown to be expressed in other tissues or cell lines were not, adds weight to the proposal that this gene is an important component of the repertoire of MT mRNA expression in the thyroid.

The detection of *MT1L* mRNA by RT-PCR and northern blotting does not necessarily infer that this gene expresses a protein, i.e. the mRNA observed may not necessarily be translated. It is possible that the mRNA produced by the *MT1L* gene, which contains a TGA ("stop") codon midway through the transcribed region, is unstable and rapidly degraded within the cell, although the fact that significant levels of *MT1L* mRNA were detected by northern blotting suggests that this is not the case. Alternatively, the mRNA may be translated into a shortened 25 amino acid protein which may be unstable and rapidly degraded by the cell. Also, it is possible that the TGA codon is from a mutated copy of a functional gene, however the fact that this sequence was confirmed from three different sources, i.e. two gene sequences and a cDNA sequence from three unrelated individuals, makes this unlikely.

Interestingly, a recent report documents the cloning of a novel human MT cDNA, *MT1R* by RT-PCR from human reticulocytes (Lambert *et al.*, 1996). This novel cDNA is almost identical to that determined for *MT1L*, the most striking differences in the coding region being that the TGA codon at position 26 of *MT1L* is a TGT (cysteine) codon in *MT1R* (see Figure 5.23). Also, the serine residue at position 6 is encoded by a TCC codon in *MT1L* but a TCG codon in *MT1R*. There are several differences between the *MT1R* cDNA 3' untranslated region and the corresponding region of the *MT1L* gene (see Figure 5.23), however the number of differences is significantly less than is usually seen between two different human MT isogenes (e.g. between *MT1L* and *MT1E*, see Figure 5.23). In all there are 15 mismatches between *MT1L* and *MT1R*, but 40 mismatches between *MT1L* and the most closely related of the other MT-I genes on chromosome 16, *MT1E*. Considering the similarities between *MT1L* and *MT1R*, particularly in the coding regions, the possibility exists that *MT1L* cDNA and *MT1R* cDNA in fact arise from the same gene, and the differences are due to sequencing errors, errors incorporated during RT-PCR, or allelic differences. To reconcile this situation, the *MT1L* gene was resequenced and the presence of the inframe TGA codon in the second exon of the *MT1L* gene was reconfirmed. The paper describing *MT1R* cDNA states that it was amplified on two

```
1
MetAspProAsnCysSerCysAlaThrGlyGlySerCysSerCysAlaSerSerCysLys
ATGGACCCCAACTGCTCCTGCGCCACTGGGGGCTCCTGCTCCTGTGCCAGCTCCTGCAAG
..................G.....................................
..................T..........T.........A.G..C...G..........

61
CysLysGluCysLys * ThrSerCysLysLysSerCysCysSerCysCysProMetGly
TGCAAAGAGTGCAAATGAACCTCCTGCAAGAAGAGCTGCTGCTCCTGCTGCCCCATGGGC
..................T.....................................
..................C.....................T...........G.....

121
CysAlaLysCysAlaGlnGlyCysValCysLysGlyAlaSerGluLysCysSerCysCys
TGTGCCAAGTGTGCCCAGGGCTGCGTCTGCAAAGGGGCGTCGGAGAAGTGCAGCTGCTGT
.....................................................A.....
.....................................A.....................

181
AlaTer
GCCTGATGTGGGGACAGCCCTGCTCCCAGATGTAAACAGAGCAACCTGCACAAACCTGGA
..................A.....................................
............A.....T..T.............T...A...........-......

241
TTTTTTTTTCA-TACAACCCTGAGCG-TTTGCTACATTCCTTTTTCTATTAAATATGTAA
..........--...........A.-.........A.T.........C...........
.......AAA.A......A......CA......G....T......A..C.........G.

301
ACGACAATAAAACAGTTTTGACTTGATTCGGACCCTCCTT
..C.-......-........-....-.....G-.......
CT...........A.........T.A..TT..T.C..G.
```

Figure 5.23 Comparison of *MT1L*,*MT1R* and *MT1E* nucleotide sequences
The nucleotide sequence of the *MT1L* cDNA is shown with the predicted amino acid sequence above (three letter code). This sequence is compared with the nucleotide sequences of the *MT1R* cDNA depicted immediately below in bold (Lambert *et al.*, 1996) and the *MT1E* cDNA (Schmidt *et al.*, 1985). Mismatches are indicated; an alternative nucleotide by its letter code and a deletion as -. Dots indicate where the sequences are identical. There are 15 mismatches between the *MT1L* and *MT1R* nucleotide sequences and 40 mismatches between the *MT1L* and *MT1E* nucleotide sequences.

separate occasions, but it is unclear whether this was from two individuals. Obviously the possibility exists that *MT1R* and *MT1L* are 2 separate genes, but such a gene has not been found in the functional locus mapped to chromosome 16q13. It is possible however, that the *MT1R* gene is a distinct gene situated in another position in the genome, although there is some evidence against this, since Karin *et al.* (1984a) showed that all human MT genes capable of conferring cadmium resistance are on chromosome 16, and most likely in the one locus.

Several lines of evidence have demonstrated that the *MT1L* gene produces mRNA, the question remains as to whether this is translated into a functional MT protein. The *MT1L* gene was cloned into eukaryotic expression vectors and transfected into mammalian cells in an attempt to express MT1L protein. The *MT1L* gene was demonstrated to produce a full length mRNA when transfected into the COS cell line under the control of the SV40 promoter. The mRNA produced by this gene was of the same size as that produced by *MT2A*. Although northern analysis of transfected cells with a general MT-I/II probe showed lower levels of expression of *MT1L* mRNA than *MT2A* mRNA, this is at least partly due to the hybridisation strength of the probe, which was shown to hybridise considerably more strongly to the *MT2A* gene than the *MT1L* gene.

Previous preliminary data suggested that the *MT1L* gene encodes a functional MT protein as the transfected gene, like *MT2A*, under the control of the SV40 promoter was capable of protecting transfected COS cells from cadmium toxicity (A.F.Holloway, B.Sc. Honours Thesis). An attempt was made to determine the size of the encoded MT1L protein by expressing it in COS cells under the control of the SV40 promoter and incubating the cells with ^{35}S-cysteine, thereby radiolabelling expressed MT proteins. However, neither MT1L nor MT2A protein (investigated in parallel) was detectable under these conditions, although endogenous MT was observed as well as significant increases in endogenous MT levels after cadmium treatment. Since MT is expressed in many cells at relatively high levels, and endogenous monkey MT mRNA and protein were easily detected in the COS cells, it is likely that the endogenous monkey MT protein masked any human MT1L and MT2A protein expressed in these cells after transfection. This suggests that significant amounts of protein were not produced by the transfected constructs and

this was in fact confirmed by the failure to detect increased MT levels by the sensitive radioimmunoassay. Notably, this assay detected an approximately 20 fold increase in the endogenous MT protein after treatment with cadmium.

To overcome this problem attempts were made to examine the MT1L protein in a cell line which does not produce endogenous MT protein. Embryonic fibroblasts from MT-I/II$^{-/-}$ transgenic mice which are incapable of expressing MT-I and -II protein (Michalska and Choo, 1993) were transfected with the *MT1L* gene and *MT2A* cDNA under the control of the SV40 promoter. However, significant levels of *MT2A* or *MT1L* mRNA were not detected by northern analysis following transfection. This vector may have been unsatisfactory in the mouse fibroblast system because of a combination of factors. Much of the effectiveness of the SVL vector relies on the fact that it is able to replicate in COS cells (Kaufman, 1990), however such replication does not occur in embryonic fibroblasts reducing the expression levels in this cell type. Furthermore, primary cell lines, such as the mouse embryonic fibroblasts prepared in this instance, usually are transfected with much lower efficiencies than established cell lines such as COS cells and therefore only a very small percentage of cells may have been transfected with the MT genes (e.g. Hawley-Nelson *et al.*, 1993).

Finally, the expression of *MT1L* and *MT2A* was examined in the MT-I/II$^{-/-}$ embryonic fibroblasts using the expression vector pBK-CMV. The CMV promoter produces transcription at levels much higher than the SV40 promoter of the SVL vector, and the pBK-CMV vector does not rely on replication of the vector for high expression levels (Kaufman, 1990). Again, MT1L or MT2A protein was not detected in transfected cells, although the cadmium induced endogenous MT in HeLa cells was easily detected. It is likely that the failure to see protein in this case was due to the inherent problem of low transfection efficiencies in such primary cell cultures.

Thus a model system has not yet been found in which any protein produced by the human *MT1L* gene could be observed. Transfection of the gene into a eukaryotic cell line appears the best approach to examine this question, although alternative approaches exist. One alternative would be to purify the protein from human tissues or cell lines, but this method has inherent problems in that although upregulation of the gene may be achieved in an expressing cell line like CEM using cadmium or zinc, any MT isolated from these cells would no doubt contain many of the MT isoforms

with MT2A being the predominant MT protein. A second alternative would be to produce the protein by *in vitro* translation, although this method is somewhat artificial and expression of a protein in this way may not necessarily imply that the protein is likely to be produced *in vivo*. However, it should be noted that mammalian selenoproteins have been investigated by *in vitro* translation (Berry *et al.*, 1991a). Thirdly, the protein could be expressed in a bacterial system, however this approach would not cover the possibility that the *MT1L* gene encodes a selenoprotein, as prokaryotic and eukaryotic cells possess different systems for the incorporation of selenocysteine residues (Rocher *et al.*, 1991), and a mammalian selenoprotein can therefore not be expressed in a bacterial system.

Although the transfection of the *MT1L* gene into a cell line appears the best method for detection of MT1L protein, there are complications involved with the detection of an MT protein by this approach. Most problematic is the masking of the expressed protein by the cell's endogenous protein. This arises primarily because most cells express MT proteins at relatively high levels, but also because endogenous MTs are induced by physiological stresses and insults and possibly also by calcium phosphate-mediated transfections (Foster *et al.*, 1989). An alternative is to use cell lines which do not express endogenous MT protein such as MT-I/II$^{-/-}$ embryonic mouse fibroblasts, however such primary cell lines have, as stated above, the complication of notoriously low transfection efficiencies. A worthwhile further study may be to use the CHO (chinese hamster ovary) cell line for transfecting the pBK-CMV-MT constructs. This cell line has the advantage that it does not generally express MT protein, and furthermore since it is an established cell line, transfection efficiencies are usually relatively high.

It remains to be determined whether the *MT1L* gene encodes a functional MT protein, but it should be noted that difficulties encountered in expressing the MT1L protein in this study were not due to the gene itself since MT2A was also never successfully detected.

The fact that *MT1L* mRNA is expressed in a highly tissue specific manner suggests that this gene is likely to produce a functional protein and the detection of *MT1L* mRNA in the thyroid and other endocrine tissues may be relevant to the possible production of a selenoprotein. The thyroid is the site of expression of several

mammalian selenoproteins, including type 1 iodothyronine de-iodinase. Furthermore, studies suggest that selenoproteins may have particular importance in the brain and in endocrine and reproductive organs (Behne *et al.*, 1988). Thus, the possibility that a MT selenoprotein is expressed in this tissue is quite conceivable.

There are now a number of well investigated mammalian selenoproteins and more examples are regularly being discovered. The manner in which a TGA codon is translated into a selenocysteine residue is becoming clearer, although the mechanisms involved are still not completely known (Stadtman, 1996). Many studies have investigated the factors involved in the recognition of a TGA codon as a selenocysteine rather than a termination signal. Unique sequence motifs termed selenocysteine-insertion sequences (SECIS) have now been identified in the 3' untranslated region (3'UTR) of the mRNA of selenoproteins and have been shown to be essential in the translation of selenoproteins (Berry *et al.*, 1991b; Shen *et al.*, 1993). Conserved 3-4 nucleotide sequences have been identified in the SECIS elements of several selenoproteins and have been shown to be necessary for the insertion of a selenocysteine residue (e.g. the sequences 'UAAA' and 'UGAU', as indicated in Figure 5.24). Furthermore, although the primary nucleotide structure of the 3'UTRs are not conserved, the SECIS elements are predicted to form stem-loop secondary structures (see Figure 5.24) which appear highly conserved (Berry *et al.*, 1993). There are differences in the efficiencies with which the predicted stem-loops of different mRNAs allow the insertion of a selenocysteine residue and this appears to mirror the order of appearance of these proteins after selenium-repletion of selenium-deficient animals (Berry *et al.*, 1993). The *MT1L* 3'UTR was investigated for the presence of stem-loop structures which may be necessary for the translation of a MT1L selenoprotein. Predicted secondary structure of the *MT1L* 3'UTR was generated using the University of Wisconsin Genetics Computer Group Fold program and is shown in Figure 5.24. The predicted secondary structure of the 3'UTR of the *MT1L* mRNA does not contain a stem-loop structure with a high degree of homology to those identified in the 3'UTRs of known selenoproteins. However, the major loop contains a "UAAA" sequence which has been found to be an element essential for selenocysteine insertion (Low and Berry, 1996).

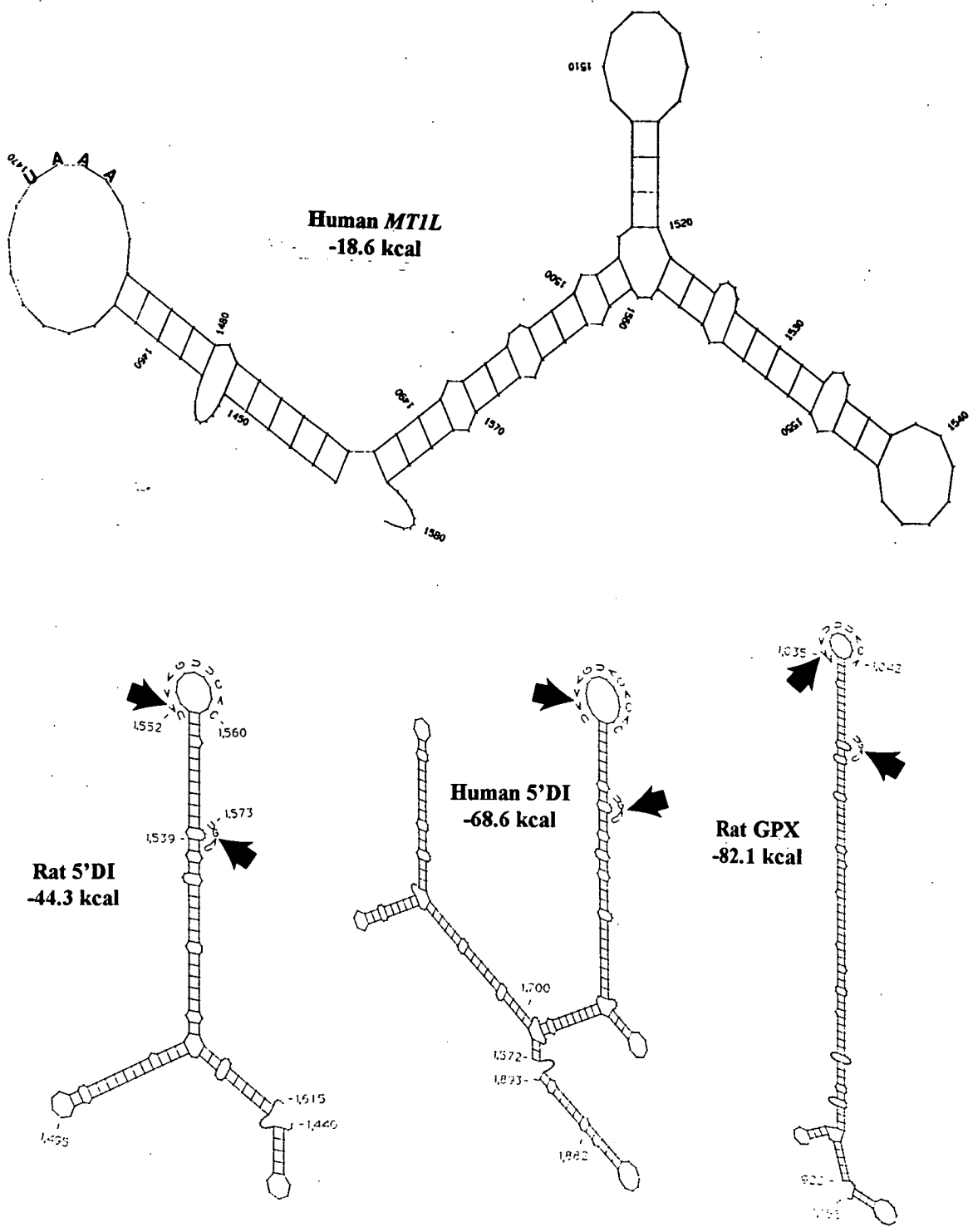


Figure 5.24 Predicted secondary structure in the 3'UTR of selected selenocysteine-encoding mRNAs and the *MT1L* mRNA.
The predicted secondary structure of the *MT1L* mRNA 3'UTR is shown. The selenocysteine encoding mRNAs are rat type I iodothyronine 5' deiodinase (5'DI), human 5'DI and rat glutathione peroxidase (GPX), taken from Berry *et al.*, 1991b. The *MT1L* mRNA was analysed using the FOLD program of the University of Wisconsin Genetics Computer Group software. The conserved sequences "UAAA" and "UGAU" are indicated with arrows.

Studies also suggest that the nucleotide following the TGA codon has an affect on whether termination or selenocysteine incorporation occurs. Although selenoproteins have been identified with all possible bases in this fourth position (Low and Berry, 1996), studies suggest that in eukaryotes termination rather than selenocysteine insertion is favoured when the base following the TGA codon is a purine (McCaughan *et al.*, 1995), as is the case with the internal TGA codon in *MT1L* (see Figure 5.2, nucleotide 994). Finally, most of the eukaryotic selenoprotein mRNAs have a TGA codon specifying a selenocysteine residue but have a codon other than TGA specifying termination of translation (Low and Berry, 1996). A MT1L selenoprotein would therefore be relatively unusual since both the selenocysteine residue and termination would be specified by a TGA codon (see Figure 5.2). Analysis of the 3'UTR and context of the internal TGA codon of the *MT1L* mRNA therefore suggests there is a low probability that this gene encodes a selenoprotein. However, such analysis only suggests the likelihood that a selenocysteine residue is specified and can not be taken as proof of whether a selenoprotein is or is not encoded.

Almost all of the known selenoproteins are involved in redox reactions, and the best studied of the mammalian selenoproteins, glutathione peroxidase, is involved in the scavenging of hydroxyl radicals. The selenol moiety has been shown to be the active centre of this enzyme (Forstrom *et al.*, 1978), and it is suggested that under normal physiological conditions a selenol moiety is more reactive than the corresponding thiol moiety (Stadtman, 1996). It is intriguing that MTs have also been implicated in the scavenging of free radicals, and it is conceivable that a MT-selenoprotein may be a more efficient free radical scavenger. Oikawa *et al.* (1991) synthesized a selenium analogue of metallothionein in which all 7 of the cysteine residues of *Neurospora crassa* metallothionein were replaced with selenocysteine residues. This protein had reduced metal binding properties, binding 3 rather than the usual 6 copper ions probably as a result of constraints on the protein structure due to the ionic radius of selenium compared with sulfur. However, it is difficult to draw inferences with respect to the metal binding properties of a mammalian metallothionein with a single selenocysteine substitution.

This study has further characterised the human *MT1L* gene at the mRNA level and demonstrated its differential expression which is possibly restricted to endocrine tissues and lymphocytes. In addition previous studies suggest that MT1L has the capacity to protect against cadmium toxicity. Therefore, the *MT1L* gene is now as well characterised as many of the human MT genes located on chromosome 16. It is likely that a MT1L protein, either a selenoprotein or a truncated MT protein would have novel properties. Such properties may be required or advantageous in endocrine tissues. Further investigation of such a protein may provide insight into its physiological role and the function of MTs as a whole.

CHAPTER 6: CONCLUDING REMARKS

Metallothioneins (MTs) are highly conserved cysteine rich proteins found throughout the animal kingdom. The regulation, protein structure and metal binding properties of MTs are well understood. However, the primary physiological role of these proteins remains to be determined. Suggested roles include heavy metal detoxification, metal transport and homeostasis and free radical scavenging. The primary aim of this study was to investigate the expression profiles and structures of MTs in two mammalian species, and to integrate this information with current ideas on MT action. MTs were examined in the sheep and in the human, both of which have complex multigene families encoding multiple MT isoforms. Firstly, the expression of MT-I and -II isoforms was investigated in the developing sheep brain. The aim was to examine MT expression at the regional and cellular level in the developing brain in order to gain insight into the factors which regulate MT expression during neural development. Secondly the cDNA encoding the brain-specific MT isoform, MT-III, was cloned from the sheep and its expression examined with respect to MT-I and -II isoforms. Thirdly, a novel human MT gene, *MT1L*, was characterised. The aim was to investigate the contribution of this isogene to MT expression in the human. The theme of this study was to correlate MT expression with its physiological role, and particular attention was paid to the function of MT in the brain. The role of MT in the brain is topical due to the recent finding of a novel brain-specific isoform, MT-III, with growth inhibitory properties. This finding also has drawn renewed attention to another enigmatic feature of MTs; the apparent requirement for the expression of multiple isoforms in higher eukaryotes.

The expression of MT-I and -II mRNA and protein in the developing sheep brain was investigated by immunocytochemistry and northern blotting, as detailed in Chapter 3. Changes in both the regional and cell type expression of MT was observed during the development of the sheep brain. The appearance of MT mRNA and protein in the foetal sheep brain was found to coincide with the migration of glial cells into the cortex and the maturation of this cell type. Furthermore, expression of MT protein was found to be confined to glial cells as it was detected in oligodendrocytes, astrocytes and radial glial cells but not in neurons. Interestingly, the expression of

MT protein in oligodendrocytes was confined to the foetal brain. Further investigation of MT protein at the cellular level determined that under normal physiological conditions MT protein was present in only a subset of glial cells at any one time. The pattern of MT expression suggested that at least under basal conditions MT regulation may be more dependent on cell-specific or localised factors than on cell location, subclass of glial cell or blood-borne factors. MT expression in the normal brain may then be dependent on the physiological state of individual cells, and factors such as the proliferative state, internal zinc concentration or redox state of the cell may be important. Although MT was found to be expressed in some dividing cells, and was localised to proliferative regions in the developing brain, MT was found not to be intrinsic to cell division. Further studies may, however, be able to correlate MT expression with the internal environment of the cell and factors such as changes in zinc status or redox state. MT protein was often expressed in glial cells forming the glial limiting membrane and in cells surrounding blood vessels raising the possibility it has a protective role in the brain. However, MT was also found in many other regions of the sheep brain, and its expression in the glial limiting membrane or around blood vessels was not always complete, suggesting that MT has other roles in the brain. This study documents in detail the expression patterns of MT during the development of the sheep brain. The sheep brain may now be used as a convenient model system to examine changes in MT expression during physiological stresses or insults. Investigation of MT under such conditions may provide a wider understanding of MT physiology in the brain.

Discovery of the brain specific MT-III isoform has generated much interest since it is structurally unique and has been found to display novel growth inhibitory properties *in vitro*. Expression of the MT-III isoform is restricted almost entirely to neural tissue and is suggested to be deficient in the Alzheimer's disease brain. Chapter 4 documents the cloning of a MT-III cDNA from the sheep brain. The putative MT-III isoform predicted by this cDNA is unique in that it contains a structural deletion of 3 amino acids which have been shown to be conserved in all MT isoforms from mammals so far examined. Furthermore, the sheep MT-III protein is missing 3 cysteine residues which are normally conserved in MT proteins. The putative sheep MT-III protein is therefore likely to have altered metal binding

properties. However, the sheep MT-III protein retains the CPCP amino acid sequence unique to MT-III isoforms to which the growth inhibitory function of these proteins has been assigned. This novel sheep MT-III isoform may then provide a valuable tool for determining the relevance of its growth inhibitory and metal binding properties *in vivo* and ultimately in determining the physiological role of MT-III in the brain. Interestingly, the work in Chapter 4 demonstrated that the expression of sheep MT-III mRNA mirrors that of MT-I and -II in the developing brain, i.e. MT-III mRNA is first detected at the same time as significant levels of MT-I and -II mRNA are observed. Furthermore, the differential expression pattern of MT-III mRNA in different regions of the adult brain closely mimics that of MT-I and -II mRNA. This is particularly interesting since it has been suggested that the MT-III isoform has a distinctly different role in the brain to MT-I and -II isoforms. The cell type expression of the MT-III isoform is particularly controversial as it has been shown to be co-expressed in glial cells with MT-I and -II in the human, but expressed in neurons in the mouse. The cellular localisation of MT-III may then be species specific and investigation of MT-III expression in a further species may shed light on this matter. The cellular localisation of MT-III in the sheep brain may be determined by a detailed *in situ* hybridisation study and suitable oligonucleotides for such a study have been identified here. Determination of the localisation of MT-III protein will require production of a MT-III specific antibody and the novel sheep MT-III protein may be ideally suited to the production of such an antibody. Future work will focus on confirming the structure of the MT-III protein and investigating its metal binding and growth inhibitory properties.

The requirement for multiple functional MT genes in higher eukaryotes is still unknown. One possibility is that their function is to provide the immediate production of large amounts of protein in times of stress or insult. Alternatively, the various isoforms may function in different tissues or cell types or in response to different stimuli. However, the discovery of a MT-III isoform with novel properties adds weight to the theory that the various MT isoforms may provide functional diversity. Investigation of the individual MT genes, their regulation, expression patterns and any unique features or properties is important in order to understand the function of MTs as a whole. Chapter 5 examines a novel human MT gene, *MT1L* located in the

functional MT locus on chromosome 16. This gene is particularly interesting due to the presence of an inframe TGA ("stop") codon in the middle of the second exon. Despite this, the *MT1L* gene was shown to produce a full length mRNA and furthermore previous work by the author suggested that the *MT1L* gene encoded a protein capable of protecting cells from cadmium toxicity. Expression of *MT1L* mRNA was examined by RT-PCR and found to be tissue specific. The data presented in Chapter 5 suggests that the expression of this gene may be confined to endocrine tissues and blood cells as *MT1L* mRNA was detected in pineal gland, pancreas, thyroid, hypothalamus and lymphocytes, but not in the human liver. A novel human MT cDNA, *MT1R*, recently cloned by Lambert *et al.* (1996) has high sequence homology with *MT1L*, however, the TGA codon in *MT1L* is a TGT (cysteine) codon in *MT1R*. The sequence of *MT1L* presented here has been confirmed in clones from three individuals. The similarities of *MT1L* cDNA to *MT1R* cDNA are intriguing and further investigation is necessary to determine whether they arise from the same gene, the differences being due to polymorphisms, or they arise from completely different genes. However, a gene encoding the *MT1R* cDNA has not been identified in the functional locus on chromosome 16q13. The possibility exists that the *MT1L* gene encodes a shortened but functional MT protein truncated at the TGA codon in the second exon. Such a protein would be almost equivalent to a normal β-domain of MT proteins. Alternatively, the *MT1L* gene may encode a selenoprotein with a single selenocysteine residue encoded by the TGA codon in the second exon. Either of these possibilities would result in a MT protein with novel properties and possibly a novel function which is particularly relevant in endocrine tissues and this requires further examination. Importantly though, the data presented in Chapter 5 indicates that the *MT1L* gene contributes to the repertoire of human MT mRNA expression, particularly in endocrine tissues and is now as well characterised as many of the human MT genes.

The data presented in this thesis then has provided a detailed study of the expression of MT in the developing and adult sheep brain, correlating MT expression with glial cell type but more importantly providing insight into the regulation of MT expression at the cellular level. The gene encoding a MT-III isoform with novel structural properties was cloned from the sheep brain. This novel MT isoform may

provide a useful tool in examining the physiological role of MT-III isoforms and their relationship to other MT proteins. Finally, a novel human MT gene, *MT1L*, with unique features was characterised at the mRNA level and found to be expressed in endocrine tissues. The expression of any protein encoded by this gene is likely to have novel properties and warrants further investigation.

Abel, J. and Ruiter, N. (1989) Inhibition of hydroxyl-radical-generated DNA degradation by metallothionein. *Toxicol. Lett.* **47**, 191-196.

Adams, R.P.L (1980) Cell culture for biochemists, 2nd edition, Elsevier, Amsterdam.

Andersen, R.D., Piletz, J.E., Birren, B.W. and Herschman, H.R. (1983) Levels of metallothionein messenger RNA in fetal, neonatal and maternal rat liver. *Eur. J. Biochem.* **131**, 497-500.

Andersen, R.D., Taplitz, S.J., Wong, S. and Bristol, G. (1987) Metal-dependent binding of a factor *in vivo* to the metal-responsive elements of the metallothionein 1 gene promoter. *Mol. Cell. Biol.* **7**, 3574-3581.

Andersen, R.D., Taplitz, S.J., Oberbauer, A.M., Calame, K.L. and Herschman, H.R. (1990) Metal-dependent binding of a nuclear factor to the rat metallothionein-I promoter. *Nucl. Acids Res.* **18**, 6049-6055.

Andrews, G.K., Adamson, E.D. and Gedamu, L. (1984) The ontogeny of expression of murine metallothionein: comparison with the α-fetoprotein gene. *Dev. Biol.* **103**, 294-303.

Andrews, G.K., Gallant, K.R. and Cherian, M.G. (1987) Regulation of the ontogeny of rat liver metallothionein mRNA by zinc. *Eur. J. Biochem.* **166**, 527-531.

Andrews, G.K., Huet-Hudson, Y.M., Paria, B.C., McMaster, M.T., De, S.K. and Dey, S.K. (1991) Metallothionein gene expression and metal regulation during preimplantation mouse embryo development (MT mRNA during early development). *Dev. Biol.* **145**, 13-27.

Andrews, P.A., Murphy, M.P. and Howell, S.B. (1987) Metallothionein-mediated cisplatin resistance in human ovarian carcinoma cells. *Cancer Chemother. Pharmacol.* **19**, 149-154.

Angel, P., Poting, A., Mallick, U., Rahmsdorf, H.J., Schorpp, M. and Herrlich, P. (1986) Induction of metallothionein and other mRNA species by carcinogens and tumour promoters in primary human skin fibroblasts. *Mol. Cell Biol.* **6**, 1760-1766.

Arey, L.B. (1965) Developmental anatomy: A textbook and laboratory manual of embryology. W.B.Saunders Company, USA.

Aruoma, O.I., Halliwell, B. and Dizdaroglu, M. (1989) Iron ion-dependent modifications of bases in DNA by the superoxide radical-generating system hypoxanthine-xanthine oxidase. *J. Biol. Chem.* **264**, 13024-13028.

Åstrom K. E. (1967) On the early development of the isocortex in fetal sheep. *Prog. Brain Res.* **26**, 1-59.

Bahnson, R.R., Banner, B.F., Ernstoff, M.S., Lazo, J.S., Cherian, M.G., Banerjee, D. and Chin, J.L. (1991) Immunohistochemical localisation of metallothionein in transitional cell carcinoma of the bladder. *J. Urol.* **46**, 1518-1520.

Bakka, A. and Webb, M. (1981) Metabolism of zinc and copper in the neonate: Changes in the concentrations and contents of thionein-bound Zn and Cu with age in the livers of the newborn of various mammalian species. *Biochemical Pharmacology* **30**, 721-725.

Bakka, A., Endresen, L., Johnson, A.B., Edminson, P.D. and Rugstad, H.E. (1981) Resistance against cis-dichlorodiammineplatinum in cultured cells with a high content of metallothionein. *Toxicol. Appl. Pharmacol.* **61**, 215-226.

Banerjee, D., Onosaka, S. and Cherian, M.G. (1982) Immunohistochemical localization of metallothionein in cell nucleus and cytoplasm in rat liver and kidney. *Toxicology* **24**, 95-105.

Basu, A. and Lazo, J.S. (1990) A hypothesis regarding the protective role of metallothioneins against the toxicity of DNA interactive anticancer drugs. *Toxicol. Lett.* **50**, 123-135.

Basu, A. and Lazo, J.S. (1991) Suppression of dexamethasone-induced metallothionein expression and *cis*-diamminedichloroplatinum (II) resistance by v-mos. *Cancer Res.* **51**, 893-896.

Bauer, J., Ganter, U., Abel, J., Strauss, S., Jonas, U., Weib, R., Gebicke-Haerter, P., Volk, B. and Berger, M. (1993) Effects of interleukin-1 and interleukin-6 on metallothionein and amyloid precursor protein expression in human neuroblastoma cells. *J. Neuroimmunol.* **45**, 163-173.

Bauman, J.W., Liu, J., Liu, Y.P. and Klaassen, C.D. (1991) Increase in metallothionein produced by chemicals that induce oxidative stress. *Toxicol. Appl. Pharmacol.* **110**, 347-354.

Beach, L.R. and Palmiter, R.D. (1981) Amplification of the metallothionein I gene in cadmium-resistant mouse cells. *Proc. Natl. Acad. Sci. USA* **78**, 2110-2114.

Beattie, J.H., Richards, M.P. and Self, R. (1993) Separation of metallothionein isoforms by capillary zone electrophoresis. *J. Chromatogr.* **632**, 127-135.

Behne, D., Hilmert, H., Scheid, S., Gessner, H. and Elger, W. (1988) Evidence for specific selenium target tissues and new biologically important selenoproteins. *Biochim. Biophys. Acta* **966**, 12-21.

Benveniste, E.N., Sparacio, S.M., Norris, J.G., Grenett, H.E. and Fuller, G.M. (1990) Induction and regulation of interleukin-6 gene expression in rat astrocytes. *J. Neuroimmunol.* **30**, 201-212.

Berry, M.J., Banu, L. and Larsen, P.R. (1991a) Type I iodothyronine deiodinase is a selenocysteine-containing enzyme. *Nature* **349**, 438-440.

Berry, M.J., Banu, L., Chen, Y., Mande, S.J., Kieffer, J.D., Harney, J.W. and Larsen, P.R. (1991b) Recognition of UGA as a selenocysteine codon in Type I deiodinase requires sequences in the 3' untranslated region. *Nature* **353**, 273-276.

Berry, M.J., Banu, L., Harney, J.W. and Larsen, P.R. (1993) Functional characterization of the eukaryotic SECIS elements which direct selenocysteien insertion at UGA codons. *EMBO J.* **12**, 3315-3322.

Blaauwgeers, H.G.T., Sillevis Smitt, P.A.E., De Jong, J.M.B.V. and Troost, D. (1993) Distribution of metallothionein in the human central nervous system. *Glia* **8**, 62-70.

Blaauwgeers, H.G.T., Sillevis Smitt, P.A.E., De Jong, J.M.B.V. and Troost, D. (1994) Localisation of metallothionein in the mammalian central nervous system. *Biol. Signals* **3**, 181-187.

Blalock, T.L., Dunn, M.A. and Cousins, R.J. (1988) Metallothionein gene expression in rats: tissue-specific regulation by dietary copper and zinc. *J. Nutr.* **118**, 222-228.

Böck, A., Forchhammer, K., Heider, J. and Baron, C. (1991) Selenoprotein synthesis: an expansion of the genetic code. *TIBS* **16**, 463-467.

Boulanger, Y., Goodman, C.M., Forte, C.P., Fesik, S.W. and Armitage, I.M. (1983) Model for mammalian metallothionein structure. *Proc. Natl. Acad. Sci. USA* **80**, 1501-1505.

Breathnach, R. and Chambon, P. (1981) Organization and expression of eukaryotic split genes coding for proteins. *Ann. Rev. Biochem.* **50**, 249-383.

Bremner, I. (1987) Nutritional and physiological significance of metallothionein, in Experentia Supplementum 52, (Kägi, J.H.R. and Kojima, Y. eds), pp 81-108. Birkhauser Verlag, Basel.

Bremner, I. and Beattie, J.H. (1990) Metallothionein and the trace minerals. *Ann. Rev. Nutr.* **10**, 63-83.

Bremner, I., Williams, R.B. and Young, B.W. (1977) Distribution of copper and zinc in the liver of the developing fetus. *Br. J. Nutr.* **38**, 87-92.

Bremner, I., Mehra, R.K. and Sato, M. (1987) Metallothionein in blood, bile and urine, in Experentia Supplementum 52, (Kägi, J.H.R. and Kojima, Y. eds) pp 507-517. Birkhauser Verlag, Basel.

Bühler, R.H.O. and Kägi, J.H.R. (1974) Human hepatic metallothioneins. *FEBS Lett.* **39**, 229-234.

Byravan, S., Foster, L.M., Phan, T., Verity, A.N. and Campagnoni, A.T. (1994) Murine oligodendroglial cells express nerve growth factor. *Proc. Natl. Acad. Sci. USA* **91,** 8812-8816.

Cagen, S.Z. and Klaassen, C.D. (1979) Protection of carbon tetrachloride-induced hepatotoxicity by zinc: Role of metallothionein. *Toxicol. Appl. Pharmacol.* **51**, 107-116.

Caroni, P. and Schwab, M.E. (1989) Co-distribution of neurite growth inhibitors and oligodendrocytes in rat CNS: appearance follows nerve fibre growth and precedes myelination. *Dev. Biol.* **136,** 287-295.

Carter, A.D., Felber, B.K., Walling, M.J., Jubier, M.F., Schmidt, C.J. and Hamer, D.H. (1984) Duplicated heavy metal control sequences of the mouse metallothionein-I gene. *Proc. Natl. Acad. Sci. USA* **81**, 7392-7396.

Chauvin, P.J., Cherian, M.G., Wysocki, G.P. and Pringle, G.A. (1992) A comparison of metallothionein expression in human normal and neoplastic salivary tissues using immunohistochemistry. *Toxicologist* **12**, 379.

Chelly, J., Concordet, J.P., Kaplan, J.C. and Kahn, A. (1989) Illegitimate transcription: Transcription of any gene in any cell type. *Proc. Natl. Acad. Sci USA* **86**, 2617-2621.

Cherian, M.G. (1977) Studies of the synthesis and metabolism of zinc-thionein in rats. *J. Nutr.* **107**, 965-972.

Cherian., M.G. (1993) Nuclear and cytoplasmic localization of metallothionein in human liver during development and in tumor cells in Experentia Supplementum 52, (Kägi, J.H.R. and Kojima, Y. eds) pp 175-188. Birkhauser Verlag, Basel.

Cherian, M.G. (1994) The significance of the nuclear and cytoplasmic localization of metallothionein in human liver and tumor cells. *Environ. Health Perspect.* **102**, 131-135.

Cherian, M.G., Yu, S. and Redman, C.M. (1981) Site of synthesis of metallothionein in rat liver. *Can. J. Biochem.* **59**, 301-306.

Cherian, M.G., Templeton, D.M., Gallant, K.R. and Banerjee, D. (1987) Biosynthesis and metabolism of metallothionein in rat during perinatal development in Experentia Supplementum 52, (Kägi, J.H.R. and Kojima, Y. eds) pp 499-505. Birkhauser Verlag, Basel.

Chernaik, M.L. and Huang, P.C. (1991) Differential effect of cysteine-to-serine substitutions in metallothionien on cadmium resistance. *Proc. Natl. Acad. Sci. USA* **88**, 3024-3028.

Chin, J.L., Banerjee, D., Kadhim, S.A., Kontozoglou, T.E., Chauvin, P.J. and Cherian, M.G. (1993) Metallothionein in testicular germ cell tumors and drug resistance. *Cancer Res.* **72**, 3029-3035.

Chomczynski, P. and Sacchi, N. (1987) Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159.

Choudhuri, S., Kramer, K.K., Berman, N.E., Dalton, T.P., Andrews, G.K. and Klaassen, C.D. (1995) Constitutive expression of metallothionein genes in mouse brain. *Toxicol. Appl. Pharmacol.* **131**, 144-154.

Chubatsu, L.S. and Meneghini, R. (1993) Metallothionein protects DNA from oxidative damage. *Biochem. J.* **291,** 193-198.

Chubatsu, L.S., Gennari, M. and Meneghini, R. (1992) Glutathione is the antioxidant responsible for resistance to oxidative stress in V79 Chinese hamster fibroblasts rendered resistant to cadmium. *Chem. Biol. Interact.* **82**, 99-110.

Cismowski, M.J. and Huang, P.C. (1991) Effect of cysteine replacement at positions 13 and 50 on metallothionein structure. *Biochemistry* **30**, 6626-6632.

Compere, S.J. and Palmiter, R.D. (1981) DNA methylation controls the inducibility of the mouse metallothionein-I gene in lymphoid cells. *Cell* **25**, 233-240.

Cormack, D.H. (1984) Introduction to histology. J.B.Lippincott Company, Philadelphia

Cousins, R.J. (1985) Absorption, transport and hepatic metabolism of copper and zinc: special reference to metallothionein and caeruloplasmin. *Physiol. Rev.* **65**, 238-309.

Cousins, R.J. and Leinart, A.S. (1988) Tissue-specific regulation of zinc metabolism and metallothionein genes by interleukin 1. *FASEB J.* **2**, 2884-2890.

Cousins, R.J. and Lee-Ambrose, L.M. (1992) Nuclear zinc uptake and interactions and metallothionein gene expression are influenced by dietary zinc in rats. *J. Nutr.* **122**, 56-64.

Coyle, P., Philcox, J.C. and Rofe, A.M. (1993a) Corticosterone enhances the zinc and interleukin-6 mediated induction of metallothionein in cultured rat hepatocytes. *J. Nutr.* **123**, 1464-1470.

Coyle, P., Philcox, J.C. and Rofe, A.M. (1993b) Metallothionein induction in freshly isolated rat hepatocytes. *Biol. Trace Elem. Res.* **36**, 35-49.

Coyle, P., Philcox, J.C. and Rofe, A.M. (1993c) Clinical significance of metallothionein: A dealer in heavy metals. *Clin. Biochem. Res.* **14**, 118-125.

Coyle, P., Philcox, J.C. and Rofe, A.M. (1995) Metallothionein induction in cultured rat hepatocytes by arthritic rat serum, activated macrophages, interleukin-6, interleukin-11 and leukaemia inhibitory factor. *Inflamm. Res.* **44**, 475-481.

Crawford, B.D., Enger, M.D., Griffith, B.B., Griffith, J.K., Hanners, J.L., Longmire, J.L., Munk, A.C., Stallings, R.L., Tesmer, J.G., Walters, R.A. and Hildebrand, C.E. (1985) Coordinate amplification of metallothionein I and II genes in cadmium-resistant chinese hamster ovary cells: Implications for mechanisms regulating metallothionein gene expression. *Mol. Cell. Biol.* **5**, 320-329.

Culotta, V.C. and Hamer, D.H. (1989) Fine mapping of the mouse metallothionein gene metal response element. *Mol. Cell Biol.* **9**, 1376-1380.

Culotta, V.C., Joh, H.D., Lin, S.J., Slekar, K.H. and Strain, J. (1995) A physiological role for *Saccharomyces cerevisiae* copper/zinc superoxide dismutase in copper buffering. *J. Biol. Chem.* **270**, 29991-29997.

Dalton, T., Palmiter, R.D. and Andrews, G.K. (1994) Transcriptional induction of the mouse metallothionein-I gene in hydrogen peroxide-treated Hepa cells involves a composite major late transcription factor/antioxidant response element and metal response promoter elements. *Nucl. Acid Res.* **22**, 5016-5023.

Danielson, K.G., Ohi, S. and Huang, P.C. (1982) Immunochemical detection of metallothionein in specific epithelial cells of rat organs. *Proc. Natl. Acad. Sci. USA* **79**, 2301-2304.

De, S.K., McMaster, M.T. and Andrews, G.K. (1990) Endotoxin induction of murine metallothionein gene expression. *J. Biol. Chem.* **256**, 15267-15274.

Dorian, C., Gattone II, V.H. and Klaassen, C.D. (1992) Accumulation and degradation of the protein moiety of cadmium-metallothionein (CdMT) in the mouse kidney. *Toxicol. Appl. Pharmacol.* **117**, 242-248.

Dudek, E.J., Peak, J.G., Roth, R.M. and Peak, M.J. (1993) Isolation of V79 fibroblast cell lines containing elevated metallothionein levels that have increased resistance to the cytotoxic effects of ultraviolet-A radiation. *Photochem. Photobiol.* **58**, 836-840.

Durnam, D.M. and Palmiter, R.D. (1981) Transcriptional regulation of the mouse metallothionein-I gene by heavy metals. *J. Biol. Chem.* **256**, 5712-5716.

Durnam, D.M. and Palmiter, R.D. (1984) Induction of metallothionein-I mRNA in cultured cells by heavy metals and iodoacetate: evidence for gratuitous inducers. *Mol. Cell. Biol.* **4**, 484-491.

Durnam, D.M., Perrin, F., Gannon, F. and Palmiter, R.D. (1980) Isolation and characterization of the mouse metallothionein-I gene. *Proc. Natl. Acad. Sci. USA* 77, 6511-6515.

Durnam, D.M., Hoffman, J.S., Quaife, C.J., Benditt, E.P., Chen, H.Y. and Palmiter, R.J. (1984) Induction of mouse metallothionein-I mRNA by bacterial endotoxin is independent of metals and glucocorticoid hormones. *Proc. Natl. Acad. Sci. USA* **81**, 1053-1056.

Dziegielewska, K.M., Reader, M., Matthews, N., Brown, W.M., Møllgard, K. and Saunders, N.R. (1993) Synthesis of the foetal protein fetuin by early developing neurons in the immature neocortex. *J. Neurocytol.* **22,** 266-272.

Ebadi, M. (1986a) Characterisation of a metallothionein-like protein in rat brain. *Biol. Trace Elem. Res.* **11**, 101-116.

Ebadi, M. (1986b) Biochemical alteration of a metallothionein-like protein in developing rat brain. *Biol. Trace Elem. Res.* **11**, 117-128.

Eichholtz-Wirth, H., Born, R., Reidel, G. and Hietel, B. (1993) Transient cisplatin-resistant murine fibrosarcoma cell characterized by increased metallothionein content. *J. Cancer Res. Clin. Oncol.* **119**, 227-233.

Elmes, M.E., Haywood, S. and Jasani, B. (1991) A histochemical and immunocytochemical study of hepatic copper and metallothionein in the pre- and post-natal rat. *J. Pathol.* **164**, 83-87.

Endresen, L., Bakka, A. and Rugstad, H.E. (1983) Increased resistance to chlorambucil in cultured cells with a high concentration of cytoplasmic metallothionein. *Cancer Res.* **43**, 2918-2926.

Erickson, J.C., Sewell, A.K., Jensen, L.T., Winge, D.R. and Palmiter, R.D. (1994) Enhanced neurotrophic activity in Alzheimer's disease cortex is not associated with down-regulation of metallothionein-III (GIF). *Brain Res.* **649**, 297-304.

Erickson, J.C., Masters, B.A., Kelly, E.J., Brinster, R.L. and Palmiter, R.D. (1995) Expression of human metallothionein-III in transgenic mice. *Neurochem. Int.* **27**, 35-41.

Fornace Jr., A.J., Schalch, H. and Alamo Jr., I. (1988) Coordinate induction of metallothionein I and II in rodent cells by UV irradiation. *Mol. Cell Biol.* **8**, 4716-4720.

Forstrom, J.W., Zakowski, J.J. and Tappel, A.L. (1978) Identification of the catalytic site of rat liver glutathione peroxidase as selenocysteine. *Biochemistry* **17**, 2639-2644.

Foster, R., Jahroudi, N., Varshney, U. and Gedamu, L. (1988) Structure and expression of the human MT-1G gene. *J. Biol. Chem.* **263**, 11528-11535.

Foster, R., Olsson, P.E. and Gedamu, L. (1989) Calcium phosphate-mediated transfection alters metallothionein gene expression in response to Cd^{2+} and Zn^{2+}. *Mol. Cell. Biol.* **9**, 4105-4108.

Fowler, B.A., Hildebrand, C.E., Kojima, Y. and Webb, M. (1987) Nomenclature of metallothioneins in Experentia Supplementum 52, (Kägi, J.H.R. and Kojima, Y. eds) pp 19-22. Birkhauser Verlag, Basel.

Fredrickson, C.J. (1989) Neurobiology of zinc and zinc-containing neurons. *Int. Rev. Neurobiol.* **31**, 145-238.

Friedman, R.L. and Stark, G.R. (1985) α-Interferon-induced transcription of HLA and metallothionein genes containing homologous upstream sequences. *Nature* **314**, 637-639.

Furey, W.F., Robbins, A.H., Clancy, L.L., Winge, D.R., Wang, B.C. and Stout, C.D. (1986) Crystal structure of Cd, Zn metallothionein. *Science* **231**, 704-710.

Gasull, T., Giralt, M., Hernandez, J., Martinez, P., Bremner, I. and Hidalgo, J. (1994) Regulation of metallothionein concentrations in rat brain: effect of glucocorticoids, zinc, copper and endotoxin. *Am. J. Physiol.* **266**, E760-E767.

Gick, G.G. and McCarty, K.S. (1982) Amplification of the metallothionein-I gene in cadmium- and zinc-resistant Chinese hamster ovary cells. *J. Biol Chem.* **257**, 9049-9053.

Giorno, R. (1984) A comparison of two immunoperoxidase staining methods based on the avidin-biotin interaction. *Diagn. Immunol.* **2**, 161-166.

Glanville, N., Durnam, D.M. and Palmiter, R.D. (1981) Structure of mouse metallothionein-I and its mRNA. *Nature* **292**, 267-269.

Grady, D.L., Moyzis, R.K. and Hildebrand, C.E. (1987) Molecular and cellular mechanisms of cadmium resistance in cultured cells in Experentia Supplementum 52, (Kägi, J.H.R. and Kojima, Y. eds) pp 447-456. Birkhauser Verlag, Basel.

Grunstein, M. and Hogness, D.S. (1975) Colony hybridisation: A method for the isolation of cloned DNAs that contain a specific gene. *Proc. Natl. Acad. Sci. USA* **72**, 3961-3965.

Haerslev, T., Jacobsen, G.K. and Zedeler, K. (1995) The prognostic significance of immunohistochemically detectable metallothionein in primary breast carcinomas. *APMIS* **103**, 279-285.

Haile-Meskel, H., Cherian, M.G., Martinez, V.J., Veinot, L.A. and Frei, J.V. (1993) Metallothionein as an epithelial proliferative compartment marker for DNA flow cytometry. *Mod. Pathol.* **6**, 755-760.

Hall, A.C., Young, B.W. and Bremner, I. (1979) Intestinal metallothionein and the mutual antagonism between copper and zinc in the rat. *J. Inorg. Biochem.* **11**, 57-66.

Hall, P.A., Levinson, D.A., Woods, A.L., Yu, C.C., Kellock, D.B.and Watkins, J.A. (1990) Proliferating cell nuclear antigen (PCNA) immunolocalization in paraffin sections: an index of cell proliferation with evidence of deregulated expression in some neoplasms. *J.Pathol.* **162**, 285-294.

Hamer, D.H. (1986) Metallothionein. *Ann. Rev. Biochem.* **55**, 913-951.

Hart, B.A., Voss, G.W., Shatos, M.A. and Doherty, J. (1990) Cross-tolerance to hyperoxia following cadmium aerosol pretreatment. *Toxicol. Appl. Pharmacol.* **103**, 255-270.

Harvey, M., Sands, A.T., Weiss, R., Hegi, M.E., Wiseman, R.W., Pantazis, P., Giovanella, B.C., Tainsky, M.A., Bradley, A. and Donehower, L.A. (1993) *In vitro* growth characteristics of embryo fibroblasts isolated from p53-deficient mice. *Oncogene* **8**, 2457-2467.

Hawley-Nelson, P., Ciccarone, V., Gebeyehu, G., Jessee, J. and Felgner, P.L. (1993) Lipofectamine reagent: A new, higher efficiency polycationic liposome transfection reagent. *Focus* **15**, 73-79

Heguy, A., West, A., Richards, R.I. and Karin, M. (1986) Structural and tissue-specific expression of the human metallothionein IB gene. *Mol. Cell Biol.* **6**, 2149-2157.

Hempe, J.M. and Cousins R.J. (1992) Cysteine-rich intestinal protein and intestinal metallothionein: An inverse relationship as a conceptual model for zinc absorption in rats. *J. Nutr.* **122**, 89-95.

Hempe, J.M., Carlson, J.M. and Cousins, R.J. (1991) Intestinal metallothionein gene expression and zinc absorption in rats are zinc responsive but refractory to dexamethasone and interleukin 1a. *J. Nutr.* **121**, 1386-1389.

Hewicker-Trautwein, M. and Trautwein, G. (1993) An immunohistochemical study of the fetal sheep neocortex and cerebellum with antibodies against nervous system-specific proteins. *J. Comp. Path.* **109**, 409-421.

Hidalgo, J., Borrás, M., Garvey, J.S. and Armario, A. (1990) Liver, brain and heart metallothionein induction by stress. *J. Neurochem.* **55**, 651-654.

Hidalgo, J., Campmany, L., Marti, O. and Armario, A. (1991) Metallothionein-I induction by stress in specific brain areas. *Neurochem. Res.* **16**, 1145-1148.

Hidalgo, J., Garcia, A., Oliva, A.M., Giralt, M., Gonzalez, B., Milnerowicz, H., Wood, A. and Bremner, I. (1994) Effect of zinc and glucocorticoids on metallothionein levels of cultured neurons and astrocytes from rat brain. *Chem. Biol. Interact.* **93**, 197-219.

Hoadley, J.E., Leinart, A.S. and Cousins, R.J. (1988) Relationship of 65Zn absorption kinetics to intestinal metallothionein in rats: Effects of zinc depletion and fasting. *J. Nutr.* **118**, 497-502.

Hozumi, I., Inuzuka, T., Hiraiwa, M., Uchida, Y., Anezaki, T., Ishiguro, H., Kobayashi, H., Uda, Y., Miyatake, T. and Tsuji, S. (1995) Changes of growth inhibitory factor after stab wounds in rat brain. *Brain Res.* **688**, 143-148.

Huber, K.L. and Cousins, R.J. (1988) Maternal zinc deprivation and interleukin-1 influence metallothionein gene expression and zinc metabolism of rats. *J. Nutr.* **118**, 1570-1576.

Hughes, S.M., Lillien, L.E., Raff, M.C., Rohrer, H. and Sendtner, M. (1988) Ciliary neurotrophic factor induces type-2 astrocyte differentiation in culture. *Nature* **335**, 70-73.

Hunziker, P.E. and Kägi, J.H.R. (1985) Isolation and characterization of six human hepatic isometallothioneins. *Biochem. J.* **231**, 375-382.

Hunziker, P.E. and Kägi, J.H.R. (1987) Human hepatic metallothioneins: Resolution of 6 isoforms in Experentia Supplementum 52, (Kägi, J.H.R. and Kojima, Y. eds) pp 257-264 Birkhauser Verlag, Basel.

Ibáñez, E., Vidal, F. and Hidalgo, J. (1995) Early mouse preimplantation development is unaffected by microinjection of metallothionein antibodies. *Zygote* **3**, 81-84.

Imagawa, M., Chiu, R. and Karin, M. (1987) Transcription factor AP-2 mediates induction by two different signal-transduction pathways: Protein kinase C and cAMP. *Cell* **51**, 251-260.

Imagawa, M., Ishikawa, Y., Shimano, H., Osada, S. and Nishihara, T. (1995) CTG triplet repeat in mouse growth inhibitory factor/metallothionein III gene promoter represses the transcriptional activity of the heterologous promoters. *J. Biol. Chem.* **270**, 20898-20900.

Imbert, J., Zafarullah, M., Culotta, V.C., Gedamu, L. and Hamer, D. (1989) Transcription factor MBF-I interacts with metal regulatory elements of higher eucaryotic metallothienein genes. *Mol. Cell. Biol.* **9**, 5315-5323.

Imbra, R.J. and Karin, M. (1987) Metallothionein gene expression is regulated by serum factors and activators of protein kinase C. *Mol. Cell. Biol* **7**, 1358-1363.

Itano, Y., Noji, S., Koyama, E., Taniguchi, S., Taga, N., Takahashi, T., Ono, K. and Kosaka, F. (1991) Bacterial endotoxin-induced expression of metallothionein genes in rat brain, as revealed by *in situ* hybridization. *Neurosci. Lett.* **124**, 13-16.

Itoh, M., Ebadi, M. and Swanson, S. (1983) The presence of zinc binding proteins in the brain. *J. Neurochem.* **41**, 823-829.

Jacobson, M. (1991) Developmental neurobiology, 3rd edition. Plenium Press, New York.

Jahroudi, N., Foster, R., Price-Haughey, J., Beitel, G. and Gedamu, L. (1990) Cell-type specific and differential regulation of the human metallothionein genes. *J. Biol. Chem.* **265**, 6506-6511.

Janeczko, K. (1994) Age-dependent changes in the proliferative response of S100 protein-positive glial cells to injury in the rat brain. *Int. J. Devel. Neuroscience* **12**, 431-440.

Jasani, B. and Elmes, M. E. (1991) Immunohistochemical detection of metallothionein in Methods in Enzymology 205, (Riordan, J.F. and Vallee, B.L. eds) pp95-107. Academic Press Inc, San Diego.

Juurlink, B.H.J. (1994) Type-2 astrocytes have much greater susceptibility to heat stress than type-1 astrocytes. *J. Neurosci. Res.* **38,** 196-201.

Kadonaga, J.T., Jones, K.A. and Tjian, R. (1986) Promoter-specific activation of RNA polymerase II transcription by Sp1. *TIBS* **11**, 20-23.

Kägi, J.H.R. (1991) Overview of metallothionein in Methods in Enzymology 205, (Riordan, J.F. and Valle, B.L.eds) pp 613-626. Academic Press Inc, San Diego.

Kägi, J.H.R. (1993) Evolution, structure and chemical activity of class I metallothioneins:an overview in Metallothionein III, Biological roles and medical implications, (Suzuki, K.T., Imura, N. and Kimura, M. eds) pp 29-56. Birkhauser Verlag, Basel.

Kägi, J.H.R. and Vallee, B.L. (1960) Metallothionein: a cadmium- and zinc-containing protein from equine renal cortex. *J. Biol. Chem.* **235**, 3460-3465.

Kägi, J.H.R. and Kojima, Y. (1987) Chemistry and biochemistry of metallothionein in Experentia Supplementum 52, (Kägi, J.H.R. and Kojima, Y. eds) pp 25-62. Birkhauser Verlag, Basel.

Kägi, J.H.R. and Schäffer, A. (1988) Biochemistry of metallothionein. *Biochemistry* **27**, 8509-8515.

Kägi, J.H.R., Himmelhoch, S.R., Whanger, P.D., Bethune, J.L. and Vallee, B.L. (1974) Equine hepatic and renal metallothioneins. *J. Biol. Chem.* **249**, 3537-3542.

Kaina, B., Lohrer, H., Karin, M. and Herrlich, P. (1990) Overexpressed human metallothionein IIA gene protects chinese hamster ovary cells from killing by alkylating agents. *Proc. Natl. Acad. Sci. USA* **87**, 2710-2714.

Karasawa, M., Nishimura, H., Tohyama, C., Hashiba, H. and Kuroki, T. (1991) Localisation of metallothionien in hair follicles of normal skin and the basal cell layer of hyperplastic epidermis: Possible association with cell proliferation. *J. Invest. Dermatol.* **97**, 97-100.

Karin, M. (1985) Metallothioneins: Proteins in search of function. *Cell* **41**, 9-10.

Karin, M. and Richards, R.I. (1982) Human metallothionein genes - primary structure of the metallothionein-II gene and a related pseudogene. *Nature* **299**, 797-802.

Karin, M., Andersen, R.D., Slater, E., Smith, K. and Herschman, H.R. (1980) Metallothionein mRNA induction in HeLa cells in response to zinc or dexamethasone is a primary induction response. *Nature* **286**, 295-297.

Karin, M., Slater, E.P. and Herschman, H.R. (1981) Regulation of metallothionein synthesis in HeLa cells by heavy metals and glucocorticoids. *J. Cell Physiol.* **106**, 63-74.

Karin, M., Cathala, G. and Nguyen-Huu, M.C. (1983) Expression and regulation of a human metallothionein gene carried on an autonomously replicating shuttle vector. *Proc. Natl. Acad. Sci. USA* **80**, 4040-4044.

Karin, M., Eddy, R.L., Henry, W.M., Haley, L.L., Byers, M.G. and Shows, T.B. (1984a) Human metallothionein genes are clustered on chromosome 16. *Proc. Natl. Acad. Sci. USA* **81**, 5494-5498.

Karin, M., Haslinger, A., Holtgreve, H., Richards, R.I., Krauter, P., Westphal, H.M. and Beato, M. (1984b) Characterisation of DNA sequences through which cadmium and glucocorticoid hormones induce human metallothionein-II_A gene. *Nature* **308**, 513-519.

Karin, M., Haslinger, A., Heguy, A., Dietlin, T. and Cooke, T. (1987) Metal-responsive elements act as positive modulators of human metallothionein-IIA enhancer activity. *Mol. Cell Biol.* **7**, 606-613.

Kasahara, K., Fujiwara, Y., Nishio, K., Ohmori, T., Sugimoto, Y., Komiya, K., Matsuda, T. and Saujo, N. (1991) Metallothionein content correlates with the sensitivity of human small-cell lung cancer cell lines to cisplatin. *Cancer Res.* **51**, 3237-3242.

Kaufman, R.J. (1990) Vectors used for expression in mammalian cells in Methods in Enzymology 185, (Goeddel, D.V. ed) pp 487-511. Academic Press Inc., San Diego.

Kay, J., Cryer, A., Darke, B.M., Kille, P., Lees, W.E., Norey, C.G. and Stark, J.M. (1991) Naturally occurring and recombinant metallothioneins: structure, immunoreactivity and metal-binding functions. *Int. J. Biochem.* **23**, 1-5.

Kelley, S.L., Basu, A., Teicher, B.A., Hamer, D.H., Hacker, M.P. and Lazo, J.S. (1988) Overexpression of metallothionein confers resistance to anticancer drugs. *Science* **241**, 1813-1815.

Kelly, E.J. and Palmiter, R.D. (1996) A murine model of Menkes disease reveals a physiological function of metallothionein. *Nature genetics* **13**, 219-222.

Koewn, W.A., Campbell, C.R. and Kucherlapati, R.S. (1990) Methods for introducing DNA into mammalian cells in Methods in Enzymology 185, (Goeddel, D.V. ed) pp 527-537 Academic Press Inc., San Diego.

Klein, D., Scholz, P., Drasch, G.A., Müller-Höcker, J. and Summer, K.H. (1991) Metallothionein, copper and zinc in fetal and neonatal human liver: changes during development. *Toxicol. Lett.* **56**, 61-67.

Kobayashi, H., Uchida, Y., Ihara, Y., Nakajima, K., Kohsaka, S., Miyatake, T. and Tsuji, S. (1993) Molecular cloning of rat growth inhibitory factor cDNA and the expression in the central nervous system. *Mol. Brain Res.* **19**, 188-194.

Koizumi, S., Otaki, N. and Kimura, K. (1982) Estimation of thionein synthesis in cultured cells by slab gel electrophoresis. *Ind. Health* **20**, 101-108.

Koizumi, S., Suzuki, K. and Otsuka, F. (1992) A nuclear factor that recognizes the metal-responsive elements of human metallothionein II_A gene. *J. Biol. Chem.* **267**, 18659-18664.

Kojima, Y., Berger, C., Vallee, B.L. and Kagi, J.H.R. (1976) Amino acid sequencing of equine renal metallothionein-IB. *Proc. Natl. Acad. Sci. USA* **73**, 3413-3417.

Kondo, Y., Kuo, S.M. and Lazo, J.S. (1994) Interleukin 1B-mediated metallothionein induction and cytoprotection against cadmium and cis-diamminedichloroplatinum. *J. Pharmacol. Exp. Ther.* **270**, 1313-1318.

Kondo, Y., Woo, E.S., Michalska, A.E., Choo, K.H.A. and Lazo, J.S. (1995) Metallothionein null cells have increased sensitivity to anticancer drugs. *Cancer Research* **55**, 2021-2023.

Lambert, E., Kille, P. and Swaminathan, R. (1996) Cloning and sequncing a novel metallothionein I isoform expressed in human reticulocytes. *FEBS lett.* **389**, 210-212.

Larsen, W.J. (1993) Human embryology. Churchill Livingstone Inc., New York.

Lazarini, F., Deslys, J.P. and Dormont, D. (1991) Regulation of the glial fibrillary acidic protein, beta-actin and prion protein mRNAs during brain development in mouse. *Brain Res. Mol. Brain Res.* **10**, 343-346.

Lazo, J.S., Kondo, Y., Dellapiazza, D., Michalska, A.E., Choo, A. and Pitt, B.R. (1995) Enhanced sensitivity to oxidative stress in cultured embryonic cells from transgenic mice deficient in metallothionein I and II genes. *J. Biol. Chem.* **270**, 5506-5510.

Leber, A.P. and Miya, T.S. (1976) A mechanism for cadmium- and zinc-induced tolerance to cadmium toxicity: involvement of metallothionein. *Toxicol. Appl. Pharmacol.* **37**, 403-414.

Lee, S.C., Liu, W., Dickson, D.W., Brosnan, C.F. and Berman, J.W. (1993) Cytokine production by human fetal microglia and astrocytes. *J. Immunol.* **150**, 2659-2667.

Lee, W., Haslinger, A., Karin, M. and Tjian, R. (1987) Activation of transcription by two factors that bind promoter and enhancer sequences of the human metallothionein gene and SV40. *Nature* **325**, 368-373.

Lehman-McKeeman, L.D., Andrews, G.K. and Klassen, C.D. (1988) Ontogeny and induction of hepatic isometallothioneins in immature rats. *Toxicol. Appl. Pharmacol.* **92**, 10-17.

Lewis, S.A., Balcarek, J.M., Krek, V., Shelanski, M. and Cowan, N.J. (1984) Sequence of a cDNA clone encoding mouse glial fibrillary acidic protein: Structural conservation of intermediate filaments. *Proc. Natl. Acad. Sci. USA* **81,** 2743-2746.

Li, T.Y., Kraker, A.J., Shaw, C.F.III and Petering, D.H. (1980) Ligand substitution reactions of metallothioneins with EDTA and apo-carbonic anhydrase. *Proc. Natl. Acad. Sci USA* 77 6334-6338.

Liang, L., Fu, K., Lee, D.K., Sobieski, R.J., Dalton, T. and Andrews, G.K. (1996) Activation of the complete mouse metallothionein gene locus in the maternal deciduum. *Molecular Reproduction and Development* **43**, 25-37.

Liu, Y., Liu, J., Iszard, M.B., Andrews, G.K., Palmiter, R.D. and Klaassen, C.D. (1995) Transgenic mice that overexpress metallothionein-I are protected from cadmium lethality and hepatotoxicity. *Toxicol. Appl. Pharmacol.* **135**, 222-228.

Lohrer, H. and Robson, T. (1989) Overexpression of metallothionein in CHO cells and its effect on cell killing by ionizing radiation and alkylating agents. *Carcinogenesis* **10**, 2279-2284.

Lohrer, H., Robson, T., Grindley, H., Foster, S. and Hall, A. (1990) Differential effects on cell killing in metallothionein overexpressing CHO mutant cell lines. *Carcinogenesis* **11**, 1937-1941.

Low, S.C. and Berry, M.J. (1996) Knowing when not to stop: selenocysteine incorporation in eukaryotes. *TIBS* **21**, 203-208.

Margoshes, M. and Vallee, B.L. (1957) A cadmium protein from equine kidney cortex. *J. Am. Chem Soc.* **79**, 4813.

Makar, T.K., Nedergaard, M., Preuss, A., Gelbard, A.S., Perumal, A.S. and Cooper. A.J.L. (1994) Vitamin E, ascorbate, glutathione disulfide, and enzymes of glutathione metabolism in cultures of chick astrocytes and neurons: evidence that astrocytes play an important role in antioxidative processes in the brain. *J. Neurochem.* **62,** 45-53.

Markossian, K.A., Melkonyan, V.Z., Paitan, N.A. and Nalbandyan, R.M. (1988) On the transfer between dopamine β-monooxygenase and Cu-thionein. *Biochem. Biophys. Res. Commun.* **153**, 558-563.

Masters, B.A., Kelly, E.J., Quaife, C.J., Brinster, R.L. and Palmiter, R.D. (1994a) Targeted disruption of metallothionein I and II genes increases sensitivity to cadmium. *Proc. Natl. Acad. Sci. USA* **91**, 584-588.

Masters, B.A., Quaife, C.J., Erickson, J.C., Kelly, E.J., Froelick, G.J., Zambrowickz, B.P., Brinster, R.L. and Palmiter, R.D. (1994b) Metallothionein III is expressed in neurons that sequester zinc in synaptic vesicles. *J. Neurosci.* **14**, 5844-5857.

Mayo, K.E. and Palmiter, R.D. (1981) Glucocorticoid regulation of metallothionein-I mRNA synthesis in cultured mouse cells. *J. Biol .Chem.* **256**, 2621-2624.

Mayo, K.E. and Palmiter, R.D. (1982) Glucocorticoid regulation of the mouse metallothionein I gene is selectively lost following amplification of the gene. *J. Biol. Chem.* **257**, 3061-3067.

McCaughan, K.K., Brown, C.M., Dalphin, M.E., Berry, M.J. and Tate, W.P. (1995) Translation termination efficiency in mammals is influenced by the base following the stop codon. *Proc. Natl. Acad. Sci. USA* **92**, 5431-5435.

Mehra, R.K. and Bremner, I. (1983) Development of a radioimmunoassay for rat liver metallothionein-I and its application to the analysis of rat plasma and kidneys. *Biochem. J.* **213**, 459-465.

Mehra, R.K. and Bremner, I. (1984) Metallothionein-I in the plasma and liver of neonatal rats. *Biochem. J.* **217**, 859-862.

Mello-Filho, A.C., Chubatsu, L.S. and Meneghini, R. (1988) V79 Chinese hamster cells rendered resistant to high cadmium concentrations also become resistant to oxidative stress. *Biochem. J.* **256**, 475-479.

Mercer, J.F.B. and Grimes, A. (1986) Variation in the amounts of hepatic copper, zinc and metallothionein mRNA during development in the rat. *Biochem. J.* **238**, 23-27.

Michalska, A. and Choo, K.H.A. (1993) Targeting and germ-line transmission of a null mutation at the metallothionein I and II loci in the mouse. *Proc. Natl. Acad. Sci USA* **90**, 8088-8092.

Min, K.S. Mukai, S., Ohta, M., Onosaka, S. and Tanaka, K. (1992) Glucocorticoid inhibition of inflammation-induced metallothionein synthesis in mouse liver. *Toxicol. Appl. Pharmacol.* **113**, 293-298.

Morton, K.A., Jones, B.J., Sohn, M., Schaefer, A.E., Phelps, R.C., Datz, F.L. and Lynch, R.E. (1992) Uptake of cadmium is diminished in transfected mouse NIH/3T3 cells enriched for metallothionein. *J. Biol. Chem.* **267**, 2880-2883.

Morton, K.A., Jones, B.J., Sohn, M.H., Datz, F.L. and Lynch, R.E. (1993) Enrichment for metallothionein does not confer resistance to cisplatin in transfected NIH/3T3 cells. *J. Pharmacol. Exp. Ther.* **267**, 697-702.

Mueller, P.R., Salser, S.J. and Wold, B. (1988) Constitutive and metal-inducible protein: DNA interactions at the mouse metallothionein I promoter examined by *in vivo* and *in vitro* footprinting. *Genes Dev.* **2**, 412-427.

Murphy, D., McGown, A.T., Crowther, D., Mander, A. and Fox, B.W. (1991) Metallothionein levels in ovarian tumors before and after chemotherapy. *Br. J. Cancer.* **63**, 711-714.

Naganuma, A., Satoh, M., Kojima, Y. and Imura, N. (1985) Protective effect of metallothionein inducing metals on lethal toxicity of *cis*-diamminedichloroplatinum in mice. *Toxicol. Lett.* **24**, 203-207.

Naganuma, A., Satoh, M. and Imura, N. (1987) Prevention of lethal and renal toxicity of cis-diamminedichloroplatinum (II) by induction of metallothionein synthesis without compromising its antitumour activity in mice. *Cancer Res.* **47**, 983-987.

Nagle, W.W. and Vallee, B.L. (1995) Cell cycle regulation of metallothionein in human colonic cancer cells. *Proc. Natl. Acad. Sci. USA* **92**, 579-583.

Nakajima, K. and Suzuki, K. (1995) Immunochemical detection of metallothionein in brain. *Neurochem. Int.* **27**, 73-87.

Nakajima, K., Adachi, M, Kimura, M., Kobayashi, K. and Suzuki, K. (1989) Immunocytochemical studies of metallothionein I in brain, retina, peripheral nerve and paraneuron. *J. Trace. Elem. Exp. Med.* **2**, 104.

Nakajima, K., Suzuki, K., Otaki, N and Kimura, M. (1991) Detection of metallothionein in brain in Methods in Enzymology 205, (Riordan, J.F. and Vallee, B.L. eds) pp 387-395. Academic Press Inc., San Diego.

Nartey, N.O., Banerjee, D. and Cherian, M.G. (1987a) Immunohistochemical localisation of metallothionein in cell nucleus and cytoplasm of fetal and human liver and kidney and its changes during development. *Pathology* **19**, 233-238.

Nartey, N., Cherian, M.G. and Banerjee, D. (1987b) Immunohistochemical localization of metallothionein in human thyroid tumors. *Am. J. Pathol.* **129**, 177-182.

Naruse, S., Igarashi, S., Furuya, T., Kobayashi, H., Miyatake, T. and Tsuji, S. (1994) Structures of the human and mouse growth inhibitory factor-encoding genes. *Gene* **144**, 283-287.

Nielson, K.B. and Winge, D.R. (1984) Preferential binding of copper to the β domain of metallothionein. *J. Biol. Chem.* **259**, 4941-4946.

Nielson, K.B. and Winge, D.R. (1985) Independence of the domains of metallothionein in metal binding. *J. Biol. Chem.* **260**, 8698-8701.

Nielson, K.B., Atkin, C.L. and Winge, D.R. (1985) Distinct metal-binding configurations in metallothionein. *J. Biol. Chem.* **260**, 5342-5350.

Nishimura, H., Nishimura, N. and Tohyama, C. (1989) Immunohistochemical localization of metallothionein in developing rat tissues. *J. Histochem. Cytochem.* **37**, 715-722.

Nishimura, H, Nishimura, N. and Tohyama, C. (1990) Localization of metallothionein in the genital organs of the male rat. *J. Histochem. Cytochem.* **38**, 927-933.

Nishimura, H., Nishimura, N., Kobayashi, S. and Tohyama, C. (1991) Immunohistochemical localization of metallothionein in the eye of rats. *Histochemistry* **95**, 535-539.

Nishimura N., Nishimura H., Ghaffar A. and Tohyama C. (1992) Localization of metallothionein in the brain of rat and mouse. *J. Histochem. Cytochem.* **40**, 309-315.

Nordberg, G.F., Piscator, M. and Lind, B. (1971) Distribution of cadmium among fractions of mouse liver. *Acta Pharmacol. Toxicol.* **29**, 456-470.

Nordberg, G.F., Goyer, R. and Nordberg, M. (1975) Comparative toxicity of cadmium-metallothionein and cadmium chloride on mouse kidney. *Arch. Pathol.* **99**, 192-197.

Ochi, T. Effects of glutathione depletion and induction of metallothionein on the cytotoxicity of an organic hydroperoxide in cultured mammalian cells. *Toxicology* **50**, 257-268.

Ohtake, H. and Koga, M. (1979) Purification and characterization of zinc-binding protein from the liver of the partial hepatectomized rat. *Biochem. J.* **183**, 683-690.

Oikawa, T., Esaki, N., Tanaka, H. and Soda, K. (1991) Metalloselenonein, the selenium analogue of metallothionein: Synthesis and characterization of its complex with copper ions. *Proc. Natl. Acad. Sci USA* **88**, 3057-3059.

Otvos, J.D. and Armitage, I.M. (1980) Structure of the metal clusters in rabbit liver metallothionein. *Proc. Natl. Acad. Sci. USA* 77, 7094-7098.

Paliwal, V.K. and Ebadi, M. (1989) Biochemical properties of metallothionein isoforms from bovine hippocampus. *Exp. Brain Res.* **75**, 477-482.

Paliwal, V.K., Iversen, P.L. and Ebadi, M. (1990) Regulation of zinc metallothionein II mRNA synthesis in rat brain. *Neurochem. Int.* **17**, 441-447.

Palmiter, R.D. (1987) Molecular biology of metallothionein gene expression in Experientia Supplementum 52, (Kägi, J.H.R. and Kojima, Y. eds) pp 63-80. Birkhauser Verlag, Basel.

Palmiter, R.D. (1994) Regulation of metallothionein genes by heavy metals appears to be mediated by a zinc-sensitive inhibitor that interacts with a constitutively active transcription factor, MTF-1. *Proc. Natl. Acad. Sci USA* **91**, 1219-1223.

Palmiter, R.D. and Findley, S.D. (1996) Cloning and functional characterization of a mammalian zinc transporter that confers resistance to zinc. *EMBO J.* **14**, 639-649.

Palmiter, R.D., Findley, S.D., Whitmore, T.E. and Durnam, D.M. (1992) MT-III, a brain specific member of the metallothionein gene family. *Proc. Natl. Acad. Sci USA* **89**, 6333-6337.

Palmiter, R.D., Sandgren, E.P., Koeller, D.M., Findley, S.D. and Brinster, R.L. (1993) Metallothionein genes and their regulation in transgenic mice in Metallothionein III, Biological roles and medical implications, (Suzuki, K.T., Imura, N. and Kimura, M. eds) pp 399-406. Birkhauser Verlag, Basel.

Panemangalore, M., Banerjee, D., Onosaka, S. and Cherian, M.G. (1983) Changes in the intracellular accumulation and distribution of metallothionein in rat liver and kidney during postnatal development. *Dev. Biol.* **97**, 95-102.

Paynter, J.A., Camakaris, J. and Mercer, J.F.B. (1990) Analysis of hepatic copper, zinc, metallothionein and metallothionein-Ia mRNA in developing sheep. *Eur. J. Biochem.* **190**, 149-155.

Penschow, J.D. and Coghlan, J.P. (1994) Hybridization histochemistry using radiolabeled oligodeoxyribonucleotide probes in Methods in Molecular Biology 33, (Choo, K.H.A. ed) pp 277-292, Humana Press Inc, Totowa, NJ.

Peters, J.M., Duncan, J.R., Wiley, L.M., Rucker, R.B. and Keen, C.L. (1995) Effect of metallothionein antisense oligonucleotide on embryo development. *Reproductive Toxicology* **9**, 123-130.

Peterson, M.G. and Mercer, J.F.B. (1988) Differential expression of four linked sheep metallothionein genes. *Eur. J. Biochem.* **174**, 425-429.

Peterson, M.G., Hannan, F. and Mercer, J.F.B. (1988) The sheep metallothionein gene family. *Eur. J. Biochem.* **174**, 417-424.

Philcox, J.C., Coyle, P., Michalska, A., Choo, K.H.A. and Rofe, A.M. (1995) Endotoxin-induced inflammation does not cause hepatic zinc accumulation in mice lacking metallothionein gene expression. *Biochem. J.* **308**, 543-546.

Pitt, B.R., Brookens, M.A., Steve, A.R., Atlas, A.B., Davies, P., Kuo, S. and Lazo, J.S. (1992) Expression of pulmonary metallothionein genes in late gestational lambs. *Pediatr. Res.* **32**, 424-430.

Ponte, P., Ng, S.Y., Engel, J., Gunning, P. and Kedes, L. (1984) Evolutionary conservation in the untranslated regions of actin mRNAs: DNA sequence of a human β-actin cDNA. *Nucleic Acids Res.* **12**, 1687-1696.

Poutney, D.L., Fundel, S.M., Faller, P., Birchler, N.E., Hunziker, P. and Vašák, M. (1994) Isolation, primary structures and metal binding properties of neuronal growth inhibitory factor (GIF) from bovine and equine brain. *FEBS lett.* **345**, 193-197.

Pulido, P., Kagi, J.H.R. and Vallee, B.L. (1966) Isolation and some properties of human metallothionein. *Biochemistry* **5**, 1768-1777.

Quaife, C.J., Findley, S.D., Erickson, J.C., Froelick, G.J., Kelly, E.J., Zambrowicz, B.P. and Palmiter, R.D. (1994) Induction of a new metallothionein isoform (MT-IV) occurs during differentiation of stratified squamous epithelia. *Biochemistry* **33**, 7250-7259.

Radtke, F., Heuchel, R., Georgiev, O., Hergersberg, M., Gariglio, M., Dembic, Z. and Schaffner, W. (1993) Cloned transcription factor MTF-I activates the mouse metallothionein I promoter. *EMBO J.* **12**, 1355-1362.

Raff, M.C. (1989) Glial cell diversification in the rat optic nerve. *Science* **243**, 1450-1455.

Renan, M.J. and Dowman, P.I. (1989) Increased radioresistance of tumour cells exposed to metallothionein-inducing agents. *Radiat. Res.* **120**, 442-455.

Reynolds, M.L. and Møllgard, K. (1985) The distribution of plasma proteins in the neocortex and early allocortex of the developing sheep brain. *Anat. Embryol.* **171**, 41-60.

Richards, M.P. and Cousins, R.J. (1975) Mammalian zinc homeostasis: requirement for RNA and metallothionein synthesis. *Biochem. Biophys. Res. Commun.* **64**, 1215-1223.

Richards, M.P. and Beattie, J.H. (1993) Characterization of metallothionein isoforms. Comparison of capillary zone electrophoresis with reversed-phase high-performance liquid chromatography. *J. Chromatogr.* **648**, 459-468.

Richards, R.I., Heguy, A. and Karin, M. (1984) Structural and functional analysis of the human metallothionein-IA gene: Differential induction by metal ions and glucocorticoids. *Cell* **37**, 263-272.

Riordan, J.R. and Richards, V. (1980) Human fetal liver contains both zinc- and copper-rich forms of metallothionein. *J. Biol. Chem.* **255**, 5380-5383.

Rising, L., Vitarella, D., Kimelberg, H.K. and Aschner, M. (1995) Metallothionein induction in neonatal rat primary astrocyte cultures protects against methylmercury cytotoxicity. *J. Neurochem.* **65**, 1562-1568.

Robbins, A.H., McRee, D.E., Williamson, M., Collett, S.A., Xuong, N.H., Furey, W.F., Wang, B.C. and Stout, C.D. (1991) Refined crystal structure of Cd, Zn metallothionein at 2.0 Å resolution. *J. Mol. Biol.* **221**, 1269-1293.

Robson, T., Hall, A. and Lohrer, H. (1992) Increased sensitivity of a Chinese hamster ovary cell line to alkylating agents after overexpression of the human metallothionein II-A gene. *Mutat. Res.* **274**, 177-185.

Rocher, C., Faucheu, C., Hervé, F., Bénicourt, C. and Lalanne, J.L. (1991) Cloning of murine SeGpx cDNA and synthesis of mutated GPx proteins in *Escherichia coli*. *Gene* **98**, 193-200.

Rofe, A.M., Philcox, J.C., Haynes, D.R., Whitehouse, M.W. and Coyle, P. (1992) Changes in plasma zinc, copper, iron and hepatic metallothionein in adjuvant-induced arthritis treated with cyclosporin. *Biol. Trace Elem. Res.* **34**, 237-248.

Rushmore, T.H., Morton, M.R. and Pickett, C.B. (1991) The antioxidant responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. *J. Biol. Chem.* **266**, 11632-11639.

Sadhu, C. and Gedamu, L. (1989) Metal-specific posttranscriptional control of human metallothionein genes. *Mol. Cell Biol.* **9**, 5738-5741.

Sadler, J.W. (1990) Langman's medical embryology, 6th edition. Williams and Wilkins, USA.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular cloning, a laboratory manual, 2nd edition. Cold Spring Harbor Laboratory Press, USA.

Sato, M., Sasaki, M. and Hojo, H. (1992) Tissue specific induction of metallothionein synthesis by tumour necrosis factor α. *Res. Commun. Chem. Pathol. Pharmacol.* **75**, 159-172.

Sato, M., Sasaki, M. and Hojo, H. (1995) Antioxidant roles of metallothionein and manganese superoxide dismutase induced by tumour necrosis factor-α and interleukin-6. *Arch. Biochem. Biophys.* **316**, 738-744.

Satoh, M., Naganuma, A. and Imura, N (1988) Metallothionein induction prevents toxic side effects of cisplatin and adriamycin used in combination. *Cancer Chemother. Pharmacol.* **21**, 176-178.

Satoh, M., Kloth, D.M., Kadhim, S.A., Chin, J.L., Naganuma, A., Imura, N. and Cherian, M.G. (1993a) Modulation of both cisplatin nephrotoxicity and drug resistance in murine bladder tumor by controlling metallothionein synthesis. *Cancer Res.* **53**, 1829-1832.

Satoh, M., Kondo, Y., Mita, M., Nakagawa, I., Naganuma, A. and Imura, N. (1993b) Prevention of carcinogenicity of anticancer drugs by metallothionein induction. *Cancer Res.* **53**, 4767-4768.

Schechinger, T., Hartmann, H.J. and Weser, U. (1986) Copper transport from Cu(I)-thionein into apo-caeruloplasmin mediated by activated leucocytes. *Biochem. J.* **240**, 281-283.

Schilder, R.J., Hall, L., Monks, A., Handel, L.M., Fornace, A.J., Ozols, R.F., Fojo, A.T. and Hamilton, T.C. (1990) Metallothionein gene expression and resistance to cisplatin in human ovarian cancer. *Int. J. Cancer* **45**, 416-422.

Schmid, K.W., Greeff, M., Hittmair, A., Tötsch, M., Öfner, D., Dockhorn-Dworniczak, B., Böcker, W. and Jasani, B. (1994) Metallothionein expression in normal, hyperplastic and neoplastic thyroid follicular and parafollicular C cells using monoclonal antimetallothionein antibody E9. *Endocr. Pathol.* **5**, 114-122.

Schmidt, C.J. and Hamer, D.H. (1986) Cell specificity and an effect of ras on human metallothionein gene expression. *Proc. Natl. Acad. Sci. USA* **83**, 3346-3350.

Schmidt, C.J., Jubier, M.F. and Hamer, D.H. (1985) Structure and expression of two human metallothionein-I isoform genes and a related pseudogene. *J. Biol. Chem.* **260**, 7731-7737.

Schroeder, J.J. and Cousins, R.J. (1990) Interleukin-6 regulates metallothionein gene expression and zinc metabolism in hepatocyte monolayer cultures. *Proc. Natl. Acad. Sci. USA* **87**, 3137-3141.

Seagrave, J., Hildebrande, C.E. and Enger, M.D. (1983) Effects of cadmium and glutathione metabolism in cadmium sensitive and cadmium resistant Chinese hamster cell lines. *Toxicology* **29**, 101-107.

Sequin, C. and Prevost, J. (1988) Detection of a nuclear protein that interacts with a metal regulatory element of the mouse metallothionein I gene. *Nucl. Acids Res.* **16**, 10547-10560.

Searle, P.F., Davison, B.L., Stuart, G.W., Wilkie, T.M., Norstedt, G. and Palmiter, R.D. (1984) Regulation, linkage, and sequence of mouse metallothionein I and II genes. *Mol. Cell Biol.* **4**, 1221-1230.

Sewell, A.K., Jensen, L.T., Erickson, J.C., Palmiter, R.D. and Winge, D.R. (1995) Bioactivity of metallothionein-3 correlates with its novel β domain sequence rather than metal binding properties. *Biochemistry* **34**, 4740-4747.

Shaikh, Z.A. and Lucas, O.J. (1970) Induction of cadmium binding protein. *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **29**, 298.

Shaik, Z.A. and Nolan, C.V. (1987) Comparison of cadmium-saturation assay and radioimmunoassay for the determination of metallothionein concentration in tissues in Experentia Supplementum 52, (Kägi, J.H.R. and Kojima, Y. eds) pp 343-349. Birkhauser Verlag, Basel.

Shellard, S.A., Fichtinger-Schepman, M.J., Lazo, J.S. and Hill, B.T. (1993) Evidence of differential cisplatin-DNA adduct formation, removal and tolerance of DNA damage in three human lung carcinoma cell lines. *Anti-Cancer Drugs* **4**, 491-500.

Shen, Q., Chu, F.F. and Newburg, P.E. (1993) Sequences in the 3'-untranslated region of the human cellular glutatione peroxidase gene are necessary and sufficient for selenocysteine incorporation at the UGA codon. *J. Biol. Chem.* **268**, 11463-11469.

Sobocinski, P.Z., Canterbury, W.J., Knutsen, G.L. and Hauer, E.C. (1981) Effect of adrenalectomy on cadmium- and turpentine-induced hepatic synthesis of metallothionein and alpha 2-macrofetoprotein in rat. *Inflammation*, **5**, 153-164.

Stadtman, T.C. (1996) Selenocysteine. *Annu. Rev. Biochem.* **65**, 83-100.

Stennard, F.A., Holloway, A.F., Hamilton, J. and West, A.K. (1994) Characterisation of six additional human metallothionein genes. *Biochim. Biophys. Acta* **1218**, 357-365.

Stennard, F.A., Stewart, T.C. and West, A.K. (1995) Effect of prior, low-level cadmium exposure *in vivo* on metallothionein expression in cultured lymphocytes. *J. Appl. Toxicol.* **15**, 63-67.

Stuart, G.W., Searle, P.F., Chien, H.Y., Brinster, R.L. and Palmiter, R.D. (1984) A 12-base-pair DNA motif that is repeated several times in metallothionein gene promoters confers metal regulation to a heterologous gene. *Proc. Natl. Acad. Sci. USA* **81**, 7313-7322.

Stuart, G.W., Searle, P.F. and Palmiter, R.D. (1985) Identification of multiple metal regulatory elements in mouse metallothionein-I promoter by assaying synthetic sequences. *Nature* **317**, 828-831.

Suzuki, K, Nakajima, K., Kawaharada, U., Hara, F., Uehara, K., Otaki, N., Kimura, M. and Tamura, Y. (1992a) Localization of metallothionein in the brain of *Macaca fascicularis*. *Acta Histochem. Cytochem.* **25**, 609-616.

Suzuki, K., Nakajima, K., Kawaharada, U., Uehara, K., Hara, F., Otaki, N., Kimura, M. and Tamura, Y. (1992b) Metallothionein in the human brain. *Acta Histochem. Cytochem.* **25**, 617-622.

Suzuki K., Nakajima K., Otaki N. and Kimura M. (1994) Metallothionein in developing human brain. *Biol. Signals* **3**, 188-192.

Suzuki, T., Umeyama, T., Ohma, C., Yamanaka, H., Suzuki, K., Nakajima, K. and Kimura, M. (1991) Immunohistochemical study of metallothionein in normal and benign prostatic hyperplasia of human prostate. *The Prostate* **19**, 35-42.

Tamai, K.T., Gralla, E., Ellerby, L., Valentine, J.S. and Thiele, D.J. (1993) Yeast and mammalian metallothioneins functionally substitute for yeast copper-zinc superoxide dismutase. *Proc. Natl. Acad. Sci. USA* **90**, 8088-8092.

Templeton, D.M., Banerjee, D. and Cherian, M.G. (1985) Metallothionein synthesis and localisation in relation to metal storage in rat liver during gestation. *Can. J. Biochem. Cell Biol.* **63**, 6-22.

Thiele, D.J. (1992) Metal-regulated transcription in eukaryotes. *Nucleic Acids Res.* **20**, 1183-1191.

Thomas, J.P., Bachowski, G.J. and Girotti, A.W. (1986) Inhibition of cell membrane lipid peroxidation by cadmium- and zinc-metallothioneins. *Biochim. Biophys. Acta* **884**, 448-461.

Thornalley, P.J. and Vašák, M. (1985) Possible role for metallothionein in protection against radiation-induced oxidative stress. Kinetics and mechanism of its reaction with superoxide and hydroxyl radicals. *Biochim. Biophys. Acta* **827**, 36-44.

Tohyama, C., Suzuki, J.S., Hemelraad, J., Nishimura, N. and Nishimura, H. (1993) Induction of metallothionein and its localization in the nucleus of rat hepatocytes after partial hepatectomy. *Hepatology* **18**, 1193-1201.

Tsuji, S., Kobayashi, H., Uchida, Y., Ihara, Y. and Miyatake, T. (1992) Molecular cloning of human growth inhibitory factor cDNA and its down-regulation in Alzheimer's disease. *EMBO J.* **11**, 4843-4850.

Tsujikawa, K., Imai, T., Kakutani, M., Kayamori, Y., Mimura, T., Otaki, N., Kimura, M., Fukuyama, R. and Shimizu, N. (1991) Localization of metallothionein in nuclei of growing primary cultured adult rat hepatocytes. *FEBS* **283**, 239-242.

Tu, G.F., Cole, T., Duan, W. and Schreiber, G. (1989) The nucleotide sequence of transthyretin cDNA from a sheep choroid plexus cDNA library. *Nucl. Acids. Res.* **17**, 6384.

Uchida, Y. and Ihara, Y. (1995) The N-terminal portion of growth inhibitory factor is sufficient for biological activity. *J. Biol. Chem.* **270**, 3365-3369.

Uchida, Y., Takio, K., Tatani, K., Ihara, Y. and Tomonaga, M. (1991) The growth inhibitory factor that is deficient in Alzheimer's disease brain is a 68 amino acid metallothionein-like protein. *Neuron* **7**, 337-347.

Udom, A.O. and Brady, F.O. (1980) Reactivation *in vitro* of zinc-requiring apo-enzymes by rat liver zinc-thionein. *Biochem.J.* **187**, 329-335.

Umeyama, T., Saruki, K. Imai, K. Yamanaka, H., Suzuki, K. Ikei, N., Kodaira, T., Nakajima, K., Saitoh, H. and Kimura, M (1987) Immunohistochemical demonstration of metallothionein in the rat prostate. *Prostate* **10**, 257-264.

Vallee, B.L. (1979) Metallothionein: historical review and perspectives in Metallothionein (Kägi, J.H.R and Nordberg, G.F. eds) pp19-40.Birkhauser Verlag, Basel.

Varshney, U., Jahroudi, N., Foster, R. and Gedamu, L. (1986) Structure, organization and regulation of human metallothionein I_F gene: Differential and cell-type-specific expression in response to heavy metals and glucocorticoids. *Mol. Cell. Biol.* **6**, 26-37.

Vašák, M., Galdes, A., Hill, H.A.O., Kägi, J.H.R., Bremner, I. and Young, B.W. (1980) Investigation of the structure of metallothioneins by proton nuclear magnetic resonance spectroscopy. *Biochemistry* **19**, 416-425.

Vašák, M., Kägi, J.H.R. and Hill, H.A.O. (1981) Zinc(II), cadmium(II) and mercury(II) thiolate transitions in metallothionein. *Biochemistry* **20**, 2852-2856.

Vasconcelos, M.H., Tam, S.C., Beattie, J.H. and Hesketh, J.E. (1996) Evidence for differences in the post-transcriptional regulation of rat metallothionein isoforms. *Biochem. J.* **315**, 665-671.

Vibulsreth, S., Hefti, F., Ginsberg, M.D., Dietrich, W.D. and Busto, R. (1987) Astrocytes protect cultured neurons from degeneration induced by anoxia. *Brain Res.* **442**, 303-311.

Ward, J.F., Blakely, W.F. and Joner, E.I. (1985) Mammalian cells are not killed by DNA single-strand breaks caused by hydroxyl radicals from hydrogen peroxide. *Radiat. Res.* **103**, 383-392.

Webb, M. (1987) Toxicological significance of metallothionein in Experentia Supplementum 52, (Kägi, J.H.R. and Kojima, Y. eds) pp 109-136. Birkhauser Verlag, Basel.

West, A.K., Stallings, R., Hildebrand, C.E., Chiu, R., Karin, M. and Richards, R.I. (1990) Human metallothionein genes: Structure of the functional locus at 16q13. *Genomics* **8**, 513-518.

Winge, D.R. and Garvey, J.S. (1983) Antigenicity of metallothionein. *Proc. Natl. Acad. Sci. USA* **80**, 2472-2476.

Winge, D.R. and Miklossy, K.A. (1982) Domain nature of metallothionein. *J. Biol. Chem.* **257**, 3471-3476.

Wlostowski T. (1993) Involvement of metallothionein and copper in cell proliferation. *BioMetals* **6**, 71-76.

Wood, D.P., Klein, E., Fair, W.R. and Chaganti, R.S.K. (1993) Metallothionein gene expression in bladder cancer exposed to cisplatin. *Mod. Pathol.* **6**, 33-35.

Yagle, M.K. and Palmiter, R.D. (1985) Coordinate regulation of mouse metallothionein I and II genes by heavy metals and glucocorticoids. *Mol. Cell. Biol.* **5**, 291-294.

Yang, Y.Y., Woo, E.S., Reese, C.E., Bahnson. R.R., Saijo, N. and Lazo, J.S. (1994) Human metallothionein isoform gene expression in cisplatin-sensitive and resistant cells. *Mol. Pharmacol.* **45**, 453-560.

Young, J.K. Garvey, J.S. and Huang, P.C. (1991) Glial immunoreactivity for metallothionein in the rat brain. *Glia* **4**, 602-610.

Young, J.K. (1994) Glial metallothionein. *Biol. Signals* **3**, 169-175.

Yuguchi, T., Kohmura, E., Yamada, K, Sakaki, T., Yamashita, T., Otsuki, H., Kataoka, K., Tsuji, S. and Hayakawa, T. (1995a) Expression of growth inhibitory factor mRNA following cortical injury in rat. *J. Neurotrauma* **12**, 299-306.

Yuguchi, T., Kohmura, E., Yamada, K, Sakaki, T., Yamashita, T., Otsuki, H., Wanaka, A., Tohyama, M., Tsuji, S. and Hayakawa, T. (1995b) Changes in growth inhibitory factor mRNA expression compared with those of c-jun mRNA expression following facial nerve transection. *Mol. Brain Res.* **28**, 181-185.

Zalups, R.K., Fraser, J. and Koropatnik, J. (1995) Enhanced transcription of metallothionein genes in rat kidney: effect of uninephrectomy and compensatory renal growth. *Am. J. Physiol.* **268**, F643-F650.

Zheng, H., Berman, N.E. and Klaassen, C.D. (1995) Chemical modulation of metallothionein I and III mRNA in mouse brain. *Neurochem. Int.* **27**, 43-58.

ADDENDUM

Page vi, line 2	Replace "Chatacterisation" with "Characterisation"
Page x, line 4	Replace "MT-II and -II null mice" with "MT-I and -II null mice"
Page 2, line 19	Replace "subtisilin" with "subtilisin"
Page 3, line 4	Replace "were" with "where"
Page 3, line 5	To the phrase "...MT-III and MT-IV were discovered only recently" Add ",although MT-IV expression has not been confirmed at the protein level."
Page 11, line 3	Replace "Bremner *et al.*, 1984" with "Mehra and Bremner, 1984"
Page 13, line 13	Replace "MT-I/I$^{I-/-}$ "with "MT-I/II$^{-/-}$"
Page 15, line 26	Replace "illicit" with "elicit"
Page 15, line 28	Replace "..related to the role of these proteins..." with "...related to the role of MT proteins..."
Page 17, line 20	Replace "...Elisa assay, and a silver saturation method." with "...Elisa assay, a silver saturation method and a Cadmium haem assay"
Page 20, line 8	Replace "where" with "were"
Page 44, line 3	To the phrase "Embryonic mouse fibroblasts were prepared..." add ", from MT-I/II$^{-/-}$ transgenic mouse (Michalska and Choo, 1993)..."
Page 46, line 6	Replace "167 ml" with "167 µl"

Page 53

Add " *2.10.5 Collection of tissues*

Fetal sheep tissues were collected by Dr K. Dziegielewska from ewes of known gestation according to NH&MRC guidelines. Details of the procedure are outlined in Reynolds and Mollgard, 1985. Immediately after dissection tissues were either frozen in liquid nitrogen and stored at -80°C for RNA isolation, or fixed in Bouin's or paraformaldehyde fixative.

Page 55, line 27

Replace "(E120-E133)" with "(E120-E133, where E denotes the gestational age in days)"

Figure 3.10, line 9

Replace "...(eg arrow in C) but there is no cross reactivity with FITC (eg arrow in D)." with "...(eg arrow in D) but there is no cross reactivity with FITC (eg arrow in C)."

Page 82, line 31

Remove "the probe was specific for the MT-III isoform"

Page 88, line 10

Replace "expression is restricted to the brain" with "expression is probably restricted to the brain"

Page 101, line 3

To the phrase "MT1L mRNA was detected in 2 of the 3 human thyroid samples examined (Figure 5.13)." Add "..., which is more evident on the original autoradiograph."

Add References

Holloway, A.F. (1992) Human metallothionein genes, *MT1J* and *MT1L*. *B.Sc. Honours Thesis.*

Stennard, F.A. (1995) Human metallothionein genes. *Ph.D Thesis.*

Reynolds, M.L. and Mollgard, K. (1985) The distribution of plasma proteins in the neocortex and early allocortex of the developing sheep brain. *Anat. Embryol.* **171**, 41-60.