1990

Expression Of Growth Factor Inducible Genes

Paul Waterhouse

I found that spp, calcyclin, MEP, MRP and transin exhibit unique spatial and temporal patterns of expression in the female reproductive tissues. Calcyclin mRNA increased 5-10 fold over the level seen in virgin uterus, peaking on day 10. spp mRNA was undetectable in virgin uterus and increased to a maximal level, $2\frac{1}{2}$ times higher than that of calcyclin, on day 11. *In situ* hybridization revealed that spp was expressed in the decidua capsularis as well as in granulated metrial gland cells. Calcyclin expression in adjacent sections was a 'diffuse band' with the highest intensity in the decidua capsularis and decidua lateralis.

Studies with artificial deciduomas showed that the presence of an embryo was not necessary, and that the hormonal environment in a pregnant animal was sufficient for expression of all these genes in the maternal tissues.

EXPRESSION OF GROWTH FACTOR INDUCIBLE GENES

by

Paul Waterhouse

Department of Biochemistry

Submitted in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

Faculty of Graduate Studies

The University of Western Ontario

London, Ontario

November, 1989

ISBN 0-315-55280-8

ABSTRACT

cDNA libraries were constructed using the Okayama/Berg cloning strategy, and full length cDNA clones for the growth factor inducible genes TIMP, MRP, MEP and calcyclin were isolated, and used to study the temporal and spatial patterns of expression of these genes. The TIMP and MRP cDNAs were used to express the murine proteins in *E. coli*. The native proteins were found to be expressed only at low levels, although fusion proteins with MRP and TIMP sequences at the carboxyl terminus of ß-galactosidase were expressed at high levels in *E. coli*. The purified fusion proteins were used to raise MRP and TIMP specific antisera.

To determine the effect of an upstream overlapping ORF, TIMP transcripts either containing or lacking the AUG of the 5' ORF were generated by SP6 polymerase, and translated in reticulocyte lysates. The *in vitro* translated TIMP was immunoprecipitable with TIMP antiserum. TIMP was translated up to 10-fold more efficiently from the transcript lacking the upstream AUG.

RNA was isolated from the reproductive tissues dissected from female mice on each day of pregnancy and analysed on Northern and slot blots. TIMP mRNA levels were highest on day 7, and then decreased with a specific time course in each of placenta, decidua and uterus during pregnancy. A burst of TIMP expression was observed in the amnion on days 10 and 18. The TIMP mRNA levels in ovary were high in virgin mice, and low throughout gestation until day 18 when they increased to a level 25-fold higher than seen in any other tissue. This burst of TIMP expression coincided with an induction of transin-2 in the ovary.

I found that spp, calcyclin, MEP, MRP and transin exhibit unique spatial and temporal patterns of expression in the female reproductive tissues. Calcyclin mRNA increased 5-10 fold over the level seen in virgin uterus, peaking on day 10. spp mRNA was undetectable in virgin uterus and increased to a maximal level, $2\frac{1}{2}$ times higher than that of calcyclin, on day 11. *In situ* hybridization revealed that spp was expressed in the decidua capsularis as well as in granulated metrial gland cells. Calcyclin expression in adjacent sections was a 'diffuse band' with the highest intensity in the decidua capsularis and decidua lateralis.

Studies with artificial deciduomas showed that the presence of an embryo was not necessary, and that the hormonal environment in a pregnant animal was sufficient for expression of all these genes in the maternal tissues.

ACKNOWLEDGEMENTS

I thank all the members of the D.T.D. lab for creating a working environment and sharing their knowledge of molecular biology; especially Rama Khokha for exciting if not always fruitful collaborations, and encouragement with my endeavours. I am grateful to D.T.D. for his constant encouragement to do the best work possible, and to keep at it when when prospects weren't so bright. D.T.D. was a constant source of both anguish and amusement, even though I don't understand his sense of humour. Marilyn McLeod gave emergency assistance at the last moment with the *in situ* hybridizations, and Beth Orphan was generous with photographic and typing assistance when her workload would allow it.

I also wish to thank the members of P.K. Lala's lab for friendly advice and help with the mice. In particular Ranjit S. Parhar skillfully dissected from mice most of the tissues used in chapter 5, and Jeff Lysiak was a knowledgeable instructor of mouse surgery and cryosectioning.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

Tables

NOMENCLATURE

A	adenine
aa	amino acid
ACE	10 mM $NaCH_3COO^-$ pH 5.1, 50 mM NaCl, 3 mM EDTA
BNL	murine embryonic liver cell line
bp	basepair(s)
bFGF	basic fibroblast gowth factor
BSA	bovine serum albumin
C	cytosine
CCD	cell cycle dependent
cAMP	cyclic adenosine 5' monophosphate
cDNA	complementary deoxyribonucleic acid
CIP	calf intestinal phosphatase
CNS	central nervous system
cpm	counts per minute
d	day
DEP	diethylpyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside 5'-triphosphate
DTT	dithiothreitol
e1	elastase-1
E. coli	*Escherichia coli*
ECM	extracellular matrix
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal grov[redacted]factor
FGF	fibroblast growth factor
g	gravity
G	guanine
GMG cells	granulated metrial gland cells
GTP	guanosine 5'-triphosphate
h	hour
H3	histone 3
H4	histone 4
Hox	homeobox
IGF-1	insulin-like growth factor 1
IPTG	isopropyl ß-D-thio-galactopyranoside
kbp	kilobase pair(s)
kDa	kilodalton(s)
LB	Luria-Bertani broth
LTR	long terminal repeat
MEM	minimal Eagle's medium
MEP	major excreted protein
MMP	matrix metalloproteinase
MMTV	mouse mammary tumor virus
M_r	relative molecular mass
mRNA	messenger ribonucleic acid
MRP	mitogen regulated protein
NDV	Newcastle disease virus
nt	nucleotide
O/N	overnight
ORF	open reading frame
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor

PEPCK	phosphoenol pyruvate carboxy kinase
PFP	purified fusion protein
PKC	protein kinase C
PP	post partum
RGD	arginine-glycine-aspartic acid
rt	room temperature
SDS	sodium dodecyl sulfate
spp	secreted phosphoprotein
T	thymine
TAT	tyrosine aminotransferase
TBS	tris-buffered saline
TCP	total cell protein
TE	10 mM Tris·HCl pH 8.0, 1 mM EDTA buffer
TGF-ß	transforming growth factor-ß
TIMP	tissue inhibitor of metalloproteinases
TPA	12-O-tetradecanoyl phorbol-13-acetate
Tris	Tris(hydroxymethyl) aminomethane
tRNA	transfer ribonucleic acid
U	unit(s)
YIGSR	tyrosine-isoleucine-glycine-serine-arginine

CHAPTER 1 - INTRODUCTION

1.1.1 Cell Cycle Dependent Gene Expression

In the classical mammalian cell cycle, synthesis of DNA (S) and mitosis (M) are two discrete events separated by two gaps, G1 and G2 (Howard and Pelc, 1953). Quiescent cells are in a resting state referred to as G0, and can be stimulated to enter G1 of the cell cycle by the addition of serum growth factors. Stages S, G2 and M are of a determinate length, and the variability of the length of G1 is responsible for different cell cycle times of daughter cells (Pardee *et al.*, 1978). Brooks *et al.* (1980) described a probabilistic model where a cell's progression through the cell cycle is controlled by random events A and L that mark the beginning of 2 processes. The A state is similiar to G1, whereas the random event at the beginning of the L state of the Brooks model may take place at any time in the ceil cycle and is linked to the centriole cycle.

Experimental study of the mammalian cell cycle involves the stimulation of quiescent tissue culture cells with growth factors. These cells pass several control points and reach S phase about 12 hours (h) later. The first control point is termed competence, which is achieved by the addition of platlet derived growth factor (PDGF) or fibroblast growth factor (FGF) (Pledger *et al.*, 1977). Competent cells no longer require PDGF or FGF and 'progress' to the control point 'V' in ~ 6 h, in the presence of epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1) (Leof *et al.*, 1981). Progression to the next control point, termed the restriction point (R) requires efficient protein synthesis (Rossow *et al.*, 1979) and the continued presence of IGF-1 (Leof *et al.*, 1981), but does not require new transcription (Campisi and Pardee, 1984). Cells initiate DNA synthesis

about 2 h later, when and if they pass the last control point 'W' at the G1-S boundary (Das, 1981). For cycling cells the state of competence is achieved in the previous cell cycle (Scher *et al.*, 1979), and the only growth factor required to progress from G1 to S is IGF-1 (Campisi and Pardee, 1984).

In order to understand the cell cycle, it is important to define the role of cell cycle dependent (CCD) genes, and how their expression is regulated, because a subset of these genes will play a role in cell cycle regulation and also play a role in cellular transformation. One method that has been used sucessfully to identify CCD genes is differential screening of a cDNA library with probes made from mRNA isolated from cells in different growth states (reviewed in Denhardt *et al.*, 1986). Accordingly, Ferrari and Baserga (1987) detected 0.3-1.0% of the genes as being growth factor inducible. Linzer and Nathans (1983) have suggested that the number of cell cycle regulated genes is 50-100. In a more recent report, 82 independent growth factor inducible sequences were isolated by differential screening of a cDNA library (Almendral *et al.*, 1988).

The specific expression of histone genes at S phase is a good example of the levels at which cell cycle dependent genes can be regulated. A five-fold increase in the rate of histone H3 transcription leads to a 50-fold increase in the cytoplasmic level of H3 mRNA (DeLisle *et al.*, 1983), partly resulting from increased stability of histone mRNAs in S phase (Heintz et al., 1983). A stem loop structure at the 3' end of H3 mRNA was required to couple stability of the mRNA with DNA replication (Levine *et al.*, 1987). This structure appeared to be the target of a nuclease since shorter histone mRNAs, lacking 5 or 12 nucleotides from the 3' end, were observed when

the transcripts were being rapidly degraded after DNA replication had stopped (Ross *et al.*, 1986).

Fusion of ß-globin coding sequences downstream of the human H3 promoter and leader sequence conferred the histone-like properties of hydroxyurea sensitivity and cycloheximide superinduction onto the ß-globin transcripts (Morris *et al.*, 1986). Analysis of deletion mutants revealed several features of the human histone H4 promoter region, such as the specific binding of a protein H4TF-1 to the region -79 to -102, and several GC-rich repeat sequences between -60 and -105 that play a role in transcriptional efficiency (Daily *et al.*, 1986). The histone-specific hexamer GACTTC was shown to be necessary for maximal H4 transcription, and may be involved in the coordinate cell cycle dependent expression of the histone genes (Sive *et al.*, 1986).

1.1.2 Regulation of Transcription

Serum inducible genes such as c-*fos* and c-*jun* are activated early and independently of new protein synthesis, while the transcription of more slowly activated genes requires protein synthesis as evidenced by their inhibition in the presence of cycloheximide (Lau and Nathans, 1987). Some of the early genes encode transcription factors. The protein product Jun is part of the AP-1 transcription complex, and is responsible for the sequence specific interaction with DNA (Chiu *et al.*, 1988), which is potentiated by Fos via a 'leucine zipper' motif (Landschultz *et al.*, 1988; Kouzarides *et al.*, 1988). Synthetic peptides of the Fos and Jun leucine zipper regions form heterodimers with a 1000-fold preference over homodimers, demonstrating the specificity of the interaction (O'Shea *et al.*, 1989).

Induction of the later genes by cAMP or TPA (via protein kinase C (PKC)) mediated pathways involves response elements in the gene that bind different transcription factors (reviewed in Karin, 1989). The AP-1 recognition site (among others) directs induction by TPA and not cAMP, while the AP-2 site mediates induction by both agents (Imagawa *et al.*, 1987). The interactions can be more complex, e.g. the proenkephalin promoter contains binding sites for AP-1, AP-2, AP-4 and ENKTF-1. Interestingly, this promoter is not induced by TPA alone and requires a phospho-diesterase inhibitor in combination (Comb *et al.*, 1988).

Transcription factors Oct-1 and Oct-2 bind to the octamer sequence element ATGCAAAT found in a wide variety of promoters and enhancers (reviewed in Schaffner, 1989). Specificity is conferred by tissue specific factors which bind adjacent sequence motifs. A TATA box in conjunction with the histone H2B octamer binds the lymphocyte specific Oct-2, but is inactive in non-lymphoid cells (Müller *et al.*, 1988). The CCAAT and GC boxes are found adjacent to the octamer in housekeeping genes. The region of homology between Oct-1 and Oct-2 contains a homeobox (Scheidereit *et al.*, 1988), which is important in regulating development.

1.1.3 Oncogenes

Stimuli which determine the growth, quiescence or differentiation state of a cell arise from a combination of hormonal elements such as growth factors and structural elements including the extracellular matrix (ECM) and neighbouring cell surfaces. The signals for many of these events are the proteins encoded by proto-oncogenes, which are the cellular homologues of the viral genes responsible for the neoplastic properties of RNA tumour virus transformed cells (Bishop, 1981). Proto-oncogenes can become

activated into oncogenes through mutations which effect either their level of expression (e.g. *myc, fos, int*) or structural changes in the encoded proteins (*ras, src,* p53; Balmain, 1985). A co-operative effect of oncogenes was demonstrated by the ability of cloned *ras* to transform only cells previously immortalized by over-expression of *myc* (Land *et al.*, 1983; Newbold and Overell, 1983). Upregulation of ras^H with a mutation at codon 12 is sufficient to transform primary rodent cells, although the fully transformed cells also had a variety of chromosomal abberations (Spandidos and Wilke, 1984).

Expression of *myc* or *ras* in various tissues in transgenic mice (reviewed in Groner *et al.*, 1987) revealed a tissue and developmental specificity for transformation by each of these genes. The elastase-1 (e1) tissue specific promoter was used to demonstrate that the pancreatic acinar cells were efficiently transformed by *ras* and not by *myc* (Quaife *et al.*, 1987). However, *myc* was more effective than *ras* at producing tumours in the mammary gland when expressed under control of the whey acidic protein (Wap) promoter (Andres *et al.*, 1987) which is known to be activated at a late stage in pregnancy. There was a higher incidence of mammary tumours (50% of mice within 6 months) when *ras* was under control of the mouse mammary tumour virus long terminal repeat (MMTV LTR) which is expressed in mammary epithelium at earlier stages (Sinn *et al.*, 1987). The tumour potential of *myc* in mammary tissue was not dependent on the state of differentiation. Tissues from an F1 cross of MMTV LTR-*myc* and MMTV LTR-*ras* transgenic mice in which *ras* was tumorigenic displayed accelerated tumor formation when both *ras* as well as *myc* were overexpressed (Sinn *et*

al., 1987).

The normal p21 gene product of *ras* has GTPase activity that is impaired by a mutation at codon 12 (McGrath *et al.*, 1984). Loss of the GTPase activity results in an inability by p21 to activate yeast adenylate cyclase (Broek *et al.*, 1985).

The c-*myc* gene product is a nuclear protein that binds strongly to DNA (Watt *et al.*, 1985; Rapp *et al.*, 1986) and is involved in DNA synthesis (Studzinski *et al.*, 1986). *Myc* expression is very complex and regulated at several levels. In this context, there are three promoters, along with two additional upstream open reading frames (ORF) in the additional 5' sequence from P0 and P1 (Bentley and Groudine, 1986). There are also three noncontiguous regions of the *myc* gene which show high levels of transcription in the antisense direction (Nepveu and Marcu, 1986), a negative element present in the promoter region (Remmers *et al.*, 1985) and a positive modulator in exon 1 (Yang *et al.*, 1986). The 3' region of *myc* mRNA contains two AU-rich sequences which serve as sites for endonucleolytic degradation after the poly A tail is removed (Brewer and Ross, 1988), and further, the protein contains PEST regions (Rogers *et al.*, 1986) which are associated with unstable proteins.

In MMTV induced tumors the provirus integrates within 30 kb of and activates expression from *int*-1 (Nusse *et al.*, 1984) or *int*-2 (Peters *et al.*, 1983). Expression of *int* mRNA increases from undetectable levels to 1-10 copies per tumor cell (Fung *et al.*, 1985). Normal expression of *int*-1 is restricted to the neural tube of day (d) 8-13 embryos, and to the testis of post-puberal mice (Schackleford and Varmus, 1987). *Int*-2 is expressed

up to d 7.5 of embryonic development (Jakobovits *et al.*, 1986). Regions of *int*-1 and *int*-2 share about 50% similarity at the amino acid level, and *int*-2 is also similar to basic FGF (Dickson and Peters, 1987).

Other transforming members of the FGF family include *hst*/KS3 (Delli-Bovi *et al.*, 1987) and FGF5 (Zhan *et al.*, 1988). These two proteins, along with *int*-2 protein, contain hydrophobic leader sequences which facilitate their secretion and provide an autocrine feedback loop. Over expression of these FGF-like proteins with leader sequences correlates positively with tumour growth potential. Interestingly, three additional forms of basic FGF with N-terminal extensions of 4.7, 5.3 and 6.4 kiloDaltons (kDa) are synthesized by a human hepatoma cell line. These FGFs, which also retain the FGF activity, result from initiation of translation at CUG codons 5' to the AUG start site (Florkeiwicz and Sommer, 1989). Some other oncogenes and growth factor related products are *v-sis* and PDGF (Doolittle *et al.*, 1983); *v-erb*-B and the EGF receptor (Downward *et al.*, 1983); *v-fms* and their CSF-1 receptor (Scherr *et al.*, 1985); *v-ros* has homology to the insulin receptor (Ebina *et al.*, 1985) and *c-erb*-A codes for the glucocorticoid receptor (Sap *et al.*, 1986).

1.1.4 Tissue-Specific Gene Expression

Some examples of promoters that direct tissue specific expression of cloned genes are given above. The co-ordinate regulation of a set of tissue-specific structural genes is more efficient than independent regulation of each gene. A deletion on mouse chromosome 7 resulted in low levels of expression of a subset of liver-specific genes including glucose-6-phosphatase, tyrosine aminotransferase (TAT) and phosphoenol pyruvate

carboxykinase (PEPCK). The deletion does not affect the structure of these genes, since they are located on other chromosomes (Cori *et al.*, 1983; Peterson *et al.*, 1985; Loose *et al.*, 1986). The PEPCK mRNA level was less than 10% of normal while the transcription was only reduced to 25-50% of the normal level, suggesting that post transcriptional mechanisms were also affected by the chromosome 7 deletion (Loose *et al.*, 1986). A developmental aspect of this liver-specific regulation is that TAT was not inducible by glucocorticoids in foetal liver, but became inducible within 3 h after birth (Perry *et al.*, 1983).

Albumin and α-fetoprotein are related proteins that have different developmental patterns of regulation. Both genes are expressed in the foetal liver, yolk sac and gut. The level of α-fetoprotein drops drastically after birth while albumin expression continues unchanged. The post-partum decline of α-fetoprotein (and another gene, H19) expression is conferred by *raf* (Pachnis *et al.*, 1984). The unlinked allele *Rif* controls the induction of α-fetoprotein during liver regeneration (Balayew and Tilghman, 1982). An exogenous α-fetoprotein gene with 7 kb of 5' sequence was expressed correctly in transgenic mice, demonstrating that the sequences required for regulation by *Rif* and *raf* were present in this region (Krumlauf *et al.*, 1985).

Homeotic genes, first identified in Drosophila, have been extensively studied and provide an excellent example of tissue and development specific regulation. The 'homeobox' is a 180-base pair (bp) segment of DNA coding for a helix-turn-helix motif in the protein which by itself has DNA binding ability (Pabo and Sauer, 1984). Enhanced binding and specificity is likely conferred by protein-protein interactions. The *Drosophila* homeotic

sequence has been used to isolate other homeobox genes in *Drosophila*, mouse (McGinnis *et al.*, 1984a; 1984b) and human (Levine *et al.*, 1984).

Direct evidence that homeobox genes encode specific transcription factors has come from sequence analysis of cloned transcription factors. Cloning of the human lymphocyte Ig specific transcription factor Oct-2 revealed a helix-turn-helix motif with 33% similiarity to the concensus homeodomain (Ko *et al.*, 1988). Purified Oct-2 was found to bind the Ig specific octamer sequence and to the *Drosophila* '*engrailed*' consensus sequence (Scheidereit *et al.*, 1988). A homeodomain was also identified in the 33 kDa transcription factor Pit-1, which is responsible for the cell type expression of the recently diverged growth hormone and prolactin genes (Ingraham *et al.*, 1988).

In *Drosophila*, homeobox genes determine the number and polarity of body segments (see Gehring and Hiromi, 1986). The murine homeobox genes isolated so far belong to three classes that are designated by a number and the prefix '*Hox*' for the *Antp*-like, '*En*' for *engrailed*-like and '*Cdx*' for the *caudal*-like class of *Drosophila* genes. The spatial and temporal pattern of murine homeobox expression is consistent with their playing a role in the determination of cell fate. Most Hox- and En-genes are expressed primarily in the nervous system as well as in other tissues of mesodermal and ectodermal origin (reviewed in Holland and Hogan, 1988a). *Cdx*-1 is expressed exclusively in the intestinal epithelium (derived from endoderm) beginning at d 14 of gestation and continuing into adulthood (Duprey *et al.*, 1988). The expression of *Hox*-1.5 is clearly higher in the posterior than in the anterior presomitic mesoderm (Gaunt, 1987), and *Hox*-

1.5 is detectable at a slightly earlier stage than *Hox*-3.1 (Gaunt, 1988). A typical pattern of spatial expression has sharp invariant anterior limit, with a less defined posterior boundary whose position changes during development. The central nervous system (CNS) and the adjacent mesodern sometimes show the same anterior-posterior limits of expression (Holland and Hogan, 1988b); an exception is *Hox*-3.1 which has a more anterior boundary in the nervous system than in the prevertebrae (Le Mouellic *et al.*, 1988).

Short and long versions of *Xenopus* XlHbox protein 1 are generated by translation of two related mRNAs. The long mRNA is transcribed from an upstream promoter and contains an AUG which is 5' to, and in frame with, the start codon for the short protein (Cho *et al.*, 1988). Specific antibodies that recognize either the long, or both the long and short version of the XlHbox 1 protein also recognize the murine homologues. Immunostaining disclosed that the short protein always had a more anterior position in the embryo than the long protein. Like other homeobox genes, each of these proteins also had a more anterior boundary in the CNS than in the adjacent mesoderm, and expression in the CNS persisted longer in development (Oliver *et al.*, 1988).

The promoter elements recognized by the homeobox containing Oct-2 and Pit-1 transcription factors have been identified, although the genes controlled by homeodomains that determine mammalian regionalization have yet to be found. Indications that (in *Drosophila*) homeobox proteins can recognize more than one site, and furthermore that more than one protein can bind a specific site, lead to the proposal that both the affinity and concentration of homeobox proteins regulate the gene activity (Levine and Hoey, 1988). As in *Drosophila*, an interesting aspect of murine homeobox

regulation is the colinearity between the anterior-posterior pattern of expression and the position of a particular *Hox*-gene in its cluster. *Hox*-1.6 is at the 3' end of the cluster, and has the most anterior border of expression of the *Hox*-1.1 to 1.6 cluster (Duboule and Dollé, 1989). An even more definitive gradient of expression has been shown for the *Hox*-2 cluster. Each succesive gene from 5' to 3' has a more anterior boundary in the CNS (Graham *et al.*, 1989). It is clearly evident that there is a specific spatial and temporal expression of the homeobox genes. Some effect tissue specific development, while others promote regionalization.

1.1.5 Cancer

A decreased requirement for growth factors leads to a growth advantage for the transfomed cell. Growth factors increase the expression of some proto-oncogenes (*myc*, *fos*; Muller *et al.*, 1984). Inappropriate expression of either *myc* or *fos* leads to the same growth advantage as stimulation by growth factors. Expression of oncogenes related to growth factors or their receptors transforms the cell through related mechanisms.

The proto-oncogenes are essential for cell growth, while the homeotic genes are responsible for pattern formation and differentiation. Although a role for homeobox genes in cancer has not been demonstrated, they are expressed during differentiation of murine teratocarcinoma cells (Colberg-Poley *et al.*, 1985), which can be induced to form muscle or nerve (McBurney *et al.*, 1982). Teratocarcinoma cells develop normally when placed in the strong individuation field of a blastocyst (Mintz *et al.*, 1975).

Just as important as the mutations that lead to activation of proto-oncogenes is the way a cell perceives its environment, since this influences growth of the cell as well as its response to growth factors. Ingber and

Folkman (1989a) postulate that the ECM conveys regulatory information to (endothelial) cells through both mechanical and chemical mechanisms. The insoluble ECM provides a surface on which cells can spread and generate tension within the cytoskeleton. In the presence of growth factor, endothelial cells were induced to proliferate when the insoluble ECM provided a surface on which they could spread; and they differentiated toward capillary tube formation when lower ECM densities permitted cell shortening (Ingber and Folkman, 1989b). Specific sequences in ECM proteins are also capable of mediating these effects. In this context, the sequence arginine-glycine-aspartic acid (RGD) in the laminin A chain is involved in cell attachment. Grant *et al.* (1989) showed that the addition of a synthetic peptide with the sequence YIGSR, which is in the B chain of laminin, induced capillary tube formation by endothelial cells.

1.2 TIMP

Tissue inhibitor of metalloproteinases is a specific inhibitor of the metalloproteinases, including enzymes such as collagenase(s), gelatinase and stromelysin. Other naturally occuring metalloproteinase inhibitors are the M_r 750,000 α_2-macroglobulin, which constitutes 95% of the collagenase inhibitor activity in human plasma (Woolley *et al.*, 1976); a group of low M_r (10,000-20,000) inhibitors found in extracts of bone, cartilage and aorta (Harris *et al.*, 1984); M_r 6,000 and 12,000 proteins present in the culture medium of chick skin (Shinkai *et al.*, 1977) and a 66,000 M_r protein secreted by rabbit chondrocytes (Morris, 1989).

TIMP was identified as a potent collagenase inhibitor present in the culture medium of foetal rabbit bone explants (Sellers *et al.*, 1977). It was later shown to also inhibit the degradation of gelatin and proteoglycans by

two other metalloproteinases (Sellers *et al.*, 1979) and was subsequently named TIMP (Cawston *et al.*, 1981). TIMP was first isolated from explant and tissue culture media, and later from serum, amniotic fluid, cerebrospinal fluid, vitreous humour synovial fluid and was shown to be stored in platelets (reviewed in Cawston, 1986). TIMP has been purified to homogeneity from several of these sources including explant and tissue culture cell media as well as amniotic fluid.

The M_r of mature TIMP is 28,000 in SDS-polyacrylamide gels. The size of the core protein predicted from the amino acid sequence is M_r 23,144 for human TIMP (Docherty *et al.*, 1985) and M_r 22,500 for murine TIMP (Edwards *et al.*, 1986). There are two potential N-glycosylation sites in both murine and human TIMP amino acid sequences. During processing, the M_r 2,500 signal peptide is cleaved and carbohydrates representing 20% of the secreted molecule by weight (Kishi and Hakakawa, 1984) are added. The 12 cysteines in mature TIMP form 6 disulfide bridges; free sulfhydryl groups were not detected in active TIMP (Kishi and Hakakawa, 1984), although a free sulfhydryl group has been reported to be necessary for the activity of another collagenase inhibitor isolated from polymorphonuclear leukocytes (Macartney and Tscheche, 1984). The presence of disulfide bridges is consistent with the sensitivity of TIMP activity to reducing agents and its remarkable stability at low pH or when heated (90°C for 1 h).

TIMP binds in a 1:1 stoichiometry with active metalloproteinases, and forms an M_r 54,000 complex with collagenase (Cawston *et al.*, 1983), an M_r 43,000 complex with proteoglycanase (Galloway *et al.*, 1983) and an M_r 86,000 complex with gelatinase (Murphy *et al.*, 1985a). The binding is essentially irreversible with a K_i of 1.4×10^{-10} M (Cawston *et al.*, 1983).

The role of TIMP was first proposed to be 'a ubiquitous inhibitor' that acted as a fail-safe mechanism to prevent tissue resorption by limiting collagenase activity to the site of its secretion (Reynolds *et al.*, 1977). TIMP has been localized by immunofluorescent staining to the areas of wound healing that are actively undergoing tissue remodelling (see 4.1). The time course of appearance of TIMP lagged one day behind the appearance of collagenase, suggesting a coordinated expression of these two proteins.

A drop in TIMP levels is associated with some pathological conditions. Collagenase activity was observed to be higher and TIMP levels were depressed in the synovial fluid from arthritic joints. This is due, at least in part, to a mononuclear cell factor (MCF) and a synovial factor which stimulates the production of proteinases and prostaglandin E by cartilage and bone cells. Corticosteroids such as hydrocortisone, which are used to treat rheumatoid arthritis, act by inhibiting MCF action and stimulating TIMP production by these cells (McGuire *et al.*, 1982). The proteases secreted by the neutrophils that invade inflamed joints can degrade TIMP (Okada *et al.*, 1988).

TIMP also plays a role in controlling the invasive and metastatic potential of cells. An inverse correlation was demonstrated between TIMP levels and the invasive potential of intracranial tumors (Halaka *et al.*, 1983). The addition of purified TIMP blocked the invasion of metastatic cells *in vitro* in an amnion invasion assay (Thorgeirson *et al.*, 1982; Schultz *et al.*, 1988); and intraperitoneal injections of TIMP drastically reduced the lung colonization potential of B16F10 melanoma cells injected subcutaneously in nude mice (Schultz *et al.*, 1988). 'Normal' Swiss 3T3 cells acquired a tumorigenic and metastatic propensity when their secreted

TIMP levels were reduced 50% by an antisense RNA expression vector, demonstrating that small changes in the balance between TIMP and metalloproteinases can have dramatic consequences (Khokha *et al.*, 1989).

Changes in TIMP and metalloproteinase activities influence the extracellular matrix (ECM) deposition and degradation. In fibroblast cells in tissue culture, transforming growth factor-ß (TGF-ß) stimulates TIMP expression either alone (Overall *et al.*, 1989) or in synergism with growth factors (Edwards *et al.*, 1987a). At the same time TGF-ß blocks induction of collagenase, resulting in conditions which favor extracellular matrix deposition. Similarly, the naturally occuring all-*trans*- and all-*cis*-retinoic acids increase TIMP and decrease collagenase activity secreted into the medum by fibroblasts (Clark *et al.*, 1987). Conversely to TGF-ß, interleukin-1α suppresses TIMP synthesis and induces collagenase in rabbit cervical fibroblasts, resulting in a net degradation of ECM components (Ito *et al.*, 1988). The induction of collagenase was also sensitive to 17-ß-estradiol. Murphy *et al.* (1985b), however, reported an induction of TIMP in human fibroblasts by interleukin I. TIMP mRNA levels are also elevated by serum, PDGF, FGF, TPA, Newcastle disease virus (NDV), and dsRNA (Edwards *et al.*, 1985a; Gewert *et al.*, 1987; Denhardt *et al.*, 1987).

TIMP is a single copy gene in both human and mouse, and resides on the X chromosome (Spurr *et al.*, 1987; Mullins *et al.*, 1988). The control elements identified so far in the mouse gene reside in the first intron. This region was responsible for conferring virus inducibility, and also enhanced constitutive expression from an upstream promoter (Coulombe *et al.*, 1988). Edwards *et al.* (1986) observed a progressive time dependent shortening

of the poly A tails in serum-induced TIMP mRNA which may reflect aging of the mRNA and may play a role in the mRNA half life.

1.3 MRP

Mitogen regulated protein was first detected, by ^{35}S-labelling, as a 30,000-39,000 M_r glycoprotein that was secreted into the medum following stimulation of Swiss 3T3 cells with serum, FGF or EGF (Nilsen-Hamilton *et al.*, 1980). The non-glycosylated core protein has an M_r of 22,000. A partial length MRP cDNA was isolated by immunoscreening of a λgt 11 expression library (Parfett *et al.*, 1985). The nucleotide sequence of the MRP cDNA was identical to another cDNA called proliferin, which wa- isolated by differential screening of a cDNA library made from serum stimulated cells (Linzer and Nathans, 1983). The two reported proliferin cDNAs differ from each other at four positions, resulting in three amino acid substitutions. PLF-1 was generated from fibroblast mRNA, and PLF-2 from placental mRNA (Linzer *et al.*, 1985).

The stimulation of MRP synthesis by growth factors is due primarily to an increase of transcription. Although incompletely processed transcripts are present in the nuclei of resting cells, there is no detectable mRNA in the cytoplasm (Linzer and Wilder, 1987). MRP mRNA as well as the secreted protein levels increase from 6 to 18 h after stimulation by growth factors, and the induction is blocked by transcriptional inhibitors (Nilsen-Hamilton *et al.*, 1980; Parfett *et al.*, 1985). Two PLF-1 promoters which have been isolated are serum inducible in a CAT assay. The 15-fold higher level of transcription from one of the two promoters may be due to the presence of a histone H2B-like element at -94 to -85 (Linzer and Mordacq, 1987). Connor *et al.* (1989) showed that a third promoter (*plf3*)

was also responsive to TGF-α, and contained a negative regulatory element at -1101 to -1073. The stability of the protein appears to be controlled by its degradation in the lysosomes (Nilsen-Hamilton *et al.*, 1981).

Synthesis of MRP is associated with the process of immortalization. Primary mouse embryo fibroblasts (MEFs) do not make MRP (Parfett *et al.*, 1985). However, when they are cultured by the 3T3 regime (3 x 10^5 cells per 5 cm dish passed every 3 d) MRP mRNA accumulates in the cytoplasm and MRP is secreted starting about passage 10 as the cells recover from crisis, (Edwards *et al.*, 1987). The only known *in vivo* site of MRP synthesis is the trophoblast giant cells of the mid-gestational placenta (Lee *et al.*, 1988). It has been proposed that MRP acts as an autocrine growth factor, although this has yet to be demonstrated. Recombinant murine MRP/PLF-1 from CHO cells binds to the mannose-6-phosphate receptor, which is known to function in directing acid hydrolases to the lysosomal compartment, although MRP isolated from placenta fails to do so (Lee and Nathans, 1988). The over-expression of PLF-1 in both muscle and 10T½ derived myoblasts inhibits their differentiation into myotubes, although over-expression of PLF-2 has no such effect (Wilder *et al.*, 1989). A specific carbohydrate moiety of PLF-1 may be necessary for its function, or alternatively, over-expression of PLF-1 may indirectly interfere with differentiation by overloading the lysosomes. The implication of this is not clear since PLF-2 is expressed at higher levels than PLF-1 in tissue culture cells and placenta (Wilder and Linzer, 1987).

1.4 spp

The cDNA for secreted phosphoprotein (spp) was isolated as a TPA-inducible mRNA by differential screening of a TPA-treated JB6 epidermal

cell library, and was initially designated 2ar. TPA induction of spp mRNA is biphasic. The first peak was seen 6 h after addition of TPA to subconfluent exponentially growing JB6 cells, and the second round of induction was seen if the cells were allowed to reach confluence in the presence of TPA (Smith and Denhardt, 1987). The mRNA was also induced in quiescent murine fibroblasts stimulated with serum, PDGF, FGF, EGF (Smith and Danhardt, 1987) or embryonal carcinoma-derived growth factor (Nomura *et al.*, 1988), and by TGF-ß and 1,25-dihydroxyvitamin D_3 in rat osteosarcoma cells (Yoon *et al.*, 1987; Noda *et al.*, 1988; Prince and Butler, 1987). On the other hand, dexamethasone decreased the level of osteopontin mRNA (=spp) in the osteosarcoma cells (Noda *et al.*, 1988), and the induction of spp by TPA was inhibited by retinoic acid and dexamethasone in JB6 cells (Smith and Denhardt, 1989). The maximal spp mRNA level in fibroblasts was seen by 6 h post induction.

spp was shown to be the murine equivalent of rat osteopontin based on sequence similiarity and the conservation of several features in the protein's primary structure (Craig *et al.*, 1989). The open reading frame in the 2ar cDNA encodes an M_r 32,350 protein. However, the secreted glycoprotein has an M_r of 57,000 to 65,000 on reducing SDS-polyacrylamide gels. The anomolous migration may be due in part to carbohydrates, and also to a high degree of phosphorylation and a high proportion of acidic amino acids. spp is a member of a family of adhesion proteins, including collagens and fibronectin, which contain the tripeptide RGD. This sequence is recognized by a family of receptors called integrins. Each RGD containing protein is specifically recognized by at least one receptor (Ruoslahti and Pierschbacher, 1987). Rat osteopontin functions as an

attachment and spreading factor for osteoblasts when it is used to coat polystyrene plates, and this attachment is inhibited by peptides specifically containing the RGD sequence. This led to the speculation that osteopontin is playing a role in guiding or attaching cells to areas of ossification (Oldberg *et al.*, 1986). The osteopontin-mediated attachment activity is also displayed by fibroblasts and one of three epidermal lines tested (reviewed in Butler, 1989).

Osteopontin expression is seen at an early stage of bone formation. Specific antibody detects osteopontin in preosteoblasts and osteoblasts at stages of cartilage-to-bone transition, before the process of mineralization takes place, in calvaria, developing tibia, and in future mandible and clavicle (Mark *et al.*, 1987; 1988a). Strong immunostaining of osteopontin seen in the Golgi is consistent with its being a secreted protein. Osteopontin mRNA is also detectable in rat and mouse calvaria, as well as in foetal mouse backbone and forelimbs (Yoon *et al.*, 1987; Nomura *et al.*, 1988). When compared to the bone specific protein osteonectin, osteopontin is expressed later in development, and by a cell type different then that responsible for osteonectin (Yoon *et al.*, 1987; Nomura *et al.*, 1988).

In addition to bone, spp/osteopontin mRNA and/or protein has also been detected in predentin, kidney, the granulated metrial gland (GMG) cells of the decidua and placenta of pregnant mice, inner ear, brain, (Yoon *et al.*, 1987; Nomura *et al.*, 1988; Mark *et al.*, 1988a; 1988b), the ventral and dorsal skin of lactating mice (D.T.Denhardt, personal communication), and in human blood and milk (Senger *et al.*, 1988; 1989). The role of this protein may be the same or it may be specialized in these diverse tissues.

Elevated spp mRNA levels are correlated with the metastatic ability of both the *ras*^{H}-transformed 10T½ cells and the non-*ras* transformed 6BL4b cells in both spontaneous and experimental metastasis assays in nude mice (Craig, 1989). Also increased secretion of pp69 (osteopontin) by transformed rat cell lines (Craig *et al.*, 1988), and of transformation-associated phosphoprotein (the human homologue) by human osteosarcoma lines has been correlated to their tumorigenicity (Senger and Peruzzi, 1985). Elevated levels of the protein were detected in the serum of patients with disseminated carcinomas, but not in patients with non-metastatic tumors (Senger *et al.*, 1988).

The lack of a 'normal' ECM in transformed cells coupled with an elevated spp expression may enhance or facilitate the attachment of malignant cells that are invading the secondary site. Both the invasion of tumor cells into the amniotic membrane *in vitro* (Gehlson *et al.*, 1988) and the *in vivo* experimental lung metastasis in nude mice by malignant cells (Humphries *et al.*, 1986) are blocked by synthetic peptides containing the RGD attachment sequence.

spp is a single copy gene. The sequence of the promoter region revealed that there are potential binding sites for transcription factors AP-1, AP-2, AP-3 and AP-5, as well as sequences similiar to palindromic response elements for estrogen, glucocorticoid and retinoic acid in addition to TATA and CAT boxes. Chimaeric constructs of regions of the promoter and the CAT reporter gene revealed the location of a positive transcriptional element between -253 and -543, and a negative regulatory element between -543 and -570 (Craig, 1989). The TPA responsive element was localized to -253 and +79.

1.5 Calcyclin

Murine calcyclin was isolated as a cDNA (5B10) whose cognate mRNA level increased 4-fold by 18 h after serum stimulation of quiescent fibroblasts (Edwards and Denhardt, 1985). The cDNA was identified as the murine homologue of human calcyclin based on similiarity at the nucleotide level (Guo *et al.*, 1989). The 0.6 kb mRNA has an ORF which encodes an M_r 8,000 protein that has been visualized by *in vitro* translation of hybrid selected (Edwards *et al.*, 1985a) or SP6 generated mRNA (Guo *et al.*, 1989). There is no signal peptide in the sequence (Guo *et al.*, 1989), and the protein has recently been shown to copurify with the prolactin receptor from a breast cancer cell line (Murphy *et al.*, 1988).

Calcyclin is a member of the S100 Ca^{++}-binding protein family whose members are related by sequence similiarity. Other proteins in the S100 family are the α and ß p100 subunits (Kuwano *et al.*, 1984), the p11 subunit of calpactin I (Saris *et al.*, 1987), the vitamin D_3-dependent intestinal calcium-binding protein (Fullmer and Wasserman, 1981), and a migration inhibitory protein secreted by activated macrophages (Lagasse and Clerk, 1988) that is equivalent to the cystic fibrosis antigen (Dorin *et al.*, 1987; in Rogers, 1989). Another CCD gene (18A2), which is maximally expressed in S phase, has 54% simiarity to calcyclin at the amino acid level (Jackson-Grusby *et al.*, 1987). The function of calcyclin is unknown, although the related proteins have a variety of functions ascribed to them. The S100 proteins likely modulate the activity of specific activator proteins in accordance with the Ca^{++} levels. Disulfide bonded S100ß dimers have a neurite-promoting activity, function in microtubule disassembly, and also the inhibition of PKC-dependent phosphorylation of tau and pp80. Calpactin

mediated cytoskeleton/membrane interactions may be modulated by p11; the rat homologue of 18A2 is expressed at a high level during differentiation of epithelial stem cells to myoepithelium (reviewed in Kligman and Hilt, 1988).

Calcyclin mRNA is regulated in a CCD manner, and is induced following serum induction of quiescent tissue culture cells (Edwards *et al.*, 1985a). The mRNA is abundant in tissues that normally contain proliferating cells, such as the placenta, decidua and uterus of pregnant mouse, skin, stomach, heart, lung, intestine, and whole embryo. The mRNA is also detectable in kidney, brain, spleen, testis, prostate and vesicular gland (Guo *et al.*, 1989). Elevated levels of calcyclin mRNA are associated with chronic, but not with acute leukemias (Ferrari *et al.*, 1987), most human breast cancer cell lines (Murphy *et al.*, 1988) and mouse epidermis treated with TPA (Guo *et al.*, 1989).

There are about 8 copies of the mouse calcyclin gene (Guo *et al.*, 1989), while a single copy of the human calcyclin gene resides on chromosome 1 (Ferrari *et al.*, 1987). Sequence analysis of the human calcyclin promoter revealed the presence of a TATA box, a GC box, and an SV40-like enhancer along with other enhancer sequences. Transient expression assays using various chimaeric promoter-CAT constructs have shown an EGF negative regulatory element extending from -1371 to -1194, and serum-inducible sequences just upstream of the promoter (Ghezzo *et al.*, 1988). Synthetic promoters were used to map the PDGF/serum response element to be between -42 to -32 (Ghezzo *et al.*, 1989).

1.6 MEP and Transin

MEP is the major excreted protein of transformed mouse fibroblasts cells (Gottesman, 1978). The acid activatable proteolytic activity of MEP

has a substrate specificity similiar to that of cathepsin L (Gal and Gottesman, 1986) and its identity as murine procathepsin L was confirmed by its sequence similiarity to the human liver cathepsin L (Mason *et al.*, 1986). The 1600 nucleotide mRNA codes for a 39,000 M_r glycoprotein which is processed to 29,000 M_r by removal of the pro region (Gal *et al.*, 1985). A second cleavage produces an active form with a 21,000 M_r heavy chain and a 7,000 M_r light chain (Portnoy *et al.*, 1986). The protease is active on the ECM proteins fibronectin, laminin and collagen (Kirschke *et al.*, 1982; Mason *et al.*, 1986b).

Secreted MEP levels were shown to be correlated with experimental and spo taneous metastatic propensity of ras^H transformed 10T½ cells (Denhardt *et al.*, 1987), although over-expression of MEP in NIH3T3 cells was not sufficient to mimic the transformed phenotype (Kane *et al.*, 1988). Regulated expression of MEP in a tumorigenic but non-metastatic line would more clearly evaluate its role in metastasis. The related cysteine protease cathepsin B [cathepsin L can accommodate larger hydrophobic side chains at the active site (Kirschke *et al.*, 1988)] is present at much higher levels in malignant rather than the surrounding normal human breast tissue (Poole *et al.*, 1978), and is especially predominant in the smallest and fastest growing tumors (Sloane, 1981). Although there is a correlation, cysteine proteinases are not always associated with invasive tumors. However, this may reflect their instability in the purification procedures (Mullins and Rorhlich, 1983).

The 1,900 nucleotide (nt) rat transin mRNA (Matrisian *et al.*, 1985) encodes a 60 kDa protein, which is the rat equivalent of human stromelysin

(Whitham *et al.*, 1986). Transin shares 71% similarity at the amino acid level with transin-2; both were isolated as oncogene induced mRNA species (Breathnach *et al.*, 1987). There is also about 55% amino acid sequence similarity between human stromelysin and type I collagenase (Whitham *et al.*, 1986; Goldberg *et al.*, 1986). At the level of gene organization (exon number and size) transin and transin-2 are strikingly similar to each other as well as to rabbit synovial collagenase and human fibroblast type I collagenase (Collier *et al.*, 1988).

Transin/stromelysin is secreted as a proenzyme that upon activation preferentially degrades proteoglycans, and is also active on epithelial basement membrane and ECM components including laminin, fibronectin and collagen type IV, but not collagen types I-III (interstitial collagens) (Wilhelm *et al.*, 1987). Transin also enhances type IV collagenase activity through limited proteolysis (Murphy *et al.*, 1987). The active form of transin is inhibited by TIMP (Galloway *et al.*, 1983; Herron *et al.*, 1986a).

Transin mRNA is induced by EGF, interleukin 1ß, and transformation with v-*src* or *ras*^{H}. The EGF-mediated induction of transin transcription is blocked by cycloheximide and by actinomycin D (Matrisian *et al.*, 1986a), TGF-ß (Machida *et al.*, 1988), and by agents which raise the cAMP levels (Kerr *et al.*, 1988).

Collagenase and transin are expressed, and are co-inducible by phorbol esters in human foetal skin fibroblasts, colon, cornea and gingiva (Wilhelm *et al.*, 1986), and also in rabbit synovial cells and fibroblasts (Gross *et al.*, 1984; Frisch *et al.*, 1987). The co-induction of collagenase and stromelysin in rabbit capillary endothelial cells by TPA is accompanied

also increased levels of TIMP and two other inhibitors of M_r 19,000 and 22,000, resulting in little or no net increase in degradative ability (Herron *et al.*, 1986b). Transin mRNA is more abundant in malignant than in benign mouse carcinoma tissues (Matrisian *et al.*, 1986b). In contrast, human endothelial cells and a melanoma strain secrete high levels of collagenase but undetectable amounts of stromelysin. Furthermore, stromelysin production is not inducible by phorbol esters in these cells (Wilhelm *et al.*, 1987).

The concerted action of proteases (largely matrix metalloproteinases (MMP) which may be activated by serine or cysteine proteases) and their inhibitors is required for the deposition of new ECM components necessary for tissue remodelling, wound healing and cell migration (Mullins and Rohrlich, 1983). Uncontrolled destruction of the basement membrane or ECM has dire consequences and is associated with pathological conditions such as rheumatoid arthritis and metastasis.

1.7 Purpose of Thesis.

The focus of our laboratory was to study cell cycle dependent gene expression. Several partial length cDNAs had been isolated for which the cognate mRNA showed increased abundance following serum stimulation of quiescent tissue culture cells. My aim was to isolate full length cDNA clones for these inducible genes in order to undertake studies of their molecular characterization and regulation, as well as biochemical studies on the protein overexpressed in *E. coli*. Antisera were available for some proteins whose synthesis was induced by serum stimulation. An expression library was constructed so that the respective cDNA clones could be isolated using the

available antisera.

Each result chapter presents another step towards this end. First is a description of how the full length cDNA clones of inducible genes were isolated and used to generate polyclonal antisera specific for the cDNA encoded protein product. An *in vitro* transcription/translation system was used to show that an ORF upstream of, and overlapping the TIMP coding region (revealed by the nucleotide sequence of the full length cDNA clone), reduced the efficiency of TIMP translation. During the course of this work it was shown that TIMP can block the invasion of malignant cell lines (section 1.2). Foetal trophoblasts are as invasive as malignant cells and the pregnant animal is an ideal system to determine if TIMP plays a similiar role of blocking the invasion of foetal trophoblasts into maternal tissues. The development of the tissues which support pregnancy represent a model to study the expression of other growth inducible genes and to determine their roles *in vivo*. A study was undertaken to elucidate the spatial and temporal patterns of expression of the serum inducible genes TIMP, MRP, MEP, the transins 1 and 2, calcyclin and spp in the mouse female reproductive tissues during pregnancy.

CHAPTER 2 - MATERIALS AND METHODS

2.1 Bacterial Strains.

The strains of *Escherichia coli* used were: RR1 and JM103 for maintenance of plasmids; JA221 for expression from the ompA secretion vector and NF1 for expression of ß-galactosidase fusion proteins. The genotype of RR1 is F^-, *hsd*S20 (r_B,m_B), *ara*-14, *pro*A2, *lac*Y1, *gal*K2, *rps*L20 (Sm^r), *xyl*-5, *mtl*-1, *sup*E44 , λ^- and can be transformed efficiently with plasmid DNA (Bolivar *et al.*, 1977). JM103, has the genotype Δ (*lac pro*), *thi*, *str*A, *sup*E, *end*A *sbc*B, *hsd*R^-, F'*tra*D36, *pro*AB, *lac*I^q, Z Δ*M*15 (Messing *et al.*, 1983). JA221 has the genotype *lpp*$^-$, *hds*M^+, *trp*E$^-$, *leu*B6, *lac*Y, *rec*A1/F', *lac*I^q, *lac*$^+$, *pro*$^+$ (Nakamura and Inouye, 1979). NF1 is a *lac*Z^- am strain which contains the defective prophage λ *N*am7 *N*am53 *c*I857 ΔH1 (Bernard *et al.*, 1979; obtained from K. Stanley). RR1 and NF1 were grown in Luria-Bertani media (Miller, 1972). JM103 was grown in yeast-tryptone broth (2% bacto-tryptone, 0.5% yeast extract, 0.5% NaCl). JA221 was grown in M9 media with supplements (Grayeb *et al.*, 1984) for maintenance and [^{35}S]methionine labelling, and in the supplemented 19 plus 0.2% casamino acids for inducing expression under non-labelling conditions. Cultures were grown at 37°C with aeration unless indicated otherwise. Strains containing plasmids were grown in medium with 100 µg/ml ampicillin. Stocks were stored in medum containing 15% glycerol at -70°C.

2.2 Nucleic Acid Purification

2.2.1 Handling of Nucleic acids

All glassware, plasticware and buffers used for the manipulation of DNA were autoclaved if they had not been obtained sterile. Glassware used for RNA manipulations was acid-washed and baked, and the buffers were

autoclaved with diethylpyrocarbonate (DEP) when possible. DNA was kept in TE buffer at 4°C for short term, and at -20°C for long term storage. RNA was stored at -70°C in DEP-treated water. Working stocks of DNA and RNA were kept on ice except for the period of incubation.

Following isolation or enzyme treatment, the DNA or RNA was either analyzed directly or purified further by extraction with phenol or phenol/ chloroform, and two ethanol precipitations. The phenol or phenol/ chloroform/isoamyl alcohol at a ratio of 25/24/1 was first equilibrated with 0.1 M Tris·HCl pH 8.0 for DNA, or with ACE buffer (10 mM sodium acetate pH 5.1, 50 mM NaCl, 3 mM EDTA) for RNA, then saturated with argon gas and stored at 4°C. Ethanol precipitations were performed by the addition of ammonium acetate to 2 M, followed by $2\frac{1}{2}$ volumes of ethanol and incubation at -20°C. The DNA precipitate was centrifuged, and the pellet resuspended in TE. This was made to 0.3 M sodium acetate and the DNA precipitated a second time with ethanol. The DNA pellet was dried and dissolved in TE. For RNA, both precipitations were from 0.3 M sodium acetate, and the pellets were dissolved in DEP-water. The concentration of nucleic acid was determined spectrophotometrically.

2.2.2 Purification of Plasmid DNA

Cells were grown overnight O/N in medium containing antibiotic. Large scale plasmid preparations were by the method of Clewell and Helinski (1969) and purified on CsCl gradients (Maniatis *et al.*, 1982). Plasmid mini-preps were by the method of Birnboim and Doly (1979). Contaminating RNA was removed by incubation with 40 µg/ml RNAase A for 30 min at 37°C, followed by phenol/chloroform extraction and ethanol precipitation.

2.2.3 Isolation of RNA

To isolate cytoplasmic RNA from tissue culture cells, the plasma

membrane was disrupted and the nuclei removed by centrifugation. The cytoplasmic proteins were denatured with urea, and then extracted with phenol (Edwards *et al.*, 1985b). The RNA was finally recovered by ethanol precipitation.

To isolate total RNA from tissue, frozen tissue was homogenized using a Polytron (Brinkman Instruments) in 1 g/5 ml (= 1 volume) of 5 M guanidinium isothiocyanate with fresh 1% ß-mercoptoethanol. This was diluted with one volume of water and centrifuged at 14,000 g for 15 min to remove debris. One volume of 10 M LiCl was mixed with the supernatant, and incubated O/N at 4°C. The precipitate was collected by centrifugation at 14,000 g for 1 h, resuspended in 2 volumes of 2 M urea-3 M LiCl using a pasture pipet, and centrifuged again at 14,000g for 1 h. The pellet was dissolved in ½ volume of TE-0.5% SDS, and incubated with 50 µg/ml proteinase K at 55°C for 30 min or until clear. This solution was made 1X ACE and was extracted twice with an equal volume of phenol equilibrated with ACE buffer. Sodium acetate was added to a concentration of 0.3 M and the RNA precipitated with 2½ volumes of ethanol at -20°C O/N. The ethanol precipitate was dissolved in a small volume of water, made to 1X ACE, and extracted with an equal volume of phenol, phenol/chloroform and reprecipitated. The ethanol precipitate was dissolved in a small volume of water and the concentration determined. The RNA was stored at -70°C.

2.3 *In Vitro* DNA Manipulations

Restriction endonucleases and other DNA modifying enzymes were purchased from Bethesda Research Laboratories, Boehringer-Mannheim, New England Biolabs, Pharmacia LKB Biotechnology and Promega Corporation, and were used according to supplier's instructions. A 2-5 fold excess of

restriction endonuclease was used to ensure complete digestion.

The 3' recessed ends of restricted DNA were made blunt with Klenow fragment of DNA polymerase I. A typical reaction contained 1-25 μg of DNA in 25 μl of reaction buffer (50 mM Tris·HCl pH 7.6, 10 mM $MgCl_2$) with 5 units (U) of enzyme and 0.2 mM of all 4 dNTPs, and was incubated at rt for 15 min. S1 nuclease was also used to make blunt the 5' overhanging ends. DNA (10-20 μg) was incubated with 5 U of S1 in 100 μl of buffer (50 mM Na acetate pH 4.5, 200 mM NaCl and 1 mM $ZnSO_4$) for 15 min at 15°C.

Nuclease *Bal*31 was used to degrade DNA termini in a controlled manner. 10 μg of restricted DNA was treated with 1 U of *Bal*31 in 100 μl of buffer (12 mM $CaCl_2$, 12 mM $MgCl_2$, 200 mM NaCl, 20 mM Tris·HCl pH 8.0, 1 mM EDTA) at 30°C for 3 min.

To prevent self ligation without an insert, the 5' ends of vector DNA were routinely dephosphorylated with calf intestinal phosphatase (CIP). 10-20 μg of DNA was incubated with one unit of CIP at 37°C for 30 min in 50 mM Tris·HCl pH 9.0, 1 mM $MgCl_2$. For blunt ends an additional 1 U of CIP was then added and the mixture incubated at 56°C for 30 min.

DNA fragments were ligated with T4 DNA ligase at rt for 1 h for fragments with sticky ends, or O/N at 14°C for fragments with blunt ends. cDNA inserts to be subcloned were ligated at a molar ratio of 2:1 - 3:1 of insert to vector, in a buffer of 50 mM Tris·HCl pH 7.6, 10 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 1 mM ATP and 0.1 mg/ml bovine serum albumin (BSA), with ligase at 50 U/ml for sticky ends, and at 200 U/ml for blunt ends.

2.4 Gel Electrophoresis

DNA resuspended in TE with 7% sucrose, 0.5 M urea, 0.03% bromophenol blue and 0.03% xylene cyanol, was analyzed on 0.8-1.0% agarose

(SeaKem) gels. The running buffer contained 50 mM Tris·HCl, pH 8.0, 5 mM sodium acetate, 0.5 mM EDTA, and 0.5 μg/ml ethidium bromide. DNA was visualized and photographed under short wave ultraviolet light. For isolation of DNA fragments from agarose gels, high purity low melting temperature agarose (BioRad) was used. The fragment of interest was excised with a scalpel, the gel melted and extracted twice with phenol and then with phenol/chloroform (Weislander, 1979) and the DNA purified on an Elutip column (Schleicher and Schuell), and recovered by ethanol precipitation. Small DNA fragments were also analyzed on 6-12% polyacrylamide gels run in Tris-borate buffer (Maniatis *et al.*, 1982). DNA fragments were isolated from polyacrylamide gels by the method of Maxam and Gilbert (1977).

RNA was analyzed on 1.1% agarose gels containing formaldehyde or on 10% polyacrylamide gels containing 7 M urea (Maniatis *et al.*, 1982). The restriction fragments of either pBR322 or λ DNA provided the size markers used on DNA and RNA gels.

Denaturing SDS-polyacrylamide gel electrophoresis of proteins was performed according to Laemmli (1970), using 4.5% stacking gels and 8-12% running gels. Gels containing ^{35}S-labelled proteins were impregnated with 2,5-diphenyloxazole (Bonner and Laskey, 1974) and fluorographed at -70°C, using pre-flashed Kodak XAR film. Molecular weight markers (Bio-Rad) used were phosphorylase B (92 kDa), BSA (69 kDa), ovalbumin (46 kDa) carbonic anhydrase (30 kDa), lactoglobulin A (18 kDa) and lysozyme (14 kDa) (Bio-Rad). Methylated ^{14}C-labelled markers were ovalbumin, carbonic anhydrase and lactoglobulin A (Dupont-NEN).

2.5 Transformation of *E. coli* with DNA

DNA was introduced into *E. coli* by the method of Hanahan (1983). Transformed cells were selected by growth on plates of agar containing the appropriate medium and 100 μg/ml carbenicillin. Transformed colonies were screened directly by colony hybridization with the appropriate ^{32}P-labelled probe, or plasmid mini-preps of individual clones were made and analyzed.

2.6 Preparation of Radiolabelled DNA

^{32}P-labelled DNA probes were made by a nick-translation procedure modified from Rigby *et al.* (1977). 150 ng of DNA was added to 10 μl of reaction mixture (50 mM Tris·HCl, pH 8.0, 5 mM $MgCl_2$, and all four dNTPs. Typically 20-50 μCi each of 1 or 2 [α-^{32}P]dNTPs (800 or 3000 Ci/mmol; DuPont-NEN or ICN Radiochemicals) was used along with the complementary unlabelled dNTPs (20 μM each). This mixture was incubated with DNase I (25 ng; Worthington) for 3 min at rt, and then with 2.5 U of *E.coli* DNA polymerase I for 2 h at 15°C. The reaction was stopped by the addition of EDTA to 20 mM and incubation at 75°C for 10 min. The labelled DNA was separated from unincorporated dNTPs by column chromatography using Sephadex G-50 medium (Pharmacia) in TE buffer (Maniatis *et al.*, 1982). The specific activity of probes was determined by TCA precipitation and was always $>10^8$ cpm/μg.

End-labelled DNA fragments were used as markers on Northern and Southern blots. DNA with 5' protruding ends was labelled by filling in the ends with Klenow fragment as described in section 2.3, using a required [α-^{32}P]dNTP at 1 μCi/μl. Alternatively, T4 polynucleotide kinase was used to label dephosphorylated ends. To kinase blunt ends, the buffer contained 50 mM Tris·HCl pH 9.5, 10 mM $MgCl_2$, 5 mM DTT, > 1μM [γ-^{32}P]ATP (specific

activity > 800 Ci/mmol), and 2 U of T4 DNA polynucleotide kinase. To kinase 5' protruding ends the pH of the buffer was 7.6. The reaction mixture was incubated at 37°C for 30 min and the labelled DNA fragments isolated on a column as above.

2.7 Hybridizations

2.7.1 DNA and RNA Blot Analysis

DNA and RNA were transferred from agarose gels to either nitrocellulose (0.45 μm, BA-85, Schleicher and Schuell) or GeneScreen Plus (DuPont-NEN) filters. Agarose gels containing DNA were soaked in 0.5 M NaOH, 1.5 M NaCl for 30 min to denature the DNA, and then in 0.5 M Tris·HCl pH 7.4, 1.5 M NaCl for 30 min to neutralize the NaOH. DNA was transferred to the filter by capillary action (Southern, 1975) in 20X SSC for nitrocellulose or 10X SSC for GeneScreen Plus (1X SSC is 0.15 M NaCl, 0.015 M sodium citrate). RNA was transferred from the formaldehyde gels without any treatment. RNA was slotted onto Genescreen Plus with the Schleicher and Schuell Minifold II apparatus as recommended. Wet Genescreen Plus filters were exposed to ultraviolet light to covalently bind the nucleic acids (Church and Gilbert, 1984). Filters were baked *in vacuo* at 80°C for 2 h. Hybridization of nitrocellulose filters was performed as described by Edwards and Denhardt (1985), except that poly(rA) was omitted from the hybridization solution. GeneScreen Plus filters were prehybridized in 50% formamide, 10% dextran sulfate, 1% SDS, 1 M NaCl, 100 μg/ml denatured salmon sperm DNA for 4 h at 42°C, and then hybridized for at least 16 h in the same solution with the denatured probe at 1-3 x 10^6 cpm/ml). Blots were washed to a final stringency of 42°C, 55°C or 65°C in 0.1x SSC, depending on the probe. Probes were stripped from the

Genescreen Plus blots as described (Denhardt *et al.*, 1987; Knandjian, 1987) before reprobing. Autoradiography was at -70°C with a fluorescent screen. The specific hybridization signals were quantified by scanning autoradiograms with an LKB UltraScan XL densitometer.

2.7.2 Quantification of mRNA Levels

The level of specific mRNA in the total RNA preparations was determined by laser densitometry at 633 nm (LKB UltraScan XL) of the specific bands on autoradiograms of slot blots. The autoradiograms were in the linear range of exposure, and the relative mRNA levels were determined from the area under the curves representing each of the slots. Several precautions were taken to ensure the accuracy of these determinations. The samples were first analyzed on Northern blots to evaluate the integrity of the specific mRNAs. Identical replicate slot blots containing all of the RNA samples were made at the same time. 16 μg of each of the RNAs was diluted in 1.6 ml of 17% formaldehyde/10X SSC and heated to 55°C for 15 min to denature the RNA. 200 μl was loaded on each of 5 slot blots; 100 μl on one slot blot to be probed for 18S levels; and 400 μl on one slot blot to be probed for low abundance mRNAs. All of the tissue RNA samples were contained on a set of two slot blots, which were probed, washed and exposed together. Loading differences between RNA samples from different tissues were corrected by comparison to the signal for 18S rRNA. To compare the levels of calcyclin and spp mRNA, the blots were hybridized and washed under identical conditions and exposed for the same length of time.

2.7.3 Colony Hybridization

Bacteria were seeded onto a filter using a filtration device (Schleicher and Schuell), grown at 37°C on agar/medium plates with 100 μg/ml

carbenicillin, and the plasmids amplified for 24 h with 170 μg/ml chloramphenicol. The filters were then processed according to Grunstein and Hogness (1975), with an additional 1 min lysis in 10% SDS prior to the alkali denaturation. The proteinase K and chloroform washes were also omitted. The colonies on the filters were probed as for Southern blots.

2.8 Immunodetection

2.8.1 Isolating Antigens

Three antigens were used to immunize rabbits; BNL cell total cell protein (TCP), and two fusion proteins with MRP or TIMP sequences at the carboxyl end of ß-galactosidase.

BNL is an embryonic liver cell line. Exponentially growing cells were harvested, washed and resuspended and the cells disrupted in phosphate buffered saline (PBS) containing 0.5% Triton X-100, 0.05% SDS using a Dounce homogenizer. The cell suspension was incubated at 37°C for 15 min with 100 μg/ml RNAase A and 50 μg/ml DNAase I. The proteins were precipitated with 10 volumes of acetone, centrifuged, and resuspended in PBS. The protein concentration was determined using a Bio-Rad kit.

MRP and TIMP cDNAs were ligated into pEX3 to generate pMRPX and pTIMPX which directed the expression of the fusion proteins MRPX and TIMPX (see figure 3.14). *E. coli* strain NF1 carrying pMRPX or pTIMPX was grown at 30°C to an OD_{600} of 0.5, and then induced at 42°C for 90 min. The cells were collected and resuspended in ice cold 50 mM Tris pH 8.0, 50 mM EDTA, and 25% (w/v) sucrose (6 ml per 100 ml of culture). The cells were lysed by incubating with 1 mg/ml lysozyme for 10 min, and then 0.5% Triton X-100 for a further 10 min. An equal volume of RIPA buffer (25 mM Tris·HCl pH 7.2, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% Na desoxycholate and 0.1% SDS) was added and kept at 4°C for 20 minutes,

with occasional swirling. The mixture was sonicated with two 30 second bursts on a Fisher sonic dismembranator using the medium probe at 60% of maximum, and cooling on ice. The fusion protein was collected by centrifugation at 13,000g and washed once with 50% RIPA buffer. 100 ml of bacteria yielded 1-2 mg of fusion protein that was 90-95% pure by SDS-polyacrylamide gel analysis.

2.8.2 Antibody production

New Zealand white male rabbits were immunized subcutaneously with 2 mg of BNL cell TCP or MRPX or TIMPX fusion protein (section 2.8.1) emulsified with Freund's complete adjuvant. Booster immunizations were made in Freund's incomplete adjuvant at 4 week intervals for a total of 4 injections for the BNL cell TCP, or 6 injections for the fusion proteins. The specific antiserum used here was obtained 8-10 days after the last injection. Preimmune serum obtained from each animal before the series of injections was consistently negative.

The anti-ß-galactosidase antibodies were removed by affinity chromatography. 20 mg of ß-galactosidase (Sigma) was coupled to 0.7 g of cyanogen bromide-activated Sepharose 4B following the manufacturer's instructions (Pharmacia). 10 ml of antiserum was passed twice over a 2 ml column in a 5 ml syringe, the column was washed with 1 ml of PBS, and the flowthrough fraction saved as specific antisera.

2.8.3 Immunoprecipitations

[^{35}S]methionine labelled proteins were immunoprecipitated from tissue culture media as described by Denhardt *et al.* (1987). To immunoprecipitate ^{35}S-labelled *in vitro* translated proteins, 50 µl of reticulocyte lysate was made 2% SDS, 100 mM ß-mercaptoethanol and heated

at 90°C for 2 minutes. This was diluted to 500 µl with IP buffer (50 mM Tris·HCl pH 7.4; 150 mM NaCl; 0.1% NP40) and divided into two 250 µl aliquots. Each aliquot received 20 µl of either anti-TIMP antisera or preimmune serum, and was incubated at 4°C for 2 h. An excess of Protein A-Sepharose CL-4B (Pharmacia) was added. After rotating for 2 h at 4°C the immunoprecipitate was collected by centrifugation, washed twice with the IP buffer, and resuspended in Laemmli sample buffer. The sample was boiled for 2 min, centrifuged to remove the sepharose, and a volume of supernatant equivalent to 10 µl of lysate was electrophoresed on a 10% polyacrylamide SDS minislab gel.

2.8.4 Western Blot Analysis

Proteins were electroblotted from SDS-polyacrylamide gels onto nitrocellulose using a Bio-Rad Mini Trans-Blot Cell as recommended (after Towbin *et al.*, 1979). The blots were stained with Amido Black (0.1% in 50% methanol, 10% acetic acid) and processed for immunodetection by incubation in Tris buffered saline (TBS; 20 mM Tris·HCl, pH 7.4, 150 mM NaCl) for 5 min and then in TBS with 0.05% Tween 20 for 1 h to block non-specific adsorption. The primary antisera were used at a dilution of 1/500 in TBS-Tween 20 for 30 min. After washing 5 x 10 min in TBS and then 1 h in TBS-Tween 20, the blots were incubated for 2 h with 1 µCi/ml ^{125}I-labelled protein A. The blots were washed in TBS and the immunodetected proteins visualized by autoradiography.

2.8.5 Colony Immunodetection

Bacteria were seeded onto nitrocellulose filters, using a filtration device (Schleicher and Schuell), at a density of 3,000-4,000 per 10 cm filter, and overlayed on agar/medium with antibiotic. The clones were grown O/N at 30°C, and induced at 42°C for 3 h. The colonies were lysed

directly on the filters by placing the filter on Whatman 3MM paper soaked with 1% SDS, in a chloroform atmosphere for 20 min. The filters were then incubated for 1 h in 50 mM Tris·HCl pH 7.4, 200 mM NaCl, 10 mM $MgCl_2$, 1 µg/ml lysozyme and 2.5 µg/ml DNAase I for 1 h, and then in TBS-Tween (section 2.8.3) to block nonspecific binding sites. The filters were subsequently incubated with antisera as described for Western blotting (2.8.3), except the secondary antibody was peroxidase-linked goat anti-rabbit Ig (Boehringer-Mannheim). Positive colonies were visualized by incubation with 0.7 mg/ml 4-chloro-1-napthol (from a stock of 3 mg/ml in methanol) in TBS with 0.05% H_2O_2.

2.9 Radiolabelling Cellular Proteins

2.9.1 Pulse Labelling Bacteria

JA221 containing TIMP-*omp*A constructs were grown at 37°C in M9 supplemented with 4 mg/ml glucose, 20 µg/ml tryptophan, 20 µg/ml leucine, 2 µg/ml thiamine containing 50 µg/ml ampicillin to a density of 1 x 10^7 /ml and then induced with 2 mM isopropyl ß-D-thio-galactosidase (IPTG). After 1 h induction, the cells were labelled for 15 min with 20 µCi of [^{35}S]methionine (DuPont-NEN) per 0.5 ml of culture. The cells were chilled to 4°C, centrifuged, and resuspended in Laemmli sample buffer.

2.9.2 Conditioned Tissue Culture Media

Exponentially growing BNL cells were labelled 24 h after passageing. The medium (minimal Eagle's medium (MEM)/10% bovine calf serum) was replaced with methionine-free Dulbecco's modified Eagle's medium (Gibco) containing 3% dialysed serum. After 1 h at 37°C, [^{35}S]methionine was added to a specific activity of 200 µCi/ml, and the cells incubated for 4 h at 37°C. The medium was removed, centrifuged to remove cell debris, and stored at -20°C.

2.10 *In Vitro* Expression of TIMP

2.10.1 Construction of expression vectors

The SP6/TIMP RNA expression vectors were made by blunt end ligation of TIMP cDNA fragments into the blunted *Hind*III site of pSP64 (Melton *et al.*, 1984), adjacent to the SP6 promoter, using standard cloning procedures (Maniatis *et al.*, 1982). The fragment used to generate vector pTIMP-N (see figure 4.1) contained 821 bp of TIMP cDNA and the poly A tail, and was obtained by an *Nci*I/*Pvu*II restriction of 16C8b in pW7 (Edwards *et al.*, 1986). The fragment used to generate pTIMP-F was obtained by a partial *Fok*I restriction of the *Nci*I/*Pvu*II TIMP fragment. This fragment lacked the 5' 37 bp of TIMP cDNa sequence, including the ATG of the upstream ORF.

2.10.2 *In Vitro* Transcription

Gradient purified pTIMP-N and pTIMP-F were linearized by restriction with *Eco*RI, and purified by phenol extraction and ethanol precipitation. Five μg of linearized vector was added to a final volume of 50 μl of SP6 *in vitro* transcription cocktail as specified by the manufacturer (Promega), except that the final NTP concentrations were 2 mM each. To generate capped transcripts, 1 mM m^7G (5')ppp(5')G was added to the reaction mix, and the GTP reduced to 0.1 mM. A 3 μl aliquot of the complete reaction mixture was added to 10 μCi of dried [α^{32}P]UTP. After incubation at 40°C for 1 h, the transcripts were purified by phenol extraction and ethanol precipitation. The mRNA was dissolved in DEP-treated water to a final concentration of 100 ng/μl, dialysed against DEP-water on polycarbonate membranes (Millipore, VM 0.05 μm) for 30 minutes and stored at -20°C.

Aliquots of these RNA samples from each step in the purification were analyzed on a Northern blot to ensure that there was no loss or degradation of the RNA. The proportion of acid-insoluble cpm at the end of the transcription reaction determined the weight of RNA transcribed.

2.10.3 *In Vitro* Translation

For *in vitro* translation of TIMP, 100 ng of *in vitro* transcribed mRNA was treated with 2 mM CH_3HgOH for 2 min at 4°C to denature the RNA, added to a final volume of 25 µl of rabbit reticulocyte lysate (Promega; DuPont-NEN) containing 25 µCi of [^{35}S]methionine, and incubated at 30°C for 15 min, unless otherwise indicated. All samples were kept at 4°C before and immediately after the 30°C incubation. In samples to be glycosylated, 2.0 µl of dog pancreatic microsomes (Promega) were added to 25 µl of lysate.

To analyze the translation products, 2 µl of lysate was diluted to 10 µl in sample buffer, boiled for 2 min and electrophoresed on a 10% polyacrylamide SDS gel, and fluorographed (2.4). The TIMP bands on fluorograms exposed for various lengths of time were quantitated by laser densitometry (section 2.7.2).

To determine the [^{35}S]methionine incorporation a 2.0 µl aliquot of lysate was incubated in 0.1 ml of 1 M NaOH, 1.5% H_2O_2 at 37°C for 10 minutes. After the addition of 0.4 ml of 25% trichloroacetic acid/2% casamino acids the samples were incubated on ice for 30 minutes. The precipitates were collected on Whatman GF/C filters by vacuum filtration, washed with 2 ml of 5% trichloroacetic acid, 2 ml of ethanol, dried and analyzed by standard scintillation counting techniques.

2.11 *In Situ* Hybridization

2.11.1 Sample Preparation

Outbred CD-1 mice of timed gestation (the day of plugging is taken as d0) were sacrificed and the uterus containing the embryos was immediately placed in ice cold PBS containing 4% paraformaldehyde, 0.1% gluteraldehyde. The uterus and amniotic sac were perforated to allow the fixative to penetrate to the inner tissues. After fixing for 2 h, the samples were cryoprotected by immersing in 5% sucrose/PBS for 1 h, 10% sucrose/ PBS for 1 h and then in 18% sucrose/PBS ON, all at 4°C. Portions of the uterus containing an embryo were blotted dry, and oriented in OCT medium (Tissue-Tek) in a 1.5 ml Eppendorf tube (with the bottom cut off and sealed with parafilm) and flash frozen in isopentane cooled in liquid nitrogen The samples were stored at -70°C, and sectioned within one month.

The usual precautions to avoid RNAase contamination (2.2.1) were followed. Frozen sections, in the transverse plane, of 8-10 μm thickness were cut (Leitz Kryostat 1720) at -27°C. Adjacent sections were collected on glass slides for histological staining, and on chrome-alum coated slides (coated twice, by dipping in 0.5% gelatin / 0.5% chromium potassium sulfate) for *in situ* hybridizations. The sections were allowed to air dry, and the sections for *in situ* hybridizations were then post-fixed in 4% paraformaldehyde/PBS for 5 min at rt, washed 2 x 10 min in PBS, and dehydrated in a graded ethanol series (4 min in each of 70%, 90%, 95%, 100% ethanol, each containing 0.3 M sodium acetate), and stored at -70°C.

2.11.2 Probe preparation

Strand specific probes of ^{35}S-labelled RNA were generated from linearized vectors of pSP64 containing TIMP or MRP cDNA inserts, or of

pGEM3 containing spp or calcyclin cDNA inserts using SP6 polymerase (or T7 polymerase for the sense strand of calcyclin) according to the manufacturer's specifications (Promega). The vectors were linearized with the appropriate restriction enzymes adjacent to the insert/vector junction to allow transcription of the whole cDNA (591 bp *StuI*/*Acc*I TIMP fragment; 757 bp *Pst*I/*Hin*cII MRP fragment; *Sal*I/*Pvu*II bp calcyclin fragment), except for spp which was linearized at the *Pvu*II site in the middle of the cDNA to allow transcription of a 551 nucleotide sense strand, and a 445 nucleotide antisense strand. The quality of the transcripts was checked on Northern blots. [α-^{35}S]UTP (DuPont-NEN) was used to label the TIMP, MRP and spp transcripts. [α-^{35}S]GTP was used to label the calcyclin transcripts, since a poly U tract at the beginning of the calcyclin antisense sequence resulted in a high percentage of short (~100 nucleotides) transcripts which led to a high background. The probes were precipitated with ethanol to remove the unincorporated nucleotides. The specific activity of the probes, determined by trichloroacetic acid precipitation, was 1-2 x 10^9 cpm/μg. Probes were stored in 1 M DTT at -70°C for up to one month.

2.11.3 Hybridizations

The procedure was a modification of Higgins and Wilson (1987) obtained from C. Naus. Sections were rehydrated, incubated with 2 μg/ml proteinase K in 50 mM Tris·HCl pH 7.6, 5 mM EDTA for 10 min at rt, washed 2 x 10 min in PBS, fixed for 5 min in 4% paraformaldehyde, washed 2 x 10 min in PBS, dehydrated through a graded ethanol series (2.11.1) and air dried.

Sections were prehybridized in 50% formamide, 5X Denhardt's solution (0.1% each of Ficoll, polyvinylpyrrolidone and BSA), 25 mM PIPES pH 6.8

(Piperazine-N,N'-bis[2-ethanesulfonic acid]), 25 mM EDTA, 750 mM NaCl, 0.2% SDS, 40 mM DTT, 250 µg/ml denatured salmon sperm DNA and 250 µg/ml yeast tRNA for 2 h at 48°C in a humidified chamber. In addition the hybridization solution contained 10% dextran sulfate and 1-2 x 10^7 cpm/ml of specific probe. The sections were hybridized O/N at 48°C with 75 µl of hybridization solution, under a coverslip sealed with Lepage's plastic cement.

The slides were washed 2 x 5 min in 4X SSC, 10 mM ß-mercaptoethanol and 2 x 10 min in 4X SSC at rt. Non-specifically bound probe was degraded with 2 µg/ml RNAase A (in 10 mM Tris·HCl pH 8.0, 0.5 M NaCl and 1 mM EDTA) for 15 min at 37°C. The final washes were RNAase buffer for 30 min at 37°C, 2X SSC for 30 min at rt and 0.1X SSC for 30 min at 45°C.

These sections were counterstained with Harris's haematoxylin, coated with autoradiographic emulsion (Kodak NTB-2 diluted with an equal volume of water), and exposed for 5-75 days at 4°C in the presence of dessicant. The emulsion was developed for 2 min at 18°C in fresh D19 (Kodak) diluted 1:1 with water. The sections were examined and photographed with a Wild-Leitz photomicroscope, using Kodak technical Pan film 2415.

2.12 Tissue Samples

2.12.1 Time Course

Timed pregnancies were set up for outbred CD-1 mice (Charles River), the day of appearance of a vaginal plug being taken as day 0. Tissues were isolated at 2 pm on each day of pregnancy (see figure 5.1C). The organs were dissected and placed directly into vials in liquid nitrogen, or into ice cold MEM while being further dissected. At a gestational age of longer than 8 days, each of the amnion, placenta, all of the decidua and the uterus were isolated from the other tissues, and pooled. Up to day 8, the

ectoplacental cone in the gut region of the embryo was taken as 'placenta'. Total RNA was isolated as described (2.2.3).

2.12.2 Artificial Deciduomas

CD-1 female mice were rendered sterile in the left horn of the uterus by oviduct resection, while anaesthetized with avertin (2.5% v/v 2 methyl-2-butanol (Fisher), 1.25%w/v 2,2,2-tribromoethanol (Aldrich); 0.01 ml/g body weight). The mice were allowed to recover for two weeks before mating. To generate an artificial deciduoma, 10 µl of Planters peanut oil was injected into the lumen of the left horn on day 4.5, again under anaesthesia. Surgery was performed following the guidelines of the Canadian Council on Animal Care.

CHAPTER 3 - CLONING AND EXPRESSION OF TIMP AND MRP IN *E.coli*

3.1 INTRODUCTION

The starting point of my work was to isolate full length cDNA clones corresponding to growth regulated genes. Full length cDNAs allow a detailed analysis of the nucleotide and predicted polypeptide sequences, which can reveal similiarities to other genes as well as the nature of the gene product such as size, hydrophobicity, secretion and glycosylation signals. The cDNA can be used to over-express the protein product and generate lots of an otherwise scarce protein, to study its function, to study mutant protein forms, or to use as antigen to generate specific antisera. The cDNA sequence corresponding to the 5' end of the mRNA is also useful in locating the promoter region of the corresponding genomic clone.

Short cDNA clones lack 5' base pairs because of incomplete synthesis of the first strand of cDNA representing the 5' end of the template mRNA. The use of S1 nuclease to cleave the terminal hairpin, formed during synthesis of the second strand, also contributes to shortening of the cDNAs. Okayama and Berg (1982) devised a cloning strategy in which several steps differed from the classical method for generating cDNAs and resulted in the more efficient generation of full length cDNAs. This approach (see figure 3.1) involves a covalent joining of the cDNA to the vector primer, which is probably responsible for generating a high number of clones per μg of mRNA, and there is no S1 nuclease step. Furthermore, oligo[dC] tailing of the 3' end of the first strand of cDNA (at the 5' end of the mRNA) is a positive selection for full length reverse transcripts, since terminal transferase adds tails to blunt ends more efficiently than to 3' recessed ends (Roychoudhury *et al.*, 1976). Efficient synthesis of the

second strand of cDNA is primed from RNA oligonucleotides, generated by RNAase H digestion of the mRNA cDNA duplex, rather than by 'hairpin' formation of the first strand cDNA in the classical cloning strategy. This avoids alkali digestion of the template mRNA, and eliminates handling of single stranded DNA. Vector priming also leads to the cDNA being in a specific orientation in the plasmid, a feature which I have used in the work described below to ensure that cDNAs in the expression library were in the sense orientation.

There exist a plethora of vectors to express foreign genes in bacteria, yeast and in higher eukaryotic cells as well as animals. *E. coli* is attractive as a host for expression of foreign genes because of its economy and simplicity. Foreign proteins have been expressed at a high level, up to 30% of total bacterial protein on stained gels (Queen, 1983). Biologically functional adenovirus E1A protein was expressed in *E. coli* to about 5% of total protein (Ferguson *et al.*, 1984). Similiarly, human α_1-antitrypsin expressed up to 15% of the total cell protein, and displayed normal activity in the anti-elastase assay (Courtney *et al.*, 1984).

Expression of cDNA has also been used to identify specific clones in 'expression' libraries. For example, Young and Davis (1983) identified and isolated a yeast RNA polymerase II clone from a λgt11 expression library in *E. coli*. Specific antiserum was used to identify a tropomyosin cDNA in a chicken smooth muscle-pUC8 plasmid expression library (Helfman *et al.*, 1983).

I constructed a murine cDNA expression library in *E. coli* using the Okayama and Berg (1982) cloning strategy to orient the cDNA in the sense orientation behind the λP_R promoter and cro ATG translational start signal.

I then isolated four full length cDNAs encoding TIMP, MRP, calcyclin and MEP. Expression of the TIMP and MRP cDNAs in *E. coli* under optimized conditions resulted in low levels of the native TIMP and MRP proteins. TIMP and MRP were expressed at high levels, as fusion proteins with ß-galactosidase, which were subsequently purified and used to generate specific antisera.

3.2 RESULTS

3.2.1 Construction of cDNA libraries.

There were several growth regulated genes for which we wanted to isolate full length cDNA clones. One approach to finding cDNAs of interest was to use specific antibodies to screen cDNA expression libraries in *E. coli*. A mammalian cDNA cloned in the correct orientation and reading frame into a prokaryotic expression system will be expressed as the protein product under appropriate conditions. Two other features aimed for in the library were a) that there be full length cDNAs, and b) that low abundance mRNAs be represented.

I devised a cloning strategy that would have all three of these features (figure 3.1). The Okayama-Berg (1982) cloning strategy yields a high number of clones per µg of starting mRNA, and is efficient at making full length transcripts. The efficiency of this strategy is due to the first strand cDNA being covalently attached to the primer-vectorand that there is no S1 nuclease digestion. Further, the dC tailing of the cDNA selects for full length reverse transcripts since a blunt end is more efficiently recognized by terminal transferase than a 3' recessed end. The cDNA synthesized on the 'primer' is also in the correct orientation to be expressed

from the λP_R and cro ATG which is on the 'linker' fragment. The gene for the λcI857 ts repressor resides on the linker fragment which is isolated from a pCQV2 (Queen, 1983) derivative which had a *Kpn*I linker inserted in the *Bam*HI site. I chose to use this fragment as the linker because the repressor is not titrated out by a high copy number since it is encoded by the same plasmid. Expression from λP_R will be efficiently repressed until the cI857 is inactivated by incubation at 42°C. It is important that expression of the cDNA be regulatable in case the product is toxic to the host; constitutive expression at a high level would interfere with normal growth of the host. The final vector contains all of the information needed for regulatable expression and can be transfected into the *E. coli* host of choice such as a protease-deficient or high transformation efficiency strain.

Two cDNA libraries were constructed. Library 'G' was made using intact mRNA to be screened for specific full length cDNAs using ^{32}P-labelled nucleotide probes. Library E was to be screened with specific antisera to detect expressing clones containing the cDNA of interest, and was made using mRNA which had been treated with exonuclease II to remove sequences from the 5' region of the mRNA which may be deleterious to expression of the coding region in *E coli*. Figure 3.2 shows the extent of exonuclease II digestion of the treated mRNA prep compared to the intact mRNA. This shortening was reflected in the cDNA synthesized as seen in figure 3.3 which shows a size profile of the two libraries. A stained gel does not reveal the extent to which full length cDNAs were made in library G. The smear shows that cDNAs up to 1.5 kilobase pairs (kbp) in length were synthesized.

Figure 3.1 Construction of a cDNA expression library.

Ori, pBR322 origin of replication; Amp^R, ß-lactamase gene; P_R, λ rightward transcriptional promoter; c1857, temperature sensitive repressor of P_R. The primer is made from pBR322:SV40 0.71-0.86 (Berg A), and the linker from pCQV2-K. Homopolymer dT or dG tails are added at the unique *Kpn*I sites in each plasmid. The unwanted tails are removed by restriction enzyme cleavage, and the primer and linker isolated by gel electrophoresis. A molar excess of poly A^+ mRNA is annealed with dT-tailed primer. The primer efficiently primes first strand cDNA synthesis on the mRNA template by reverse transcriptase. Oligo(dC) tails are added to the 3' end of the cDNA. Less than full-length reverse transcripts are selected against at this step because of poor tailing of recessed 3' ends. Digestion with *Eco*RI produces a sticky end complementary to the non-tailed end of the linker. A 2-fold molar excess of linker is added to circularize the cDNA-containing molecule. The strong G-C base pairing joins the molecules which are then ligated at the *Eco*RI sticky ends. The RNA strand is replaced with DNA to improve the transformation efficiency. The polypeptide encoded by the cDNA will be expressed (at 42°C) as a fusion product with the six amino acids encoded by the sequence upstream of the G-C 'tails'. Only 1 in 3 of the cDNAs will be in the correct reading frame to code for a 'sense' protein.

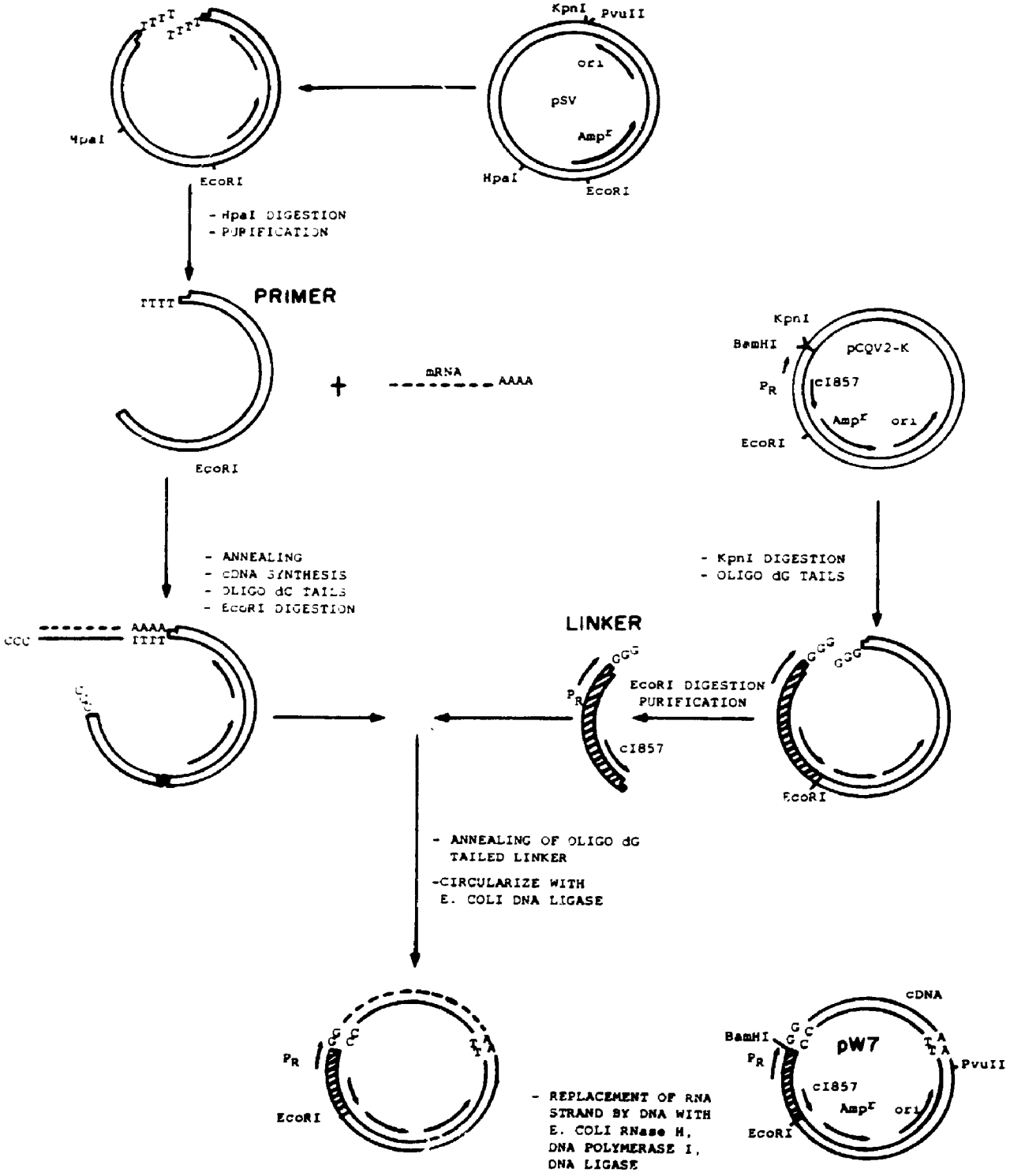

KpnI
PvuII
ori
pSV
Amp^r
HpaI
EcoRI
HpaI
EcoRI
- HpaI DIGESTION
- PURIFICATION
PRIMER
TTTT
EcoRI
mRNA
AAAA
KpnI
BamHI
pCQV2-K
P_R
cI857
Amp^r
ori
EcoRI
- ANNEALING
- cDNA SYNTHESIS
- OLIGO dC TAILS
- EcoRI DIGESTION
- KpnI DIGESTION
- OLIGO dG TAILS
LINKER
AAAA
TTTT
EcoRI DIGESTION
PURIFICATION
P_R
cI857
EcoRI
- ANNEALING OF OLIGO dG TAILED LINKER
-CIRCULARIZE WITH E. COLI DNA LIGASE
P_R
EcoRI
- REPLACEMENT OF RNA STRAND BY DNA WITH E. COLI RNase H, DNA POLYMERASE I, DNA LIGASE
cDNA
BamHI
pW7
P_R
cI857
Amp^r
ori
EcoRI
PvuII

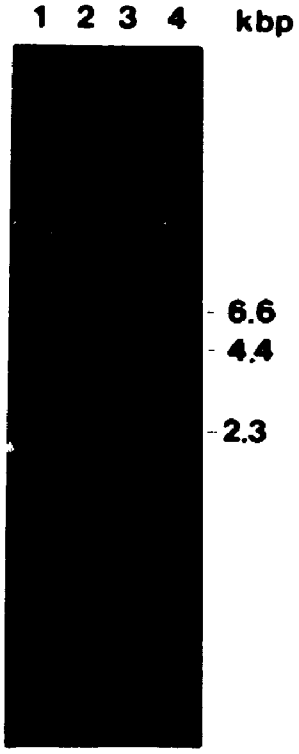
Figure 3.2 Analysis of mRNA used to generate the libraries.

Northern blot analysis of the mRNA samples used to generate cDNA in expression libraries E and G. Poly A^+ mRNA isolated from BNL cells was analysed before and after enzyme treatments to determine the extent of degradation. Untreated mRNA is in lane 3; mRNA treated with tobacco acid pyrophosphatase to remove the 5' cap, and calf intestinal phosphatase is in lane 2; mRNA subsequently digested with exonuclease II and used to make library E is in lane 1. The blot was probed with ^{32}P-labelled ß-actin cDNA. The extent of 5' shortening of the mRNAs is shown by the smear in lane 1.

Figure 3.3 cDNA profile of the two libraries.

Size analysis of the cDNA inserts in the cDNA expression libraries E and G. The lanes contain *Eco*RI restricted plasmid DNA from library E (1); library G (2); vector primer (3); and *Hin*dIII-λ molecular weight markers (4).


1 2 3


1 2 3 4
kbp
6.6
4.4
2.3

Libraries G and E were generated by transfecting the recombinant molecules (figure 4.1) into the high transformation efficiency *E. coli* strain MM294 using the Hanahan (1983) transfection protocol. A plasmid preparation of library E was also transfected into the *E. coli* strain JM103 for immunological screening. Low abundance mRNAs should be represented since libraries G and E in MM294 each contained more than 10^6 individual clones. The E sublibrary in JM103 contained about $2x10^5$ individual clones.

3.2.2 Isolation of full length cDNAs.

Full length cDNAs were being sought for the growth regulated genes 16C8 and 5B10, for which partial length cDNAs had been isolated earlier (Edwards *et al.*, 1985a) based on elevated levels of the cognate mRNA in mouse embryo fibroblasts following serum stimulation. During construction of libraries G and E, partial length cDNAs were isolated from a λgt11 expression library which corresponded to MEP (Denhardt *et al.*, 1986) and MRP (Parfett *et al.*, 1985). I used these partial length cDNAs to identify and isolate the corresponding full length cDNAs from library G.

To identify full length cDNA clones, library G was screened with ^{32}P-labelled nick translated cDNA inserts from the partial length clones. Colonies giving positive signals were picked , replated and rescreened until the clones picked were from well isolated colonies. Plasmid DNA containing the cDNA insert was purified and analysed on Southern blots (figures 3.4-3.7) to determine the size of the cDNA insert by comparison to molecular size markers. The size of the vector alone is 3.3 kbp. After positive identification of the cDNAs on Southern blots, more accurate size determinations were made by analysing the *Bam*HI-*Pvu*II released inserts on

ethidium bromide stained polyacrylamide gels (results not shown).

The cDNAs for 16C8 and MRP (figure 3.4) were full length and the longest cDNA for 5B10 was almost full length (lane 6 in figure 3.5). The estimated size for the TIMP cDNA was 950 bp, and TIMP mRNA migrates at 900 nucleotides. The MRP cDNA was sized at 1050 bp, and the mRNA is 950 nucleotides in length. The 5B10 cDNA was 500 bp in length, which is somewhat shorter than the 680 nucleotide long mRNA. The longest MEP cDNA (MEP-H; figure 3.6) isolated from library G appeared to be full length at first, but was later shown to contain an extra vector sequence at the 5' end of the cDNA which arose from an artifactual cloning event involving an *Eco*RI site in the MEP cDNA. A full length MEP cDNA clone (MEP-A; figure 3.7) was isolated from library CL22 (from J. Smith) which was constructed with the Okayama-Berg strategy but using a different linker (Kowalski *et al.*, 1985) using mRNA from JB6 cells treated with TPA.

Comparison of the nucleotide sequence of the full length cDNAs to sequences present in Genebank identified 16C8 as the murine homologue of human TIMP (tissue inhibitor of metalloproteinase) (Edwards *et al.*, 1986), and 5B10 as the murine homologue of human calcyclin (Guo *et al.*, 1989). The MEP protein had already been well characterized and the nucleotide sequence of the λgt11 MEP clone and of MEP-H revealed the similiarity between MEP and cysteine proteinases (Denhardt *et al.*, 1986).

These full length cDNAs were used in experiments described later in this thesis, and also in work published elsewhere. The MRP cDNA was used to express MRP polypeptide in *E. coli* (section 3.2.4), and the 5' part of the cDNA was used to identify a genomic clone containing the promoter and upstream region of one of the MRP genes (Conner *et al.*, 1989). The

Figure 3.4 Southern blot analysis of full length TIMP and MRP cDNAs.

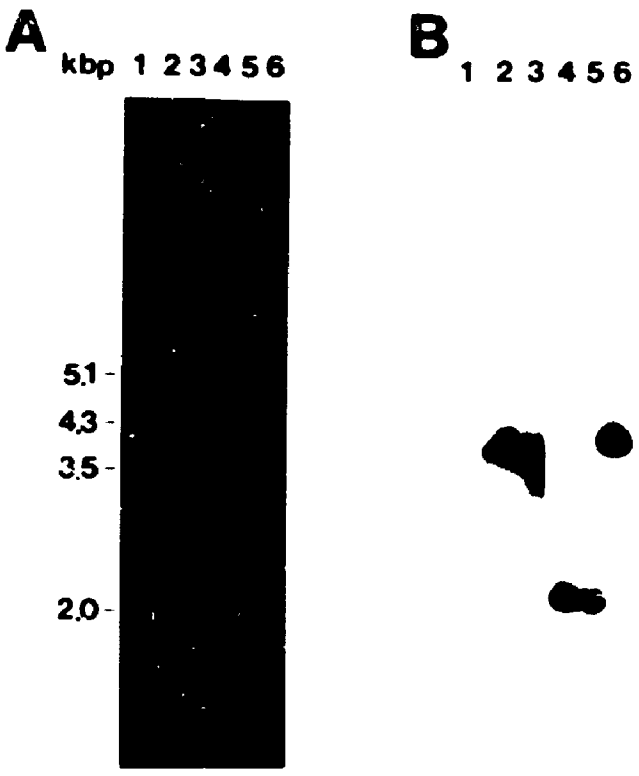
A. Plasmid DNA was restricted, electrophoresed (0.2 μg/lane) on a 0.8% agarose gel, and stained with ethidium bromide. The lanes contain MRP-21 restricted with *Hind*III (2) and MRP-31 restricted with *Hind*III (3); 16C8-B restricted with *Pst*I (4); *Pst*I and *Bam*HI (5); *Hind*III (6);. The molecular weight markers (1) are a *Hind*III - *Eco*RI restriction of λ DNA. There is a unique *Hin*dIII site in the vector, a unique *Bam*HI site in the vector adjacent to the 5' end of the cDNA. *Pst*I cuts once in the vector and once in the TIMP cDNA. Interpretation of these fragments is complicated by the presence of a second non-TIMP cDNA clone - the lower band in lane 6 and the middle band in lane 4 - which contaminated the original isolate.

B. A Southern blot of the gel was probed with ^{32}P-labelled cDNA inserts from existing partial length MRP (Parfett *et al.*, 1985) or 16C8 (Edwards *et al.*, 1985) cDNA clones. The lanes are as in panel A. The positive signals confirm the identity of the full length clones.

Figure 3.5 Southern blot analysis of 5B10 and 31H4 cDNAs.

A. Plasmid DNA was restricted with *Bam*HI, electrophoresed on a 0.8% agarose gel, and stained with ethidium bromide. The lanes contain *Hin*cII-λ markers (1); or 0.2 μg plasmid DNA from 5B10-1 (2); 5B10-2 (3); 5B10-3 (4); 5B10-4 (5); 5B10-5 (6); 5B10-6 (7); 5B10-7 (8) and 5B10-8 (9).

B. A Southern blot of the gel was probed with ^{32}P-labelled cDNA inserts from the original 5B10 clone (Edwards *et al.*, 1985). The lanes are as in panel A. The resulting autoradiogram shows that clones 5B10-1, -2, -4, -5 and -6 each contain a 5B10 cDNA insert.


A
kbp 1 2 3 4 5 6
5.1
4.3
3.5
2.0
B
1 2 3 4 5 6

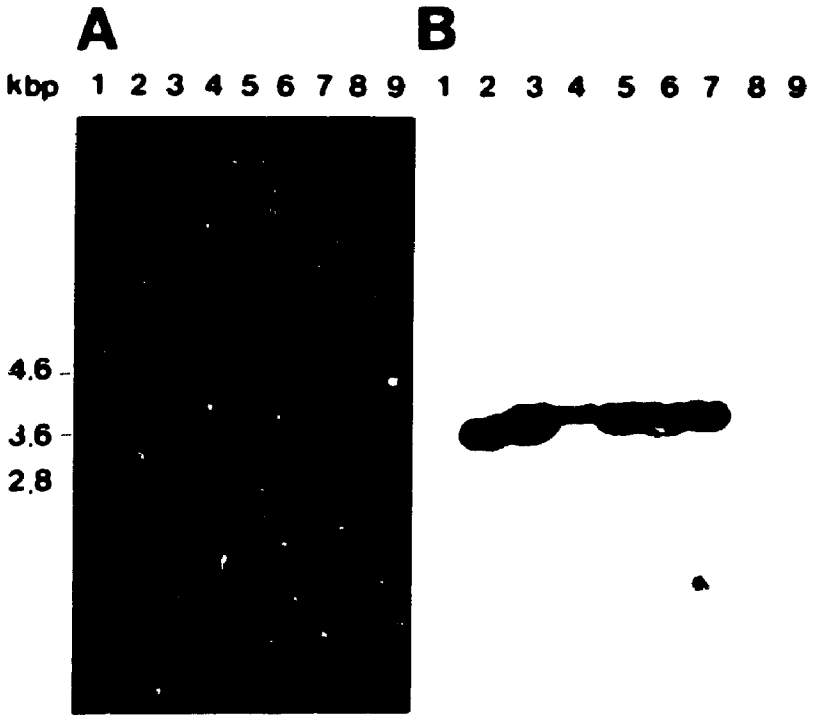

A
B
kbp 1 2 3 4 5 6 7 8 9 1 2 3 4 5 6 7 8 9
4.6
3.6
2.8

Figure 3.6 Southern blot analysis of an MEP cDNA.

A. Plasmid DNA of clone MEP-H, isolated from expression library G, was restricted and electrophoresed on a 0.8% agarose gel. The lanes contain *Hind*III-λ (1); MEP-H not restricted with enzyme (2); MEP restricted with *Eco*RV (3) or *Stu*I (4), which each have unique sites in the MEP cDNA and no sites in the vector.

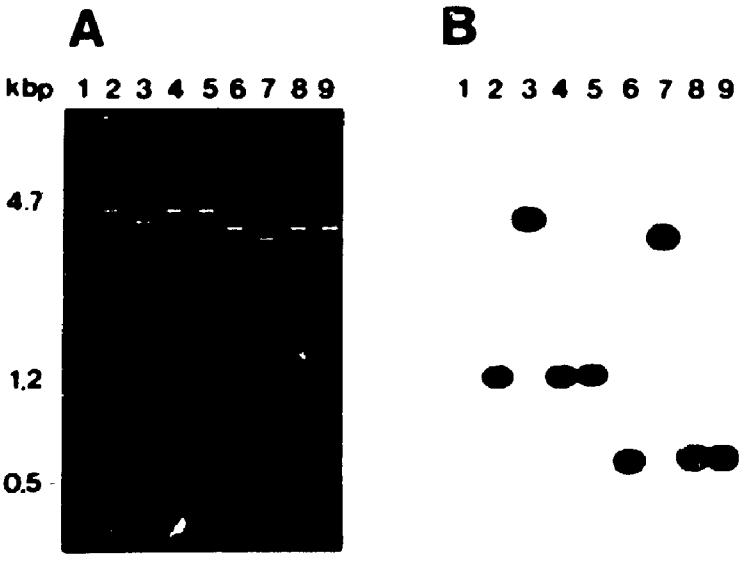
B. A Southern blot of the gel was probed with a ^{32}P-labelled cDNA insert obtained from a λgt11-MEP clone (Denhardt *et al.*, 1986).

Figure 3.7 Southern blot analysis of full length MEP cDNAs.

A. Plasmid DNA was restricted and 0.5 μg electrophoresed on 0.8% agarose gel, and stained with ethidium bromide. The lanes contain λ molecular weight markers (1); *Bam*HI restricted plasmid DNA from clones MEP-A (2); MEP-B (3); MEP-C (4); MEP-D (5); *Bam*HI-*Stu*I restricted plasmid from clones MEP-A (6); MEP-B (7); MEP-C (8); and MEP-D (9). Two *Bam*HI sites in the full length cDNA release a 1200 bp MEP fragment. This fragment is cut in half by *Stu*I which has one recognition site in the cDNA.

B. A Southern blot of the gel was probed with a ^{32}P-labelled MEP cDNA insert from nucleotide 0 to 496 in Denhardt *et al.* (1986), which corresponds to the 3' half of the full length MEP cDNA.


A
B
kbp 1 2 3 4
1 2 3 4
4.4
2.3


A
B
kbp 1 2 3 4 5 6 7 8 9
1 2 3 4 5 6 7 8 9
4.7
1.2
0.5

full length 16C8 (TIMP) cDNA was expressed in *E. coli* (section 3.2.5) and used to study a mechanism of regulating TIMP at the translational level (chapter 4). TIMP cDNA was also expressed in the antisense orientation to down-modulate TIMP expression in Swiss 3T3 cells, which revealed a role of TIMP as a tumor suppressor gene (Khokha *et al.*, 1989). I have used these full length cDNA clones of MEP, MRP, TIMP and calcyclin to study the expression of these genes in the uterus, decidua, placenta and amnion during pregnancy of CD1 mice (chapter 5).

3.2.3 Expression of cDNAs in 'expression' library E.

Several full length cDNA clones were isolated from library G, however, library E was not as useful as an 'expression' library. The E sublibrary in JM103 was screened with two polyclonal antisera specific for MRP and MEP (from M. Nilsen-Hamilton) and with an antiserum raised against total cell protein (TCP) of BNL cells which was intended to evaluate the expression library. Nitrocellulose filters containing about 3,000 colonies, which had been grown at 28°C and induced at 42°C for 2 hours, were screened immunologically as described. No MRP or MEP expressing clones were identified. Since the mRNA encoding MRP and MEP each represented more than 0.1% of the total mRNA present in serum-stimulated cells, screening of 70,000 colonies should ensure that MEP and MRP mRNAs were represented in the clones screened.

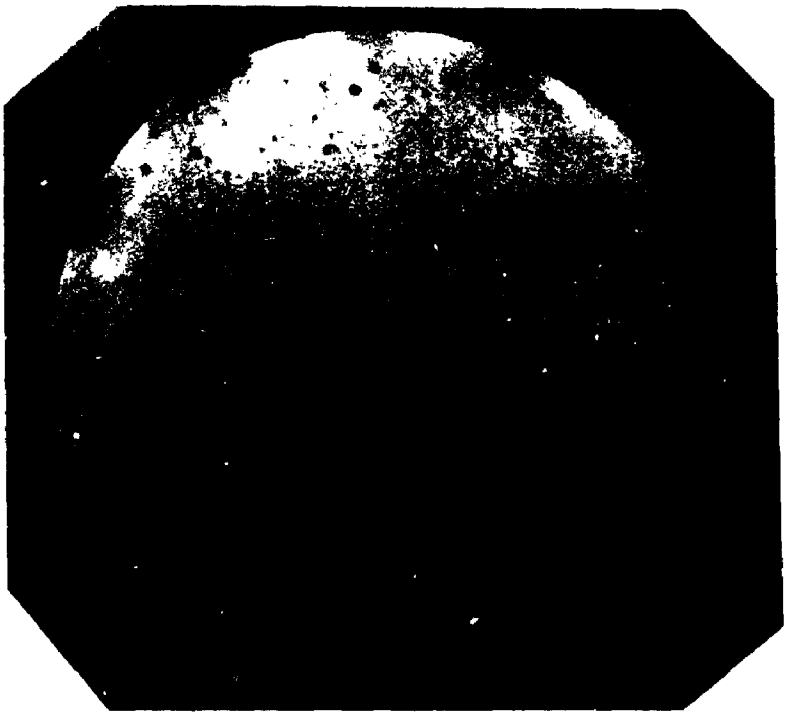
A rabbit antiserum raised against TCP was used to detect specific clones in the library that were expressing eukaryotic sequences. About 1/2000 clones had a positive signal. Figure 3.8 shows a second screening of one of the positive clones. A Western blot of 3 such clones shows the specific cDNA encoded proteins which are induced by incubation at 42°C

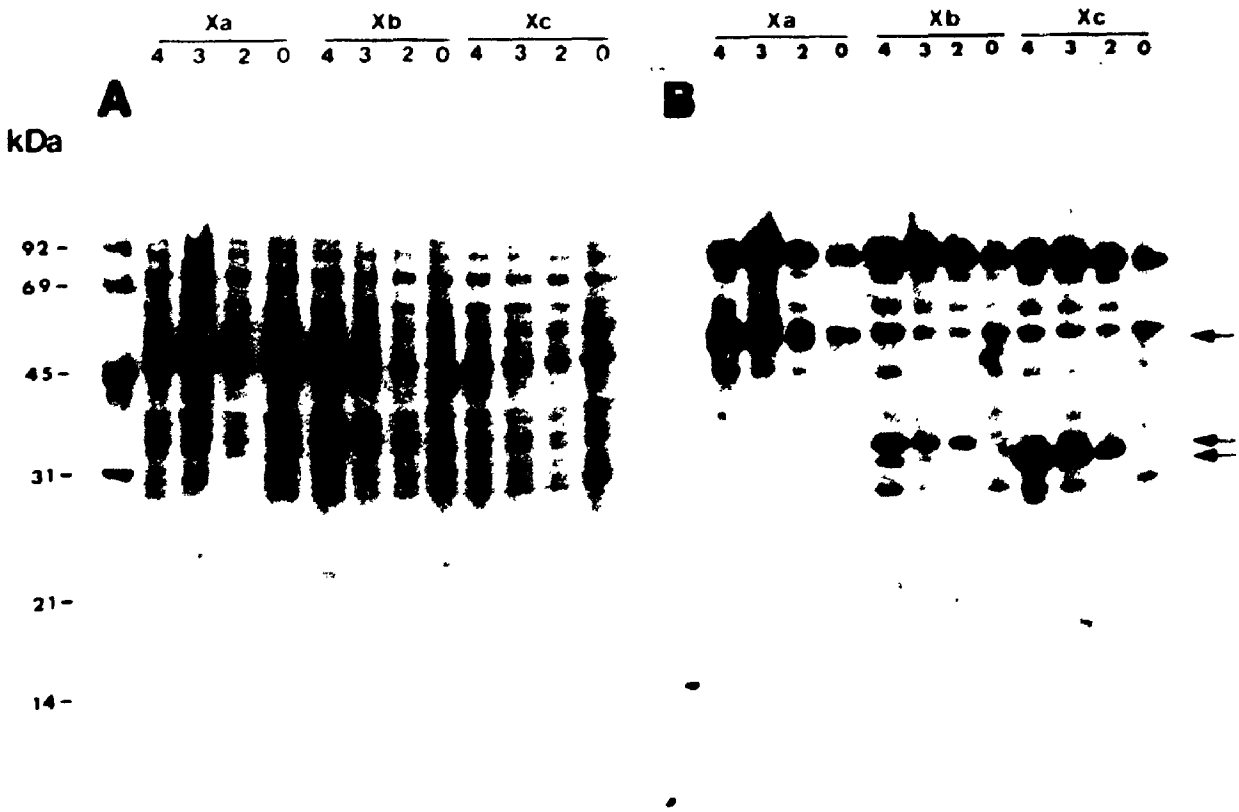
Figure 3.8 Immunodetection of expressing clones.

This is a second screening of a positive clone. Colonies were grown on nitrocellulose paper at 28°C, and then induced at 42°C for 3 h. Expressing clones were identified by probing sequentially with anti-TCP antibody, peroxidase coupled to goat anti rabbit-Ig, and then peroxidase substrate. The anti-TCP antiserum was raised in rabbits against BNL cell TCP and used to evaluate the expression library. The clone with the strong positive signal seen here is sample Xc in figure 3.9.

Figure 3.9 Western blot analysis of three clones expressing the cDNA protein product in *E. coli*.

Total protein extract of expressing *E. coli* clones grown at 28°C, and then induced at 42°C for 0, 2, 3 or 4 h were electrophoresed on 12% acrylamide-SDS gels and blotted to nitrocellulose paper. Panel A shows the TCP pattern of clones Xa, Xb and Xc on the blot stained with amido black. Panel B shows an autoradiogram of the blot. Specific proteins being expressed at 42°C were identified by incubating the blot sequentially with anti-TCP antisera and ^{125}I-protein A. The arrows indicate the cDNA specific proteins.




Xa
Xb
Xc
4 3 2 0 4 3 2 0 4 3 2 0
A
B
kDa
92-
69-
45-
31-
21-
14-

(figure 3.9B). None of these proteins were produced at a high enough level to be detected by staining the blot with amido black (figure 3.9A). The M_r of the proteins on the Western blot demonstrates that long cDNAs can be expressed in this expression system.

3.2.4 Expression of MRP in *E. coli*.

MRP was expressed in *E. coli* by engineering fragments of the MRP coding region directly behind λP_R and cro ATG (figure 3.10). The 5' end of the MRP cDNA was digested with Bal 31 exonuclease to generate a heterogenous population of 5' ends which were then ligated behind the λ expression cassette of pCQV2, and transfected into JM103. One third of the cDNAs are expected to be in the correct reading frame to express a sense peptide. Clones expressing MRP were identified by immunodetection of colonies after induction at 42°C for 2 hours. The positive clones were isolated, and the MRP specific proteins visualized on Western blots following induction of the host bacteria at 42°C (figure 3.11B). The largest MRP polypeptide produced in these clones had an M_r of 21,000 which is smaller than the 29,000 M_r full length polypeptide (Nilsen-Hamilton *et al.*, 1980). The cDNA encoded MRP polypeptides were not detectable on stained gels or on the stained blot (figure 3.11A). The level of MRP expression was increased about 2 fold by inserting a transcriptional terminator after the cDNA sequence, or by expressing the cDNA in the protease-deficient *E. coli* strain SG13069 (Gottesman and Gottesman, 1981), although the protein was still not detectable on a Coomassie stained gel (results not shown).

3.2.5 Expression of TIMP in *E. coli*.

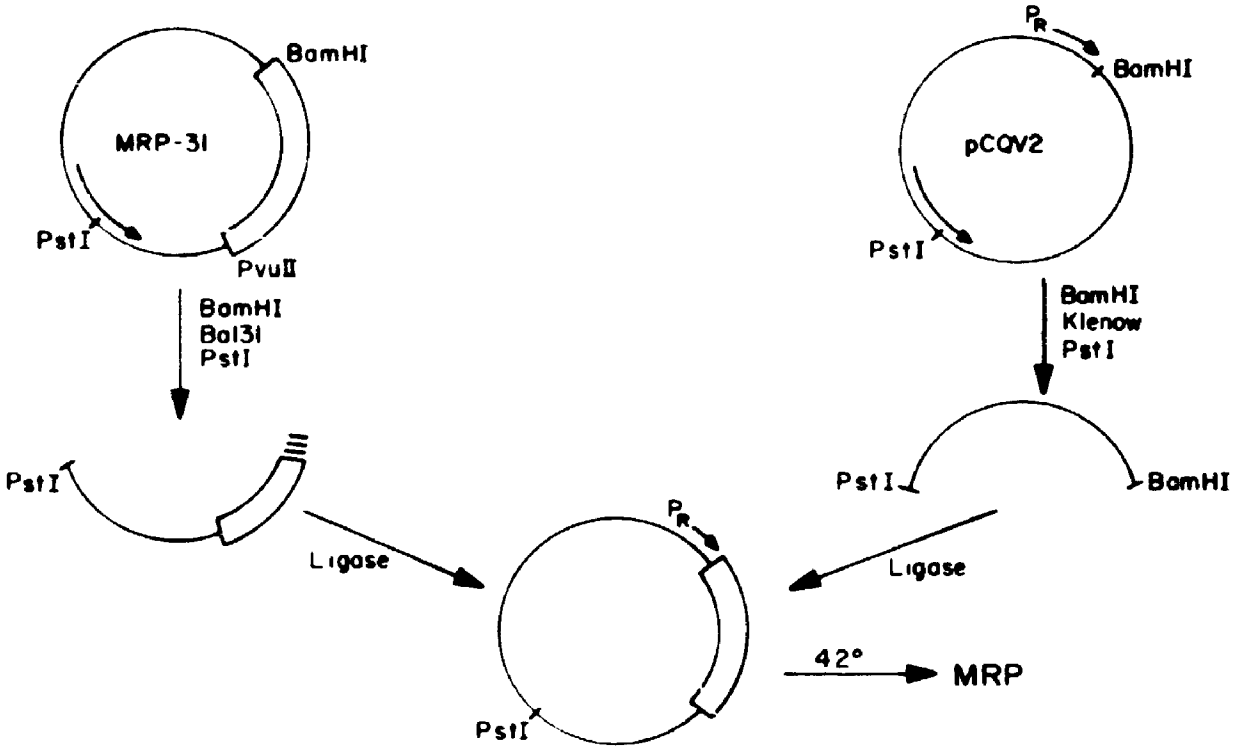
To over-express TIMP in *E. coli*, the cDNA sequence coding for the secreted portion of the protein was cloned into the *Eco*RI site of the pIN-

Figure 3.10 Engineering the expression of MRP in *E. coli*.

Restricted plasmid DNA with an exposed 5' end of MRP cDNA was incubated with nuclease *Bal*31 and then *Pst*I to generate a set of fragments with a heterogeneous population of 5' ends that were then ligated downstream of the pCQV2 transcriptional promoter and ATG start site. The ligated DNA was transformed into *E. coli* and the resulting clones screened immunologically for MRP synthesis at 42°C.

Figure 3.11 Western blot analysis of MRP expressing clones.

Control (pCQV2 alone) and MRP expressing clones A to G were grown at 28°C, and then induced at 42° for 0, 2, 3, or 4 h as designated, electrophoresed on 12% polyacrylamide-SDS gels and blotted to nitrocellulose paper. Panel A shows a Coomassie blue stained gel. Panel B shows the same seven clones on an autoradiogram of a Western blot which was probed sequentially with rabbit anti-MRP and ^{125}I-protein A. The arrows indicate the position of the MRP-specific polypeptides. The 14 kDa band giving a strong signal on the autoradiogram is lysozyme, which was used to lyse the cells before electrophoresis. ^{125}I-protein A binds to lysozyme.


BamHI
MRP-31
PstI
PvuII
BamHI
Bal31
PstI
PstI
Ligase
PR
PstI
42°
MRP
PR
BamHI
pCQV2
PstI
BamHI
Klenow
PstI
PstI
BamHI
Ligase

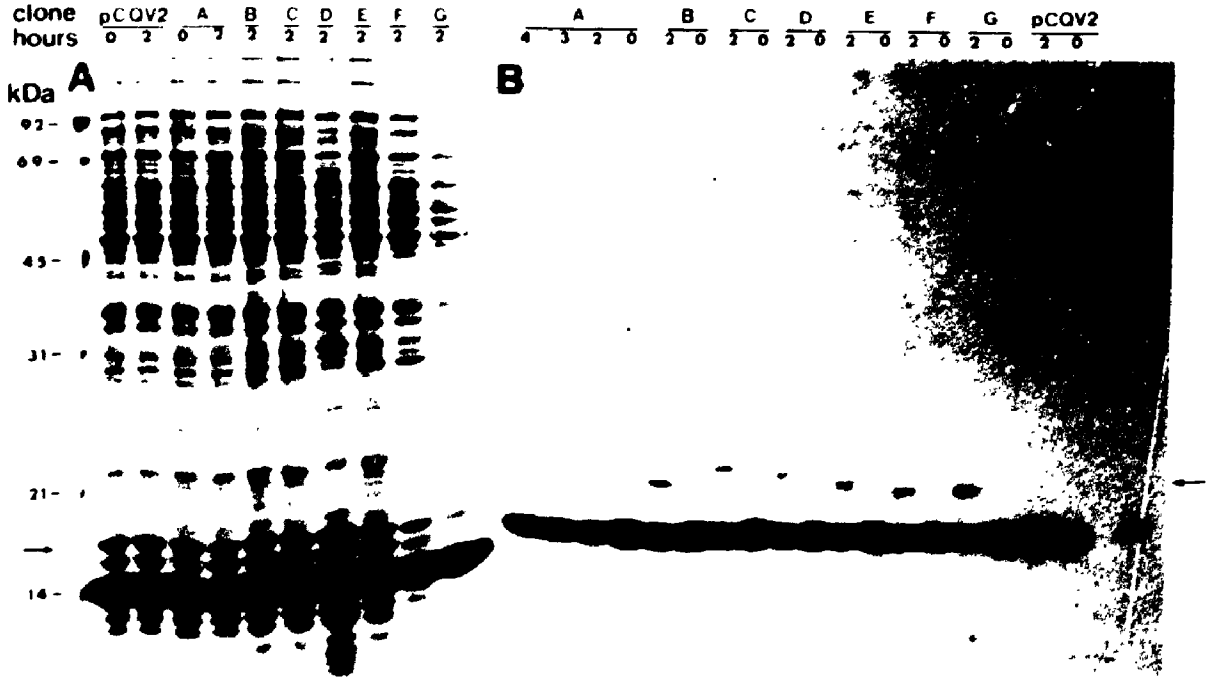

clone
hours
pCQV2
A
B
C
D
E
F
G
kDa
A
92-
69-
45-
31-
21-
14-
B
A
B
C
D
E
F
G
pCQV2

Figure 3.12 Construction of a TIMP secretion vector.

The 591-bp *Stu*I-*Acc*I fragment of TIMP cDNA encoding the secreted portion of TIMP was ligated into the *Eco*RI(blunted)-*Bam*HI sites of pIN-III-ompA$_2$, and transformed into *E. coli* JA221 which carries the lacI^Q repressor. Induction of the lpp promoter with IPTG directs the synthesis and secretion of 'mature' TIMP polypeptide with two extra amino acids (AlaPro) at the amino terminus. The arrowhead indicates where the signal sequence is cleaved.

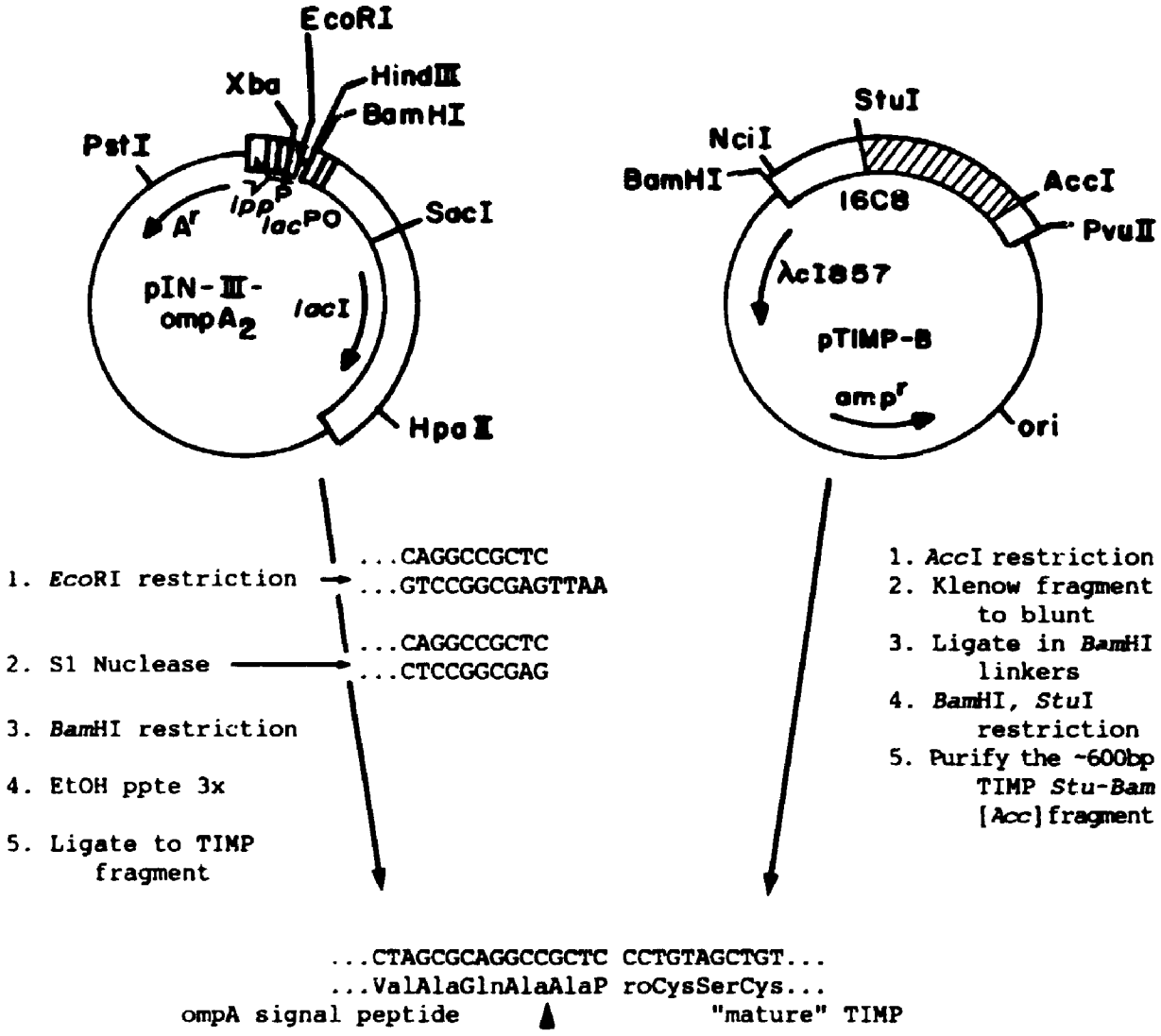
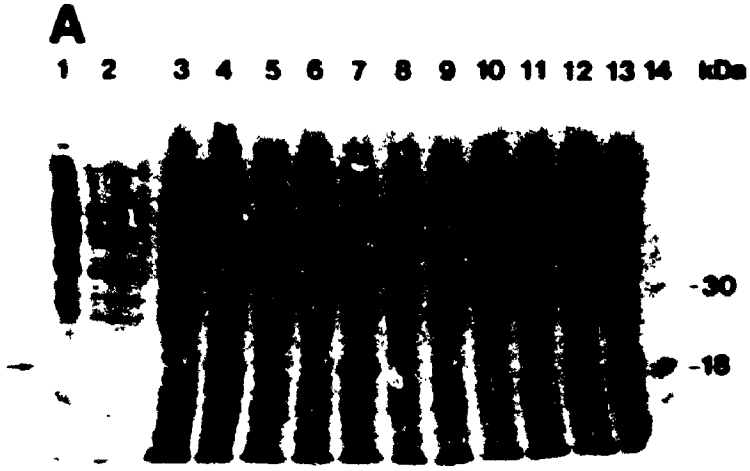
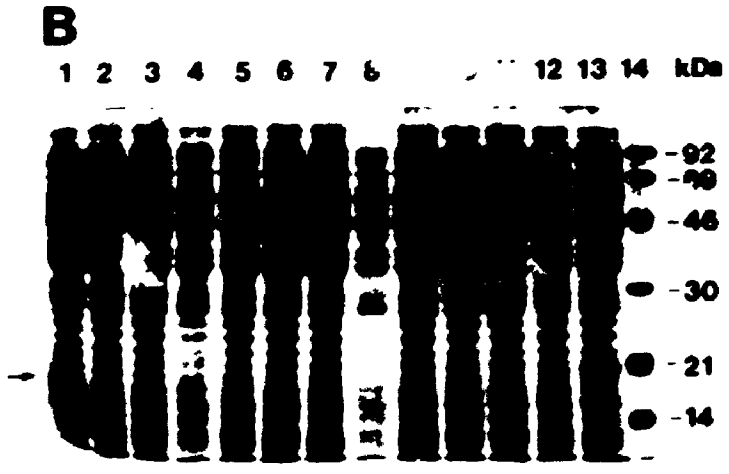

EcoRI
Xba
HindIII
BamHI
PstI
SacI
Ar
lpp
lacPO
pIN-III-
ompA2
lacI
HpaII
StuI
NciI
BamHI
AccI
16C8
PvuII
λcI857
pTIMP-8
ampr
ori
1. EcoRI restriction
...CAGGCCGCTC
...GTCCGGCGAGTTAA
2. S1 Nuclease
...CAGGCCGCTC
...CTCCGGCGAG
3. BamHI restriction
4. EtOH ppte 3x
5. Ligate to TIMP fragment
1. AccI restriction
2. Klenow fragment to blunt
3. Ligate in BamHI linkers
4. BamHI, StuI restriction
5. Purify the ~600bp TIMP Stu-Bam [Acc] fragment
...CTAGCGCAGGCCGCTC CCTGTAGCTGT...
...ValAlaGlnAlaAlaP roCysSerCys...
ompA signal peptide
"mature" TIMP

Figure 3.13 Synthesis of TIMP in JA-221.

Clones containing the $ompA_2$-TIMP secretion vector were identified by screening with a ^{32}P-labelled cDNA probe, isolated, grown to a density of 2 x 10^5/ml and induced with 2 mM IPTG. Uninduced samples were grown for the same length of time without IPTG. **A.** After 1 h induction in minimal media, cells were labelled with [^{35}S]methionine for 15 minutes. Cell pellets were resuspended in Laemli buffer, boiled, electrophoresed on a 10% polyacrylamide gel and processed for autoradiography. The uninduced samples are clone 1 in lane 2; clone 6 in lane 8; and positive control A3-98 which is Staphylococcal nuclease A protein (from Takahara *et al.*, 1985) in lane 12. The induced samples are clone 1 in lane 1; clone 2 in lane 3; clone 3 in lane 4; clone 4 in lane 5; clone 5 in lane 6; clone 6 in lane 7, clone 7 in lane 9; clone 8 in lane 10; $ompA_2$ vector in lane 11; A3-98 positive control in lane 13; and molecular size markers in lane 14. The arrow indicates the position of the pulse-labelled TIMP in lanes 4 and 7.

B. After 2 h of induction in rich medium, cells were boiled in Leamli buffer, electrophoresed on a 10% polyacrylamide-SDS gel and stained with Coomassie blue. The samples are the same as for panel A. The arrow indicates the M_r · the TIMP specific band seen in panel A.


A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 kDa
30
18


B
1 2 3 4 5 6 7 8 12 13 14 kDa
92
66
46
30
21
14

III-ompA$_2$ *E. coli* secretion vector (figure 3.12). The strategy was that the ompA signal sequence would direct the mature TIMP polypeptide to the periplasmic space where it would be protected from cytoplasmic proteases, and would also facilitate purification.

Clones of JA221 carrying the TIMP secretion vector were screened for synthesis of the TIMP protein (figure 3.13). [^{35}S]methionine labelling of IPTG-induced cells showed that two of the clones were synthesizing a polypeptide with the same M_r as mature unglycosylated TIMP (lanes 4 and 7 in figure 3.13A). These TIMP polypeptides were not detectable, however, on a Coomassie stained gel when the same clones were induced with IPTG in rich medium (figure 3.13B). The staphylococcal nuclease A protein produced by clone A3-98 (from Takahara *et al.*, 1985) is visible on the stained gel (lane 13 in figure 3.13B). The JA221-TIMP clones were not synthesizing sufficient TIMP to permit purification for biochemical analysis, or to use as an antigen to generate TIMP-specific antisera.

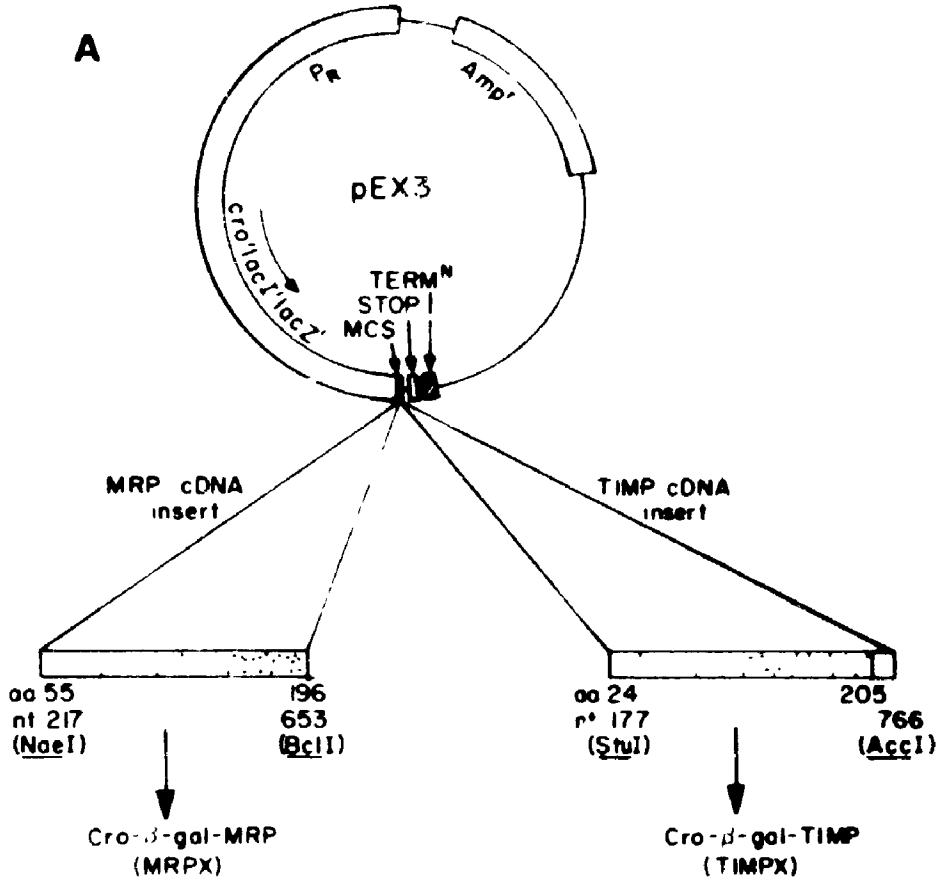
3.2.6 Production of TIMP and MRP fusion proteins.

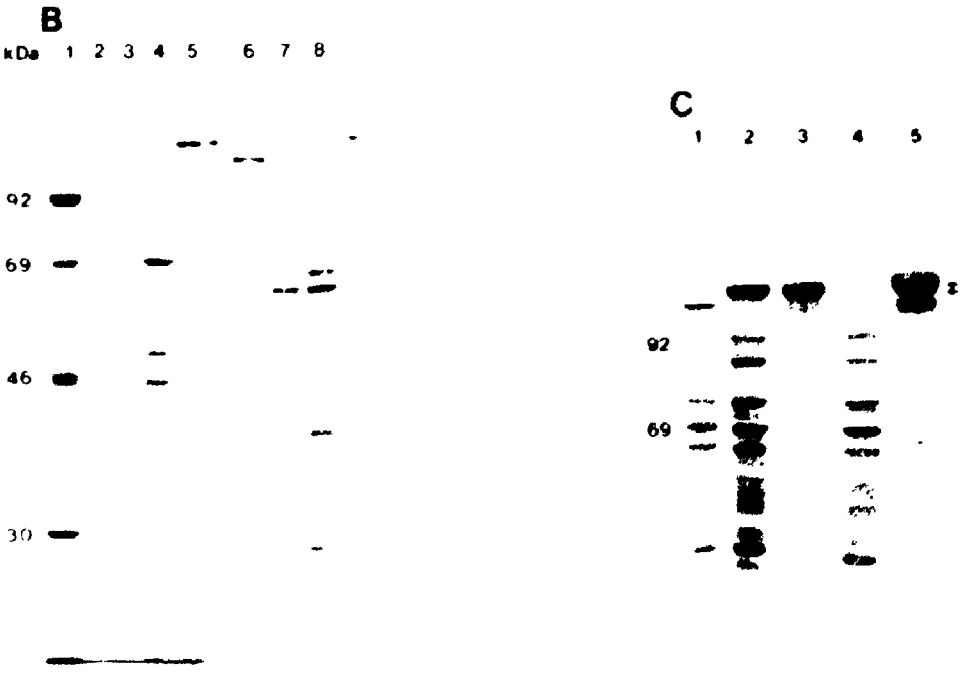
One of the purposes of expressing specific proteins in *E. coli* was to isolate enough protein, free of other contaminating mammalian proteins, to generate specific antisera. A second approach to achieve this aim was to express them as fusion proteins with ß-galactosidase using the pEX vector system (Stanley and Luzio, 1984). Expression of the cDNA as a fusion protein increases the stability of the cDNA encoded polypeptide in *E. coli* and facilitates purification. Regions of the TIMP and MRP coding sequence were engineered into pEX3 in the correct reading frame for expression of the cDNA portions as a fusion protein at the carboxyl terminus of ß-galactosidase (figure 3.14A). The MRPX and TIMPX constructs were

Figure 3.14 Production of cro-ß-galactosidase-TIMP and cro-ß-galactosidase-MRP fusion proteins.

A. Construction of the fusion protein vectors. cDNA fragments containing coding sequence were cloned into the multiple cloning site of pEX3 vectors. The *Stu*I-*Acc*I fragment of TIMP cDNA was cloned into the *Bam*HI site of the pEX3 multiple cloning site (MCS). The *Nae*I-*Bcl*I fragment of MRP cDNA was cloned into the *Sma*I site of pEX3. Expression from the λ P_R and cro ATG generates a fusion protein with the TIMP or MRP sequence at the carboxyl terminus. The vector contains stop codons in all 3 reading frames (STOP) and a transcription termination signal ($TERM^N$) to ensure efficient synthesis of a discrete protein product.

B. Induction of fusion protein synthesis. *E. coli* NF1 carrying either pEX3, the pTIMPX or pMRPX fusion constructs were grown at 28°C (repressive temperature), and then induced at 42°C to allow expression of the fusion proteins. TCP was electrophoresed on an 8% polyacrylamide SDS gel, and the gels were stained with Coomassie blue. The samples are molecular weight markers in lane 1; pEX3 grown at 30°C in lane 2; pEX3 grown at 42°C in lanes 3 and 6; MRPX grown at 30°C in lane 4; MRPX grown at 42°C in lane 5; TIMPX grown at 30°C in lane 7; and TIMPX grown at 42°C in lane 8. **C.** Purification of the fusion proteins. Total cell protein (TCP) extracts and purified fusion proteins (PFP), were electrophoresed as in Fig. 4.1B to determine the extent of purification. Lane 1 is pEX3 TCP; lane 2 is MRPX TCP; lane 3 is MRPX PFP; lane 4 is TIMPX TCP; and lane 5 is TIMPX PFP. The arrows show TIMPX and MRPX fusion proteins.


A
Pr
Amp'
pEX3
cro'lacI'lacZ'
TERM^N
STOP
MCS
MRP cDNA
insert
TIMP cDNA
insert
aa 55
nt 217
(NaeI)
196
653
(BclI)
aa 24
nt 177
(StuI)
205
766
(AccI)
Cro-β-gal-MRP
(MRPX)
Cro-β-gal-TIMP
(TIMPX)


B
kDa 1 2 3 4 5 6 7 8
92
69
46
30
C
1 2 3 4 5
92
69

transfected into *E. coli* strain NF1 which carries the lambda cI857 ts repressor to regulate expression of the fusion protein. The fusion proteins were expressed by incubating the cells at 42°C, and the cell lysates analysed on SDS polyacrylamide gels (figure 3.14B). Both the TIMPX and MRPX fusion proteins had a higher M_r than ß-galactosidase, and represented a significant portion of the total cell protein. The fusion proteins were purified by lysing and solubilizing the induced cells, and recovering the insoluble fusion proteins by centrifugation. The extent of purification was checked on SDS polyacrylamide gels (figure 3.14C).

3.2.7 Immunoprecipitation of MRP and TIMP.

MRP- and TIMP-specific antisera generated by injecting the fusion proteins into New Zealand white rabbits were used to immunoprecipitate MRP from [^{35}S]methionine-labelled cell culture media (figure 3.15A), and *in vitro* translated TIMP from reticulocyte lysates (figure 3.15B). The MRPX antiserum effectively immunoprecipitated glycosylated MRP. The immunoprecipitation was more efficient when the ß-galactosidase antibodies had been absorbed out with a ß-galactosidase affinity column. The antiserum to MRPX also did not immunoprecipitate a high molecular weight protein that was precipitated by antiserum raised to glycosylated MRP from tissue culture cells, presumably an unrelated species which had contaminated the MRP preparation used as antigen.

The anti-TIMPX antiserum immunoprecipitated both the non-glycosylated and glycosylated forms of TIMP synthesized *in vitro* (figure 3.15B), however the efficiency was low. The antisera immunoprecipitated less than 10% of the TIMP in the reticulocyte lysate, and did not detect TIMP secreted by Swiss 3T3 cells, either by immunoprecipitation of

Figure 3.15 Immunoprecipitation of MRP and TIMP.

The figures are autoradiograms of 12% polyacrylamide-SDS gels on which were electrophoresed samples of MRP (A) and TIMP (B) both before and after immunoprecipitation. **A.** [^{35}S]methionine-labelled conditioned medum from BNL cells is in lane 1; the immunoprecipitates from conditioned media are in lanes 2-4; using antisera raised against MRP purified from tissue culture media (from M. Nilsen-Hamilton) is in lane 2; antisera to MRPX is in lane 3; and antisera to MRPX which was preadsorbed to remove anti ß-galactosidase antibodies is in lane 4. **B.** [^{35}S]methionine-labelled *in vitro* translation products in reticulocyte lysate with (lanes 4-7) or without (lanes 1-3) dog pancreatic microsomes. Reticulocyte lysate (2 µl) incubated with TIMP mRNA is in lane 1; the immunoprecipitate (50 µl) using anti-TIMPX antisera is in lane 2; and using preimmune sera is in lane 3. Reticulosyte lysate without TIMP mRNA, with dog pancreatic microsomes to process the *in vitro* translation products is in lane 4; and the immunoprecipitate using anti TIMPX antisera is in lane 5; lysate with TIMP mRNA is in lane 6; and the immunoprecipitate using anti-TIMPX antisera is in lane 7. The anti-TIMP antisera specifically recognizes both nonglycosylated and glycosylated forms of TIMP (arrows), although the efficiency of immunoprecipitation is poor.


A
1 2 3 4 kDa
46
30
18
B
1 2 3 4 5 6 7 kDa
-46
-30
-18

[^{35}S]methionine-labelled cell culture medium, or by Western blot analysis of the medium or amniotic fluid.

3.3 DISCUSSION

Full length cDNA clones for 16C8 and MRP were identified in library G by screening with nucleotide probes. Sequencing of the cDNAs revealed that 16C8 was murine TIMP (Edwards *et al.*, 1986) and 5B10 (lacking ~150 bp of 5' untranslated region) was murine calcyclin (Guo *et al.*, 1989). The longest MEP cDNA isolated from library G was missing sequence 5' to an *Eco*RI site, presumably because that sequence in the RNA/DNA duplex was cleaved during the cloning procedure (Fig. 3.1). A full length MEP cDNA was isolated from a CL22/TPA treated library (from J. Smith) which was generated using a *Hind*III instead of an *Eco*RI restriction in the Okayama-Berg strategy (Kowalski *et al.*, 1985). The sequence of the TIMP cDNA is identical to the genomic sequence, except for the 5' most 27 bp of the cDNA which differ from the genomic sequence (Edwards *et al.*, 1986; Gewart *et al.*, 1987). This sequence may be part of a transcript from an as yet uncharacterized promoter further upstream, which is then spliced to produce the transcript that was cloned here. Alternatively, it may be derived from an anomolous cloning event, at the 5' end of the cDNA. The sequence was not found in genebank.

Specific clones were not identified in the expression library E by immunodetection, even though expression of specific cDNA encoded proteins was detected with the anti-TCP antibody (Fig. 3.8). The signal (intensity) of the native MRP band on the Western blot (Fig. 3.11) was much less than the signal seen for the proteins identified by the TCP antibody (Fig. 3.9).

In addition, these MRP-expressing clones were generated by ligating the MRP coding sequence directly downstream of the λP_R promoter (Fig. 3.10), without the intervening dG tails used in the Okayama-Berg strategy. The dG tail will code for several glycine residues in a row, and since the tRNA which recognizes GGG represents only 10% of the Gly-tRNAs (Ikemura, 1981), this region can be expected to further reduce the level of expression of MRP in the expression library.

MRP synthesis was detectable in clones that had the coding region ligated directly beside the λP_R and *cro* ATG. The rgest native MRP polypeptide expressed by one of these clones had a M_r of 21,000 (Fig. 3.11B) which is the same size as the polypeptide backbone of the mature secreted protein (Nilsen-Hamilton *et al.*, 1980). The failure to detect longer proteins may be due to the signal sequence interfering with efficient translation in *E. coli*. In addition, a full length cDNA would not be expressed since there is an in-frame TAG five codons upstream of the start site (Linzer *et al.*, 1985).

A much higher level of MRP expression would be necessary if it was to be isolated and purified for any biochemical or functional studies. The two fold increase in MRP level in a *lon*$^-$ *htpR*- strain, or after introducing a transcriptional terminator (results not shown) suggest that other protease systems (Maurizi *et al.*, 1985) were involved in degrading the murine protein. It could well be that other proteins with more inherent stability in *E. coli* would have been detected in this library.

The eukaryotic proteins Myc (Watt *et al.*, 1985), adenovirus E1A (Ferguson *et al.*, 1984), p53 (Queen, 1983) and bovine α_1-antitrypsin

(Courtney *et al.*, 1984) to name a few are stable enough to be expressed at high levels in bacteria. Somatomedin C was expressed in *E. coli* and the accumulated protein increased from undetectable in wild type, up to 5% of TCP in a *lon*$^-$ *htpR*$^-$ strain (Buell *et al.*, 1985). The stability of some eukaryotic proteins and not others, while not completely understood, is not a surprise.

The first step in studying mutated forms of TIMP, towards elucidating its mechanism of action, was to over-express TIMP in *E. coli* to an extent that it could be easily purified. The strategy was to use a secretion vector to transport the native TIMP into the periplasmic space where it would be protected from the cytoplasmic proteases.

The level of [^{35}S]methionine incorporation into TIMP compared to staphylococcal nuclease A (Fig. 3.13A lanes 4, 7 and 13) suggested that TIMP was being efficiently synthesized. Nuclease A produced in this system represented 10% of TCP (Takahara *et al.*, 1985). Unlike the nuclease A, however, the TIMP band was not detectable on a Coomassie stained gel (Fig. 3.13B lanes 4, 7 and 13). Enrichment of proteins in the periplasmic space, or longer induction times did not improve the yield of TIMP (results not shown). Since the level of [^{35}S]methionine incorporated into TIMP during the pulse labelling indicates that the protein was being efficiently synthesized, the problem was likely an instability of the protein. Although the protein was sequestered in the periplasmic space, other protease systems would be encountered there. Other mammalian proteins were shown to accumulate to a higher level in mutant hosts which are deficient in these proteases The N-terminal amino acid can also have a stabilizing or destabilizing effect on the proteins in prokaryotes (Bachmair *et al.*, 1986).

This may not be the case for TIMP as N-terminal alanine, predicted for the processed TIMP (figure 3.12), is associated with the most stable proteins in bacteria and is therefore not likely to be responsible for the rapid degradation of TIMP.

Native TIMP and MRP were not overproduced sufficiently to allow purification and subsequent biochemical characterization. Synthesis of these sequences as fusion proteins with ß-galactosidase allowed overproduction and purification of enough protein to generate antisera that specifically recognized the murine proteins. The eukaryotic sequences were likely protected here because the fusion protein formed an insoluble precipitate (Stanly and Luzio, 1984) which also facilitated purification.

Both the MRP- and TIMP- specific antisera recognized the murine proteins. The MRP antiserum raised to the fusion protein immunoprecipitated glycosylated MRP secreted by tissue culture cells as efficiently as the antiserum raised against purified murine MRP (figure 3.15A). The anti-TIMP antiserum recognized both non-glycosylated and glycosylated TIMP although the antibodies were either low affinity or low titer since a vast excess of antisera immunoprecipitated only 5-10% of the *in vitro* translated TIMP (figure 3.15B, lane 1 and 2). The antiserum was able to detect the *in vitro* translated TIMP since it has a very high specific activity. The usefulness of a specific antibody is to study the protein distribution *in vivo*. Unfortunately, this antiserum did not immunoprecipitate detectable amounts of TIMP from the medium of ^{35}S-labelled tissue culture cells overproducing TIMP (line 16S1, from R. Khokha), nor did it detect TIMP on Western blots of concentrated medium from the overproducing cells (results not shown).

The reason for a poor antigenic response to TIMP in the rabbits (I had the same result with antisera made in guinea pigs; data not shown) may be due to a high level of conservation between the species. As well as the similiarity between mouse and human TIMP at the sequence level, TIMP effectively inhibits MPPs from other species. Rabbit bone TIMP inhibits human fibroblast collagenase and gelatinase (Murphy *et al.*, 1982); human fibroblast TIMP inhibits rabbit collagenase (Murphy *et al.*, 1985b); human recombinant TIMP inhibits mouse collagenase (Thomson *et al.*, 1987); and both mouse and human fibroblast TIMP inhibit porcine collagenase (Overall *et al.*, 1989). The antiserum specific for human TIMP (which does not recognize murine TIMP) was raised in sheep (Hembry *et al.*, 1985). Perhaps TIMP is less conserved between mouse and goat or sheep, and the murine antigen would have elicited a better response in one of those animals.

CHAPTER 4 - REGULATION OF TIMP AT THE TRANSLATIONAL LEVEL

4.1 INTRODUCTION

A gene can be regulated at several steps during expression, including transcriptional and post-transcriptional, translational and post-translational levels. Each of these levels may play a part in the overall regulation of TIMP expression. The focus of this chapter is the regulation of TIMP expression at the translational level.

The co-ordinate expression of TIMP and MMPs is important in physiological processes such as wound healing and tissue remodeling, and in pathological conditions such as arthritis and metastasis. TIMP and collagenase were both localized by immunofluorescence to the area of scar tissue that was judged to be actively remodeling (Hembry and Ehrlich, 1986). Chowcat *et al.* (1988) also showed that collagenase and TIMP were localized to the suture line following surgery, and furthermore that each followed a specific time course of appearance during wound healing. Elevated levels of MMPs, without a corresponding increase of TIMP, in arthritic cartilage are likely responsible for the associated tissue destruction (McGuire *et al.*, 1981). Antirheumatic drugs such as dexamethasone exert their effect, at least in part, through increased TIMP levels (Hunter *et al.*, 1984). A 50% reduction in TIMP levels conferred oncogenic and metastatic propensity on 'normal' Swiss 3T3 cells (Khokha *et al.*, 1989), demonstrating again that small changes in the MMP/TIMP ratio can have a dramatic effect.

Expression of TIMP mRNA in murine fibroblasts is induced by serum, PDGF, FGF, TPA and double-stranded RNA (Edwards *et al.*, 1985a; Gewart *et al.*, 1986; Denhardt *et al.*, 1987). The level of induction by these agents is at least 10-fold, whereas the *in vivo* changes noted above are on

the order of two-fold. Multiple species of TIMP mRNA have been detected in Swiss 3T3 cells and mouse embryo fibroblasts by S1 analysis (Edwards *et al.*, 1986), and in NDV-infected L cells by RNAase protection and primer extension (Coulombe *et al.*, 1988). The longest TIMP transcript has an upstream ORF which overlaps the TIMP coding region in a different reading frame. Edwards *et al.* (1986) showed that the shorter TIMP transcript is serum inducible while the longer transcript is expressed constitutively at low levels.

The TIMP start codon has a very strong initiation context (ACC**ATG**A), whereas the sequence around the upstream AUG is suboptimal (CGG**ATG**G) (Kozak, 1986a). Purines at positions -3 and +4 are especially important for efficient initiation; pyrimidines at -2 and -1 also contribute to efficient initiation. I postulated that a portion of the initiating ribosomes should bypass the first AUG in the long transcript and initiate at the TIMP start codon due to 'leaky scanning' as proposed by Kozak (1986b). At the same time, translation of the overlapping upstream ORF would have the potential to interfere with initiation at the TIMP start codon.

I have used an *in vitro* transcription/translation system to show that the presence of the upstream sequence, which includes the 5' AUG, reduces the efficiency of TIMP translation and inhibits processing of TIMP by the microsomes. These data suggest that the presence of the upstream ORF modulates TIMP expression from the longer transcript and that this may be of particular importance in quiescent cells. Induction of a shorter TIMP mRNA, which lacks the upstream AUG, could be an effective way of amplifying TIMP expression.

4.2 RESULTS

4.2.1 Transcription of TIMP mRNAs.

The SP6 promoter can be used to generate specific transcripts with biological activity (Melton *et al.*, 1984). TIMP cDNA fragments with or witho t the ATG for the upstream ORF were cloned into the *Hin*dIII site of pSP64, adjacent to the SP6 promoter to minimize the contribution of SP64 sequence to the transcripts. The resulting constructs, and the encoded RNA and protein products, are shown in figure 4.1. pTIMP-N and pTIMP-F were used to generate the corresponding TIMP mRNA species. Transcript TIMP-N contains the coding region and 104 nucleotides at the 5' end, encompassing the AUG of an open reading frame which overlaps the TIMP ORF. Transcript TIMP-F has had 37 nucleotides (*Nci*I-*Fok*I) removed from the 5' untranslated region. The mRNAs transcribed *in vitro* were examined on a denaturing polyacrylamide gel. The autoradiogram in figure 4.2 shows that essentially all of the transcripts in the TIMP-N and TIMP-F preparations were full length. The purified RNA preparations, adjusted to a final concentration of 100 ng/µl in DEP water, were used for *in vitro* translation in reticulocyte lysates.

4.2.2 *In vitro* translation of the TIMP transcripts.

Figure 4.3 shows the kinetics of translation with respect to the amount of RNA, and time of incubation at 30°C. The optimal concentration was 100 ng of transcript per 25 µl final volume, and 15 minutes was the optimal time for translation of these TIMP transcripts. A comparison of uncapped and capped species of TIMP-N and TIMP-F mRNA translated in [^{35}S]methionine-containing reticulocyte lysate followed by electrophoretic and fluorographic analysis showed that the presence of the 5' cap resulted in enhanced

Figure 4.1 Diagram of the *in vitro* transcription/translation system for TIMP expression.

The double line represents the SP64-TIMP vectors which were constructed as described in Methods. Transcription from the SP6 promoter (shown by the arrow) produces an mRNA (dashed line) with the 5' most AUG being the start codon of the upstream ORF in transcript TIMP-N, or with the 5' AUG being the TIMP start codon in transcript TIMP-F. The bold line below the RNA represents the proteins encoded by the ORFs. The sequence around the translational start sites show that the TIMP AUG is in a favourable initiation context, and that the 5' AUG is in a poor context.

Figure 4.2 Analysis of SP6 directed TIMP transcripts.

The figure shows an autoradiogram of a gel of [^{32}P]UTP-labelled TIMP transcripts fractionated by electrophoresis in a 7 M urea 6% polyacrylamide gel. Denatured ^{32}P-labelled *Alu*I pBR322 markers of the indicated size in nucleotides (nt), are in lane 1. The capped TIMP transcripts were synthesized in the presence of [^{32}P]UTP as described in Methods. Lane 2 contains 34 ng of the capped TIMP-N mRNA, and lane 3 contains 42 ng of the capped the TIMP-F mRNA.

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

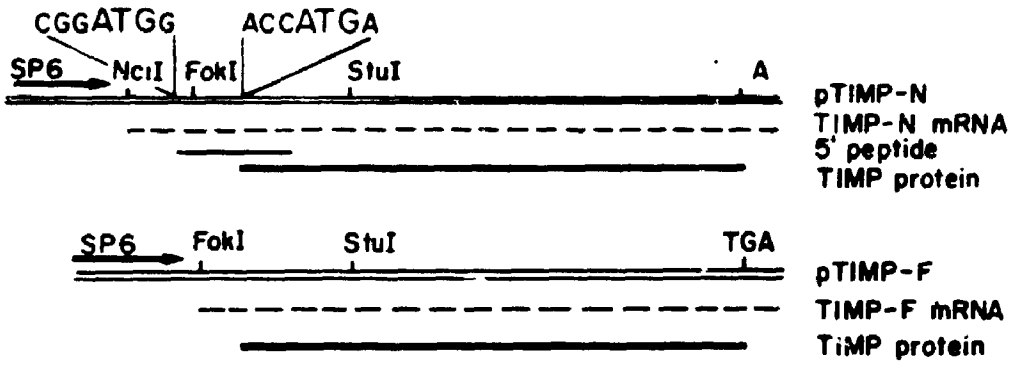
AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.


CGGATGG
ACCATGA
SP6
NciI
FokI
StuI
A
pTIMP-N
TIMP-N mRNA
5' peptide
TIMP protein
SP6
FokI
StuI
TGA
pTIMP-F
TIMP-F mRNA
TiMP protein

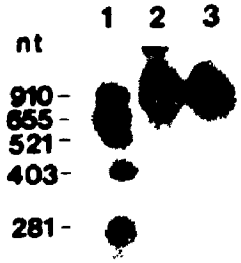
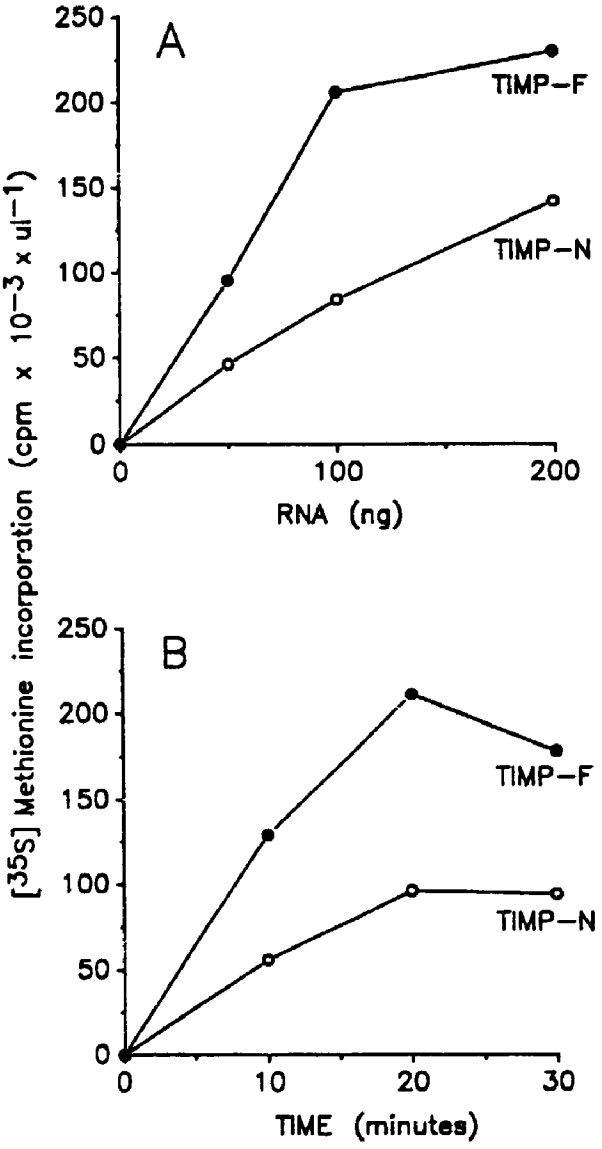

1
2
3
nt
910
655
521
403
281

Figure 4.3 Translational efficiency of TIMP-N and TIMP-F mRNA species in rabbit reticulocyte lysate.

A. Dose response of TIMP in *in vitro* translation. The indicated amounts of TIMP-N and TIMP-F mRNA were denatured with CH_3HgOH and added to a final volume of 25 µl of reticulocyte lysate. After incubation at 30°C for 30', the trichloroacetic acid-insoluble [^{35}S]methionine incorporation was determined.

B. Time course of TIMP translation. 100 ng of TIMP mRNA was treated with CH_3HgOH, and added to 25 µl of lysate, incubated at 30°C for the time indicated, and the acid insoluble [^{35}S]methionine incorporation determined.


A
250
200
150
100
50
0
0
100
200
RNA (ng)
TIMP–F
TIMP–N
[35S] Methionine incorporation (cpm x 10−3 x ul−1)
B
250
200
150
100
50
0
0
10
20
30
TIME (minutes)
TIMP–F
TIMP–N

translation of both TIMP messages (figure 4.4A).

4.2.3 Effect of the upstream ORF on *in vitro* translation of TIMP.

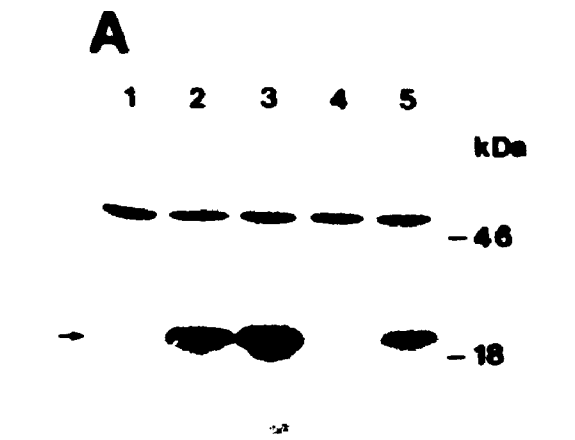
TIMP was translated several-fold more efficiently in the reticulocyte lysate primed with the 5' truncated TIMP-F transcript. Both capped and uncapped transcripts showed an increase in the efficiency of translation (figures 4.4A and B). Densitometry of the TIMP bands on the fluorogram shown in figure 4.5 revealed the extent to which translation of TIMP increased in the absence of the upstream segment (table 4.1); capped TIMP-F was translated three-fold more efficiently than capped TIMP-N. Absence of the cap decreased the translational efficiency of TIMP-F, and especially of TIMP-N, resulting in an overall nine-fold difference. The difference in translational efficiency was reproducible both with the different preparations of capped transcripts, and when the *in vitro* translation products were radiolabelled with [^{35}S]cysteine (figure 4.4B). The faint bands above the TIMP are likely aggregates of the monomer. [^{35}S]cysteine was used to label the translation products processed with microsomes since all of the cysteines are in the secreted part of the protein, whereas 2 of the 6 methionines are lost in the signal sequence.

Non-glycosylated and glycosylated forms of TIMP are readily distinguishable as 22,000 and 28,000 M_r species on SDS polyacrylamide gels. TIMP synthesized from the shorter TIMP-F transcript was efficiently processed by dog pancreatic microsomes to a 28,000 M_r species (figure 4.4B, lanes 6 and 9). In the presence of microsomes the level of TIMP translated and processed from transcript TIMP-N was ten-fold lower than that translated from TIMP-F (figure 4.4B, lanes 5 and 8; table 4.1).

Figure 4.4 SDS-PAGE analysis of TIMP *in vitro* translation products.

A. SP6-derived TIMP mRNA (100 ng) in 25 µl of reticulocyte lysate was incubated at 30°C for 15 minutes. 2 µl of lysate from each of the samples were electrophoresed on a 10% polyacrylamide-SDS gel and fluorographed. Lane 1 shows reticulocyte lysate with no added mRNA. Lane 2, uncapped TIMP-F transcript; lane 3, capped TIMP-F; lane 4, uncapped TIMP-N; and lane 5, capped TIMP-N. The arrow indicates the *in vitro* translated TIMP. The molecular weight of the ^{14}C-labelled marker proteins as indicated are lactoglobulin (18 kDa) and ovalbumin (46 kDa).

B. The samples are reticulocyte lysate with no added mRNA, lanes 1, 4 and 7; capped TIMP-N mRNA, lanes 2,5 and 8; and capped TIMP-F mRNA, lanes 3,6 and 9. Lanes 1-3 were translated in the presence of [^{35}S]methionine, and lanes 4-9 in the presence of [^{35}S]cysteine. Dog pancreatic microsomes were added to the lysate in lanes 7 to 9. The arrows indicate the positions of the unprocessed and processed forms of TIMP. The molecular weight of the ^{14}C-labeled marker proteins as indicated are lactoglobulin (18 kDa), carbonic anhydrase (30 kDa) and ovalbumin (46 kDa). The 24 kDa protein in lane 1 is an endogenous translation product which is prominent in this batch of lysate when translated with [^{35}S]methionine.


A
1 2 3 4 5
kDa
46
18

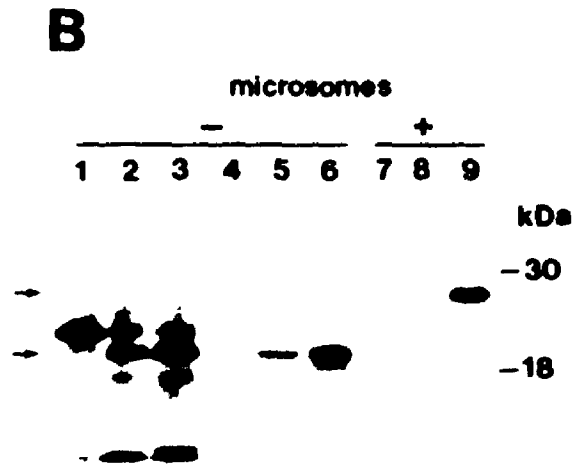

B
microsomes
–
+
1 2 3 4 5 6 7 8 9
kDa
30
18

TABLE 4.I

Quantification of TIMP protein produced by translation *in vitro* of TIMP-N and TIMP-F mRNAs.[a]

mRNA species	±CAP	1 -	1 +	2 +	2 +	3[c] +
TIMP-N[b]		0.2	1.0	1.0	1.0	1.0
TIMP-F		1.9	3.3	3.1	2.4	10.7

[a]Relative protein levels were determined by densitometry of the TIMP specific bands on the fluorograms in figures 4.4 A (column 1) and B (columns 2 and 3). The values in column 1 were derived from figure 4.4A lanes 2-5. The values in column 2 were derived from figure 4.4B lanes 2, 3, 5 and 6; and those in column 3 from figure 4.4B lanes 8 and 9.

[b]For the purpose of comparison, the values for the capped TIMP-N mRNAs in figures 4.4 A and B were normalized to 1.0.

[c]The difference of efficiency observed in the presence of microsomes is due largely to a drop in the value for TIMP-N (figure 4.4A, lane 8).

4.2.4 Immunoprecipitation of *in vitro* translated TIMP.

An antiserum raised against recombinant murine TIMP (section 3.2.7) immunoprecipitated both glycosylated and non-glycosylated forms of *in vitro* translated TIMP (figure 3.15B). Translation products endogenous to the reticulocyte lysate, particularly prominent in some experiments (e.g. lanes 4 and 6 in figure 3.15B, and lane 4 in figure 4.4B) were not immunoprecipitated by the anti-TIMP antiserum.

4.3 DISCUSSION

To assess the possible role of the short upstream ORF in the translation of TIMP, I examined the translational efficiency of an *in vitro* transcribed truncated TIMP mRNA. The truncated mRNA species is unable to produce the putative regulatory peptide in reticulocyte lysates. Using the optimized translation conditions, TIMP was translated at least three-fold more efficiently from the truncated transcript. The trend was the same for both uncapped and capped transcripts, and a ten-fold difference in translational efficiency was observed when microsomes were added to process the TIMP. This difference in translational efficiency was seen in replicate experiments, using independent preparations of the transcript. I favour the idea that the cause of this difference is the production of a protein by the ORF upstream of the TIMP start codon, although it cannot be excluded that other factors such as increased ribosomal fall off or RNA breakdown could be responsible. Two possible secondary structures in the 5' end of TIMP mRNA, each with a free energy of ~ -19 kilocalories, bury the TIMP AUG start codon in the stem region, although these structures should be easily melted out by the initiating complexes (Kozak, 1988). The upstream

AUG does not participate in secondary structure, and will be accessible for initiation.

The longest *in vivo* TIMP transcript is expressed constitutively at low levels in mouse primary embryo fibroblasts. Increases in TIMP mRNA levels in response to various agents are due to an increase of only the shorter species (Edwards *et al.*, 1986). Kozak (1989) points out that the upstream AUG on many growth control and proto-oncogene mRNAs is lost during rearrangements that accompany activation. Both long and short *lck* transcripts, which arise from two promoters, are found in leukemic and transformed cells while only the long transcript from the upstream promoter is seen in the normal cells (Sartor *et al.*, 1989). Marth *et al.* (1988) demonstrated that *lck* transcripts lacking the AUGs normally found in the 5' untranslated region were translated more efficiently leading to the high level of pp56^{lck} associated with transformation. The highly conserved first exon in the c-*myc* transcript of both mouse (Eladari *et al.*, 1986) and human (Stanton *et al.*, 1986) has an upstream overlapping ORF which, along with other secondary structural feaures, is thought to play a role in the regulation of *myc* translation. In a similar manner as shown here, the TIMP transcripts lacking the upstream AUG are translated more efficiently than the constitutively expressed long species of TIMP mRNA.

The difference in translational efficiency of TIMP from the two transcripts is predicted by the 'leaky' scanning mechanism (Kozak, 1986b). The 5' most AUG in the shorter TIMP-F transcript is the TIMP start codon which has a strong initiation context, resulting in a high translational efficiency. A portion of the 40S initiation complexes will initiate at the suboptimal 5' AUG in the TIMP-N transcript, while some will bypass and

initiate at the TIMP start site. The overall effect is a decrease in the translational efficiency of TIMP from the longer transcript. The extent of the decrease of TIMP translational efficiency in the presence of the upstream overlapping ORF is similar to the 5-10 fold drop of proinsulin synthesis *in vivo* in the presence of an overlapping upstream ORF with a poor initiation context (Kozak, 1986a).

The putative dicistronic TIMP mRNA has the potential to code for two biologically active proteins. We did not detect synthesis of a 5' peptide encoded by the upstream AUG, which would be 4 kDa and very basic. There is only one methionine (the start codon), and three cysteines in the predicted amino acid sequence of the upstream ORF, compared with 6 methionines and 12 cysteines in TIMP. The protein was not detected even on long exposures of the autoradiograms. This may be due to a low level of [^{35}S]methionine or [^{35}S]cysteine incorporation and a low translational efficiency, due to the poor initiation context.

The long TIMP transcript is expressed at a low level in quiescent mouse embryo fibroblasts. The sequence upstream of the 5' ATG reported for the long cDNA (Edwards *et al.*, 1986) differs from that reported for the same region of the genomic clone (Gewert *et al.*, 1987), although both sequences contain the upstream AUG and the same results would be expected for mRNAs containing the sequence seen in the genomic clone. Serum and NDV induce TIMP transcripts that lack the AUG of the 5' ORF (Edwards *et al.*, 1986; Coulombe *et al.*, 1988). Induction of the shorter species coupled with more efficient translation is an effective means of amplifying the expression of an otherwise poorly translated mRNA. Additionally, the 5' ORF may function to control the level of secreted TIMP in quiescent cells.

CHAPTER 5 - EXPRESSION OF GROWTH RELATED GENES IN FEMALE REPRODUCTIVE TISSUES DURING PREGNANCY

5.1 INTRODUCTION

In mouse the developing blastocyst implants in the uterine stroma at 4.5 days post-coitum; day 0 is defined as the day of appearance of a vaginal plug. Parturition is on day 18.5, and a litter size of 12 or more pups is not uncommon in the outbred strain of CD1 mice. Placenta and decidua are two tissues which arise, respectively from the embryo and the uterus, to support the growth of the conceptus during gestation (see figure 5.1 B and C). Transfer of substances across the placenta must be sufficient for the growth of the foetus. The trophoblast component of the placenta is also responsible for an immune regulatory function (Billington and Bell, 1983) and synthesis of peptide and steroid hormones (Patillo *et al.*, 1983). Decidualization is the response of the uterus to the embryo and is most prominent from the time of implantation until the formation of a definitive placenta. The decidua provides nourishment to the early blastocyst, and also plays an active role in protecting the maternal tissues from the highly invasive foetal trophoblasts.

Decidualization follows a complex temporal and spatial sequence of events with differentiation of the stromal cells occurring in different regions at different times. The decidual response is triggered by implantation of the blastocyst into the uterine stroma on day 4.5. After implantation, a wave of mitotic activity precedes differentiation of cells in the endometrium to form the antimesometrial cells of the primary decidual zone. By day 7 the decidua basalis has formed adjacent to the metrial gland region, and by day 10 the antimesometrial decidua has regressed to form the decidua capsularis (see figure 5.1B and C). The decidua lateralis lies between the decidua

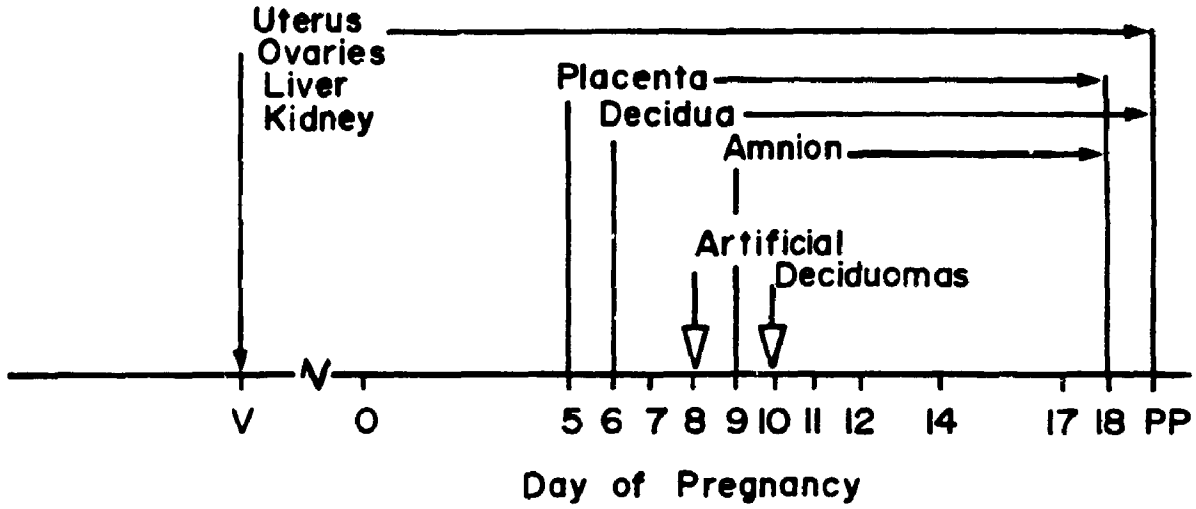
Figure 5.1 Schematic representation of a cross section through a pregnant mouse uterus.

A. This flow chart illustrates the times of gestation at which tissues were dissected from pregnant CD1 mice. Day 0 is the day of appearance of a vaginal plug. Tissues were also taken from virgin (V), and post partum (PP) animals. For example, the uterus, ovaries, kidneys and liver were dissected from virgin mice, from pregnant mice on all of the days indicated on the chart except day 0, and from a mouse on the first day post partum.

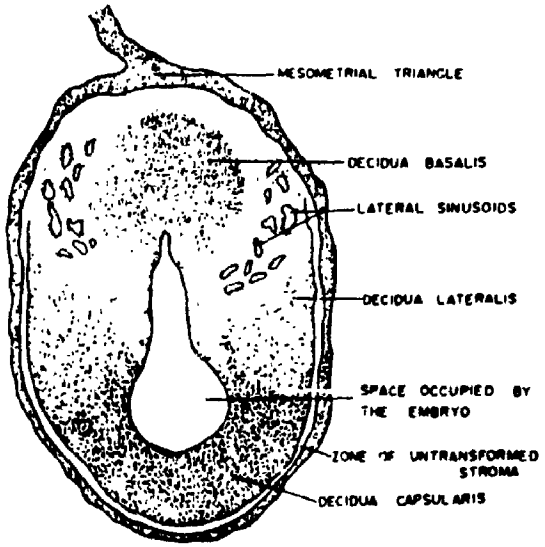
B. A schematic diagram of a cross section through the uterus at day 8 of gestation. Note the three distinct zones of decidua.

C. A cross section through the uterus at day 12. A definitive placenta has developed adjacent to the decidua basilis. The placenta and amnion are of foetal origin. B and C are adapted from Kearns (1984).

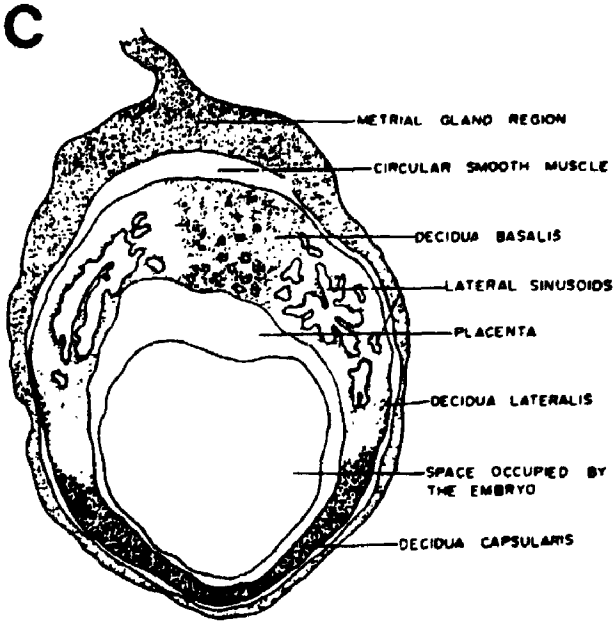
A


TISSUES PROCESSED FOR RNA
Uterus
Ovaries
Liver
Kidney
Placenta
Decidua
Amnion
Artificial
Deciduomas
V
O
5 6 7 8 9 10 11 12 14 17 18 PP
Day of Pregnancy

B


MESOMETRIAL TRIANGLE
DECIDUA BASALIS
LATERAL SINUSOIDS
DECIDUA LATERALIS
SPACE OCCUPIED BY THE EMBRYO
ZONE OF UNTRANSFORMED STROMA
DECIDUA CAPSULARIS

C


METRIAL GLAND REGION
CIRCULAR SMOOTH MUSCLE
DECIDUA BASALIS
LATERAL SINUSOIDS
PLACENTA
DECIDUA LATERALIS
SPACE OCCUPIED BY THE EMBRYO
DECIDUA CAPSULARIS

basalis and the decidua capsularis and is intermediate in composition. The ectoplacental cone of the embryo invades the decidua basalis on day 7.5, and differentiates to form the definitive placenta by day 11. During this period of time, blood vessels surrounding the decidua basalis become progressively enlarged and form lateral sinusoids. From day 12 on, the decidua basalis regresses as the fetus continues to grow (reviewed in Bell, 1984).

The antimesometrial cells of the primary decidual zone (day 4.5) are large basophilic multinucleate cells. The mesometrial cells in th decidua basalis are smaller and mononucleate. Granulated metrial gland cells are found in large numbers in the metrial gland region and the decidua basalis. These cells contain distinctive cytoplasmic granules up to 5 μm in diameter (Stewart and Peel, 1977), and are mono or binucleate.

Artificial deciduomas can be induced to develop in the uterus of nonpregnant mice by manipulating the hormonal environment, and by providing a mechanical stimulus that is the equivalent of blastocyst implantation. Injection of a small amount of oil, or a traumatic stimulus such as scratching, inside the uterus will induce the formation of an artificial deciduoma in ovariectomized mice primed with hormones. The timing is critical. A hormonal regime consisting of regular injections of progesterone, and a surge of oestradiol induce the stromal cells of the uterine lining to undergo a round of mitosis on day 4.5 at which time they will respond to an artificial stimulus by decidualization (Das and Martin, 1978).

The ectoplacental cone consists of a core of rapidly dividing trophoblast cells that are covered by a layer of secondary giant cells (Rossant and Ofer, 1977). The definitive placenta consists of three main trophoblast subpopulations. The cellular layer of the labyrinthine

trophoblast maintains the foetal interface in the murine placenta. Trophoblast giant cells form a continuous barrier at the maternal boundary of the definitive placenta, and the spongiotrophoblasts form a compact tissue through which maternal blood must pass en route to the labyrinth (Billington and Bell, 1983). The trophoblast layers protect the foetus from maternal immune competent cells, whereas antibody and erythrocytes pass more readily (Hunzicker *et al.*, 1984). Growth factor receptors are present on the trophoblast cell surface, and gestational hormones such as progesterone are required for trophoblast development (Wild, 1983).

The amnion is derived from the placenta, although the cell types are specific. As well as providing a closed environment for the foetus, the amnion also synthesizes hormones and may be a target for foetal hormones triggering the onset of labour (Vander-Kooy *et al.*, 1989).

The expression of TIMP, spp and MRP has been recently reported in some of the tissues that I have examined here. TIMP was detected by RNAase protection in decidua, amnion and ovary, and by *in situ* hybridization in the thecal cells of the ovary (Nomura *et al.*, 1989). *In situ* hybridization revealed that the GMG cells of the decidua expressed high levels of spp (Nomura *et al.*, 1988). MRP was similarly localized to the trophoblast giant cells of the placenta by *in situ* hybridization (Lee *et al.*, 1988).

In this chapter I present the day to day time course of expression, in the female mouse reproductive tissues, for each of TIMP, MRP, MEP, the transins, as well as the calcium binding proteins spp, calcyclin and calmodulin. For most of gestation the time course of TIMP expression was similar between the ovary and the decidua-placenta-uterus, however the

level of TIMP mRNA increased to a 25-fold higher level in the ovary in day 18 and post partum animals. The spatial and temporal expression of calcyclin and spp were complementary: spp expression increased rapidly from day 8, peaking on day 10, in the GMG cells of the metrial gland region, while calcyclin expression in the antimesometrial region of the decidua capsularis rapidly decreased after peaking on day 8. MEP and the transins were each expressed at very discrete time points in the decidua.

5.2 RESULTS

5.2.1 Isolation of RNA from mouse tissue during pregnancy.

The tissues which directly support pregnancy, as well as the ovaries, kidneys, and liver were dissected from pregnant mice on the days indicated in figure 5.1A. The time course of tissue isolation for mRNA processing was repeated a second time. The results were the same for all of the genes and tissues analyzed for both time course experiments, with the exception that the levels of transin, transin-2 and calmodulin mRNAs were not determined in the first time course experiment. Implantation of the embryo takes place on day 4.5, and so the tissues that support pregnancy were isolated starting on day 5. The figures 5.1 B and C show a schematic representation of the different tissues that support pregnancy. Although the uterus, decidua and placenta can be dissected cleanly as discrete tissues, the migration of trophoblast and GMG cells makes it impossible to compare distinct cell populations here. Before the placenta proper developed as a discrete tissue, it was isolated as the ectoplacental cone (a red spot) in the gut region of the embryo (days 5-8). The amnion (which was actually amnion and chorion) could not be isolated any earlier than day 9. The maternal kidneys and ovaries were taken so that it could be

determined if the high levels of expression reported for TIMP in ovary, and spp in kidney varied throughout pregnancy. Liver was taken as a control tissue, although none of these genes had been reported to be expressed in adult liver. Artificial deciduomas were induced to determine if the presence of the embryo was necessary for the expression of the various genes in the uterus and decidua.

Total tissue RNAs were isolated and analyzed on slot blots to quantify the level of expression, and further analyzed on Northern blots to confirm the mRNA size being expressed and to show the integrity of the RNA. To make a careful analysis of the level of expression of the various genes, multiple identical slot blots were made of the tissue RNA preparations. Several precautions were taken to ensure the accuracy of the determinations both between blots and between samples. (a) The multiple copies of the blots were made and processed at the same time, as detailed in the methods section. (b) One copy of the blots was probed with a ^{32}P-labelled 18S rRNA cDNA insert, and densitometry of the resulting autoradiogram determined that the RNA loadings were roughly equal. (c) The small differences in the 18S rRNA values were used to correct for loading errors (likely due to error in determining the concentration from the optical density of the purified RNAs) when expressing the values obtained with other probes. The mRNA levels of actin and glyceraldehyde phosphate dehydrogenase (GPD), which are commonly used as controls, each showed some tissue specific and time course specific differences in expression and so were not used to quantify expression of the other genes.

5.2.2 Expression of TIMP during pregnancy.

A specific time course of TIMP expression was seen in pregnant uterus, decidua, placenta, amnion and in the ovary. TIMP expression was

not detected in kidney or liver. The time course and level of TIMP expression was very similar in these tissues during pregnancy (figure 5.3) with the exception of ovary (figure 5.2A and B). TIMP levels in uterus, decidua and placenta peaked around day 8 to 10 and showed a decline thereafter. In amnion, there were increases in TIMP mRNA specifically on day 10 and again on day 18, the day of parturition (figure 5.3A). Slot blot analysis showed that TIMP was expressed at a 25-fold higher level in day 18 and post partum ovary than in any other tissue, and was similar to the level of expression in serum stimulated 3T3 cells (figure 5.2B). TIMP expression was quite low in some RNA preparations from the ovaries of virgin mice, possibly reflecting their state of oestrus.

The level of TIMP activity in the amniotic fluid was also determined, based on its property as a collagenase inhibitor, and is expressed as activity per μg of protein in the amniotic fluid (figure 5.3B). As the activity assay only measures the active TIMP level, there may have been much more TIMP actually complexed with metalloproteinases. There is no way of knowing which of the tissues synthesized the TIMP found in the amniotic fluid.

5.2.3 Expression of proteases.

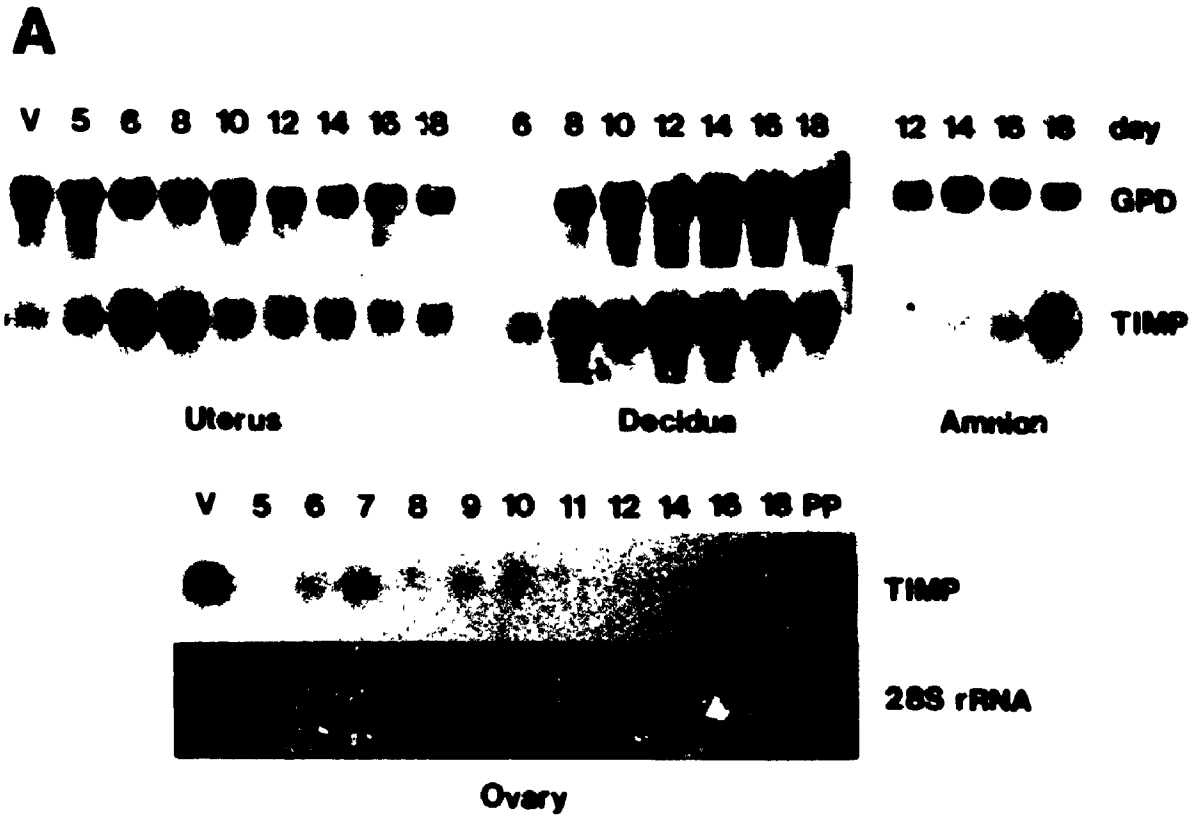
The RNA samples were also screened for the expression of several proteases. MEP (procathepsin L) mRNA was expressed in decidua, placenta and amnion. The levels were similar in each of the tissues and were equivalent to those seen in tissue culture cells. A common feature is an increase in MEP expression at the end of gestation in each of these tissues. There are also mid-gestational increases of MEP at day 8-9 in placenta and from day 10 onwards in decidua (figure 5.4A).

Expression of the transins was very specific. There was a burst of

Figure 5.2 A time course of TIMP expression in tissues supporting pregnancy.

A. Total RNA isolated from uterus, decidua, amnion and ovary were analysed by sequentially probing Northern blots with ^{32}P-labelled cDNA inserts of GPD and then TIMP. The TIMP levels in ovary are compared to the level of 28S rRNA visualized in the ethidium stained gel before transfer of the RNA to genescreen plus.

B. The level of expression of TIMP in mouse tissues and in tissue culture cells. The mRNA levels (arbitrary units) were determined by densitometry (section 2.7.2) of slot blots, containing 2μg of total RNA for each sample, which had been probed with ^{32}P-labelled TIMP cDNA. These samples were all on the same slot blot and therefore processed identically.


A
V 5 6 8 10 12 14 16 18
6 8 10 12 14 16 18
12 14 16 18 day
GPD
TIMP
Uterus
Decidua
Amnion
V 5 6 7 8 9 10 11 12 14 16 18 PP
TIMP
28S rRNA
Ovary

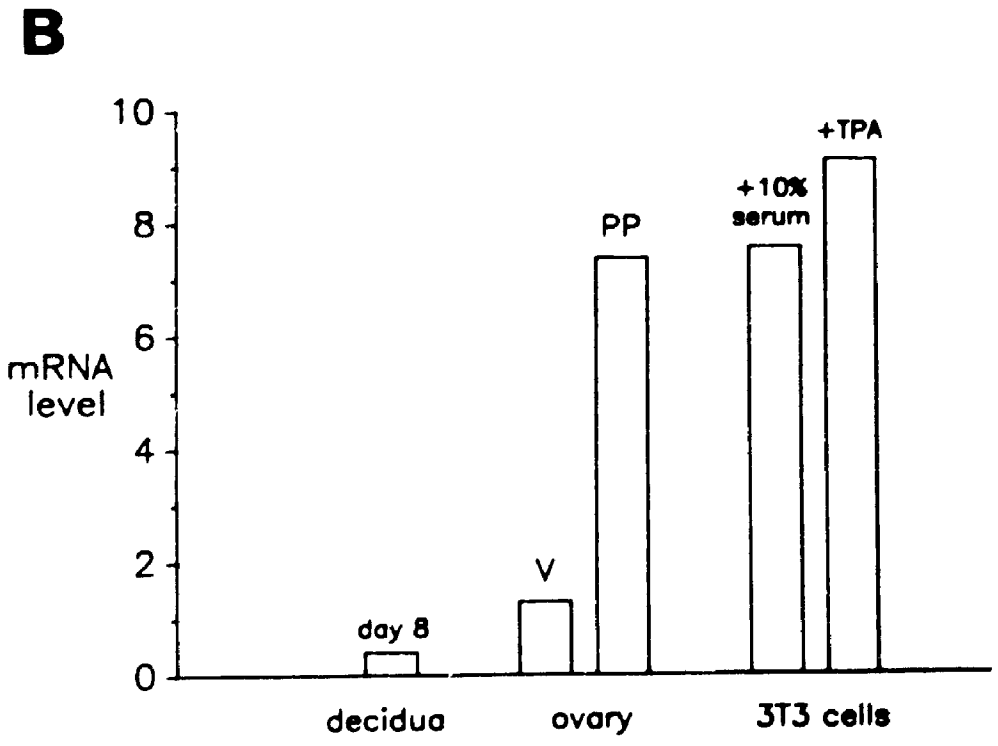
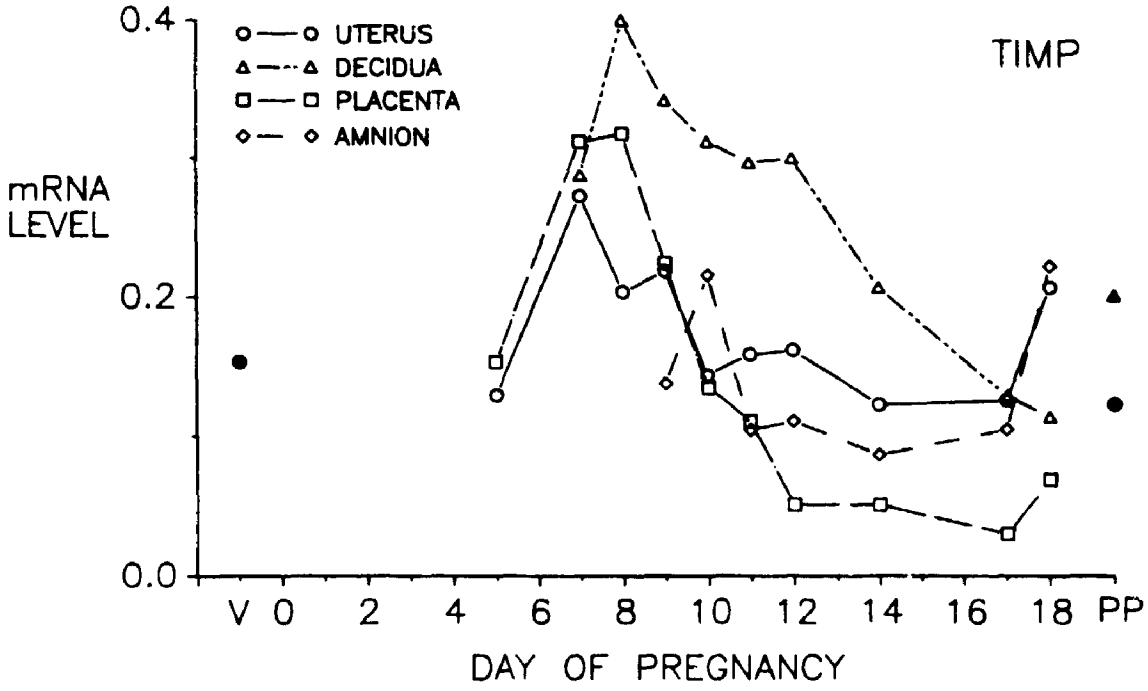

B
10
8
6
4
2
0
mRNA level
day 8
V
PP
+10% serum
+TPA
decidua
ovary
3T3 cells

Figure 5.3 Quantitation of TIMP expression in the tissues supporting pregnancy.

A. To directly compare the level of TIMP expression in these tissues, 2μg of total RNA from each time point was loaded on a slot blot and probed for TIMP expression. The level of TIMP expression was quantitated by densitometry of the autoradiogram, and the values adjusted for the level of 18S rRNA as described. The curves reflect the relative TIMP mRNA levels in each of the tissues, although an absolute value was not determined. The open symbols represent tissues taken from pregnant animals, and the closed symbols represent the same tissue (either uterus •, or decidua ▲) taken from virgin or post partum animals.

B. The level of free TIMP activity in the amniotic fluid was determined by an assay based on the collagenase inhibitory activity and was performed by R. Khokha in this lab, according to the method of (Overall *et al.*, 1989). The TIMP activity in the amniotic fluid taken from the same pregnant animals was determined, and expressed as units of TIMP / μg TCP in the amniotic fluid. One U of TIMP inhibits 2 U of collagenase by 50%. The values are the mean ± standard deviation for duplicate experiments of samples from one time course experiment.


0.4
0.2
0.0
mRNA LEVEL
UTERUS
DECIDUA
PLACENTA
AMNION
TIMP
V 0 2 4 6 8 10 12 14 16 18 PP
DAY OF PREGNANCY

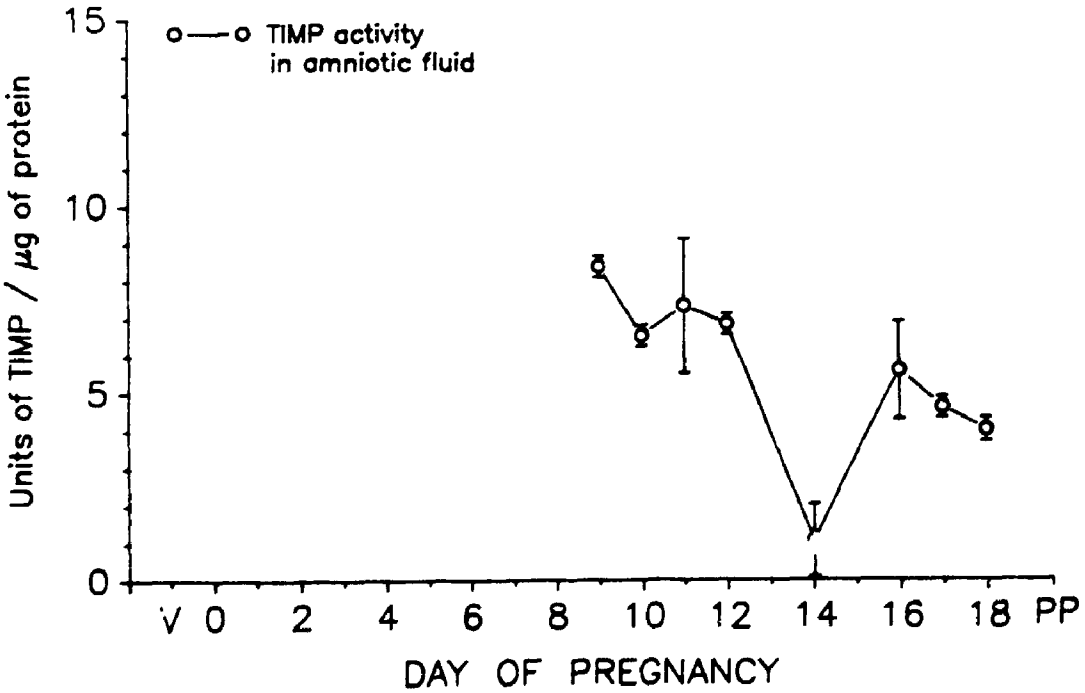
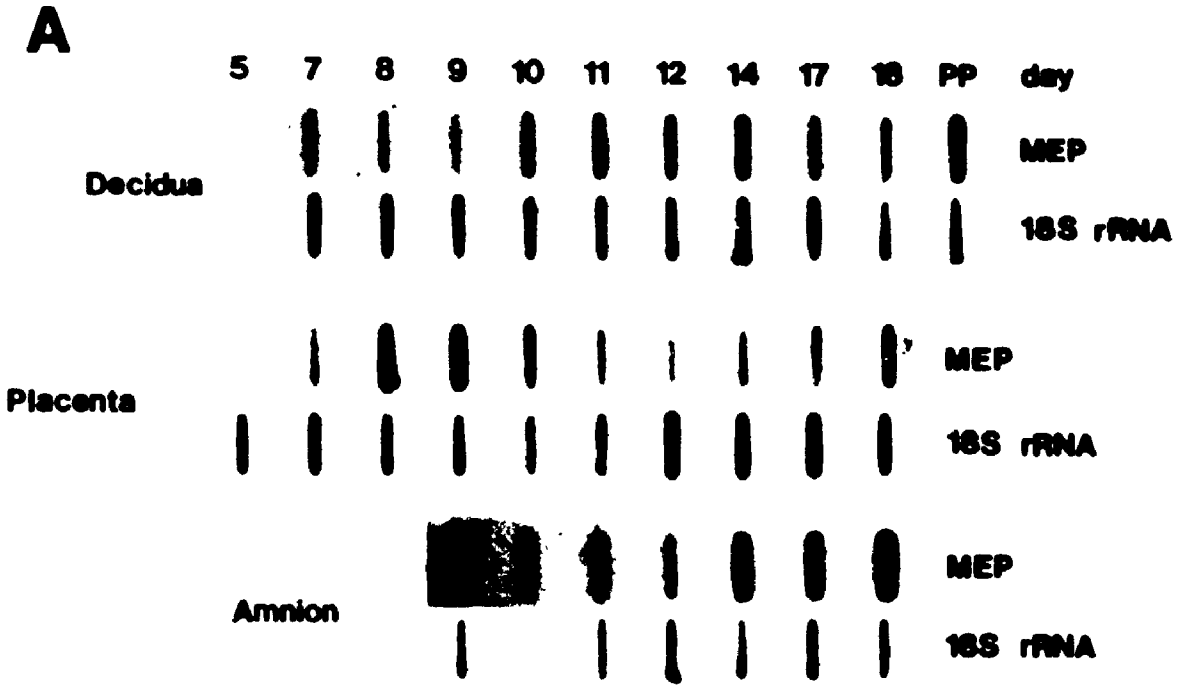
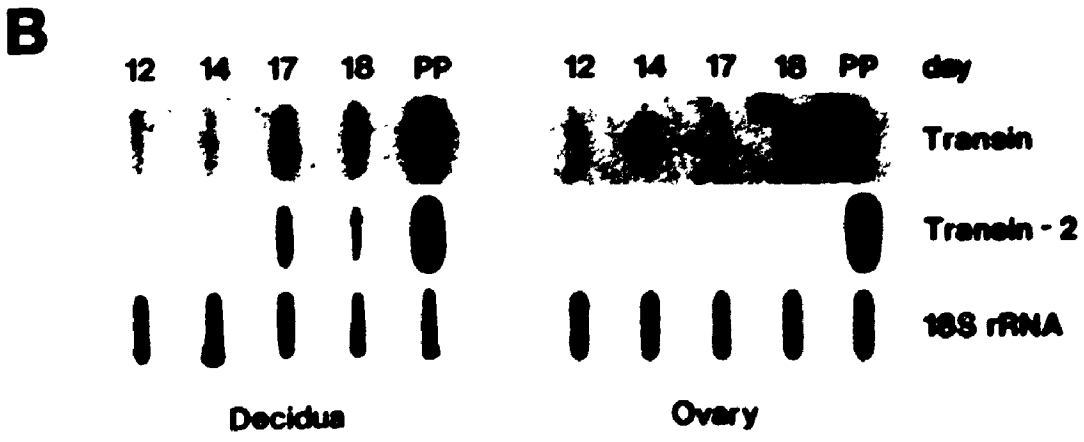

15
10
5
0
Units of TIMP / μg of protein
TIMP activity in amniotic fluid
V 0 2 4 6 8 10 12 14 16 18 PP
DAY OF PREGNANCY

Figure 5.4 Expression of the proteases MEP (procathepsin L) and Transin and Transin-2 during pregnancy.

A. Replicate slot blots containing total RNA isolated from the decidua, placenta and amnion on the indicated days were probed with the indicated ^{32}P-labelled cDNA. The samples probed with MEP are shown beside the replica probed with a cDNA to 18S rRNA.

B. These slot blots show the level of transin and transin-2 mRNA in the decidua and ovary on the indicated days of gestation. The 18S rRNA probe serves as a control for comparing the transin and transin-2 levels.


A
5
7
8
9
10
11
12
14
17
18
PP
day
Decidua
MEP
18S rRNA
Placenta
MEP
18S rRNA
Amnion
MEP
18S rRNA


B
12
14
17
18
PP
12
14
17
18
PP
day
Transin
Transin - 2
18S rRNA
Decidua
Ovary

transin-2 expression in decidua and ovary of post partum mice (figure 5.4B), and the level was higher than seen in TPA-treated 3T3 cells (results not shown). Transin was also expressed in post partum decidua, but not in the ovary. The mRNA level was much higher in TPA treated 3T3 cells than in decidua for transin-2 (results not shown).

5.2.4 Expression of MRP.

The expression of MRP in placenta has been known for a long time (Linzer *et al.*, 1985), and it was thought to be specific to the trophoblast cells of the placenta (Lee *et al.*, 1988), although unglycosylated forms of MRP are found in maternal serum during pregnancy.

Screening the tissue RNA samples taken here for expression of MRP revealed that MRP mRNA was also present in uterus and decidua on day 10 of gestation, and was expressed at a level similar to that seen in the placenta (figure 5.5). It is clear that expression in decidua and uterus is limited to day 10 (figure 5.5A), and that the mRNAs being expressed in uterus, decidua and placenta on day 10 are the same size (figure 5.5B). Since the expression of MRP in uterus and decidua was unexpected, the time course experiment was repeated three times. The burst of MRP expression in day 10 uterus and decidua was consistent. The results shown in figures 5.4A and B are from two separate time course experiments, and the level of MRP expression was just as high in the third experiment (data not shown).

Expression of MRP mRNA in these tissues was localized by *in situ* hybridization (figure 5.6). A strong MRP specific signal was seen in the layer of giant trophoblast cells surrounding the space occupied by the embryo, in both day 9 and day 10 samples. However, no uterus- or

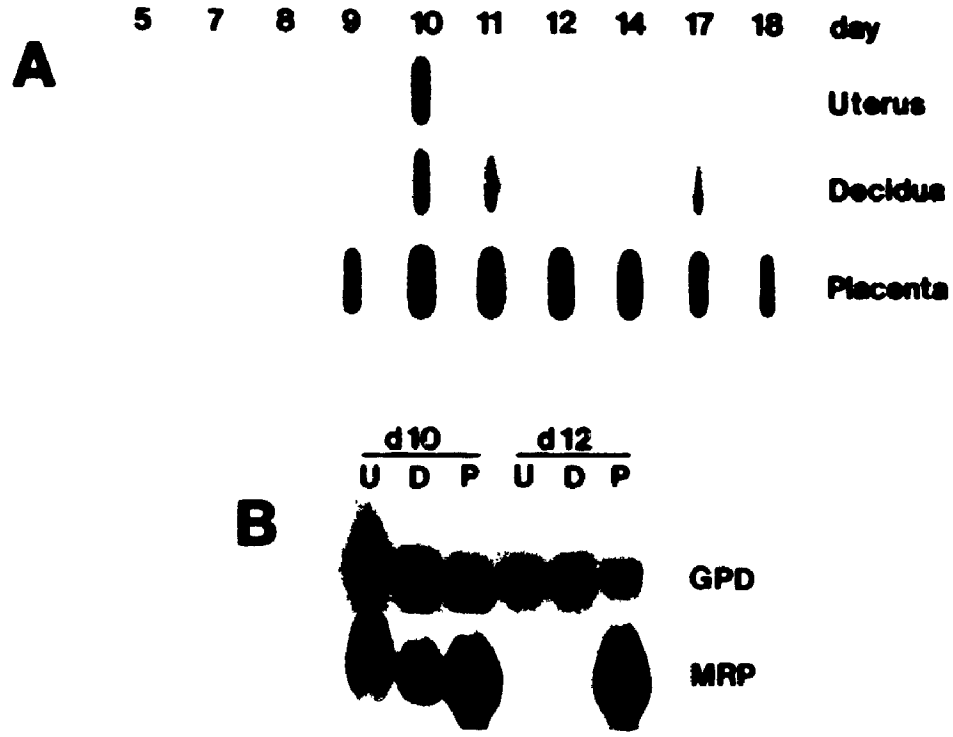
Figure 5.5 A time course of MRP expression in placenta, decidua and uterus.

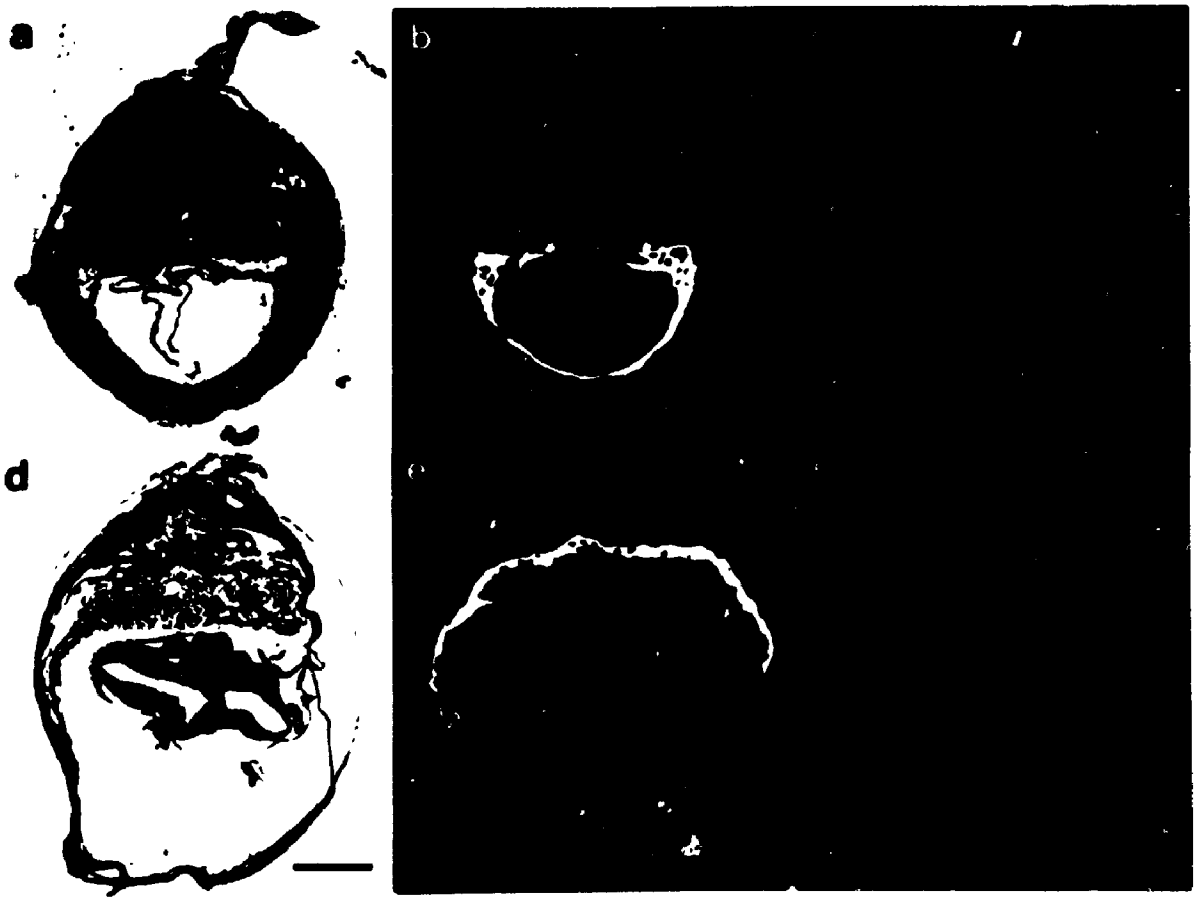
A. Slot blots with 2μg of tissue RNA per slot were probed with ^{32}P-labelled MRP cDNA insert. Equal amounts of RNA, verified by probing with an 18S rRNA probe, were loaded on each of the slots.

B. Tissue RNA samples from uterus, decidua and placenta at day 10 and day 12 of pregnancy were analysed on Northern blots probed sequentially with ^{32}P-labelled GPD and MRP cDNA inserts. These RNA samples were isolated from tissues during a different time course experiment than those in panel A.

Figure 5.6 *In situ* localization of MRP mRNA in day 8 and day 10 pregnant mice.

10μm sections in the transverse plane through the uterus of day 9 (a-c) or day 10 (d-f) pregnant mice were stained with haematoxylin, or probed with [^{35}S]UTP labelled strand specific MRP RNA probes. Antisense transcripts which recognise the mRNA strand were used to localize MRP mRNA in the adjacent sections (b, e). The controls were to incubate adjacent sections with 'sense' strand transcript (c), or to probe sections which had been pretreated with RNAase A (f). The controls of sense transcript for the day 10 section, and RNAase treatment for the day 9 section were also negative (results not shown).


A
5
7
8
9
10
11
12
14
17
18
day
Uterus
Decidua
Placenta
B
d10
d12
U D P U D P
GPD
MRP


a
b
d

decidua-specific expression of MRP could be detected in the day 10 samples that could account for the signal seen in the Northern blots, even after a long overexposure of the *in situ* slides. Thus the signal seen in the RNA blots (figure 5.5) must have come from the trophoblast cells.

5.2.5 Expression of calcyclin and spp

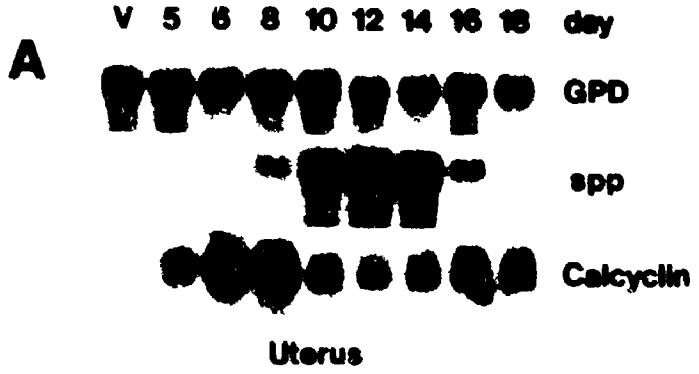
Calcyclin is a cytosolic protein of unknown function which, is expressed in a CCD manner. The localization of spp to developing bone, along with the cell attachment activity, lead to the speculation that it functions as part of the ECM to provide positional cues to migrating cells. Calcyclin and spp mRNA were expressed at high levels in uterus, decidua and placenta during pregnancy, and there were distinct spatial and temporal patterns of expression. The Northern blots in figure 5.7 illustrate that calcyclin was expressed early in uterus and decidua, and that spp mRNA levels increased dramatically just as the levels of calcyclin were declining. The mRNA level of calmodulin (another Ca^{++} binding protein) remained constant throughout the time course.

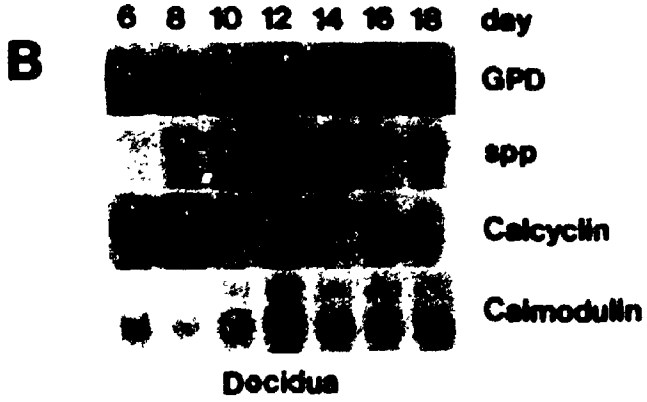
The relative levels of calcyclin and spp expression in uterus, decidua, placenta and amnion in a second time course experiment are illustrated graphically in figure 5.8. Calcyclin mRNA levels declined after peaking by day 8 or 9 in the uterus, decidua and placenta, and were expressed after day 12 in amnion. On the other hand, spp peaked at a high level between days 9 and 12 in the uterus, decidua and kidney, and was not expressed in the amnion. The level of spp expression in kidney was as high as the maximum level in decidua, and remained constant throughout the pregnancy. Calcyclin was expressed at a low level in the ovary during mid gestation (days 7-10), and in the post partum animal. spp was also expressed at a very low level in ovary throughout gestation, and at a somewhat higher level

Figure 5.7 Northern blot analysis of spp and calcyclin in uterus and decidua during pregnancy.

Northern blots of the tissue RNA samples were probed sequentially with ^{32}P-labelled cDNA inserts for GPD, calcyclin and spp. **A:** uterus; **B:** decidua. The samples were from the virgin mice (V), or mice at the indicated day of pregnancy. The decidua RNAs were also probed for expression of another Ca^{++} binding protein, calmodulin. The RNA preparations in A and B were from two separate time course experiments.

C. Slot blot analysis of cytoplasmic RNA isolated from exponentially growing tissue culture cells, probed with ^{32}P-labelled cDNA inserts as indicated. TPA was added to the tissue culture medium at 10 ng/ml for 6 h before the cells were harvested.


A
V 5 6 8 10 12 14 16 18 day
GPD
spp
Calcyclin
Uterus


B
6 8 10 12 14 16 18 day
GPD
spp
Calcyclin
Calmodulin
Decidua

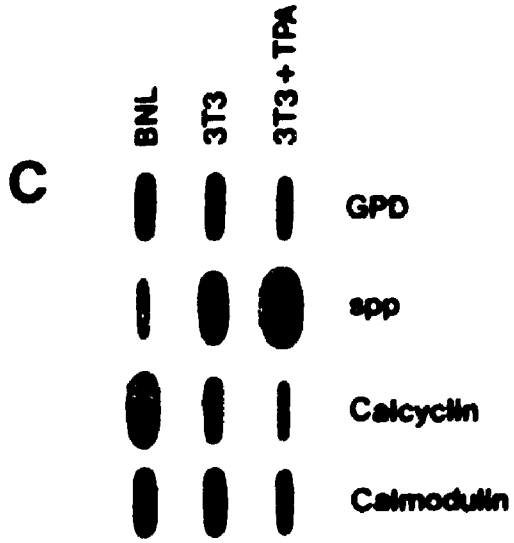
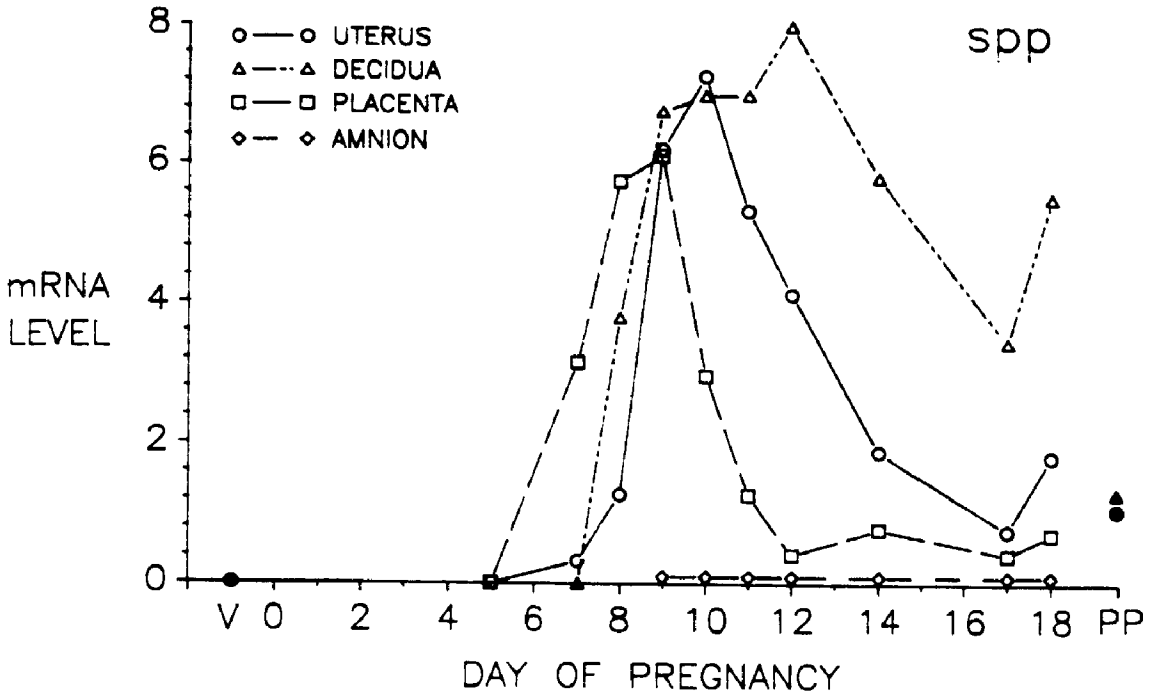
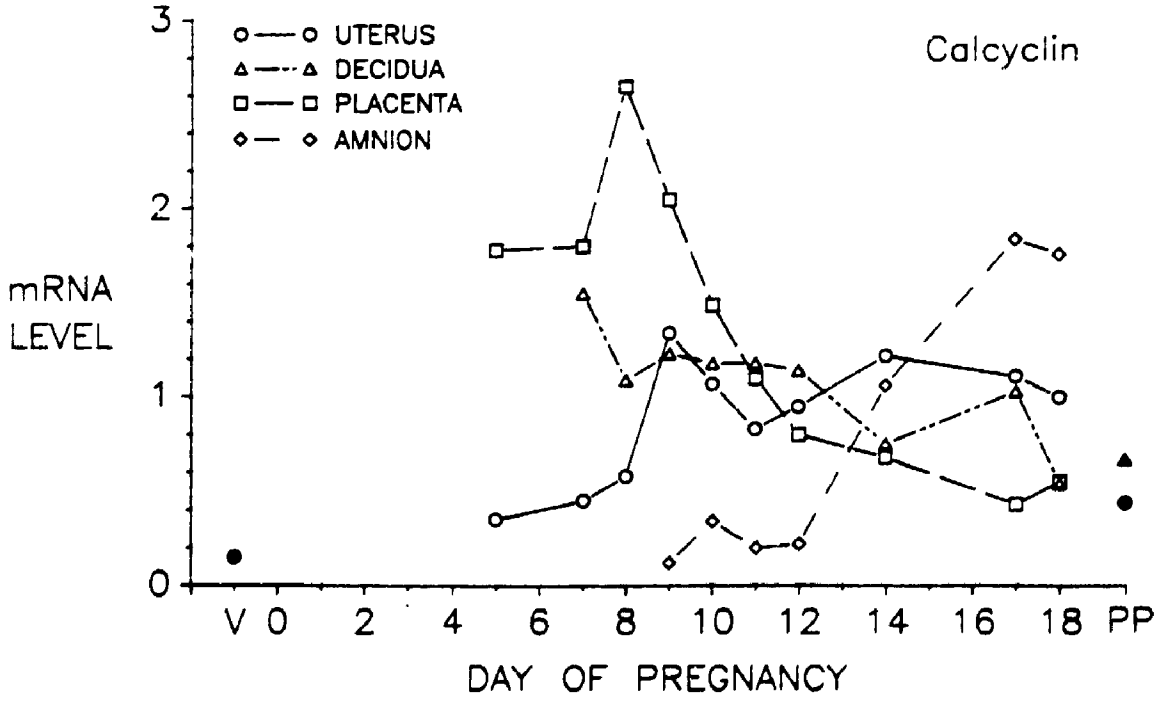

C
BNL
3T3
3T3+TPA
GPD
spp
Calcyclin
Calmodulin

Figure 5.8 Time course of calcyclin and spp expression in uterus, decidua, placenta and amnion during pregnancy.

Replicate slot blots containing 2μg of tissue RNA from each of the time points were probed with ^{32}P-labelled cDNA insert for calcyclin, spp or 18S rRNA. The levels of calcyclin and spp mRNA in each of the tissues were determined by densitometry. The relative abundance between calcyclin and spp mRNAs were determined from equal exposures of slot blots probed and washed under identical conditions. The open symbols represent tissues taken from pregnant animals, and the closed symbols represent the same tissue (either uterus •, or decidua ▲) taken from virgin or post partum animals.


UTERUS
DECIDUA
PLACENTA
AMNION
spp
mRNA LEVEL
8
6
4
2
0
V 0 2 4 6 8 10 12 14 16 18 PP
DAY OF PREGNANCY


UTERUS
DECIDUA
PLACENTA
AMNION
Calcyclin
mRNA LEVEL
3
2
1
0
V 0 2 4 6 8 10 12 14 16 18 PP
DAY OF PREGNANCY

(equivalent to post partum decidua) in the post partum ovary. Neither calcyclin nor spp were expressed in the liver.

In the tissue culture cell lines examined, the level of calcyclin mRNA was the highest in BNL cells, was less in 3T3 cells and was even lower in TPA-treated 3T3 cells. In contrast, spp mRNA was the lowest in BNLs, higher in 3T3s and the highest in TPA-treated 3T3s (figure 5.7C). The level of calmodulin mRNA did not change relative to the level of the control GPD.

In situ localization of calcyclin and spp expression in the tissues was very revealing. spp was clearly expressed at a high level by the GMG cells which migrate throughout the uterus, decidua and placenta (figure 5.9 h, k). spp was also expressed in a discrete region of the decidua capsularis (figure 5.9). This expression is not in GMG cells, and may contribute to the wide peak of spp expression seen in the tissue time course for decidua (figure 5.8).

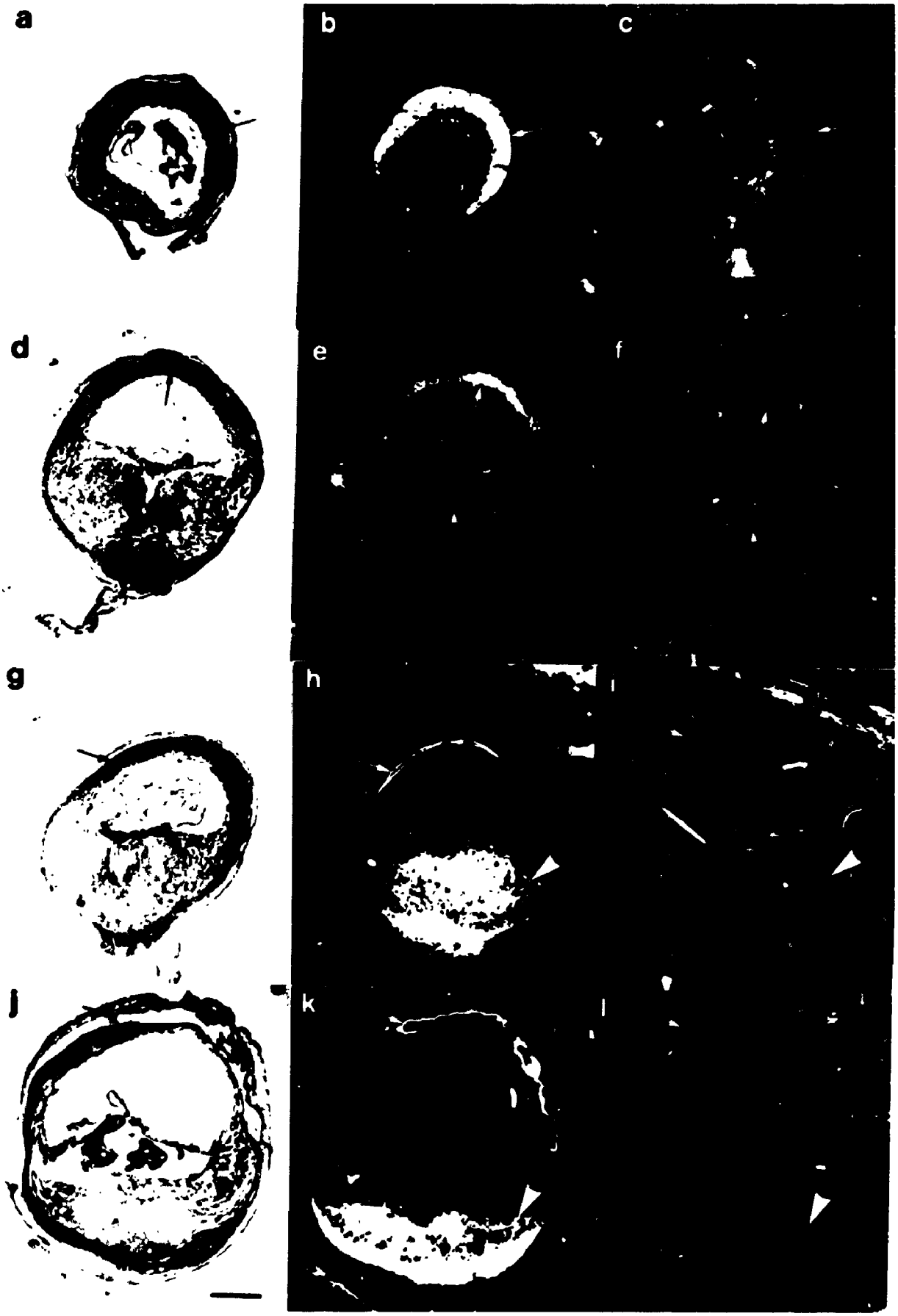
Calcyclin expression was localized by *in situ* hybridization on day 8 (figure 5.9 b), and on the day 9 sections (figure 5.9 e) adjacent to those probed for spp expression. On day 8 the calcyclin-specific signal was localized to a band running throughout the decidua. By day 9 the expression was restricted to the antimesometrial region of the decidua, and regions of the placenta (figure 5.9 e). The spatial expression pattern is clearly different than for spp, although there may be some overlap.

5.2.6 Expression in artificial deciduomas.

Artificial deciduomas were induced by injecting oil into the barren horn (which had a resected oviduct) of the uterus in day 4.5 pregnant mice. The artificial deciduomas isolated on days 8 and 10 were of the same

Figure 5.9 ***In situ*** **localization of calcyclin and spp expression.**

Adjacent 10 μm transverse sections through the uterus of day 8 (a-c), day 9 (d-i) or day 10 (j-l) pregnant mice were either stained with haematoxylin (a,d,g,j), or incubated with strand-specific [^{35}S]-labelled RNA probes for calcyclin or spp. ^{35}S-labelled antisense transcripts (b,e,h,k) were used to localize the site of expression of the specific mRNA. The controls were to pretreat the sections with RNAase A (c, i), or to probe with the 'sense' strand transcripts (f, l). The sections were then exposed to emulsion for 10 days for spp, or 21 days for calcyclin samples, and photographed using brightfield and darkfield optics. Calcyclin specific expression was seen in the decidua on days 8 (b) and 9 (e) of gestation. Some placental staining is also obvious in the day 9 sample. The spatial expression patterns of spp are shown for days 9 (h) and 10 (k) of gestation. The arrows indicate the sites in the desidua capsularis that show specific staining for calcyclin and spp. The small arrowheads indicate calcyclin signal in placenta, and the large arrowheads indicate the spp specific signal in GMG cells, which are migrating en mass towards the placenta.


a
b
c
d
e
f
g
h
i
j
k
l

consistency, but were spread throughout the uterus and lacked the spatial definition of a normal decidua. The mass was several times larger than all the deciduas from a single normal horn.

The levels of gene expression in the deciduoma and uterus in the artificially induced horn of the uterus were compared to expression in the other horn of the uterus carrying the normal pregnancy (figure 5.10). GPD, TIMP, calcyclin and spp all showed slightly decreased levels of expression in the day 10 deciduoma compared to the decidua from the normal side, and spp was slightly higher than normal in the day 8 deciduoma.

Some differences were also evident between the normal deciduas of the experimentally treated animals (from the pregnant horn of the uterus), and deciduas taken from unmanipulated, normal pregnancies (Table 5.1). TIMP and calmodulin both showed a marginal increase in the decidua from experimental animals on day 8, and a slight decrease on day 10. spp showed a decrease in the deciduas from experimental animals on day 8 and day 10 while calcyclin showed a large increase. The level of MEP mRNA was the same in day 8, but higher in day 10 experimental animals.

5.3 DISCUSSION

Examination of gene expression in the tissues that support pregnancy showed some common features, as well as some specific differences. In general the mRNA levels of TIMP, MEP, MRP spp and calcyclin each increased through mid gestation, achieving maximal levels between day 8 and day 10 in the uterus, decidua and placenta. However, the mechanisms of regulation must be complex since each of these genes also showed specific spatial and temporal pattern of expression. There was a large increase in the TIMP mRNA abundance in the ovary of day 18 and post partum mice,

Figure 5.10 Expression of TIMP, MEP, spp and calcyclin in artificial deciduomas.

Artificial deciduomas were induced in the left horn of the uterus on day 4.5 of pregnancy by injecting 10 μl of peanut oil. The uterus and deciduas were dissected out both from the normal side containing developing embryos, and from the side with the artificial deciduomas, on days 8 and 10 of pregnancy. Two μg of tissue RNA were analysed on slot blots with the probes indicated.

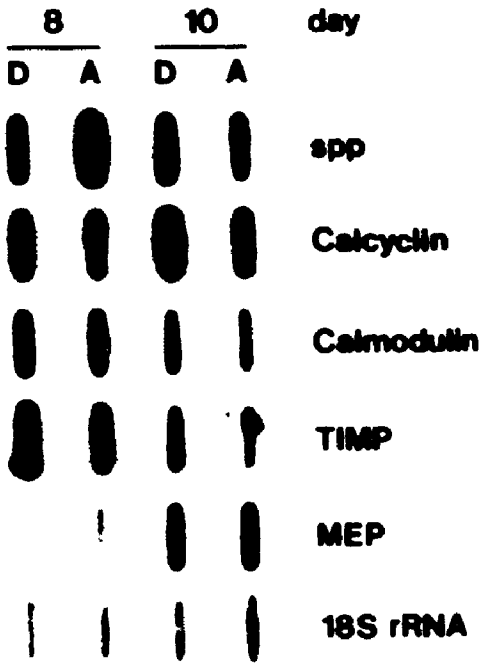

8
10
day
D
A
D
A
spp
Calcyclin
Calmodulin
TIMP
MEP
18S rRNA

TABLE 5.1

Changes in gene expression in the decidua between 'control' and 'experimentally[a] treated' mice.

	day 8		day 10	
gene	control	experimental	control	experimental
spp	3.7[b]	2.7 - 73%[c]	7.0	4.1 - 59%
Calcyclin	1.1	2.9 - 264%	1.2	3.2 - 267%
Calmodulin	1.0	1.3 - 130%	0.9	0.6 - 67%
TIMP	0.3	0.4 - 133%	0.3	0.2 - 67%
MEP	0.4	0.6 - 150%	0.9	1.8 - 200%

[a]Artificial deciduomas were generated in the left horn while the normal deciduas , used here, developed in the right horn which carried a normal pregnancy.

[b]These values were determined by densitometry of slot blots probed with the indicated cDNAs, and show the relative levels of the mRNA in the deciduas from control or experimental mice. The values between genes were normalized for the purpose of comparison, based on the intensity of the signal on the slot blot and the exposure time of the autoradiogram.

[c]The mRNA level of the experimental animal is expressed as a % of the level in the control animal.

and the later increases in MEP and calcyclin mRNAs in the amnion were specific for those genes.

The utmost care was taken to ensure the accuracy of the mRNA determinations. The RNAs were isolated and slotted under identical conditions; the individual sample loadings on slot blots were monitored by measuring the 18S rRNA levels; and the appropriate corrections made to the values expressed here. The variation of mRNA levels between mice, and even between each individual conceptus may account for some of the differences observed in separate experiments (Table 5.1, and TIMP in day 16 amnion figures 5.2A and 5.3 for example). On the whole however, the striking patterns of expression for each of the genes warrants further discussion.

TIMP mRNA levels in uterus, decidua and placenta were maximal on day 8 and then gradually declined (figure 5.2A). High levels of TIMP mRNA in day 7 decidua have been recently reported (Nomura *et al.*, 1989), although the expression in uterus and placenta, and the full time course were not examined.

The function of TIMP secreted from these tissues is to control MMPs such as collagenase, which must be participating in the ongoing tissue remodelling (Mullins and Rohrlich, 1983; Hembry and Ehrlich, 1986). A second function could be to block invasion of foetal trophoblasts into the maternal tissues. Trophoblasts are as invasive as the highly metastatic B16F10 melanoma cells (Yagel *et al.*, 1988). Altered TIMP levels have been shown to correspond to a cell's invasive properties (Khokha *et al.*, 1989), and exogenously added TIMP can block invasion *in vitro* (Thorgeirson *et al.*, 1982) and metastasis *in vivo* (Schultz *et al.*, 1988).

The level of TIMP expression in the tissues supporting pregnancy was too low to be detected by *in situ* hybridization (results not shown). The similarity between the time course of TIMP and spp mRNA levels (figures 5.3A, 5.8 and 5.10), coupled with the observations that (1) spp was immunolocalized to osteoblasts (Mark *et al.*, 1988a). Both TIMP and spp mRNAs have been localized to developing bone by *in situ* hybridization (Nomura *et al.*, 1988; 1989); and (2) spp mRNA was localized to GMG cells (Nomura *et al.*, 1988; figure 5.9 h,k) leads me to propose that TIMP is being synthesized by the GMG cells, which are also of bone marrow origin (Peel *et al.*, 1983). Further, GMG cells have been shown to have the capacity to kill trophoblast cells *in vitro* (Stewart and Mukhtar, 1988). This proposal could be confirmed if a sensitive anti-mouse TIMP antisera were available, or by analysis of RNA from isolated GMG cells.

Very specific and isolated increases of TIMP mRNA were seen in day 10 and day 18 amnion (figures 5.2A and 5.3A), and in ovary on day 18 and post partum (figure 5.2A and B). High levels of TIMP mRNA in the thecal cells of virgin ovary were previously reported (Nomura *et al.*, 1989), but I have shown here that the TIMP mRNA levels of day 18 and post partum ovary are far greater. Nomura *et al.* (1989) proposed that TIMP may function in ovary to prevent premature destruction of the corpus lutea. The function again may be to prevent inappropriate ovulation in response to fluctuating hormone levels at the end of term. This could be determined by analyzing TIMP mRNA levels in the ovary in response to injected progesterone or oestrogen, and then measuring the TIMP activity levels. It would also be informative to determine the collagenase mRNA levels at these different times.

TIMP activity in amniotic fluid showed a sudden decrease at day 14 and rose thereafter (figure 5.3B). Since this result was based on samples obtained from one time course experiment the result needs to be repeated before any significance can be attached to it. The TIMP mRNA levels did not show a corresponding drop, however a surge of collagenase or other MMP synthesis would cause a drop in active TIMP level.

The only control element so far identified in the single copy TIMP gene is an enhancer element in the first intron, which also confers virus inducibility (Coulombe *et al.*, 1988). It remains to be seen if TIMP expression is hormone inducible and if there are hormone response elements in the promoter region. The increase of TIMP mRNA in the amnion during late gestation may be due to the increased progesterone levels which rise again after day 14 (Atkinson and Hooker, 1945), either alone or in combination with growth factors from the foetus. Expression of other genes in the amnion has been shown to be controlled by external factors. Prostaglandin E_2 synthesis by human amnion at the onset of labour is triggered by cortisol, or by dexamethasone (Potestio *et al.*, 1988). TIMP synthesis is also responsive to glucocorticoids (Clark *et al.*, 1987) and so the build up of cortisol in the amniotic fluid may also be responsible for the day 18 burst of TIMP mRNA. The expression of *fos* in primary amnion cells was shown to depend on the addition of embryo derived factors (Müller *et al.*, 1986). The same case is likely true for some of the genes described here which are expressed in the amnion.

The mid-gestational nadir of MEP mRNA in placenta and decidua is consistent with the lysosomal cysteine protease playing a role in tissue remodelling at a time when the placenta proper is developing. The decidua

basalis is also undergoing changes to accommodate the growing placenta. The end of term increase of MEP mRNA would be associated with dissolution of these tissues as intact structures. The increasing MEP levels in the late gestational amnion may again be involved with tissue remodelling as the amnion expands to accommodate a growing foetus.

Transin and transin-2 mRNA expression was limited to ovary and decidua of the post partum animal. Expression of these secreted metalloproteinases in the decidua is consistent with resorption of this tissue. The two transins show some differences in regulation. Transin-2 is expressed at the same high level in both ovary and decidua, while transin is expressed at a high level in post partum decidua and at a much lower level in the post-partum ovary. These bursts of transin expression coincided with TIMP expression in these tissues, suggesting a coordinate regulation. A similar coordinate regulation was observed for TGF-ß which increased TIMP and collagenase type I expression and decreased collagenase type IV resulting in a net matrix deposition (Overall *et al.*, 1989).

Synthesis of MRP by placenta was first demonstrated by Linzer *et al.* (1985). *In situ* localization was used to demonstrate that the trophoblast giant cells of the placenta synthesized a high level of MRP (Lee *et al.*, 1988). Figure 5.5 shows MRP mRNA in the decidua and uterus on day 10, suggesting that either MRP was also being synthesized by a maternal tissue and/or that a trophoblast cell type had migrated into the decidua and uterus. *In situ* localization in day 10 sections did not reveal any MRP mRNA specifically in decidua or uterus (figure 5.6). The high level of MRP mRNA seen in the slot blot and Northern blot of day 10 uterus should have been detectable by the *in situ* technique. A trivial explanation is

contamination of the tissues during the dissection, however the utmost care was taken especially when reproducing the result. Synthesis of MRP by a maternal tissue is an attractive hypothesis to explain this discrepancy, however MRP synthesis was not detected in the day 10 artificial deciduomas.

The changes in mRNA levels of calcyclin and spp were most intriguing. Their temporal and spatial patterns of expression were almost mutually exclusive of each other. The level of calcyclin mRNA declined after reaching a maximum on day 8; spp mRNA levels increased rapidly after day 8. Calcyclin mRNA was localized by *in situ* hybridization to the decidua, especially the decidua capsularis in the antimesometrial region, as well as the placenta (figure 5.9, e). The GMG cells were the site of spp expression (figure 5.9; and Nomura *et al.*, 1988), and GMG cells are restricted to the metrial region at the opposite pole of the uterus. The band of spp expression adjacent to the smooth muscle of the uterus, between the decidua and the uterus in the antimesometrial region, is reported here for the first time.

Expression of spp in the bone-marrow derived GMG is likely under control of a specific promoter element and transcription factor similar to the Oct-2 specific factor in B cells, as well as being responsive to hormonal changes. The increase in spp expression correlated to a day 8 rise in oestrogen levels (Atkinson and Hooker, 1945), although the spp mRNA level did not stay elevated, as does the estrogen level, in late gestation. The spp mRNA level in kidney was also unaffected by changing hormone levels throughout gestation.

The human calcyclin gene is reported to have an EGF negative regulatory element (Ghezzo *et al.*, 1988). The drop in calcyclin mRNA in

response to TPA (figure 5.7C) would suggest that it also has a TPA negative response element. The decline of calcyclin expression may also be due to hormonal changes, or it may be responsive to EGF (like) growth factor(s) that are being secreted by the embryo.

The expression of another member of the S100 Ca^{++} binding protein family called oncomodulin shows a very different distribution in rat (Brewer and Ross, 1988). Expression was detected by *in situ* hybridization in the preimplantation blastocyst, the ectoplacental cone and in the amnion. Northern blot analysis showed a steady increase in oncomodulin mRNA throughout gestation in the placenta.

The artificial deciduomas were subject to the hormonal milieu of the pregnant mother, without the direct presence of the foetus. The decidual mRNA levels from the pregnant side were compared to those in the artificial deciduomas to determine the influence of the foetus on expression of these genes. The differences in specific mRNA levels between the normal and artificial deciduomas from the same animals were only slight, so it must be concluded that the factors regulating expression of these genes were largely maternal in origin. Less likely is the possibility that factors of foetal origin diffused through the maternal circulation and acted on the artificial deciduomas. More striking were the variations between the pregnant side of the experimental animals and animals carrying a full pregnancy in both horns of the uterus. These differences perhaps arise due to the lesser burden in the experimental animals.

CHAPTER 6 - SUMMARY

Several growth factor inducible genes were being studied in our laboratory to gain an understanding of their function and regulation of expression. As a first step in my work a murine cDNA expression library was constructed and the full length cDNAs for several genes were isolated by using the specific short cDNA probes which were of interest in the lab. The nucleotide sequence revealed that two of the growth inducible clones were the murine homologues of TIMP and calcyclin. The nucleotide sequence of a long MEP clone showed that it was murine procathepsin L.

The cDNAs were cloned into prokaryotic expression vectors to overexpress the encoded murine proteins in *E. coli* with an aim to purify the native TIMP and MRP proteins for subsequent biochemical analysis. An advantage of this system is that random or specifically engineered mutations can be introduced into the proteins to elucidate their function or mode of action. For example, the cysteines in TIMP could be mutated to determine the contribution from each of the six disulfide bridges. The collagen-like pro-his-pro-gln sequence near the amino terminus of TIMP could also be mutated to elucidate its role in recognition or binding of collagenase by TIMP.

We could only obtain a low level of expression in *E. coli* for the native forms of both TIMP and MRP. The pCQV2 expression cassette that was used to express MRP had been shown to overproduce human p53 to 10% of TCP (Queens, 1983). The pIN-ompA secretion vector used here to express native TIMP had been successfully used to overexpress the nuclease A protein (Takahara *et al.*, 1985) and ß-lactamase (Grayeb *et al.*, 1984). Several eukaryotic proteins such as Myc (Watt *et al.*, 1985), adenovirus

E1A (Ferguson *et al.*, 1984), bovine α_1-antitrypsin (Courtney *et al.*, 1984) and somatomedin C (Buell *et al.*, 1985) have all been expressed at high levels in *E. coli* using similar strategies.

TIMP was expressed from a secretion vector to sequester it in the periplasm away from the cytoplasmic proteases. Unfortunately only low levels were obtained using this strategy. It may have been expressed to a higher level if an *E. coli* strain defective in periplasmic proteases were used as the host. Alternatively, a higher level of expression from a stronger promoter may overwhelm the prokaryotic proteases, allowing TIMP (or MRP) to accumulate. The stability of TIMP and MRP fusion proteins, which were subsequently used to generate specific antisera, was likely due to the inaccessibility of the insoluble fusion protein aggregate to proteases. An alternative strategy could be to overexpress TIMP in yeast which may also fold and secrete the mature active protein.

A gene can be regulated at several steps during expression. The nucleotide sequence of the longest TIMP cDNA revealed the presence of an upstream overlapping ORF. The potential of this ORF to interfere with initiation of the TIMP start codon was studied in an *in vitro* transcription/ translation system. TIMP was found to be translated at least three fold more efficiently in the absence of the upstream ORF. This difference in translational efficiency is consistent with the 'leaky' scanning mechanism described by Kozak (1986b). The mechanism of blocking the initiation of TIMP translation could be more rigorously tested by mutating the upstream start codon and observing an increase of TIMP translation similar to that seen for the truncated TIMP transcript. The *in vitro* translated TIMP was not active in inhibiting collagenase, as was previously reported by Coulombe

and Skup (1988).

The development of female mouse tissues which support growth of the foetus during pregnancy was used as a model to study the expression of growth inducible genes *in vivo*. The time course of expression of TIMP in the decidua was consistent with a role in blocking the invasion of foetal trophoblast cells into maternal tissues. TIMP may also be acting in the decidua to control collagenase (or MMP) mediated tissue remodelling throughout mid and late gestation. The large increase of TIMP mRNA in ovary at the end of gestation coincided with a turn on of transin-1 and may be functioning here, as well as in the ovary of virgin mice, to block ovulation mediated by collagenase. Expression of TIMP at the end of term may be due to an increase in serum oestrogen levels. A straight forward experiment would be to test the response of TIMP levels to injected oestrogen or oestrogen agonists in virgin mice and in pregnant mice at the end of term. A combination of oestrogen and progesterone may be required. The appropriate hormone response elements have not yet been reported, and would be expected to be found in the promoter region of the TIMP gene.

The precise functions of the Ca^{++} binding proteins calcyclin and SPP are not known although SPP is postulated to act as an attachment and guiding factor for some migrating cells (Oldberg *et al.*, 1986; Butler, 1989). Calcyclin likely plays a regulatory role as do other proteins in the S100 Ca^{++} binding protein family. The spatial and temporal patterns of expression of these two proteins were mutually exclusive which may arise due to their different mechanisms of regulation. For example, calcyclin has serum response sequences in the promoter region and an FGF negative response element further upstream (Ghezzo *et al.*, 1988; 1989). The spp

promoter contains response elements for oestrogen, glucocorticoids, retinoic acid and TPA and another as yet uncharacterized negative response element further upstream (Craig, 1989). The spp mRNA levels are increased by growth factors (Smith and Denhardt, 1987), TGF-ß and vitamin D_3 (Noda *et al.*, 1988; Prince and Butler, 1987). There may also be cell type specific factors that act in a similar manner to the Oct-2 transcription factor since spp expression was restricted to GMG cells and calcyclin to a different region, in the decidua capsularis.

A greater understanding of the function of these genes could be gained by regulated expression in tissue culture cell systems or in transgenic animals that are down regulated for spp or calcyclin expression in a tissue specific manner and examination of the physiological consequences.

Almandral, J.M., Sommer, D., Macdonald-Bravo, H., Burckhardt, J., Perera, J., and Bravo, R. (1988) Complexity of the early genetic response to growth factors in mouse fibroblasts. Mol. Cell. Biol. **8**, 2140-2148.

Andres, A.-C., Schnenberger, C.-A., Groner, B., Hennighausen, L., LeMeur, M. and Gerlinger, P. (1987) Ha-*ras* oncogene expression directed by a milk protein gene promoter: tissue specificity, hormonal regulation, and tumor induction in transgenic mice. Proc. Natl. Acad. Sci. USA **84**, 1299-1303.

Atkinson, W.B. and Hooker, C.W. (1945) The day to day level of estrogen and progestin throughout pregnancy and pseudopregnancy in the mouse. Anatomical Record, 75-95.

Bachmair, A., Finley, D. and Varshavsky, A. (1986) *In vivo* half-life of a protein is a functin of its amino-terminal residue. Science **234**, 179-186.

Balmain, A. (1985) Transforming *ras* oncogenes and multistage carcinogenesis. Br. J. Cancer **51**, 1-7.

Belayew, A. and Tilghman, S.M. (1982) Genetic analysis of α-fetoprotein synthesis in mice. Mol. Cell. Biol. **2**, 1427-1435.

Bell, S.C. (1984) Decidualization: Regional differentiation and associated function. Oxford Rev. Rep. Biol. **6**, 220-271.

Bentley, D.L. and Groudine, M. (1986) A block to elongation is largely responsible for decreased transcription of c-*myc* in differentiated HL60 cells. Nature **321**, 702-706.

Bernard, H.-U., Remaut, E., Hershfield, M.V., Das, H.K., Helinski, D.R., Yanofsky, C. and Franklin, N. (1979) Construction of plasmid cloning vehicles that promote gene expression from the bacteriophage lambda p_L promoter. Gene **5**, 59-76.

Billington, W.D. and Bell, S.C. (1983) Immunobiology of mouse trophoblast. In Y.W. Lake and A. Whyte (eds.) Biology of Trophoblast, pp. 571-595.

Birnboim, H.C. and Doly, J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nuc. Acid Res. **7**, 1513-1523.

Bishop, J.M. (1981) Enemies within: the genesis of retrovirus oncogenes. Cell **23**, 5.

Bolivar, F., Rodriguex, R L., Greene, P.J., Betlach, M.C., Heyneker, H.L., Boyer, H.W., Crosa, J.H. and Falkow, S. (1977) Construction and characterization of new cloning vehicles: II. A multipurpose cloning system. Gene **2**, 95-113.

Bonner, W.M. and Laskey, R.A. (1974) A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur. J.

Biochem. **46**, 83-88.

Breathnach, R., Matrisian, L.M., Gesnel, M.C., Staub, A. and Leroy, P. (1987) Sequences coding for part of oncogene-induced transin are hig'ly conserved in a related rat gene. Nucl. Acids Res. **15**, 1139-1151.

Brewer, G. and Ross, J. (1988) Poly(A) shortening and degradation of the 3' A+U-rich sequences of human c-*myc* mRNA in a cell-free system. Mol. Cell. Biol. **8**, 1697-1708.

Broek, D., Samiy, N., Fasano, O., Fujiyama, A., Tamanoi, F., Northup, J. and Wigler, M. (1985) Differential activation of yeast adenylate cyclase by wild-type and mutant RAS proteins. Cell **41**, 763-769.

Brooks, R.F., Bennett, D.C. and Smith, J.A. (1980) Mammalian cell cycles need two random transitions. Cell **19**, 493-504.

Buell, G., Schulz, M.F., Selzer, G., Chollet, A., Movva, N.R., Semon, D., Escanez, S., Kawashima, E. (1985) Optimizing the expression in *E. coli* of a synthetic gene encoding somatomedin-C (IGF-I). Nuc. Acid Res. **13** 1923-1938.

Butler, W.T. (1989) The nature and significance of osteopontin. Collagen Rel. Res. (In press).

Campisi, J. and Pardee, A.B. (1984) Post-transcriptional control of the onset of DNA synthesis by an insulin-like growth factor. Mol. Cell. Biol. **4**, 1807-1814.

Cawston, T.E. (1986) Protein inhibitors of metallo-proteinases. In: Proteinase Inhibitors., Barrett and Salveson (eds.), pp. 589-610.

Cawston, T.E., Galloway, A., Mercer, E., Murphy, G. and Reynolds, J.J. (1981) Purification of rabbit bone inhibitor of collagenase. Biochem. J. **195**, 159-165.

Chiu, R., Boyle, W.J., Meek, J., Smeal, T., Hunter, T. and Karin, M. (1988) The c-fos protein interacts with c-Jun/WP-1 to stimulate transcription of AP-1 responsive genes. Cell **54**, 541-552.

Cho, K.W.Y., Goetz, J., Wright, C.V.E., Fritz, a., Hardwicke, J. and De Robertis, E.M. (1988) Differential utilization of the same reading frame in a *Xenopus* homeobox gene encodes two related proteins sharing the same DNA-binding specificity. EMBO J. **7**, 2139-2149.

Chowcat, N.L., Savage, F.J., Hembry, R.M. and Boulos, P.B. (1988) Role of collagenase in colonic anastomoses: a reappraisal. Br. J. Surg. **75**, 330-334.

Church, G.M. and Gilbert, W. (1984) U.V. dose for nylon. Proc. Natl. Acad. Sci. USA **81**, 1991-1995.

Clark, S.D., Kobayashi, D.K. and Welgus, H.G. (1987) Regulation of the

expression of tissue inhibitor of metalloproteinases and collagenase by retinoids and glucocorticoids in human fibroblasts. J. Clin. Invest. **80**, 1280-1288.

Clewell, D.B. and Helinski, D.R. (1969) Supercoiled circular DNA-protein complex in *Escherichia coli*: purification and induced conversion to an open circular DNA form. Proc. Natl. Acad. Sci. **62**, 1159-1166.

Colberg-Poley, A.M., Voss, S.D., Choudhury, K. and Gruss, P. (1985) Structural analysis of murine genes containing homeoobox sequences and their expression embryonal carcinoma cells. Nature **314**, 713-718.

Collier, I.E., Smith, J., Kronberger, A., Bauer, E.A., Wilhelm, S.M., Eisen, A.Z. and Goldberg, G.I. (1988) The structure of the human skin fibroblast collagenase gene. J. Biol. Chem. **263**, 10711-10713.

Comb, M., Mermod, N., Hyman, S.E., Pearlberg, J., Ross, E.M. and Goodman, H.M. (1988) Proteins bound at adjacent DNA elements act synergistically to regulate human proenkephalin cAMP inducible transcription. EMBO J. **7**, 3793-3805

Connor, A.M., Waterhouse, P., Khokha, R. and Denhardt, D.T. (1989) Characterization of a mouse mitogen-regulated protein/proliferin gene and its promoter: a member of the growth hormone/prolactin gene superfamily. Biochim. Biophys. Acta (In press).

Cori, C.F., Gluecksohn-Waelsch, S., Shaw, P.A. and Robinson, C. (1983) Correction of a genetically caused enzyme defect by somatic cell hybridization. Proc. Natl. Acad. Sci. USA **80**, 6611-6614.

Coulombe, B. and Skup, D. (1988) *In vitro* synthesis of the active tissue inhibitor of metalloproteinases encoded by a complementary DNA from virus-infected murine fibroblasts. J. Biol. Chem. **263**, 1439-1443.

Coulombe, B., Ponton, A., Daigneault, L., Williams, B.G. and Skup, D. (1988) Presence of transcription regulatory elements within an intron of the virus-inducible murine TIMP gene. Mol. Cell. Biol. **8**, 3227-3234.

Courtney, M., Buchwalder, A., Tessier, L.-H., Jaye, M., Benavente, A., Ballan, A., Kohli, V., Lathe, R., Tolstoshev and Lecocq, J.-P. (1984) High-level production of biologically active human α_1-antitrypsin in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **81**, 669-673.

Craig, A.M. (1989) Analysis of secreted phosphoprotein (2AR; osteopontin) gene expression and association with carcinogenesis. Ph.D. thesis, University of Western Ontario.

Craig, A.M., Nemir, M., Mukherjee, B.B., Chambers, A.F. and Denhardt, D.T. (1988) Identification of the major phosphoprotein secreted by many rodent cell lines as 2ar/osteopontin: enhanced expression in H-*ras*-transformed 3T3 cells. Biochem. Biophys. Res. Commun. **157**, 166-173.

Daily, L., Hanly, S.M., Roeder, R.G. and Heintz, N. (1986) Distinct

transcription factors bind specifically to two regions of the human histone H4 promoter. Proc. Natl. Acad. Sci. USA **83**, 7241-7245.

Das, M. (1981) Initiation of nuclear DNA replication: evidence for formation of committed prereplicative cellular state. Proc. Natl. Acad. Sci. USA **78**, 5677-5681.

Das, R.M. and Martin, L. (1978) Uterine DNA synthesis and cell proliferation during early decidualization induced by oil in mice. J. Reprod. Fert. **53**, 125-128.

DeLisle, A.J., Graves, R.A., Marzluff, W.F. and Johnson, L.F. (1983) Regulation of histone mRNA production and stability in serum-stimulated mouse 3T6 fibroblasts. Mol. Cell. Biol. **3**, 1920-1929.

Delli-Bovi, P., Curatola, A.M., Kern, F.G., Greco, A., Ittmann, M. and Basilico, C. (1987) An oncogene isolated by transfection of Kaposi's sarcoma DNA encodes a growth factor that is a member of the FGF family. Cell **50**, 729-737.

Denhardt, D.T., Edwards, D.R. and Parfett, C.L.J. (1986) Gene expression during the mammalian cell cycle. Biochim. Biophys. Acta **865**, 83-125.

Denhardt, D.T., Greenberg, A.H., Egan, S.E., Hamilton, R.T. and Wright, J.A. (1987) Cysteine proteinase cathepsin L expression correlated closely with the metastatic potential of H-*ras*-transformed murine fibroblasts. Oncogene **2**, 55-59.

Dickson, C. and Peters, G. (1987) Potential oncogene product related to growth factors. Nature **326**, 833.

Docherty, A.J.P., Lyons, A., Smith, B., Wright, C., Stephens, P., Harris, T.J.R., Murphy, G. and Reynolds, J.J. (1985) The cloning and expression of human tissue inhibitor of metallo-proteinases (TIMP). Nature **318**, 66-69.

Doolittle, R.F., Hunkapiller, M.W., Hood, L.E., Devare, S.G., Robbins, K.C., Aaronson, S.A. and Antoniades, H.N. (1983) Simian sarcomma virus oncogene, v-sis, is derived fromm the gene (or genes) encoding a platelet-derived growth factor. Science **221**, 275-277.

Dorin, J.R., Novak, M., Hill, R.E., Brock, D.J.H., Secher, D.S. and van Heyningen, V. (1987) A clue to the basic defect in cystic fibrosis from cloning the CF antigen gene. Nature **326**, 614-617.

Downward, J., Yarden, Y., Mayes, E., Scrase, G., Totty, N. Stockwell, P., Ullrich, A., Schlessinger, J. and Waterfield, M.D. (1983) Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences. Nature **307**, 521-557.

Duboule, D. and Dollé, P. (1989) The structural and functional organization of the murine HOX gene family resembles that of *Drosophila* homeotic genes. EMBO J. 8, 1497-1505.

Duprey, P., Chowdhury, K., Dressler, G.R., Balling, R., Simon, D., Guenet, J.-L. and Gruss, P. (1988) A mouse gene homologous to the *Drosophila* gene *caudal* is expressed in epithelial cells from the embryonic intestine. Genes & Devel. **2**, 1647-1654.

Ebina, Y., Ellis, L., Harnagin, K., Edery, M., Graf, L., Clausen, E., Ou, J., Masiazz, F., Kan, Y.K., Goldfine, I.D., Roth, R.A. and Rutter, W.J. (1985) The human insulin receptor cDNA: the structural basis for hormone-activated transmembrane signalling. Cell **40**, 747-758.

Edwards, D.R., Parfett, C.L.J. and Denhardt, D.T. (1985a) Transcriptional regulation of two serum-induced RNAs in mouse fibroblasts: equivalence of one species to B2 repetitive elements. Mol. Cell. Biol. **5**, 3280-3288.

Edwards, D.R. and Denhardt, D.T. (1985b) A study of mitochondrial and nuclear transcription with cloned cDNA probes. Exp. Cell Res. **157**, 127-143.

Edwards, D.R., Parfett, C.L.J., Smith, J.H. and Denhardt, D.T. (1987a) Evidence that post-transcriptional changes in the expression of mitogen regulated protein accompany immortalization of mouse cells. Biochem. Biophys. Res. Comm. **147**, 467-473.

Edwards, D.R., Murphy, G., Reynolds, J.J., Whitham, S.E., Docherty, A.J.P., Angel, P. and Heath, J.K. (1987b) Transforming growth factor beta modulates the expression of collagenase and metalloproteinase inhibitor. EMBO J. **6**, 1899-1904.

Edwards, D.R., Waterhouse, P., Holman, M.L. and Denhardt, D.T. (1986) A growth-responsive gene (16C8) in normal mouse fibroblasts homologous to a human collagenase inhibitor with erythroid-potentiating activity: evidence for inducible and constitutive transcripts. Nucl. Acids Res. **14**, 8863-8878.

Eladari, M.E., Syed, S.H., Guilhot, S., d'Auriol, L. and Galibert, F. (1986) On the high conservation of the human *c-myc* first exon. Biochem. Biophys. Res. Commun. **140**, 313-319.

Ferguson, B., Jones, N., Richter, J. and Rosenberg, M. (1984) Adenovirus E1a gene product expressed at high levels in *Escherichia coli* is functional. Science **224**, 1343-1345.

Ferrari, S. and Baserga, R. (1987) Oncogenes and cell cycle genes. Bioessays **7**, 9-13.

Ferrari, S., Calabretta, B., deRiel, J.K., Battini, R., Ghezzo, F., Lauret, E., Griffin, C., Emanuel, B.S., Gurrieri, F. and Baserga, R. (1987) Structural and functional analysis of a growth-regulated gene, the human calcyclin. J. Biol. Chem. **262**, 8325-8332.

Florkeiwicz, R.Z. and Sommer, A. (1989) Human basic fibroblast growth factor gene encodes four polypeptides: three initiate translation from

non-AUG codons. Proc. Natl. Acad. Sci. USA **86**, 3978-3981.

Frisch, S.M., Clark, E.J. and Werb, Z. (1987) Coordinate regulation of stromelysin and collagenase genes determined with cDNA probes. Proc. Natl. Acad. Sci. USA **84**, 2600-2604.

Fullmer, C.S. and Wasserman, R.H. (1981) The amino acid sequence of bovine intestinal calcium-binding protein. J. Biol. Chem. **256**, 5669-5674.

Fung, Y.-K. T., Schackleford, G.M., Brown, A.M.C., Sanders, G.S., and Warmus, H.E. (1985) Nucleotide sequence and expression *in vitro* of cDNA derived from mRNA of int-1, a provirally activated mouse mammary oncogene. Mol. Cell. Biol. **5**, 3337-3344

Gal, S. and Gottesman, M.M. (1986) The major excreted protein of transformed fibroblasts is an activable acid-protease. J. Biol. Chem. **261**, 1760-1765.

Gal, S. and Gottesman, M.M. (1986) The major excreted protein (MEP) of transformed mouse cells and cathepsin L have similar protease specificity. Biochem. Biophys. Res. Commun. **139**, 156-162.

Gal, S., Willingham, M.C. and Gottesman, M.M. (1985) Processing and lysosomal localization of a glycoprotein whose secretion is transformation stimulated. J. Cell Biol. **100**, 535-544.

Galloway, W.A., Murphy, G., Sandy, J.D., Gavrilovic, J., Cawston, T.E. and Reynolds J.J. (1983) Purification and characterization of a rabbit bone metalloproteinase that degrades proteoglycan and other connective tissue components. Biochem. J. **209**, 741-752.

Gaunt, S.J. (1988) Mouse homeobox gene transcripts occupy different but overlapping domains in embryonic germ layers and organs: a comparison of *Hox-3.1* and *Hox-1.5*. Development **103**, 135-144.

Gaunt, S.J. (1987) Homoeobox gene *Hox-1.5* expression in mouse embryos: earliest detection by *in situ* hybridization is during gastrulation. Development **101**, 51-60.

Gehring, W.J. and Hiromi, Y. (1986) Homeotic genes and the homeobox. Ann. Rev. Genet. **20**, 147-173.

Gewert, D.R., Coulombe, B., Castelino, M., Skup, D. and Williams, B.R.G. (1987) Characterization and expression of a murine gene homologous to human EPA/TIMP: a virus-induced gene in the mouse. EMBO J. **6**, 651-657.

Ghezzo, F., Lauret, E., Ferrari, S. and Baserga, R. (1988) Growth factor regulation of the promoter of calcyclin, a growth-regulated gene. J. Biol. Chem. **263**, 4758-4763.

Ghezzo, F., Valpreda, S., de Riel, J. and Baserga, R. (1989) Identification of serum-responsive elements in the promoter of human calcyclin, a

growth-regulated gene. DNA **8**, 171-177.

Goldberg, G.I., Wilhelm, S.M., Kronberger, A., Bauer, E.A., Grant, G.A. and Eisen, A.Z. (1986) Human fibroblast collagenase. J. Biol. Chem. **261**, 6600-6605.

Gottesman, M.M. (1978) Transformation-dependent secretion of a low molecular weight protein by murine fibroblasts. Proc. Natl. Acad. Sci. USA **75**, 2767-2771.

Gottesman, S. and Gottesman, M. (1981) Protein degradation in E. coli: the *ion* mutation and bacteriophage lambda N and cII protein stability. Cell **24**, 225-233.

Graham, A., Papalopulu, N., Krumlauf, R. (1989) The murine and Drosophila homeobox gene complexes have common features of organization and expression. Cell **57**, 367-378.

Grant, D.S., Tashiro, K.-I., Sequi-Real, B., Yamada, Y., Martin, G.R. and Kleinman, H.K. (1989) Two different laminin domains mediate the differentiation of human endothelial cells into capillary-like structures *in vitro*. Cell **58**, 933-943.

Grayeb, J., Kimura, H., Takahara, M., Hsiung, H., Masui, Y. and Inouye, M. (1984) Secretion cloning vectors in *Escherichia coli*. EMBO J. **3**, 2437-2442.

Groner, B., Schönenberger, C.-A. and Andres, A.C. (1987) Targeted expression of the *ras* and *myc* oncogenes in transgenic mice. TIGS **3**, 306-308.

Gross, R.H., Sheldon, L.A., Fletcher, C.F. and Brinckerhoff, C.E. (1984) Isolation of a collagenase cDNA clone and measurement of changing collagenase mRNA levels during induction in rabbit synovial fibroblasts. Proc. Natl. Acad. Sci. USA **81**, 1981-1985.

Grunstein, M. and Hogness, D. (1985) Colony hybridization: A method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. **72**, 3961-3965.

Guo, X., Waterhouse, P., Craig, A.M., Parfett, C.L.J., Murphy, L., Reid, R., Edwards, D.R. and Denhardt, D.T. (1989) Identification of serum-inducible mRNA (5B10) and its tissue distribution: 5B10 is the mouse homolog of human calcyclin. (in preparation).

Halaka, A.N., Bunning, R.A.D., Bird, C.C., Gibson, M. and Reynolds, J.J. (1983) Production of collagenase and inhibitor (TIMP) by intracranial tumours and dura *in vitro*. J. Neurosurg. **59**, 461-466.

Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**, 557-580.

Harris, E.D., Welgus, H.G. and Krane, S.M. (1984) Regulation of the

mammalian collagenases. Collagen Rel. Res. **4**, 493-512.

Heintz, N., Sive, H.L. and Roeder, R.G. (1983) Regulation of histone gene expression: Kinetics of accumulation and changes in the rate of synthesis and in the half-lives of individual histone mRNAs during the HeLa cell cycle. Mol. Cell. Biol. **3**, 539-550.

Helfman, D.M., Feramisco, J.R., Fiddes, J.C., Thomas, G.P. and Hughes, S.H. (1983) Identification of clones that encode chicken tropomyosin by direct immunological screening of a cDNA expression library. Proc. Natl. Acad. Sci. USA **80**, 31-35.

Hembry, R.M. and Ehrlich, H.P. (1986) Immunolocalization of collagenase and tissue inhibitor of metalloproteinases (TIMP) in hypertrophic scar tissue. Br. J. Dermatol. **115**, 409-420.

Hembry, R.M., Murphy, G. and Reynolds, J.J. (1985) Immmunolocalization of tissue inhibitor of metalloproteinases (TIMP) in human cells: characterization and use of a specific antiserum. J. Cell Sci. **73**, 105-119.

Herron, G.S., Banda, M.J., Clark, E.J., Gavrilovic, J. and Werb, Z. (1986a) Secretion of metalloproteinases by stimulated capillary endothelial cells. J. Biol. Chem. **261**, 2814-2818.

Herron, G.S., Werb, Z., Dwyer, K. and Banda, M.J. (1986b) Secretion of metalloproteinases by stimulated capillary endothelial cells. J. Biol. Chem. **261**, 2810-2813.

Higgins, G.A. and Wilson, M.C. (1987) *In situ* hybridization for mappinng the neuroanatomical distribution of novel brain mRNAs, p. 146-162. In K.L. Valentino, J.H. Eberwine and J.D. Barchas (eds.) *In Situ* Hybridization. Oxford University Press, New York.

Holland, P.W.H. and Hogan, B.L.M. (1988a) Expression of homeo box genes during mouse development: a review. Genes & Devel. **2**, 773-782.

Holland, P.W.H. and Hogan, B.L.M. (1988b) Spatially restricted patterns of expression of the homeobox-containing gene *Hox 2.1.* during mouse embryogenesis. Development **102**, 159-174.

Howard, A. and Pelc, S.R. (1953) Synthesis of desoxyribonucleic acid in normal and irradiated cells and its relation to chromosome breakage. Heredity **6**, 261-273.

Humphries, M.J., Olden, K. and Yamada, K.M. (1986) A synthetic peptide from fibronectin inhibits experimental metastasis of murine melanoma cells. Science **233**, 467-470.

Hunter, T., Duncan, S., Dew, G. and Reynolds, J.J. (1984) The effects of antirheumatic drugs on the production of collagenase and tissue inhibitor of metalloproteinases (TIMP) by stimulated rabbit articular chrondrocytes. J. Rheumatology **11**, 9-13.

Humphries, M.J., Olden, K., and Yamada, K.M. (1986) A synthetic peptide from fibronectin inhibits experimental metastasis of murine melanoma cells. Science **233**, 467-470.

Hunziker, R.D., Gambel, P. and Wegmann, T.G. (1984) Placenta as a selective barrier to cellular traffic. J. Immunol. **133**, 667-671.

Ikemura, T. (1981) Correlation between the abundance of *Escherichia coli* transfer RNAs and the occurrence of the respective codons in its protein genes. J. Mol. Biol. **146**, 1-21.

Imagawa, M., Chiu, R. and Karin, M. (19877) Transcription factor AP-2 mediates induction by two different signal-transduction pathways: protein kinase C and cAMP. Cell **51**, 251-260.

Ingber, D.E. and Folkman, J. (1989a) How does extracellular matrix control copillary morphogenesis. Cell **58**, 803-805.

Ingber, D.E. and Folkman, J. (1989b) Mechanochemical switching between growth and differentiation during fibroblast growth factor-stimulated angiogenesis *in vitro*: role of extracellular matrix. J. Cell Biol. **109**, 317-330.

Ingraham, H.A., Chen, R., Mangalam, H.J., Elsholtz, H.P., Flynn, S.E., Lin, C.R., Simmons, D.M., Swanson, L. and Rosenfeld, M.G. (1988) A tissue-specific transcription factor containing a homeodomain specifies a pituitary phenotype. Cell **55**, 519-529.

Ito, A., Goshowaki, H., Sato, T., Mori, Y., Yamashita, T. and Nagase, H. (1988) Human recombinant interleukin-1α-mediated stimulation of procollagenase production and suppression of biosynthesis of tissue inhibitor of metalloproteinase in rabbit uterine cervical fibroblasts. FEBS **234**, 326-330

Jackson-Grusby, L.L., Swiergiel, J. and Linzer, D.I.H. (1987) A growth-related mRNA in cultured mouse cells encodes a placental calcium binding protein. Nucl. Acids Res. **15**, 6677-6690.

Jakobovits, A., Shackleford, G.M., Varmus, H.E. and Martin, G.R. (1986) Two proto-oncogenes implicated in mammary carcinogenesis, *int*-1 and *int*-2, are independently regulated during mouse development. Proc. Natl. Acad. Sci. USA **83**, 7806-7810.

Kane, S.E., Troen, B.R., Gal, S., Ueda, K., Pastan, I. and Gottesman, M.M. (1988) Use of a cloned multidrug resistance gene for coamplification and overproduction of major excreted protein, a transformation-regulated secreted acid protease. Mol. Cell. Biol. **8**, 3316-3321.

Karin, M. (1989) Complexities of gene regulation by cAMP. TIGS **5**, 65-67.

Kearns, M. (1984) Ph.D. Thesis, McGill University.

Kerr, L.D., Olashaw, N.E. and Matrisian, L.M. (1988) Transforming growth

factor ß1 and cAMP inhibit transcription of epidermal growth factor- and oncogene-induced transin RNA. J. Biol. Chem. **263**, 16999-17005.

Khandjian, E.W. (1987) Optimized hybridization of DNA blotted and fixed to nitrocellulose and nylon membranes. Bio/technology **5**, 165-167.

Kirschke, H., Kembhavi, A.A., Bohley, P. and Barrett, A.J. (1982) Action of rat liver cathepsin L on collagen and other substrates. Biochem. J. **201**, 367-372.

Kirschke, H., Wikstrom, P. and Shaw, E. (1988) Active center differences between cathepsins L and B: the S_1 binding region. FEBS **228**, 128-130.

Kishi, J. and Hayakawa, T. (1984) Purification and characterization of bovine dental pulp collagenase inhibitor. J. Biochem. **96**, 395-404.

Khokha, R., Waterhouse, P., Yagel, S., Lala, P.K., Overall, C.M., Norton, G. and Denhardt, D.T. (1989) Antisense RNA-induced reduction in murine TIMP levels confers oncogenicity on Swiss 3T3 cells. Science **243**, 947-950.

Kligman, D. and Hilt, D.C. (1988) The S100 protein family. TIBS **13**, 437-443.

Ko, H.-S., Fast, P., McBride, W. and Staudt, L.M. (1988) A human protein specific for the immunoglobulin octamer DNA motif contains a functional homeobox domain. Cell **55**, 135-144.

Kouzarides, T. and Ziff, E. (1988) The role of the leucine zipper in the fos-jun interaction. Nature **336**, 646-651.

Kowalski, J., Smith, J.H., Ng, N. and Denhardt, D.T. (1985) Vectors for the direct selection of cDNA clones corresponding to mammalian cell mRNA of low abundance. Gene **35**, 45-54.

Kozak, M. (1986a) Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell **44**, 283-292.

Kozak, M. (1986b) Bifunctional messenger RNAs in eukaryotes. Cell **47**, 481-483.

Kozak, M. (1988) Leader length and secondary structure modulate mRNA function under conditions of stress. Mol. Cell. Biol. **8**, 2737-2744.

Kozak, M. (1989) The scanning model for translation: an update. J. Cell Biol. **108**, 229-241

Krieg, P.A. and Melton, D.A. (1984) Functional messenger RNAs are produced by SP6 *in vitro* transcription of cloned cDNAs. Nucl. Acids Res. **12**, 7057-7070.

Krumlauf, R., Hammer, R.E., Tilghman, S.M. and Brinster, R.L. (1985) Developmental regulation of α-fetoprotein genes in transgenic mice. Mol.

Cell. Biol. **5**, 1639-1648.

Kuwano, R., Maeda, T., Usui, H., Araki, K., Yamakuni, T., Ohshima, Y., Kurihara, T. and Takahashi, Y. (1986) Molecular cloning of cDNA of S100α subunit mRNA. FEBS **202**, 97-101

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**, 680-685.

Lagasse, E. and Clerc, R.G. (1988) Cloning and expression of two human genes encoding calcium-binding proteins that are regulated during myeloid differentiation. Mol. Cell. Biol. **8**, 2402-2410.

Land, H., Parada, L.F. and Weinberg, R.A. (1983) Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Nature **304**, 596-602.

Landschulz, W.H., Johnson, P.F. and McKnight, S.L. (1988) The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. Science **240**, 1759-1764.

Lau, L.F. and Nathans, D. (1987) Expression of a set of growth-related immediate early genes in BALB/c 3T3 cells: coordinate regulation with c-*fos* or c-*myc*. Proc. Natl. Acad. Sci. USA **84**, 1182-1186.

Lee, S.-J. and Nathans, D. (1988) Proliferin secreted by cultured cells bind to mannose 6-phosphate receptors. J. Biol. Chem. **263**, 3521-3527.

Lee, S.-J., Talamantes, F., Wilder, E., Linzer, D.I.H. and Nathans, D. (1988) Trophoblastic giant cells of the mouse placenta as the site of proliferin synthesis. Endocrinology **122**, 1761-1768.

Le Mouellic, H., Condamine, H. and Brûlet, P. (1988) Pattern of transcription of the homeo gene *Hox-3.1* in the mouse embryo. Genes & Devel. **2**, 125-135.

Leof, E.B., Wharton, W., Van Wyk, J.J. and Pledger, W.J. (1982) Epidermal growth factor (EGF) and somatomedin C regulate G1 progression of competent Balb/c 3T3 cells. Exp. Cell Res. **141**, 107-115.

Levine, B.J., Chodchoy, N., Marzluff, W.F., and Skoultchi, A.I. (1987) Coupling of replication type histone mRNA levels to DNA synthesis requires the stem-loop sequence at the 3' end of the mRNA. Proc. Natl. Acad. Sci. USA **84**, 6189-6193.

Levine, M. and Hoey, T. (1988) Homeobox proteins as sequence-specific transcription factors. Cell **55**, 537-540.

Levine, M., Rubin, G.M. and Tjian, R. (1984) Human DNA sequences homologous to a protein coding region conserved between homeotic genes of Drosophila. Cell **38**, 667-673.

Linzer, D.I.H., Lee, S.-J., Ogren, L., Talamantes, F., Nathans, D. (1985) Identification of proliferin mRNA and protein in mouse placenta.

Proc. Natl. Acad. Sci. USA **82**, 4356-4359.

Linzer, D.I.H. and Mordacq, J.C. (1987) Transcriptional regulation of proliferin gene expression in response to serum in transfected mouse cells. EMBO J. **6**, 2281-2288.

Linzer, D.I.H. and Nathans, D. (1983) Growth-related changes in specific mRNAs of cultured mouse cells. Proc. Natl. Acad. Sci. USA **80**, 4271-4275.

Linzer, D.I.H. and Wilder, E.L. (1987) Control of proliferin gene expression in serum-stimulated mouse cells. Mol. Cell. Biol. **7**, 2080-2086.

Loose, D.S., Shaw, P.A., Krauter, K.S., Robinson, C., Englard, S., Hanson, R.W. and Gluecksohn-Waelsch, S. (1986) *Trans* regulation of the phospho*enol*pyruvate carboxykinase (GTP) gene, identified by deletions in chromosome 7 of the mouse. Proc. Natl. Acad. Sci. USA **83**, 5184-5188.

Macartney, H.W. and Tschesche, H. (1984) Characterization of β_1-anticollagenase from human plasma and its reaction with PMN collagenase by disulfide thiol interchange. Eur. J. Biochem. **130**, 83-92.

Machida, C.M., Muldoon, L.L., Rodland, K.D. and Magun, B.E. (1988) Transcriptional modulation of transin gene expression by epidermal growth factor and transforming growth factor beta. Mol. Cell. Biol. **8**, 2479-2483.

Maniatis, T., Fritsch, E.F., and Sambrook (1982) Molecular cloning: A laboratory manual. Cold Spring Harbour Laboratory.

Mark, M.P., Butler, W.T., Prince, C.W., Finkelman, R.D. and Ruch, J.-V. (1988a) Developmental expression of 44-kDa bone phosphoprotein (osteopontin) and bone γ-carboxyglutamic acid (Gla)-containing protein (osteocalcin) in calcifying tissues of rat. Differentiation **37**, 123-136.

Mark, M.P., Prince, C.W., Gay, S., Austin, R.L. and Butler, W.T. (1988b) 44-kDal bone phosphoprotein (osteopontin) antigenicity at ectopic sites in newborn rats: kidney and nervous tissues. Cell Tissue Res. **251**, 23-30.

Mark, M.P., Prince, C.W., Oosawa, T., Gay, S., Bronckers, A.L.J.J. and Butler, W.T. (1987) Immunohistochemical demonstration of a 44-kD prhosphoprotein in developing rat bones. J. Histochem. Cytochem. **35**, 707-715.

Marth, J.D., Overall, R.W., Meier, K.E., Krebs, E.G. and Perlmutter, R.M. (1988) Translational activation of the *lck* proto-oncogene. Nature **332**, 171-173.

Mason, R.W., Walker, J.E. and Northrop, F.D. (1986a) The *N*-terminal amino acid sequences of the heavy and light chains of human cathepsin L.

Biochem. J. **240**, 373-377.

Mason, R.W., Johnson, D.A., Barrett, A.J. and Chapman, H.A. (1986b) Elastinolytic activity of human cathepsin L. Biochem. J. **233**, 925-927.

Matrisian, L.M., Glaichenhaus, N., Gesnel, M.-C. and Breathnach, R. (1985) Epidermal growth factor and oncogenes induce transcription of the same cellular mRNA in rat fibroblasts. EMBO J. **4**, 1435-1440.

Matrisian, L.M., Leroy, P., Ruhlmann, C., Gesnel, M.-C. and Breathnach, R. (1986a) Isolation of the oncogene and epidermal growth factor-induced transin gene: complex control in rat fibroblasts. Mol. Cell. Biol. **6**, 1679-1686.

Matrisian, L.M., Bowden, G.T., Krieg, P., Fürstenberger, G., Briand, J.-P., Leroy, P. and Breathnach, R. (1986b) The mRNA coding for the secreted protease transin is expressed more abundantly in malignant than in benign tumors. Proc. Natl. Acad. Sci. USA **83**, 9413-9417.

Maurizi, M.R., Trisler, P. and Gottesman, S. (1985) Insertional mutagenesis of the *lon* gene in *Escherichia coli*: *lon* is dispensable. J. Bacteriology **164**, 1124-1135.

Maxam, A.M. and Gilbert, W. (1977) A new method for sequencing DNA. Proc. Natl. Acad. Sci. USA **74**, 560-564.

McBurney, M.W., Jones-Villeneuve, E.M.V., Edwards, M.K.S. and Anderson, P.J. (1982) Control of muscle and neuronal differentiation in a cultured embryonal carcinoma cell line. Nature **299**, 165-167.

McGinnis, W., Levine, M.S., Hafen, E., Kuroiwa, A. and Gehring, W.J. (1984a) A conserved DNA sequence in homoeotic genes of the *Drosophila* antennapedia and bithorax complexes. Nature **308**, 428-433.

McGinnis, W., Hart, C.P., Gehring, W.J. and Ruddle, F.H. (1984b) Molecular cloning and chromosome mapping of a mouse DNA sequence homologous to homeotic genes of Drosophila. Cell **38**, 675-680.

McGrath, J.P., Capon, D.J., Goeddel, D.V. and Levinson, A.D. (1984) Comparative biochemical properties of normal and activated human *ras* p21 protein. Nature **310**, 644.

McGuire, M.K.B., Meats, J.E., Ebsworth, M.N., Murphy, G., Reynolds, J.J. and Russell, R.G.G. (1982) Messenger function of prostaglandins in cell to cell interactions and control of proteinase activity in the rheumatoid joint. Int. J. Immunopharmac. **4**, 91-102.

McGuire, M.B., Murphy, G., Reynolds, J.J. and Russell, R.G.G. (1981) Production of collagenase and inhibitor (TIMP) by normal, rheumatoid and osteoarthritic synovium *in vitro*: effects of hydrocortisone and indomethacin. Clin. Science **61**, 703-710.

Messing, J. (1983) New M13 vectors for cloning genes. Meth. Enzymol.

101, 20-78

Miller, J.H. (1972) In: Experiments in Molecular Genetics.p. 352-355. Cold Spring Harbour Laboratory, Cold Spring Harbour, N.Y.

Mintz, B. and Illmensee, K. (1975) Normal genetically mosaic mice produced from malignant teretocarcinoma cells. Proc. Natl. Acad. Sci. USA **72**, 3585-3589.

Morris, G.M. (1989) A high molecular weight collagenase inhibitor made by rabbit chondrocytes in cell culture. Matrix **9**, 127-134.

Morris, T., Marashi, F., Weber, L., Hickey, E., Greenspan, D., Bonner, J., Stein, J. and Stein, G. (1986) Involvement of the 5'-leader sequence in coupling the stability of a human H3 histone mRNA with DNA replication. Proc. Natl. Acad. Sci. USA **83**, 981-985.

Müller, R., Müller, D., Verrier, B., Bravo, R. and Herbst, H. (1986) Evidence that expression of c-*fos* protein in amnion cells is regulated by external signals. EMBO J. **5**, 311-316.

Müller, R., Bravo, R., Burckhardt, J. and Curran, T. (1984) Induction of c-*fos* gene and protein by growth factors precedes activation of c-*myc*. Nature **312**, 716-720.

Mullins, D.E. and Rohrlich, S.T. (1983) The role of proteinases in cellular invasiveness. Biochim. Biophys. Acta **695**, 177-214.

Mullins, L.J., Grant, S.G., Stephenson, D.A. and Chapman, V.M. (1988) Multilocus molecular mapping of the mouse X chromosome. Genomics **3**, 187-194.

Murphy, L.C., Murphy, L.J., Tsuyuki, D., Duckworth, M.L. and Shiu, P.C. (1988) Cloning and characterization of a cDNA encoding a highly conserved, putative calcium binding protein, identified by an anti-prolactin receptor antiserum. J. Biol. Chem. **263**, 2397-2401.

Murphy, G., Cockett, M.I., Stephens, P.E., Smith, B.J. and Docherty, A.J.P. (1987) Stromelysin is an activator of procollagenase. Biochem. J. **248**, 265-268.

Murphy, G., McAlpine, C., Poll, C. and Reynolds, J.J. (1985a) Purificatoin and characterization of a bone metalloproteinase that degrades gelatin and type IV and V collagen. Biochim. Biophys. Acta **831**, 49-5?

Murphy, G. and Reynolds, J.J. (1985) Progress towards understanding the resorption of connective tissues. BioEssays **2**, 55-60.

Murphy, G., Reynolds, J.J. and Werb, Z. (1985b) Biosynthesis of tissue inhibitor of metalloproteinases by human fibroblasts in culture. J. Biol. Chem. **260**, 3079-3083.

Murphy, G., Reynolds, J.J., Bretz, U. and Baggiolini, M. (1982) Partial purification of collagenase and gelatinase from human polymorphonuclear

leucocytes. Biochem. J. **203**, 209-221

Nakamura, K. and Inouye, M. (1979) DNA sequence of the gene for the outer membrane lipoprotein of *E. coli*: an extremely AT-rich promoter. Cell **18**, 1109-1117.

Nepveu, A. and Marcu, K.B. (1986) Intragenic pausing and anti-sense transcription within the murine c-*myc* locus. EMBO J. **5**, 2859-2865.

Newbold, R.F. and Overell, R.W. (1983) Fibroblast immortality is a prerequisite for transformation by EJ c-Ha-ras oncogene. Nature **304**, 648.

Nilsen-Hamilton, M., Shapiro, J.M., Massoglia, S.L. and Hamilton, R.T. (1980) Selective stimulation by mitogens of incorporation of ^{35}S-methionine into a family of proteins released into the medium by 3T3 cells. Cell **20**, 19-28.

Noda, M., Yoon, K., Prince, C.W., Butler, W.T. and Rodan, G.A. (1988) Transcriptional regulation of osteopontin prpduction in rat osteosarcoma cells by type ß transforming growth factor. J. Biol. Chem. **263**, 13916-13921

Nomura, S., Hogan, B.L.M., Wills, A.J., Heath, J.K. and Edwards, D.R. (1989) Developmental expression of tissue inhibitor of metalloproteinase (TIMP) RNA. Development **105**, 575-583.

Nomura, S., Wills, A.J., Edwards, D.R., Heath, J.K. and Hogan, B.L.M. (1988) Developmental expression of 2ar (Osteopontin) and SPARC (Osteopontin) RNA as revealed by *in situ* hybridization. J. Cell Biol. **106**, 441-450.

Nusse, R., van Ooyen, A., Cox, D., Fung, Y.K.T. and Varmus, H. (1984) Mode of proviral activation of a putative mammary oncogene (*int*-1) on mouse chromosome 15. Nature **307**, 131-136.

Oldberg, Å., Franzén, A. and Heinegård, D. (1986) Cloning and sequence analysis of rat bone sialoprotein (osteopontin) cDNA reveals an Arg-Gly-Asp cell-binding sequence. Proc. Natl. Acad. Sci. USA **83**, 8819-8823.

Okada, Y., Watanabe, S., Nakanishi, I., Kishi, J.-i., Hayakawa, T., Watorek, W., Travis, J. and Nagase, H. (1988) Inactivation of tissue inhibitor of metalloproteinases by neutrophil elastase and other serine proteinases. Fed. Eur. Biochem. Soc. **229**, 157-160.

Okayama, H. and Berg, P. (1982) High-efficiency cloning of full-length cDNAs. Mol. Cell. Biol. **2**, 161-170.

Oliver, G., Wright, C.V.E., Hardwicke, J. and De Robertis, E.M. (1988) Differential antero-posterior expression of two proteins encoded by a homeobox gene in *Xenopus* and mouse embryos. EMBO J. **7**, 3199-3209.

O'Shea, E.K., Rutkowski, R., Stafford III, W.F. and Kim, P.S. (1989)

Preferential heterodimer formation by isolated leucine zippers from Fos and Jun. Science **245**, 646-648.

Overall, C.M., Wrana, J.L. and Sodek, J. (1989) Independent regulation of collagenase, 72-kDa progelatinase, and metalloendoproteinase inhibitor expression in human fibroblasts by transforming growth factor-ß. J. Biol. Chem. **264**, 1860-1869.

Pabo, C.O. and Sauer, R.T. (1984) Protein-DNA recognition. Ann. Rev. Biochem. **53**, 293-321.

Pachnis, V., Belayew, A. and Tilghman, S.M. (1984) Locus unlinked to α-fetoprotein under the control of the murine *raf* and *Rif* genes. Proc. Natl. Acad. Sci. USA **81**, 5523-5527.

Pardee, A.B., Dubrow, R., Hamalin, J.L. and Kletzin, R.F. (1978) Animal cell cycle. Ann. Rev. Biochem. **47**, 715-750.

Parfett, C.L.J., Hamilton, R.T., Howell, B.W., Edwards, D.R., Nilsen-Hamilton, M. and Denhardt, D.T. (1985) Characterization of a cDNA clone encoding murine mitogen-regulated protein: regulation of mRNA levels in mortal and immortal cell lines. Mol. Cell. Biol. **5**, 3289-3292.

Pattillo, R.A., Hussa, R.O., Yorde,D.E., and Cole,L.A. (1983) Hormone synthesis by normal and neoplastic trophoblast. in Biology of Trophoblast., Y.W. Yoke and A. Whyte (eds.) pp 283-316.

Peel, S., Stewart, I.J. and Bulmer, D. (1983) Experimental evidence for the bone marrow origin of granulated metrial gland cells of the mouse uterus. Cell Tissue Res. **233**, 647-656.

Perry, S.T., Rothrock, R., Isham, K.R., Lee, K.-L. and Kenney, F.T. (1983) Development of tyrosine aminotransferase in perinatal rat liver: changes in functional rat liver: changes in functional messenger RNA and role of inducing hormones. J. Cell. Biochem. **21**, 47-61.

Peters, G., Brookes, S., Smith, R. and Dickson, C. (1983) Tumorigenesis by mouse mammary tumor virus: evidence for a common region for provirus integration in mammary tumors. Cell **33**, 369-377.

Peterson, T.C., Killary, A.M. and Fournier, R.E.K. (1985) Chromosomal assignment and *trans* regulation of the tyrosine aminotransferase structural gene in hepatoma hybrid cells. Mol. Cell. Biol. **5**, 2491-2494.

Pledger, W.J., Stiles, C.D., Antoniades, H.N. and Scher, C.D. (1977) Induction of DNA synthesis in BALB/c-3T3 cells by serum components: Reevaluation of the commitment process. Proc. Natl. Acad. Sci. USA **74**, 4481-4485.

Poole, A.R., Tiltman, K.J., Recklies, A.D. and Stoker, T.A.M. (1978) Differences in secretion of the proteinase cathepsin B at the edges of human breast carcinomas and fibroadenomas. Nature **273**, 545-547.

Portnoy, D.A., Erickson, A.H., Kochan, J., Ravetch, J.V. and Unkeless,

J.C. (1986) Cloning and characterization of a mouse c- .eine proteinase. J. Biol. Chem. **261**, 14697-14703.

Potestio, F.A., Zakar, T., Olson, D.M. (1988) Glucocorticoids stimulate prostaglandin synthesis in human amnion cells by a receptor-mediated mechanism. J. Clin. Endocrinol. Metab. **67**, 1205-1210.

Prince, C.W. and Butler, W.T. (1987) 1,25-Dihydroxyvitamin D_3 regulates the biosynthesis of osteopontin, a bone-derived cell attachment protein, in clonal osteoblast-like osteosarcoma cells. Collagen Rel. Res. **7**, 305-313

Quaife, C.J., Pinkert, C.A., Ornitz, D.M., Palmiter, R.D. and Brinster, R.L. (1987) Pancreatic neoplasia induced by *ras* expression in acinar cells of transgenic mice. Cell **48**, 1023-1034.

Queen, C. (1983) A vector that uses phage signals for efficient synthesis of proteins in *Escherichia coli* J. Molec. Appl. Genet. **2**, 1-10.

Rapp, U.R., Cleveland, J.L., Brightman, K., Scott, A. and Ihle, J.N. (1986) Abrogation of IL-3 and IL-2 dependence by recombinant murine retroviruses expressing v-*myc* oncogenes. Nature **317**, 434-438.

Remmers, E.F., Yang, J.-Q. and Marcu, K.B. (1986) A negative transcriptional control element located upstream of the murine c-*myc* gene. EMBO J. **5**, 899-904.

Reynolds, J.J. (1985) The molecular and cellular interactions involved in connective tissue destruction. Br. J. Dermatol. **112**, 715-723.

Rigby, P.W.J., Dieckmann, C., Rhodes, C. and Berg, P. (1977) Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J. Mol. Biol. **113**, 237-251.

Rogers, J. (1989) The search for functions. Nature **339**, 661-662.

Rogers, S., Wells, R. and Rechsteiner, M. (1986) Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. Science **234**, 364-368.

Ross, J., Peltz, S.W., Kobs, G. and Brewer, G. (1986) Histone mRNA degradation *in vivo*: the first detectable step occurs at or near the 3' terminus. Mol. Cell. Biol. **6**, 4362-4371.

Rossant, J. and Ofer, L. (1977) Properties of extra-embryonic ectoderm isolated from post-implantation mouse embryos. J. Embryol. Exp. Morphol. **39**, 183-194.

Rossow, P.W., Riddle, V.G.H. and Pardee, A.B. (1979) Synthesis of labile, serum-dependent protein in early G1 controls animal cell growth. Proc. Natl. Acad. Sci. USA **76**, 4446-4450.

Roychoudhury, R., Jay, E. and Wu, R. (1976) Terminal labeling and

addition of homopolymer tracts to duplex DNA fragments by terminal deoxynucleotidyl transferase. Nucl. Acids Res. **3**, 863-877.

Ruoslanti, E. and Pierschbacher (1987) New perspectives in cell adhesion: RGD and integrins. Science **238**, 491-497.

Sap, J., Munoz, A., Damm, K., Goldber, Y., Chysdael, J., Leutz, A., Beug, H. and Veunström, B. (1986) The c-*erb*-A protein is a high-affinity receptor for thyroid hormone. Nature **324**, 635-640.

Saris, C.J.M., Kristensen, T., D'Eustachio, P., Hicks, L.J., Noonan, D.J., Hunter, T. and Tack, B.F. (1987) cDNA sequence and tissue distribution of the mRNA for bovine and murine p11, the S100-related light chain of the protein-tyrosine kinase substrate p36 (calpactin I). J. Biol. Chem. **262**, 10663-10671.

Sartor, O., Gregory, F.S., Templeton, N.S., Pawar, S., Perlmutter, R.M. and Rosen, N. (1989) Selective expression of alternative *lck* mRNAs in human malignant cell lines. Mol. Cell. Biol. **9**, 2983-2988.

Schaffner, W. (1989) How do different transcription factor binding the same DNA sequence sor out their jobs? TIGS **5**, 37-39.

Scheidereit, C., Cromlish, J.A., Gerster, T., Kawakami, K., Balmaceda, C.-G., Currie, A. and Roeder, R. (1988) A human lymphoid-specific transcription factor that activates immunoglobulin genes is a homoeobox protein. Nature **366**, 551-557.

Scher, C.D., Stone, M.E. and Stiles, C.D. (1979) Platelet-derived growth factor prevents G0 growth arrest. Nature (London) **281**, 390-392.

Schultz, R.M., Silberman, S., Persky, B., Bajkowski, A.S. and Carmichael, D.F. (1988) Inhibition by human recombinant tissue inhibitor of metalloproteinases of human amnion invasion and lung colonization by murine B16-F10 melanoma cells. Cancer Res. **48**, 5539-5545.

Sellers, A., Cartwright, E., Murphy, G. and Reynolds, J.J. (1977) Evidence that latent collagenases are enzyme inhibitor complexes. Biochem J. **163**, 303-307.

Sellers, A., Murphy, G., Meikle, M.C. and Reynolds, J.J. (1979) Rabbit bone collagenase inhibitor blocks the activity of other neutral metalloproteinases. Biochem. Biophys. Res. Commun. **87**, 581-587.

Senger, D.R. and Perruzzi, C.A. (1985) Secreted phosphoprotein markers for neoplastic transformation of human epithelial and fibroblastic cells. Cancer Res. **45**, 5818-5823.

Senger, D.R., Perruzzi, C.A., Gracey, C.F., Papadopoulos, A. and Tenen, D.G. (1988) Secreted phosphoprotein associated with neoplastic transformation: close homology with plasma proteins cleaved during blood coagulation. Cancer Res. **48**, 5770-5774.

Senger, D.R., Perruzzi, C.A., Papadopoulos, A. and Tenen, D.G. (1989) Purification of a human milk protein closely similar to tumor-secreted

phosphoproteins and osteopontin. Biochim. Biophys. Acta (In press).

Shackleford, G.M. and Varmus, H.E. (1987) Expression of the proto-oncogene *int*-1 is restricted to postmeiotic male germ cells and the neural tube of mid-gestational embryos. Cell **50**, 89-95.

Shinkai, H., Kawamoto, T., Hori, H. and Nagai, Y. (1977) A complex of collagenase with low molecular weight inhibitors in culture medium of embryonic chick skin explants. J. Biochem. **81**, 261-263.

Sinn, E., Muller, W., Pattengale, P., Tepler, I., Wallace, R. and Leder, P. (1987) Coexpression of MMTV/v-Ha-*ras* and MMTV/c-*myc* genes in transgenic mice: synergistic action of oncogenes *in vivo*. Cell **49**, 465-475.

Sive, H.L. Heintz, N. and Roeder, R.G. (1986) Multiple sequence elements are required for maximal *in vitro* transcription of a human histone H2B gene. Mol. Cell. Biol. **6**, 3329-3340.

Sloane, B.F., Dunn, J.R. and Honn, K.V. (1981) Lysosomal cathepsin B: correlation with metastatic potential. Science **212**, 1151-1153.

Smith, J.H. and Denhardt, D.T. (1987) Molecular cloning of a tumor promoter-inducible mRNA found in JB6 mouse epidermal cells: induction is stable at high, but not at low, cell densities. J. Cell. Biochem. **34**, 13-22.

Smith, J.H. and Denhardt, D.T. (1989) Effects of non-phorbol tumor promoters and tumor promotion inhibitors on a TPA-inducible gene ("2ar") in mouse JB6 epidermal cells: evidence for protein kinase C involvement. J. Cell Physiol. (In press).

Southern, E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**, 503-517.

Spandidos, D.A. and Wilkie, N.M. (1984) *In vitro* malignant transformation of early passage rodent cells by a single mutated human oncogene. Nature **310**, 469.

Spurr, N.K., Goodfellow, P.N. and Docherty, A.J.P. (1987) Chromosomal assignment of the gene encoding the human tissue inhibitor of metalloproteinases to Xp11.1-p11.4. Ann. Hum. Genet. **51**, 199-194.

Stanley, K.K. and Luzio, J.P. (1984) Construction of a new family of high efficiency bacterial expression vectors: identification of cDNA clones coding for human liver proteins. EMBO J. **3**, 1429-1434.

Stanton, L.W., Schwab, M. and Bishop, J.M. (1986) Nucleotide sequence of the human N-*myc* gene. Proc. Natl. Acad. Sci. USA **83**, 1772-1776.

Stewart, I. and Mukhtar, D.D.Y. (1988) The killing of mouse trophoblast cells by granulated metrial gland cells *in vitro*. Placenta **9**, 417-425.

Stewart, I. and Peel, S. (1977) The structure and differentiation of granulated metrial gland cells of the pregnant mouse uterus. Cell tiss. Res. **184**, 517-527.

Studzinski, G.P., Brelvi, Z.S., Feldman, S.C. and Watt, R.A. (1986) Participation of c-*myc* protein in DNA synthesis of human cells. Science **234**, 467-470.

Takahara, M., Hibler, D.W., Barr, P.J., Gerlt, J.A. and Inouye, M. (1985) The *ompA* signal peptide directed secretion of Staphylococcal nuclease A by *Escherichia coli*. J. Biol. Chem. **260**, 2670-2674.

Thomson, B.M., Atkinson, S.J., Reynolds, J.J. and Meikle, M.C. (1987) Degradation of type I collagen films by mouse osteoblasts is stimulated by 1,25 dihydroxyvitamin D3 and inhibited by human recombinant TIMP (tissue inhibitor of metalloproteinases). Biochem. Biophys. Res. Commun. **148**, 596-602.

Thorgeirsson, U.P., Liotta, L.A., Kalebic, T., Margulies, I.M., Thomas, K., Rios-Candelore, M. and Russo, R.G. (1982) Effect of natural protease inhibitors and a chemoattractant on tumor cell invasion *in vitro*. J. Nat. Cancer Inst. **69**, 1049-1054.

Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci USA **76**, 4350-4354.

Tryggvason, K., Höyhtä and Salo, T. (1987) Proteolytic degradation of extracellular matrix in tumor invasion. Biochim. Biophys. Acta **907**, 191-217.

Vander-Kooy, D., Dubyak, G.R., Moore, R.M. and Moore, J.J. (1989) Adenosine triphosphate activates the phospholipase-C cascade system in human amnion cells without increasing prostaglandin production. Endocrinol. **124**, 2005-2012.

Watt, R.A., Shatzman, A.R. and Rosenberg, M. (1985) Expression and characterization of the human c-*myc* DNA-binding protein. Mol. Cell. Biol. **5**, 448-456.

Weislander, L. (1979) A simple method to recover intact high molecular weight RNA and DNA after electrophoretic separation in low gelling temperature agarose gels. Anal. Biochem. **98**, 305-309.

Whitham, S.E., Murphy, G., Angel, P., Rahmsdorf, H.-J., Smith, B.J., Lyons, A., Harris, T.J.R., Reynolds, J.J., Herrlich, P. and Docherty, A.J.P. (1986) Comparison of human stromelysin and collagenase by cloning and sequence analysis. Biochem. J. **240**, 913-916.

Wild, A.E. (1983) Trophoblast cell surface receptors. In Y.W. Lake and A. Whyte (eds.) Biology of Trophoblast. Elsevier Science Publishers, pp. 471-512.

Wilder, E.L. and Linzer, D.I.H. (1989) Participation of multiple factors, including proliferin, in the inhibition of myogenic differentiation. Mol. Cell. Biol. **9**, 430-441.

Wilder, E.L. and Linzer, D.I.H. (1986) Expression of multiple proliferin genes in mouse cells. Mol. Cell. Biol. **6**, 3283-3286.

Wilhelm, S.M., Collier, I.E., Kronberger, A., Eisen, A.Z., Marmer, B.L., Grant, G.A., Bauer, E.A. and Goldberg, G.I. (1987) Human skin fibroblast stromelysin: structure, glycosylation, substrate specificity, and differenatial expression in normal and tumorigenic cells. Proc. Natl. Acad. Sci. USA **84**, 6725-6729.

Wilhelm, S.M., Eisen, A.Z., Teter, M., Clark, S.D., Kronberger, A. and Goldberg, G. (1986) Human fibroblast collagenase: glycosylation and tissue-specific levels of enzyme synthesis. Proc. Natl. Acad. Sci. USA **83**, 3756-3760.

Woolley, D.E., Roberts, D.R. and Evanson, J.M. (1976) Small molecular weight β_1 serum protein which specifically inhibits human collagenases. Nature **261**, 325-327.

Yagel, S., Parhar, R.S., Jeffrey, J.J. and Lala, P.K. (1988) Normal nonmetastatic human trophoblast cells share *in vitro* invasive properties of malignant cells. J. Cell. Physiol. **136**, 455-462.

Yang, J.-Q., Remmers, E.F. and Marcu, K.B. (1986) The first exon of the c-*myc* proto-oncogene contains a novel positive control element. EMBO J. **5**, 3553-3562.

Yoon, K., Buenaga, R. and Rodan, G.A. (1987) Tissue specificity and developmental expression of rat osteopontin. Biochem. Biophys. Res. Commun. **148**, 1129-1136

Young, R.A. and Davis, R.W. (1983) Efficient isolation of genes by using antibody probes. Proc. Natl. Acad. Sci. USA **80**, 1194-1198.

Zhan, X., Bates, B., Hu, X. and Goldfarb, M. (1988) The human FGF-5 oncogene encodes a novel protein related to fibroblast growth factors. Mol. Cell. Biol. **8**, 3487-3495.

VITAE

NAME	WATERHOUSE, Paul David
BIRTHDATE	March 21, 1955.
BIRTHPLACE	Toronto, Canada.

EDUCATION:

1982-1990	Ph.D. (Molecular Biology) Department of Biochemistry University of Western Ontario Supervisor: Dr. D Denhardt Thesis: Expression of growth factor inducible genes.
1979-1982	M.Sc. (Cell Biology) Department of Biology University of Ottawa Supervisor: Dr. D.L. Brown Thesis: Changes in the microtubule and tubulin content of lymphocytes during activation.
1974-1979	B.Sc. Department of Biology University of Ottawa

SCHOLARSHIPS:

1981-1982; 1984-1986	Ontario Graduate Scholarship

PUBLICATIONS

1 Waterhouse, P. and J.R. McLean (1981). Slab gel electrophoresis of commercial iodine-125 fibrinogen. J. Radioanal. Chem. 65:349-353.

2 Waterhouse, P., P.J. Anderson and D.L. Brown (1983). Increases in microtubule assembly and tubulin content in mitotically stimulated mouse splenic T lymphocytes. Exp.Cell Res. 144:367-376.

3 Brown, D.L., I. Schweitzer, P. Waterhouse, P.J. Anderson and J. Little. Changes in tubulin content, MTOC activity and microtubule activity in mitogen stimulated mouse splenic lymphocytes (1983), in Intercellular communication and regulation of leukocyte function. Parker and O'Brien eds. John Wiley and Sons Ltd., England.

4 Paatero, G.I.L., D.L. Brown and P. Waterhouse (1986). Inhibition of surface immunoglobulin capping on mouse splenic lymphocytes by acetyltrimethylammonium bromide. Cell. Mol. Biol. 46:79-85.

5 Edwards, D.L., P. Waterhouse, M.L. Holman and D.T. Denhardt (1986). A growth responsive gene (16C8) in normal mouse fibroblasts homologous to a human collagenase inhibitor with erythroid potentiating activity: evidence for inducible and constitutive transcripts. Nucl. Acids Res. 14:8863-8878.

6 Denhardt, D.T., R.T. Hamilton, C.L.J. Parfett, D.R. Edwards, R. St. Pierre, P. Waterhouse and M. Nilson-Hamilton (1986). Close relationship of the major excreted protein of transformed murine fibroblasts to thiol-dependent cathepsins. Cancer Res. 46:4590-4593.

7 Denhardt, D.T., D.R.Edwards, J.Kowalski, C.L.J.Parfett and P.Waterhouse. Specialized plasmid cloning vectors for cloning cDNA. (1988), in Vectors: A survey of molecular cloning vectors and their uses. Rodriguez and Denhardt eds. Butterworths, Boston.

8 Khokha, R., P. Waterhouse, S. Yagel, P.K. Lala, C.M. Overall, G. Norton and D.T. Denhardt (1989). Antisense RNA-induced reduction in murine TIMP levels confers oncogenicity on Swiss 3T3 cells. Science 243:947-950.

9 Connor, A., P. Waterhouse, R. Khokha and D.T. Denhardt (1989). Characterization of a mouse mitogen regulated protein/proliferin gene: a member of the growth hormone/prolactin gene family. BBA 1009:75-82.

10 Waterhouse, P., R Khokha and D.T. Denhardt. Regulation of TIMP expression at the translational level. In press, J. Biol. Chem., 1990.

MANUSCRIPTS IN PREPARATION

11 Waterhouse, P., Guo, S., McLeod, M, and Denhardt, D.T. Complementary temporal and spatial patterns of expression of the calcium binding proteins calcyclin and secreted phosphoprotein in murine decidua during pregnancy.

12 Waterhouse, P., Khohka, R., Pahar, R.S., Lala P., and Denhardt, D.T. Regulation of TIMP expression in ♀ mouse reproductive tissues during pregnancy.

13 Guo, X., P. Waterhouse, A.M. Craig, C.L.J. Parfett, L. Murphy, R. Reid, D.R. Edwards and D.T. Denhardt. Identification of a serum-inducible mRNA (5B10) and its tissue distribution: 5B10 is the mouse homologue of human calcyclin.

14 Khokha R., P. Waterhouse and D.T. Denhardt. Increased proteinase expression during tumor progression of cell lines down-modulated for the expression of tissue inhibitor of metalloproteinases.

ABSTRACTS AND PRESENTATIONS

1 Waterhouse, P.D. and D.L. Brown. Synthesis of flagellar proteins in the alga Polytomella. 22nd Annual Meeting of the Canadian Federation of Biological Sciences. June, 1979.

2 Brown, D.L., K.A. Rogers and P.D. Waterhouse. Relationship of microtubule organization ir lymphocytes to the capping of surface molecules. Second International Symposium of Microtubules and Microtubule Inhibitors. Aug. 1980.

3 Waterhouse, P.D., P.J. Anderson and D.L. Brown. Tubulin and Actin contents of resting and stimulated lymphocytes. 20th Annual Meeting, American Society for Cell Biology. Nov. 1980.

4 Brown, D.L. and P.D. Waterhouse. Microtubules and the capping of lymphocyte surface receptors. 20th Annual Meeting, American Society for Cell Biology. Nov. 1980.

5 Waterhouse, P.D., P.J. Anderson and D.L. Brown. Increases in the tubulin content of lymphocytes following mitogenic stimulation. 24th Annual Meeting of the Canadian Federation of Biological Sciences. June 1981.

6 Waterhouse, P.D., P.J. Anderson and D.L. Brown. Changes in the tubulin and actin contents during mitogenic stimulation of mouse splenic lymphocytes and mouse splenic T lymphocyte populations. Annual Meeting, Ontario-Quebec Immunological Society. May 1982.

7 Waterhouse, P. and D.T. Denhardt. Cloning of cDNAs in a vector in which their expression can be regulated. Strategies for the expression of foreign genes. Oct. 1985.

8 Khokha, R., P. Waterhouse and D.T. Denhardt. Consequences of down-regulating 16c8-inhibitor of metalloproteinases. Triennial Symposium on Biology of Growth Factors. Toronto, Canada, June 1987.

9 Khokha, R., P. Waterhouse, S. Yagel, P.K. Lala, G. Norton and D.T. Denhardt. Modulation of the collagenase inhibitor (TIMP) mRNA levels by anti-sense RNA influences the invasiveness of mouse 3T3 cells. 79th Annual Meeting of the American Association for Cancer Research. New Orleans, Louisiana, May 1988.

10 Waterhouse, P., R. Khokha, A. Chambers, S. Yagel, P.K. Lala and D.T. Denhardt. The role of murine cathepsin L in metastasis. 4th International Congress of Cell Biology, Montreal, Aug. 1988.

11 Waterhouse, P., R. Khokha, A. Chambers, S. Yagel, P.K. Lala and D.T. Denhardt. The role of murine cathepsin L in metastasis. 2nd International Conference of the Metastasis Research Society and the SEK. Heidelberg, FRG. Sept. 1988.

12 Khokha, R., P. Waterhouse, S. Yagel, P.K. Lala, C.M. Overall and D.T. Denhardt. Modulation of the collagenase inhibitor (TIMP) mRNA levels by anti-sense RNA influences the invasive-ness of mouse 3T3 cells. 2nd International Conference of the Metastasis Research Society and SEK. Heidelberg, FRG. Sept. 1988.

13 Waterhouse, P., R. Khokha, A. Chambers, S. Yagel, P.K. Lala and D.T. Denhardt. The role of murine cathepsin L in metastasis. Lake Ontario Metastasis Group, Detroit. Oct. 1988.

14 Guo, X., P. Waterhouse, C.L.J. Parfett and D.T. Denhardt. The characterization of a cell-cycle-regulated cDNA clone (5B10). 5th Annual Queen's University Symposium on Gene Organization and Expression. Oct. 1988.

15 Khokha, R., Waterhouse, P., Parhar, R.S. and Denhardt, D.T. A turn on of metalloproteinases and a cysteine proteinase during tumor progression. Matrix metalloproteinase conference. Sandestin, Fl. Sept. 1989.

END

22/10/90

FIN