

**EXPRESSION AND REGULATION  
OF  
MATRILYSIN  
IN HUMAN TUMOURS**

**A dissertation submitted for the  
degree of Ph.D.**

**by**

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## Declaration

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I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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Barbara Fingleton

Date : 25<sup>th</sup> September '96

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## Abstract

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Matrilysin is a member of a multigene family of proteolytic enzymes called the matrix metalloproteinases (MMPs). It has previously been determined that matrilysin has a role to play in the development of colon cancers, however, its participation in other tumour types has not been fully explored. The aims of this research were (i) to develop an ELISA for matrilysin; (ii) to examine human breast tumour tissue by a variety of methods for expression of matrilysin; (iii) to investigate the expression of matrilysin in cancers of other tissues using cell lines; and (iv) to examine how the expression of matrilysin could be modulated by polypeptide growth factors and cytokines.

A sensitive and specific one-step sandwich ELISA was developed. This was validated and shown to have excellent reproducibility. The limit of detection was 0.45 ng/ml and the linear range was 5 - 50 ng/ml.

A pilot-scale study of ten breast tumour samples and three normal breast tissue samples demonstrated the presence of matrilysin mRNA and protein in both normal and tumour cells. Immunohistochemistry was found to be the most useful and informative method of detecting matrilysin protein. The intensity of expression varied among tumour specimens but did not correlate with stage of disease.

Matrilysin expression was examined in a variety of human tumour cell lines using RT-PCR and the matrilysin ELISA. Of the eight lines tested, only four produced the enzyme. Levels of matrilysin could be stimulated with cytokines. Interleukin-6, IGF-I and IGF-II, not previously reported as modulators of MMP activity, were among the most potent stimulators at both the mRNA and protein levels. These results were confirmed using matrilysin promoter-reporter gene constructs transfected into suitable cells.

## Abbreviations

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Abs	Absorbance
aFGF	Acidic fibroblast growth factor
AP	Alkaline phosphatase
AP-1(2)	Activator protein-1(2)
BCA	Bicinchoninic acid assay
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
bFGF	Basic fibroblast growth factor
BIA	Biospecific interaction analysis
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CAT	Chloramphenicol acetyl transferase
cDNA	Complementary DNA
C/EBP	CAAT/Enhancer binding protein
CM	Conditioned medium
CMV	Cytomegalovirus
CPRG	Chlorophenyl red $\beta$ -d galactoside
CSF	Colony stimulating factor
CV	Coefficient of variation
DAB	Diaminobenzidine
DEAE-Dextran	Diethylaminoethyl-Dextran
DEPC	Diethylpyrocarbonate
DIG	Digoxigenin
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DOTAP	N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammoniummethylsulfate
ECM	Extracellular matrix
EDC	N-ethyl-N'(diaminopropyl) carbodiimide

EDTA	Ethylenediamine tetracetic acid
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
ER	Oestrogen receptor
FCS	Foetal calf serum
FSB	Frozen storage buffer
HBS	HEPES buffered saline
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HRP	Horseradish peroxidase
IFN	Interferon
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IL	Interleukin
LB	Luria-Bertrani broth
mAb	(mouse) monoclonal antibody
MAP	Matrilysin peptide
MMLV-RT	Moloney murine leukaemia virus - reverse transcriptase
MMP	Matrix metalloproteinase
MOPS	3-[N-Morpholino]propanesulfonic acid
M <sub>r</sub>	Apparent molecular weight
mRNA	Messenger RNA
MW	Molecular weight
NBT	Nitroblue tetrazolium
NF-IL6	Nuclear factor for IL-6
OD	Optical density
OPDA	O-phenylene diamine
pAb	Polyclonal antibody
PAGE	Polyacrylamide gel electrophoresis
PAI	Plasminogen activator inhibitor
PBS	Phosphate buffered saline

PBST	Phosphate buffered saline plus Tween
PCI	Phenol : Chloroform : Isoamyl alcohol
PCR	Polymerase chain reaction
PEA	Polyoma virus enhancer
PLG	Phase light gel
r	Regression coefficient
RAP	RNA polymerase II-associated protein
RmAb	Rat monoclonal antibody
RNA	Ribonucleic acid
RT-PCR	Reverse transcription - PCR
RU	Response units
SAS	Saturated ammonium sulphate
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SP-1	Specificity protein-1
SPR	Surface plasmon resonance
SSC	Standard sodium citrate
SV40	Simian virus 40
TBE	Tris borate EDTA
TBST	Tris buffered saline plus Tween
TE	Tris EDTA
TGF- $\beta$	Transforming growth factor- $\beta$
TIE	TGF- $\beta$ inhibitory element
TIMP	Tissue inhibitor of metalloproteinases
TNF	Tumour necrosis factor
TPA	12- <i>O</i> -tetradecanoyl-phorbol-13-acetate
TRE	TPA responsive element
tRNA	Transfer RNA
uPA	Urokinase plasminogen activator
uPAR	uPA receptor
UV	Ultraviolet



## Units

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bp	Base pairs
Kb	Kilobases
KDa	KiloDaltons
μg	Microgram
μl	Microlitre
μm	Micrometre
°C	Degrees Celsius
cm	Centimetre
g	Grams
hrs	Hours
Kg	Kilogram
L	Litre
M	Molar
mA	Milliamps
mg	Milligram
ml	Millilitre
mM	Millimolar
mol	Moles
ng	Nanogram
nm	Nanometre
V	Volts
v/v	Volume per volume
w/v	Weight per volume

## **Publications and Presentations**

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### Publications

McDonnell, S. and **Fingleton, B.** (1993) Role of matrix metalloproteinases in invasion and metastasis : biology, diagnosis and inhibitors. *Cytotechnology* **12** 367 - 384.

McDonnell, S., O'Shea, H. and **Fingleton, B.** (1996) Regulation of matrilysin gene expression in human tumour cell lines by cytokines. *Proc Am Assoc Cancer Res* **37** 91.

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# **Chapter One**

## **Introduction to the MMPs**

## 1.1 INTRODUCTION

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The extracellular matrix (ECM) is a complex network composed of various proteins such as collagens, laminin and proteoglycans amongst others (Paulsson, 1992). For many years the extracellular matrix was thought to be an inert structure whose only function was to provide structural support to organisms. It is now known that ECM has a profound influence on many normal and pathological processes including embryonic development and establishment of tissue-specific functions (Boudreau *et al.*, 1995), bone remodelling, arthritis, and tumourigenesis (Peterson *et al.*, 1992).

There are 4 main classes of proteinases believed to be involved in proteolytic degradation of the extracellular matrix (Shi *et al.*, 1993): (i) serine proteinases *e.g.* plasminogen activators; (ii) cysteine proteinases *e.g.* cathepsins B and L; (iii) aspartyl proteinases *e.g.* cathepsin D and (iv) metalloproteinases *e.g.* collagenases and stromelysins. Many of these proteinases interact in a complex cascade which ultimately leads to matrix degradation. Of these different proteinases, the matrix metalloproteinases (MMPs) are believed to be the normal, physiological mediators of matrix degradation. They are secreted proteins, placing them in the proper location for extracellular matrix degradation and their enzymatic activities are most potent at pH values close to neutrality (Matrisian, 1992). Also, their combined substrate specificities include all the major macromolecules of the ECM.

### 1.2 MMP Structure and Function

The MMPs are a multigene family of matrix-degrading zinc metalloproteinases that degrade at least one component of the ECM. Molecular cloning of the various family members has revealed considerable amino acid conservation and raises the possibility that the genes arose by duplication of a single primordial gene (Matrisian, 1992). So far in humans, fourteen members of the MMP family have been identified and eleven of these have been characterised [Table 1.1]. The molecular weights and substrates of the three most recently identified MMPs (MT-MMP-2, MT-MMP-3 and MT-MMP-4) remain to be confirmed. The MMPs have been known by various names

through isolation by different groups but for the purposes of this review the designation recommended by the International Union of Biochemistry and Molecular Biology will be used (Nomenclature Committee, 1992). Therefore the 72kDa and 92kDa type IV collagenases will be referred to as gelatinase A and gelatinase B respectively. Similarly, PUMP-1 or matrin will be referred to as matrilysin. The MMP classification numbering system, as shown in Table 1.1, is also regularly used.

**Table 1.1 : Properties of human matrix metalloproteinases**

MMP	NAME	M <sub>r</sub> (latent)	M <sub>r</sub> (active)	SUBSTRATES
1	Interstitial collagenase	55	45	Fibrillar collagens Gelatin Proteoglycan
2	Gelatinase A	72	66	Denatured collagens Collagen IV, V, VII, X Elastin
3	Stromelysin-1	57	45	Proteoglycan Collagen II, IV, XI Gelatins, Laminin, Fibronectin
7	Matrilysin	28	19	As for stromelysin-1 Elastin Entactin
8	Neutrophil collagenase	75	58	As interstitial collagenase
9	Gelatinase B	92	86	As gelatinase A
10	Stromelysin-2	57	44	As stromelysin-1
11	Stromelysin-3	51	44	Similar to stromelysin-1
12	Metalloelastase	53	45/22	Elastin Type IV collagen
13	Collagenase-3	65	55	Fibrillar collagens
14	Membrane type-MMP	63	?	Gelatinase A

Analysis of the amino acid sequences reveals that these proteins contain several distinct domains that are conserved among the various members and it is these conserved regions by which they are particularly characterised [Fig 1]. The first of these is the leader sequence (approx 17 amino acids) which targets the molecule for secretion, is subsequently removed and is therefore not present in the latent enzyme (Wilhelm *et al.*, 1987). The second domain is the propeptide (approx 80 amino acids) which has been shown to be cleaved when MMPs are activated and which contains the

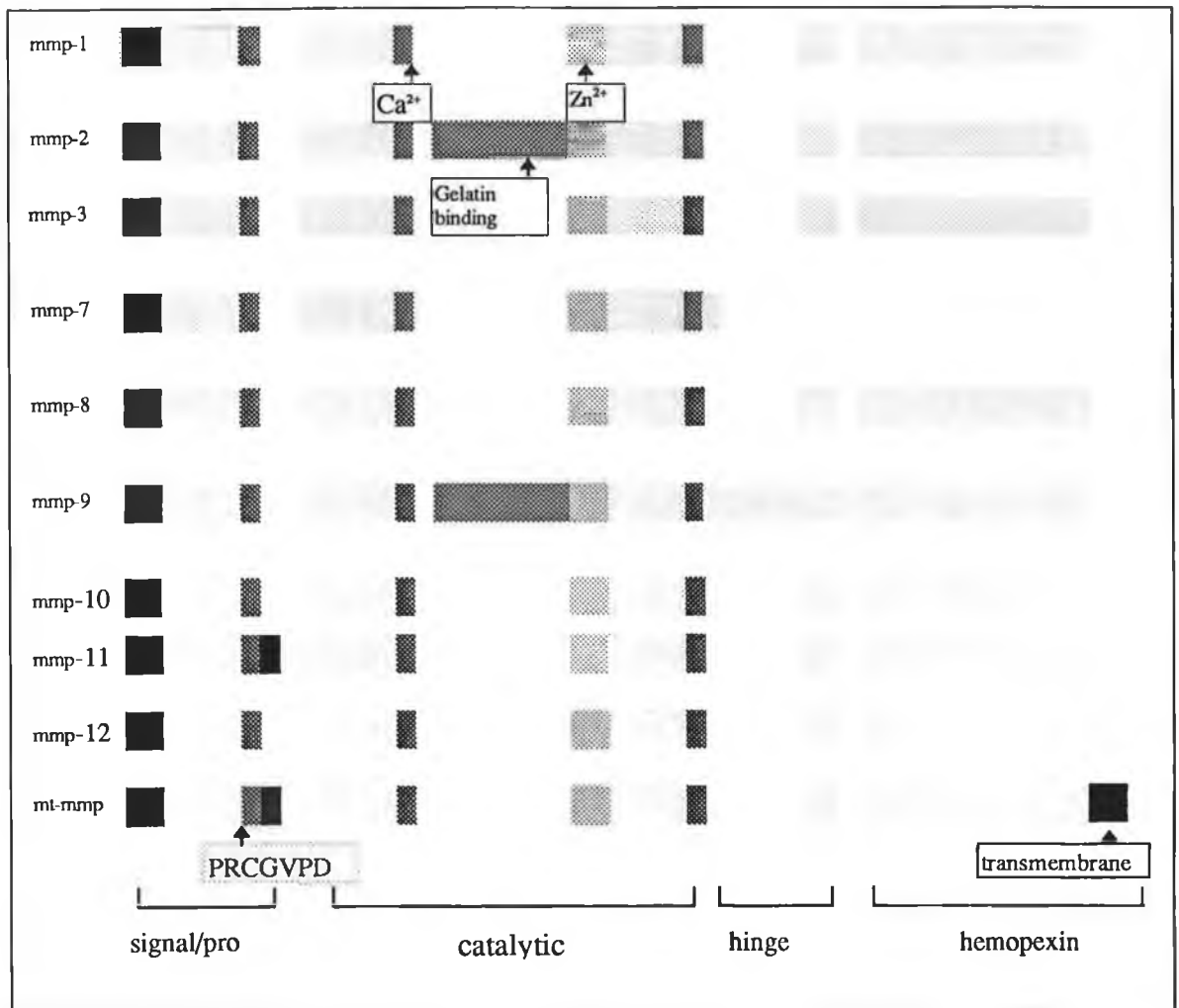
highly conserved sequence PRCGV/NPD (Grant *et al.*, 1987; Stetler-Stevenson *et al.*, 1989a; Nagase *et al.*, 1990; Freije *et al.*, 1994). The available data suggest that this region is involved in maintaining the enzyme in a latent state since mutations in this region result in an enzyme that no longer requires activation (Matrisian *et al.*, 1991). *In vitro*, this activation can be achieved by a variety of agents including: conformational perturbants such as SDS and the organomercurials; oxidants such as NaOCl, sulfhydryl alkylating agents; and, in some cases, proteolytic cleavage by plasmin or trypsin (Grant *et al.*, 1987; Wilhelm *et al.*, 1987; Stetler-Stevenson *et al.*, 1989a; He *et al.*, 1989; Springman *et al.*, 1990). There have also been a number of reports of spontaneous autoactivation (Van Wart and Birkedal-Hansen, 1990). *In vivo*, gelatinase A is activated by other MMPs, the membrane-bound MT-MMP-1 and MT-MMP-2 (Sato *et al.*, 1994; Takino *et al.*, 1995b). Two of the membrane type metalloproteinases (MT-MMP-1 and MT-MMP-2) and stromelysin-3 contain an RXKR sequence between the propeptide and catalytic domain (Sato *et al.*, 1994; Takino *et al.*, 1995b). These enzymes cannot apparently be activated by agents such as the organomercurials but require proteolytic activation by molecules such as furin (Pei and Weiss, 1995). This spectrum of activators suggests that a conformational change is required for activation and that a thiol bond is involved. The current view of MMP activation is that the N-terminal part of the molecule is folded around in the latent enzyme so that the cysteine residue in the conserved PRCGVNPD region complexes with the essential zinc molecule of the catalytic domain. Activation results when a conformation change dissociates the cysteine (Cys 73) from the zinc atom with concomitant exposure of the active site. Accordingly, when Cys<sup>73</sup> is 'on' the zinc, the activity of the enzyme is 'off'. This has been referred to as 'the cysteine switch mechanism' (Van Wart and Birkedal-Hansen, 1990).

The third domain is the catalytic domain (approx 170 amino acids) which contains the conserved sequence HExxHxxGxxH postulated to be the zinc binding domain similar to that found in the catalytic site of astacin, a close relative of the MMPs (Soler *et al.*, 1994). Studies have shown that the metalloproteinases contain two metal binding sites at which zinc binds very strongly (Soler *et al.*, 1994), one of which is the catalytic zinc and the second of which appears to have a major role in

stabilizing tertiary structure. Also present are two calcium ions, however these do not appear essential for activity but may play a role in inhibitor binding (Yuan *et al.*, 1994).

The fourth domain which is present in all members of the MMPs except matrilysin shares homology to hemopexin and vitronectin (Hunt *et al.*, 1987) and is linked to the catalytic domain by a Pro-rich sequence of 5 - 10 amino acids. This domain has been postulated to be involved in substrate specificity and indeed, specific residues within this domain (Tyr-214, Asp-235 and Gly-237) have been proposed as fundamental determinants of collagenase specificity (Sanchez-Lopez *et al.*, 1993). In fact, the presence of these particular residues aided in the identification of a specific substrate for one of the newest MMPs, collagenase-3 (Freije *et al.*, 1994). As can be seen from Table 1.1, however, matrilysin, though lacking this domain, recognises similar substrates to stromelysins -1 and -2 both of which possess this domain. Murphy and co-workers have shown that a truncated form of gelatinase A lacking this C-terminal domain had similar substrate specificities as the full length enzyme (Murphy *et al.*, 1992). The truncated gelatinase A could no longer be activated by a membrane activator (possibly MT-MMP) and did not bind tissue inhibitor of metalloproteinases-2 (TIMP-2). The conclusion, therefore, was that this domain plays an important role in the regulation of active gelatinase A by both activators and inhibitors. This theory has led to speculation that matrilysin, as the only MMP that naturally lacks this domain, is less amenable to inhibition by the normal physiological inhibitors of these proteinases, the TIMPs (Crawford and Matrisian, 1995).

The gelatinases A and B both contain an additional domain which contains three repeats of a sequence homologous to the gelatin-binding domain of fibronectin (Collier *et al.*, 1988; Wilhelm *et al.*, 1989). This domain may mediate the sequestering of these enzymes within the ECM. Gelatinase B also contains an additional domain that has some homology to collagen (Wilhelm *et al.*, 1989). Finally, the transmembrane domain consisting of approximately 24 amino acids is found at the C-terminal of the membrane-associated metalloproteinase, MT-MMP (Takino *et al.*, 1995a).



**Figure 1.1 : Domain structure of matrix metalloproteinases**

All members of the MMP family contain at least three domains : the signal, pro- and the catalytic domains. The signal domain targets the molecule for secretion, the pro-domain maintains latency until it is cleaved and the catalytic domain contains both calcium- and zinc-binding regions. Most family members also contain a domain with sequence similarity to hemopexin, while gelatinase A and B contain a gelatin-binding domain and the MT-MMPs contain putative membrane-spanning regions. Stromelysin-3 and the MT-MMPs also possess a conserved RXKR sequence which appears to allow activation of these enzymes by furin.

The MMPs may be divided into three arbitrary subclasses with respect to their substrate specificity although the distinctions between the subclasses are becoming less clear as more is known about the enzymatic activity of purified enzymes. A fourth subclass comprising the membrane-bound MMPs has also been suggested (Takino *et al.*, 1995b). The Type I collagenase subclass has 3 members, namely interstitial collagenase, neutrophil collagenase and collagenase-3. These enzymes cleave the alpha chains of types I,II and III collagens at a single site. Interstitial collagenase is produced by fibroblasts and macrophages in particular, while the expression of neutrophil collagenase is restricted to cells of the neutrophil lineage (Hasty *et al.*, 1990a). Collagenase-3 appears to be particularly associated with breast carcinoma cells and its expression was not detected in a variety of normal tissues (Freije *et al.*, 1994).

The type IV collagenase subclass contains two members : gelatinase A and gelatinase B. Both of these enzymes degrade denatured collagens (gelatins) and are the principal enzymes responsible for type IV basement membrane collagen degradation. The gelatinases have also been shown to possess some elastolytic activity (Murphy *et al.*, 1991). Expression of the zymogen form of gelatinase A is widespread and is frequently elevated and activated in malignant tumours (Monteagudo *et al.*, 1990; Brown *et al.*, 1990; Brown *et al.*, 1993). Gelatinase B, the 92 kDa enzyme, was traditionally thought of as the macrophage gelatinase but it has also been described as being expressed in transformed and tumour-derived cells, neutrophils, corneal epithelial cells, cytotrophoblasts and keratinocytes (Collier *et al.*, 1988; Wilhelm *et al.*, 1989; Pyke *et al.*, 1992; Lyons *et al.*, 1993).

The third subclass is the stromelysin subclass which contains five members, including two highly homologous enzymes stromelysin-1 and stromelysin-2, and the smallest family member, matrilysin. The fourth member of this subclass, stromelysin-3, has been cloned and shown to be associated with tumour rather than normal tissue of the breast and in various head and neck tumours (Basset *et al.*, 1990; Muller *et al.*, 1993). While its sequence specificities have not been determined, a truncated version has been shown to possess caseinolytic activity and weak activity towards fibronectin and laminin (Murphy *et al.*, 1993). The fifth member, metalloelastase, is specifically associated with mononuclear phagocytes and, as its name suggests, is a potent degrader

of elastin (Shapiro, 1994). This subclass has the widest substrate specificity with the ability to degrade proteoglycans and glycoproteins such as fibronectin and laminin. The stromelysins also appear able to degrade type IV collagen and elastin with matrilysin being the most potent elastase (Murphy *et al.*, 1991). The differential expression of the various members of the stromelysin subclass (*e.g.* stromelysin-1 is found in mature macrophages while matrilysin is found in developing pro-monocytes (Busiek *et al.*, 1992)) may help to explain why there are several family members with similar substrate specificities. More recent studies of peptide substrates for the metalloproteinases have shown subtle differences in preferred residues within the region to be cleaved (Netzel-Arnett *et al.*, 1993). A study by Navre and colleagues (Smith *et al.*, 1995) employed a novel technique of creating phage display libraries to screen thousands of possible hexapeptides which are preferentially cleaved by matrilysin or stromelysin-1. Although the two proteinases appeared to have overlapping substrate specificities, close inspection revealed that certain subsites show distinct preferences for particular residues. This may mean that although both enzymes can cleave similar substrates, the rate of proteolysis can be quite different perhaps indicating an extra regulatory mechanism. Another important substrate recognised by the stromelysins is procollagenase (MMP-1) which, upon cleavage by either stromelysin-1 or matrilysin, can be fully activated (Murphy *et al.*, 1987; Brinckerhoff *et al.*, 1990; Abramson *et al.*, 1995). Progelatinase B, while complexed to TIMP, can also be activated by matrilysin (Von Bredow *et al.*, 1996). The Glu 143 - Leu 144 bond of prourokinase is cleaved by matrilysin thus producing the low molecular weight form of the plasminogen activator (Marcotte *et al.*, 1992). These events suggest the involvement of stromelysin-1 and matrilysin in a proteolytic cascade activating other proteinases even in the presence of inhibitors. Also of physiological importance is the ability of stromelysin-1 to inactivate the serine proteinase inhibitors (serpins)  $\alpha_1$  proteinase inhibitor and  $\alpha_1$  antichymotrypsin (Mast *et al.*, 1991) thus preventing inactivation of other degradative enzymes especially associated with inflammation. Matrilysin has also been shown to degrade entactin, a basement membrane protein which bridges laminin and type IV collagen (Sires *et al.*, 1993).



### 1.3 Tissue Inhibitors of Metalloproteinases (TIMPs)

The activity of the MMPs is physiologically regulated by a specific class of natural inhibitors, the tissue inhibitors of metalloproteinases or TIMPs. The TIMPs are a multigene family (Stetler-Stevenson *et al.*, 1989b) which, at present, consists of three members : TIMP-1, TIMP-2 and TIMP-3. A possible TIMP-4 has also been recently reported (Greene *et al.*, 1996). There is an overall 38% identity between TIMP-1 and TIMP-2 at the amino acid sequence level, although the degree of similarity is higher at 68%. TIMP-3 shares a 40% identity with TIMP-1 and 45% with TIMP-2. All three contain twelve cysteine residues at virtually identical positions. These proteins bind non-covalently to active metalloproteinases in a 1:1 molar ratio and specifically inhibit their enzymatic activity. TIMP-1 also forms a complex with the inactive proform of gelatinase B (Wilhelm *et al.*, 1989) while TIMP-2 forms a complex specifically with the proform of gelatinase A (Stetler-Stevenson *et al.*, 1989b). A 9 amino acid C-terminal sequence present in TIMP-2 is thought to be responsible for its preference for interacting with gelatinase A (Willenbrock *et al.*, 1993). This sequence is also present in TIMP-3 (Uria *et al.*, 1994) but absent in TIMP-1. TIMP-1 and TIMP-2 also appear to be homologous to a growth factor activity termed 'erythroid potentiating activity'(EPA) and have been shown to possess growth promoting qualities in some models (Docherty *et al.*, 1985; Stetler-Stevenson *et al.*, 1992).

TIMP-1 is a glycoprotein (M<sub>r</sub> 30kDa) and its cDNA and amino acid sequence has been reported (Docherty *et al.*, 1985; Carmichael *et al.*, 1986) although purification and characterization of the protein from rabbit bone tissue had earlier been achieved by Cawston (1981) and by other workers from various other sources (Welgus and Stricklin, 1983; Murphy *et al.*, 1981). Early studies investigating the link between TIMP-1 expression and invasive ability in cell line models showed that TIMP-1 levels were decreased 10 - 20 fold in highly invasive as compared to the normal or poorly invasive cells (Hicks *et al.*, 1984). Using antisense TIMP-1 transfected into previously poorly invasive murine cells, Khokha and colleagues (1989) showed that an increase in invasive properties was directly linked to a reduction in TIMP-1 expression.

The second member of the TIMP family, TIMP-2 is a non-glycosylated protein ( $M_r$  23 kDa) which was isolated and cloned by several different groups (DeClerck *et al.*, 1989; Goldberg *et al.*, 1989; Stetler-Stevenson *et al.*, 1989b; Boone *et al.*, 1990). The activity of TIMP-2 has also been well characterized. It reacts stoichiometrically with active interstitial collagenase and also prevents the activation of this enzyme from its 55kDa proform to its 45kDa active form (DeClerck *et al.*, 1991a) The binding of TIMP-2 to the inactive form of gelatinase A does not prevent the enzyme becoming activated suggesting that the TIMP-2 is bound to a site other than the active site (Ward *et al.*, 1991). It has been suggested that the TIMP-2 prevents autocatalytic activation of this gelatinase (Howard *et al.*, 1991), but another possibility is that it prevents the binding of gelatinase A to MT-MMP, its apparent *in vivo* activator. Also the TIMP-2, while complexed to gelatinase A, has been shown to retain inhibitory activity to other metalloproteinases (Kolkenbrock *et al.*, 1991). The inhibitory effects of TIMP-2 on cell invasion has been demonstrated in a number of studies (DeClerck *et al.*, 1991b; Albini *et al.*, 1991).

The third human TIMP, TIMP-3 has been recently cloned by Lopez-Otin's group (Uria *et al.*, 1994) although an unpublished partial sequence had previously been lodged in the GenBank database by Apte and Olsen (Accession No. L15078). This sequence codes for a mature protein (after cleavage of a secretory domain) of 188 amino acids with a predicted  $M_r$  of 21.6 kDa. This third protein, although having a 40 - 45% identity with TIMPs 1 and 2, shares a much higher identity (82%) with ChIMP-3, a metalloproteinase inhibitor identified by Hawkes and co-workers produced by transformed fibroblasts from chickens (Pavloff *et al.*, 1992). TIMP-3 appears to be expressed in normal placental and ovarian tissues and in all breast carcinomas examined (Uria *et al.*, 1994). Whether it is functioning in breast carcinomas as an inhibitor or as a growth factor remains to be determined.

The major serum inhibitor of metalloproteinase activity is the large  $\alpha_2$ -macroglobulin protein ( $m_r$  750 kDa) which also inhibits other proteinases in a non-specific manner (Borth, 1992).

## 1.4 Regulation of the MMPs

The regulation of MMP activity is complex and occurs at several different levels : (i) at the transcriptional level, by oncogenes, tumour promoters, growth factors, glucocorticoids and adhesion molecules; (ii) at the activity level by processing of the latent precursor to an active enzyme; and (iii) total proteinase activity is modulated by the presence of TIMPs which are also independently regulated. A large number of studies have been aimed at elucidating the factors controlling expression at the transcriptional level of the MMP and TIMP genes. A representation of the information thus obtained is presented in Table 1.2. A more detailed examination of the mechanisms by which these factors regulate gene expression is given in the introductions to chapters five and six.

**Table 1.2 : Factors affecting expression of MMPs and TIMPs**

	FACTOR	CELL TYPE	MMP/TIMP	EFFECT	REFERENCE
ONCOGENES	c-Ha-ras	Rat fibroblasts	MMP-2	Stimulates	Garbisa <i>et al.</i> , 1987
	ras	Fibroblasts	MMP-3	Stimulates	Diaz-Meco <i>et al.</i> , 1991
	Ha-ras +/- v-myc	Rat fibroblasts	MMP-9	Stimulates	Bernhard <i>et al.</i> , 1994
	Ki-ras	Colon cancer line SW1417	MMP-7	Stimulates	Yamamoto <i>et al.</i> , 1995
ADHESION MOLECULES	Fibronectin receptor	Synovial fibroblasts	MMP-3, MMP-1	Stimulates	Werb <i>et al.</i> , 1989
	$\alpha_4\beta_3$	Melanoma line A375M	MMP-2	Inhibits	Seftor <i>et al.</i> , 1993
	Truncated Fibronectin	Adeno-carcinoma	MMP-7	Stimulates	Yamamoto <i>et al.</i> , 1994
	Cell contact	Rat fibroblasts	MMP-9	Stimulates	Himmelstein <i>et al.</i> , 1994
HORMONES	Dexamethasone	Articular chondrocytes	MMP-1	Inhibits	McGuire-Goldring <i>et al.</i> , 1983
	Progesterone	Rabbit fibroblasts	TIMP-1	Stimulates	Sakyo <i>et al.</i> , 1986
	$\beta$ -estradiol	Rabbit fibroblasts	TIMP-1	Stimulates	Sakyo <i>et al.</i> , 1986
	Progesterone	Human stromal fibroblasts	MMP-3	Inhibits	Osteen <i>et al.</i> , 1994
GROWTH FACTORS	EGF	Fibroblasts	MMP-3	Stimulates	McDonnell <i>et al.</i> , 1990
	TGF- $\beta$	Fibroblasts	MMP-3	Inhibits	Kerr <i>et al.</i> , 1990
	PDGF	Fibroblasts	MMP-3	Stimulates	Diaz-Meco <i>et al.</i> , 1991
	TGF- $\beta$	Hepatoma line Hep-G2	TIMP-1	Stimulates	Kordula <i>et al.</i> , 1992
	TNF- $\alpha$	Endothelial cells	MMP-3	Stimulates	Hanemaaijer <i>et al.</i> , 1993

	TGF- $\alpha$ , EGF	Keratinocytes	MMP-9, MMP-1	Stimulates	Lyons <i>et al.</i> , 1993
	EGF	Squamous cell lines	MMP-9	Stimulates	Shima <i>et al.</i> , 1993
	TNF- $\alpha$	Promyelocytic line HL-60	MMP-9	Stimulates	Ries <i>et al.</i> , 1994
	TGF- $\beta$	Epithelial cells	MMP-7	Inhibits	Bruner <i>et al.</i> , 1995
CYTOKINES	IL-6	Hepatoma line Hep-G2	TIMP-1	Stimulates	Kordula <i>et al.</i> , 1992
	IL-1	Keratinocytes	MMP-9, MMP-1	Stimulates	Lyons <i>et al.</i> , 1993
	IL-1 $\alpha$	Trabecular explants	MMP-9, MMP-3, TIMP-1	Stimulates	Samples <i>et al.</i> , 1993
	IFN- $\gamma$	Keratinocytes	MMP-1, MMP-3	Stimulates	Tamai <i>et al.</i> , 1995
	IL-4, IL-10, IFN- $\gamma$	Monocytes	MMP-7	Inhibits	Busiek <i>et al.</i> , 1995
TUMOUR PROMOTER	PMA	Fibroblasts	MMP-1, TIMP-1	Stimulates	Overall and Sodek, 1990
	PMA	Endothelial cells	MMP-1, MMP-3, MMP-9, TIMP-1	Stimulates	Hanemaaijer <i>et al.</i> , 1993
MISC.	Concanavalin A	Fibroblasts	MMP-1, MMP-2, MMP-7	Stimulates	Overall and Sodek, 1990
	Concanavalin A	Breast cancer line MDA-MB-231	MT-MMP-1	Stimulates	Yu <i>et al.</i> , 1995
	Concanavalin A	Fibroblasts	TIMP-1	Inhibits	Overall and Sodek, 1990
	Calcium phosphate crystals	Fibroblasts	MMP-1, MMP-3	Stimulates	McCarthy <i>et al.</i> , 1992
	Prostaglandin E2	Dermal Fibroblasts	MMP-1	Stimulates	Mauviel <i>et al.</i> , 1994
	$\alpha$ -difluoromethylornithine	Colon cancer line SW1116	MMP-7	Inhibits	Wallon <i>et al.</i> , 1994
	cAMP	Fibrosarcoma	MMP-2, TIMP-1, TIMP-2	Stimulates	Tanaka <i>et al.</i> , 1995
	LPS	Monocytes	MMP-7	Stimulates	Busiek <i>et al.</i> , 1995

As can be seen, there is a large variety of agents which appear to influence the expression of these proteinases and/or their inhibitors. It must be noted, however, that the majority of these studies are dealing with one factor in isolation which would not usually be the case physiologically. In some cases the effects are not direct, as for example with  $\alpha$ -difluoromethylornithine on MMP-7 expression (Wallon *et al.*, 1994) where the effect of this compound is to deplete cellular polyamines which in turn leads to the down-regulation of matrilysin. In other cases a factor which may be influenced by a number of other factors is the regulatory agent examined *e.g.* cellular cAMP levels are affected by a variety of agents such as cytokines, drugs etc. however the up-regulation of gelatinase A and TIMP levels was as a direct result of the cAMP (Tanaka *et al.*, 1995). Of particular interest are those factors which appear to differentially

regulate proteinase and inhibitor levels thus shifting the balance towards proteolysis (*e.g.* concanavalin A) or inhibition (*e.g.* progesterone). There are also examples of coordinate regulation of proteinase and inhibitor (*e.g.* IL-1 $\alpha$ ) which would suggest other regulatory processes are also important.

### **1.5 Physiological roles of MMPs**

The matrix metalloproteinases are principally known for their roles in the degeneration of bones and tissues associated with arthritis and tumour invasion and metastasis. In fact, the majority of these proteinases were first isolated and cloned from tumour-associated tissues (Liotta *et al.*, 1991a). As more expression and localization studies are performed, however, the roles of these proteinases in both 'normal' and other disease states are being elucidated. They are now implicated in a variety of events including embryo implantation and development, wound healing, endometrial remodelling during the female reproductive cycle, photoageing and inflammatory cell responses as well as in pathological conditions such as emphysema, atherosclerosis, periodontal disease and, of course, arthritis and tumour invasion (Matrisian, 1990; Woessner, 1991; Matrisian, 1992). It should be noted at this point that MMPs are not the only proteinases which play integral roles in any or all of these processes, however the contributions of the other classes of proteinases are beyond the scope of this review.

Trophoblast cells of the placenta, an organ derived from a developing foetus, have the ability to tap into the maternal circulation allowing essential exchange of nutrients and waste materials (Graham and Lala, 1991). This tapping of the maternal blood supply is dependent on a temporally and spatially controlled invasive ability exhibited by the trophoblasts (Fisher *et al.*, 1989). A number of investigators have shown that the principal mediators of trophoblast invasion are the gelatinases A and B (Fisher *et al.*, 1989; Librach *et al.*, 1991; Graham *et al.*, 1992; Cross *et al.*, 1994).

The process of tissue remodelling seen in wound repair is considered similar to processes occurring in embryonic development. Remodelling is achieved through progressive synthesis, degradation and resynthesis of extracellular matrix components

(Clark, 1988). Both interstitial collagenase and stromelysin-1 have been shown to be responsible for the degradative aspects of this remodelling process both in wound healing (Girard *et al.*, 1993; Stricklin *et al.*, 1993) and during the development and involution of mouse mammary glands (Crawford and Matrisian, 1995). Stromelysin-3 has been observed in fibroblasts during cutaneous scar formation and its expression is suggested to be a normal wound healing response (Wolf *et al.*, 1992). It is also expressed in the connective tissue of developing digits (Basset *et al.*, 1990) and in involuting mammary glands (Lefebvre *et al.*, 1992).

In adult humans, the uterine epithelium in women of reproductive age is one of the few areas where continuous growth and tissue remodelling events occur. During the proliferative (growth and remodelling) and menstrual (degradative) phases of the cycle, the enzymes matrilysin and stromelysins-1,2 and 3 are expressed in an apparently hormone-dependent manner (Osteen *et al.*, 1994) although the suppression of matrilysin during ovulation has been shown to be directly controlled by TGF- $\beta$  rather than the hormone progesterone (Bruner *et al.*, 1995). Matrilysin has also been shown to be expressed in normal exocrine glands where it is thought to act as a "pipe-cleaner" degrading proteinaceous material that could block the ducts (Saarialho-Kere *et al.*, 1995). Unlike other metalloproteinases, matrilysin expression is limited to cells of epithelial rather than stromal origin (Crawford and Matrisian, 1995). Constitutive expression of matrilysin is seen in developing promonocytes (Busiek *et al.*, 1992) and suggests its role particularly in severe inflammatory events such as cystic fibrosis (Busiek *et al.*, 1995). Matrilysin expression by mesangial cells of the kidney is also thought to contribute to glomerular inflammatory injury (Marti *et al.*, 1992).

The phenomenon of 'photoageing', that is the wrinkled appearance of sun-exposed skin is considered to be due to damage to skin collagen and elastin (Wlaschek *et al.*, 1994). A recent report has shown that expression of interstitial collagenase, gelatinase B and stromelysin-1 is induced in human skin within hours of exposure to ultraviolet B irradiation (UVB) and suggests that these enzymes are the direct cause of photoageing (Fisher *et al.*, 1996).

## 1.6 Pathological roles of MMPs

MMPs have been implicated in a number of pathological conditions either directly, where the disease is a direct result of increased levels of enzymes leading to tissue degradation, or indirectly where a link is established between the disease and loss of an MMP control mechanism. An example of the latter is Sorsby's Fundus Dystrophy, an autosomal dominant hereditary disease affecting vision (Weber *et al.*, 1994a). Point mutations in one or both alleles of the TIMP-3 gene appears to be the principal cause of the disease in affected families (Weber *et al.*, 1994b; Jacobson *et al.*, 1995). Interestingly the disease often co-segregates with emphysema, a destructive lung disease, and periodontal disease. Emphysema is characterised by the destruction of pulmonary elastic fibres and is principally associated with smokers. The elastolytic ability of macrophages, the most abundant defence cell in the lung during states of chronic inflammation (as produced in smokers), has been shown to be inhibited by TIMP-1 (Senior *et al.*, 1989). Macrophages produce two metalloproteinases with elastolytic ability, gelatinase B and metalloelastase (Shapiro *et al.*, 1993), which appear to contribute to the pulmonary destruction characteristic of emphysema (Shapiro, 1994). Periodontal diseases are characterised by the destruction of collagen fibres which provide the structural support of the tooth. Levels of active gelatinase B have been shown to be significantly higher in patients with these diseases than in controls (Teng *et al.*, 1992). Interstitial collagenase activity has also been detected in inflamed periodontal tissues and correlated with attachment loss (Villela *et al.*, 1987).

Atherosclerotic lesion development involves migration of leucocyte and smooth muscle cells, accumulation of ECM components, creation of new capillary beds and arterial enlargement during plaque evolution all of which processes require ECM remodelling. As atherosclerotic plaques develop and outgrow the compensatory arterial wall enlargement, rupture and thrombosis can occur, events which also involve ECM degradation. Libby and co-workers have demonstrated locally increased expression of gelatinase B, stromelysin-1 and interstitial collagenase in regions of atherosclerotic plaque formation (Galis *et al.*, 1994). Using an *in situ* zymographic

technique, this group demonstrated that these enzymes were present in an activated form.

Stromelysin-1 and interstitial collagenase are the principal proteinases associated with rheumatoid- and osteo-arthritis (Hasty *et al.*, 1991). Their presence and activity *in vivo* has been demonstrated by immunolocalisation using antibodies to the proteinases themselves (McCachren, 1991) and to their degradation products (Dodge and Poole, 1989). The mRNAs for these proteinases have also been localised by *in situ* hybridisation to synovial lining layer cells in patients with rheumatoid arthritis (Gravellese *et al.*, 1991). A third metalloproteinase, gelatinase B is produced by macrophages present in the inflamed joints and also appears to play a role in the cartilage destruction associated with these diseases (Hembry *et al.*, 1993).

Tumour invasion and metastasis has long been associated with metalloproteinase activity (Liotta *et al.*, 1980). Many studies have established that the invasive ability of tumour cells can be increased by upregulation of proteinase activity (Liotta *et al.*, 1983; Matrisian *et al.*, 1986; McDonnell *et al.*, 1990; Powell *et al.*, 1993) or down-regulation of proteinase inhibitor activity (Khokha *et al.*, 1989) while correspondingly, up-regulation of inhibitor activity limits invasion (Alvarez *et al.*, 1990; Frisch *et al.*, 1990; DeClerck *et al.*, 1992). Although each member of the MMP family has been implicated in tumour invasion, there are specific associations between particular proteinases and certain types of cancer. Table 1.3 lists a range of cancers and their associated MMPs. Most of the proteinases are produced by stromal fibroblasts adjacent to the tumour cells or infiltrating macrophages and are secreted apparently in response to factors produced by the tumour cells themselves (Wolf *et al.*, 1992; Kataoka *et al.*, 1993; Wang *et al.*, 1994; Borchers *et al.*, 1994). In contrast, MT-MMP-1 and matrilysin are produced exclusively by the tumour cells.



**Table 1.3 Expression of MMPs in human tumours**

<b>Metalloproteinase</b>	<b>Tumour</b>	<b>References</b>
Interstitial collagenase (MMP-1)	Gastrointestinal	McDonnell et al., 1991
	Head and neck	Okada et al., 1995
	Breast	Okada et al., 1995
Gelatinase A (MMP-2)	Lung	Urbanski et al., 1992
	Oesophageal	Shima et al., 1992
	Prostate	Pajouh et al., 1991
	Breast	Okada et al., 1995
	Colon	Levy et al., 1991 Poulsom et al., 1992
Stromelysin 1 (MMP-3)	Oesophageal	Shima et al., 1992
	Pancreatic	Bramhall et al., 1996
	Brain	Nakagawa et al., 1994
Matrilysin (MMP-7)	Gastric	McDonnell et al., 1991
	Colon	McDonnell et al., 1991 Witty et al., 1994
	Prostate	Pajouh et al., 1991 Powell et al., 1993
	Breast	Basset et al., 1990 Heppner et al., 1996
	Lymphoma	Kossakowska et al., 1993
Gelatinase B (MMP-9)	Breast	Monteagudo et al., 1990 Zucker et al., 1993
	Lung	Urbanski et al., 1992
	Skin	Pyke et al., 1991
	Ovarian	Naylor et al., 1994
	Head and Neck	Muller et al., 1991, 1993
Stromelysin 2 (MMP-10)	Breast	Basset et al., 1990 Wolf et al., 1993
	Head and Neck	Muller et al., 1993
	Basal cell carcinoma	Wolf et al., 1992 Majmuder et al., 1994
Collagenase 3 (MMP-13)	Breast	Freije et al., 1994
MT-MMP-1 (MMP-14)	Gastric	Nomura et al., 1995

A common feature of all the conditions described in the preceding paragraphs is an imbalance of proteinases and inhibitors favouring proteolysis. As stated previously, in some cases it is a specific effect of a reduction in inhibitor levels while in other cases, the degradative aspect of the disease is manifested through an increase in active proteinase levels. This is an area of great interest as there is potential for therapy with specific metalloproteinase inhibitors. The anti-invasive effects of TIMP administration to laboratory animals has already been documented (DeClerck *et al.*, 1991) but it is small synthetic molecules which have the greatest potential therapeutically. Already two such compounds are in clinical trials [British Biotechnology compounds, Baltimastat and Marimastat (Brown, 1996)] while many more are in development. Inhibitors such as Baltimastat are small molecules which fit tightly into the active site of the MMP enzymes as they mimic specific peptide residues on one side of a substrate's cleavage site. The drugs were initially developed to prevent the extensive bone and cartilage degradation seen in rheumatoid arthritis. As the role of MMPs in tumour invasion was elucidated, however, development of these synthetic inhibitors as anti-cancer agents began. These compounds are designed to complement existing cytoreductive treatments (Davies *et al.*, 1993a; Giavazzi *et al.*, 1996), their aim being the control of metastatic disease rather than eradication.

In summary, the matrix metalloproteinases are a multigene family with the ability to degrade all components of the extracellular matrix. Their expression can be regulated at the transcriptional level by a wide variety of compounds while at the activation level, their specific inhibitors, the TIMPs, and proteinase activators, often other members of the MMP family, appear to be the principal controllers. They function in both normal physiological events and in pathological conditions such as arthritis and tumour invasion. Recent developments in the design of synthetic MMP inhibitors offer a new therapeutic possibility to patients with solid tumours.

## 1.7 Thesis Outline

The research presented in this thesis has been divided into four principal areas and involves :

(A) The development of a sensitive sandwich ELISA for matrilysin using antibodies provided by our collaborators (Chapter Three).

(B) The analysis of matrilysin expression in a small number of breast tumour tissue samples. Methods of analysis investigated included RT-PCR for specific mRNA detection; and immunohistochemistry and western blotting for protein detection (Chapter Four).

(C) The investigation of the effects of a range of cytokines on expression of matrilysin and stromelysin-1 by a number of different cell lines. The effects at the mRNA level were assessed using RT-PCR while protein levels were determined using the ELISA developed in chapter three (Chapter Five).

(D) The analysis of the matrilysin promoter. Transfections with promoter-reporter gene constructs were performed with the intention of quantifying the effects of cytokines and TPA at the transcriptional level (Chapter Six).

Since the research described here covers a number of distinct but related topics, the thesis has been divided into eight sections which, it is hoped, will help clarify the results. There is a common Materials and Methods section (Chapter Two) and Bibliography (Chapter Eight). Chapters Three, Four, Five and Six each have their own introduction and results and discussion sections. Chapter One serves as a general introduction to the MMPs, while Chapter Seven provides an overall summary and conclusion.

## **Chapter Two**

### **Materials and Methods**

## 2.1 MATERIALS

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Reagents used in experimental work were of analytical grade and were purchased from Sigma Chemical Co., Poole, Dorset, England; BDH Chemicals Ltd., Poole, Dorset, England and Riedal De Haen AG, Seelze, Hannover, Germany.

Microbiological media components and PBS tablets were purchased from Oxoid Ltd., Basingstoke, Hampshire, England.

Cell culture media was obtained from Gibco BRL, Paisley, Scotland. Foetal calf serum was supplied by Biowhittaker, Verviers, Belgium. Other cell culture supplements were obtained from Sigma Chemical Co., Poole, Dorset, England. Disposable plastics for cell culture were obtained from Costar, Cambridge, MA 02140, USA. Chamber slides were obtained from Nunc, Gibco-BRL, Paisley, Scotland. The cell lines used were obtained from European Collection of Animal Cell Cultures (ECACC), Porton Down, Salisbury, Wiltshire, England.

96-well ELISA plates were purchased from Costar, Cambridge, MA 02140, USA.

The 16 amino acid matrilysin peptide (MAP peptide) [AEYSLFPNSPKWTSKV] was manufactured according to specifications by Immune Systems Ltd., Westbury-on-Trym, Bristol, England.

The VECTASTAIN ABC kit and biotinylated anti-rat antibody were obtained from Vector Laboratories, Burlingame, CA 94010, USA.

Restriction endonucleases, Taq polymerase and other components of RT-PCR assays, luciferase detection reagents and enzyme-labelled anti-mouse and anti-rabbit antibodies were purchased from Promega Corp., Southampton, Hampshire, England.

Cytokines, *in situ* hybridisation reagents, the CAT ELISA and the transfection reagent DOTAP, were purchased from Boehringer Mannheim, Hannover, Germany.

The BCA reagent for protein determination and the enzyme-labelled anti-rat antibody were obtained from Pierce Chemicals, Rockford, IL 61105, USA.

Specific primers for PCR were made to specification by R & D Systems, Abingdon, OX14 3YS, England.

pZ523 columns and the associated PLG columns for preparation of DNA were purchased from 5' → 3', Boulder, CO 80303, USA.

Specific anti-matrilysin antibodies were kindly donated by a number of groups. pAb and mAb were from Dr Paul Cannon at Syntex Research, Palo Alto, CA 94303, USA. Recombinant matrilysin was also generously donated by Dr Cannon. pAb<sub>2</sub> and RmAb were obtained from Prof Lynn Matrisian, Dept of Cell Biology, Vanderbilt University School of Medicine, Nashville, TN , USA.

CAT-promoter and luciferase-promoter constructs as well as plasmids used for probe manufacture were all kindly donated by Prof Matrisian.

Equipment used is outlined in the relevant methods section.

## 2.2 METHODS

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### 2.2.1 PROTEIN ELECTROPHORESIS

#### 2.2.1.1 Polyacrylamide Gel Electrophoresis (PAGE) (Non-Reducing)

PAGE, in the presence of sodium dodecyl sulphate (SDS) was performed using the discontinuous system described by Laemmli (1970) using 10 % or 15 % (w/v) polyacrylamide gels as necessary. The 10 % [amounts for 15 % gel in brackets] resolving gels and 3 % stacking gels were prepared as follows :

Resolving Gel :        3.3 ml [5.0 ml] 30 % (w/v) acrylamide containing 0.8 % (w/v) bisacrylamide,  
                              4.0 ml [2.3 ml] distilled water,  
                              2.5 ml [2.5 ml] 1.5 M Tris, pH 8.8 containing 0.4 % (w/v) SDS  
                              0.1 ml [0.1 ml] 10 % (w/v) SDS  
                              0.1 ml [0.1 ml] 10 % (w/v) ammonium persulphate and  
                              0.005 ml [0.005 ml] TEMED.

Stacking Gel :        1.4 ml distilled water,  
                              0.33 ml 30 % acrylamide solution  
                              0.25 ml 0.5 M Tris, pH 6.8 containing 0.4 % (w/v) SDS,  
                              0.02 ml 10 % SDS,  
                              0.02 ml 10% ammonium persulphate and  
                              0.002 ml TEMED.

Samples were dissolved in solubilisation buffer (2 % (w/v) SDS; 0.08 M Tris, pH 6.8; 10 % (w/v) glycerol; 0.2 % (w/v) Coomassie Brilliant Blue). The gel was electrophoresed in electrode buffer, pH 8.3 containing Tris (0.025 M), glycine (0.192 M) and 0.1 % (w/v) SDS at 30 mA using an Atto vertical mini-electrophoresis system until the blue dye reached the bottom of the gel.

### **2.2.1.2 Staining with Coomassie Brilliant Blue**

Gels were stained for 30 mins in 0.5 % (w/v) Coomassie Brilliant Blue in acetic acid : water : methanol (1 : 10 : 8, v/v/v), and destained overnight in the same solvent system.

### **2.2.1.3 Silver Staining**

For silver staining, the gel was fixed overnight by gentle shaking in a solution of ethanol : acetic acid : water (30 : 10 : 60 v/v/v). It was then incubated for 60 mins at room temperature in a solution of 30 % ethanol (v/v), with the solution being replaced with fresh 30 % ethanol after 30 mins. The gel was washed three times for 10 mins each wash by gentle shaking in distilled water. The water was replaced with a freshly prepared solution of 0.1 % (w/v) silver nitrate and the gel left shaking for 30 mins. After a brief wash in distilled water (20 secs), the gel was developed by gentle agitation in a freshly prepared solution of 2.5 % (w/v) sodium carbonate containing 0.02 % (v/v) formaldehyde. When the desired contrast was obtained, the reaction was quenched by adding a 1 % (v/v) acetic acid solution and followed by repeated washing in distilled water.

## **2.2.2 WESTERN BLOTTING**

Following electrophoresis as outlined above, the gel was soaked for 30 mins in cold (4°C) transfer buffer ( 0.25 M Tris, pH 8.3; 0.192 M glycine plus 20 % (v/v) methanol). A sheet of nitrocellulose cut to the same size as the gel and eight sheets of Whatman filter paper were also soaked in the transfer buffer. The proteins were transferred from the gel to the nitrocellulose using a Bio-Rad semi-dry Blotter for 42 mins at 15 Volts. After transfer, the blot (nitrocellulose) was blocked for 1 hr in blocking solution [2.5 % (w/v) dried milk dissolved in Tris Buffered Saline plus Tween [TBST] (10 mM Tris-HCl, pH 8.0; 150 mM NaCl; plus 0.05% (v/v) Tween 20)] and then incubated overnight at 4°C with primary antibody at a suitable dilution (usually 1 : 1000) in blocking solution. The following day, blots were washed three times for 10 mins each with TBST and then incubated for 3 hours gently shaking at room temperature with a suitable enzyme-labelled secondary antibody diluted according to the manufacturer's



recommendation in TBST. Following 3 x 10 min washes, the blots were developed without shaking at room temperature with the appropriate substrate solution. For alkaline phosphatase (AP)-labelled antibodies, this was BCIP (175 µg/ml) and NBT (500 µg/ml) in AP buffer (0.1M Tris, pH 9.5; 0.1 M NaCl; 5 mM MgCl<sub>2</sub>). For horseradish peroxidase (HRP)-labelled antibodies, the substrate diaminobenzidine (DAB) was prepared by dissolving one 5 mg DAB tablet in 15 ml TBS and adding fresh hydrogen peroxide to a final concentration of 0.024 %. (v/v).

## **2.2.3 ANTIBODY PURIFICATION**

### **2.2.3.1 Ammonium sulphate precipitation**

Rabbit antiserum was diluted to a final protein concentration of 3 % (w/v) as determined by BCA protein microassay (section 2.2.7) and cooled to 4°C. The pH was adjusted to 7.4 with 1 M NaOH. An equal volume of cold 100 % (w/v) saturated ammonium sulphate solution (SAS) was added dropwise with stirring to the serum and the mixture left stirring continuously at 4°C for 1 hr. The suspension was then centrifuged in an Hereaus Megafuge at 3000 rpm for 15 mins. The precipitate was washed twice with 40 % (w/v) SAS and, following centrifugation, the pellet was solubilised in a minimum volume of 0.15 M phosphate buffered saline (PBS), pH 7.4. To remove the salt, this solution was dialysed for 24 hrs at 4°C against 0.15 M PBS, pH 7.4 with repeated changes of buffer to ensure efficient dialysis.

### **2.2.3.2 Affinity Chromatography**

#### **2.2.3.2.1 Preparation of MAP affinity column**

0.7 g of CNBr-activated sepharose was suspended in 10 ml 0.001 M HCl and washed through a sintered glass funnel where it was then continuously washed for 10 mins with a total of 200 ml 0.001 M HCl. This was followed by washing with 8 ml of coupling buffer (0.1 M NaHCO<sub>3</sub>; 0.5 M NaCl, pH 8.3). 15 mg of matrilysin peptide (MAP peptide) was dissolved in 5 ml of the coupling buffer and placed in a universal container along with the washed gel. The mixture was left gently mixing on a shaking platform for 2 hrs at room temperature. The gel was then returned to a sintered glass funnel and

washed with 20 ml coupling buffer before being returned to a universal container containing 10 ml blocking buffer (0.2 M glycine in coupling buffer, pH 8.0) and left shaking for 1 hr at room temperature. To remove non-covalently bound protein, the gel was returned to the sintered glass funnel and washed with three cycles of alternating pH consisting of 30 ml 0.1 M acetate buffer, pH 4.5 containing 0.5 M NaCl, and 30 ml 0.1 M Tris buffer, pH 8.5 containing 0.5 M NaCl. The gel was suspended in 0.02 M PBS, pH 7.4 and used to pour a column into a 2 cm x 5 cm<sup>2</sup> glass chromatography column. A layer of 0.02 M PBS was added to prevent the gel from drying out.

#### 2.2.3.2.2 Use of MAP affinity column

The column was first washed with 30 ml 0.02M PBS, pH 7.4 to remove any protein that may have leached off. 2 ml fractions were collected and the absorbance measured at 280 nm [Shimadzu UV/Vis Spectrophotometer] to ensure it was less than 0.003 absorbance units before applying the sample. 1 ml of the dialysed ammonium sulphate precipitate was added slowly to the column followed by washing with 0.02 M PBS to elute unbound material from the column. 1 ml fractions were collected and monitored for the presence of protein using UV spectrophotometry (280 nm wavelength). The washing process was continued until the absorbance fell below 0.003 absorbance units signifying no protein in the fractions. The wash buffer was allowed to run into the top of the gel and dissociating buffer (0.1 M glycine-HCl, pH 2.5) was added. 1 ml fractions were collected in eppendorfs containing 100 µl of 0.1 M Trizma, pH 9.5 - this was to counteract the denaturing effects of the low pH. The fractions containing protein (monitored by BCA microassay - section 2.2.7) were pooled and dialysed for 24 hrs against 0.02 M PBS, pH 7.4 before concentration by reverse osmosis on a sucrose bed. The column was regenerated by washing with 20 ml each of (1) 0.1 M Tris, pH 8.0 , (2) 0.1 M sodium acetate, pH 4.0 and (3) 0.02 M PBS, pH 7.4.

The above process was repeated until all of the ammonium sulphate precipitate had been passed through the affinity column. After the final regeneration, the column was stored for re-use at 4°C in 0.02 M PBS containing 0.02 % (w/v) sodium azide as preservative.

## **2.2.4 ENZYME-LABELLING OF ANTIBODIES**

### **2.2.4.1 HRP-labelling using the periodate method**

5 mg purified IgG was dialysed overnight against 0.1 M sodium carbonate buffer, pH 9.2. The volume was reduced, following dialysis, to approximately 0.4 ml by reverse osmosis on a sucrose bed. A 1.7 mg quantity of HRP was dissolved in 170  $\mu$ l 0.1 M sodium carbonate buffer, pH 8.3 in a reactival. To this was added an equal volume of 0.1 M sodium carbonate buffer, pH 8.5 containing 8 mM sodium periodate and the mixture left for 15 mins at room temperature in the dark. The 0.4 ml IgG in sodium carbonate buffer, pH 9.2 was then added to the activated HRP along with 0.13 g Sephadex G-25 beads and the mixture incubated for 3 hrs at room temperature in the dark. The conjugate was eluted from the reaction mixture by centrifugation at 2000 rpm for 5 mins. To stabilise the conjugate, 40  $\mu$ l of 0.1 M NaOH containing 5 % (w/v) sodium borohydride was added to the supernatant and this reaction mixture incubated for 30 mins at room temperature in the dark, after which another 40  $\mu$ l of the sodium borohydride solution was added. The mixture was then incubated at 4°C for 1 hr. The conjugate was separated from unreacted HRP by ammonium sulphate precipitation as described in section 2.2.3.1. The final pellet was resuspended in 500  $\mu$ l 0.15M PBS, pH 7.4 and dialysed for 24 hrs against 0.15 M PBS, pH 7.4 with frequent changes of buffer. Following dialysis, the conjugate was aliquoted into 50  $\mu$ l amounts and stored at 4°C.

### **2.2.4.2 Alkaline phosphatase labelling using one-step glutaraldehyde method**

1 mg of IgG in a volume of 2.5 mls was dialysed against a large excess of 0.1 M phosphate buffer, pH 7.4 at 4°C for 24 hrs. The IgG was then placed in a small glass bottle on a magnetic stirrer. To this was added 10 mg alkaline phosphatase dissolved in 1.5 ml 0.1 M phosphate buffer. 10 % (v/v) aqueous glutaraldehyde solution was added dropwise with constant stirring to a final concentration of 0.2 % (v/v) and the mixture was left gently stirring at room temperature for 2 hrs. To saturate unreacted groups, 200  $\mu$ l of 0.1 M lysine solution in 0.1 M phosphate buffer, pH 7.4 was added and again the mixture left stirring at room temperature for 2 hrs. The conjugate was

separated from free enzyme by passage through an AcA44 Ultrogel size exclusion column equilibrated in 0.15 M PBS, pH 7.4. The conjugate which was eluted in the void volume, was dialysed overnight against 0.15 M PBS, pH 7.4, concentrated by reverse osmosis on a sucrose bed, and aliquoted into 50  $\mu$ l amounts prior to storing at 4°C.

### **2.2.5 BIACORE ANALYSIS**

This procedure was performed on the Pharmacia BIAcore System 2000 biosensor. The monoclonal antibody (mAb) was dialysed into 10 mM sodium acetate buffer, pH 5.5 at a concentration of 200  $\mu$ g/ml. It was then filtered through a 0.45  $\mu$ m filter before being immobilised onto the surface of the gold sensor chip. 35  $\mu$ l of recombinant matrilysin (rPump) or bovine serum albumin (BSA) at a concentration of 100  $\mu$ g/ml in 0.15 M PBS, pH 7.4 was then passed over the immobilised antibody at a flowrate of 5  $\mu$ l/min and the response measured. After washing with 0.15 M PBS, pH 7.4, the purified polyclonal antibody or monoclonal antibody at a concentration of 200  $\mu$ g/ml was passed over the antibody-matrilysin complex at the same flowrate and the response measured. The immobilised antibody surface was regenerated by washing through the system with 10 mM HCl.

### **2.2.6 ENZYME-LINKED IMMUNOSORBENT ASSAYS (ELISAs)**

#### **2.2.6.1 Direct ELISA for purification analysis**

The success of the purification methods was analysed using a direct ELISA system. 100  $\mu$ l of recombinant matrilysin at a concentration of 500 ng/ml in carbonate buffer, pH 9.6 (0.01 M sodium carbonate, 0.035 M sodium hydrogen carbonate) was added to each well of ten columns of a 96-well ELISA plate. The last two columns were controls and these were coated with 3 % (w/v) BSA in the same buffer. The plate was then covered and incubated at 37°C for 2 hrs. Following washing the plate five times with PBST (0.15 M PBS, pH 7.4 containing 0.05 % (v/v) Tween-20) and once with

0.15 M PBS, pH 7.4, to each well of the plate was added 200  $\mu$ l blocking solution (3 % (w/v) BSA in PBST) and the plate incubated at 37°C for 1 hr. The plate was then washed three times with PBST, inverted and shaken dry. Anti-serum, ammonium sulphate precipitate and affinity-purified antibody were diluted in PBS and 100  $\mu$ l added to appropriate wells. The plate was incubated at 37°C for 2 hrs. Control wells containing no antibody, just PBS, were also included. The plate was washed 5 times with PBST and once with PBS. Commercial HRP-labelled anti-rabbit antibody was diluted to 1 : 3000 in PBST and 100  $\mu$ l of this was added to each well. The plate was then re-covered and incubated at 37° for 30 mins following which, it was again washed 5 times with PBST and once with PBS. 100  $\mu$ l of the substrate for the enzyme (0.01 g o-phenylenediamine [OPDA] diluted in 25 ml of 0.2 M citrate buffer, pH 5.0 plus 5  $\mu$ l of 30 % (v/v) H<sub>2</sub>O<sub>2</sub>) was added to each well and the plate incubated at 37°C for 30 mins. The absorbance of each well was read at 405 nm using a Titertek Twinreader Plus microtitre plate reader.

#### **2.2.6.2 Direct ELISA for analysis of enzyme conjugates**

A 96-well ELISA plate was coated with 0.5  $\mu$ g/ml recombinant matrilysin prepared in carbonate buffer and blocked with BSA as described in section 2.2.6.1. Dilutions of the HRP-labelled antibodies were prepared in PBS and 100  $\mu$ l of these added to appropriate wells. Following incubation at 37°C for 1 hr and washing 5 times with PBST and once with PBS, 100  $\mu$ l of the substrate OPDA (prepared as described in section 2.2.6.1) was added to each well. The plate was then incubated at 37°C for 30 mins and the absorbances read at 405 nm.

#### **2.2.6.3 Two-step sandwich ELISA**

The wells of a 96-well ELISA plate were coated with 100  $\mu$ l of the monoclonal anti-matrilysin (mAb) at its optimal dilution in carbonate buffer, pH 9.6 and the plate incubated at 37°C for 2 hrs. Following washing 5 times with PBST and once with PBS, the plate was blocked by the addition of 200  $\mu$ l of 3 % (w/v) BSA in PBST and incubated at 37°C for 1 hr. Dilutions of the standard recombinant matrilysin were then

added to duplicate wells (100µl/well) and the plate incubated for 1.5 hrs at 37°C. The plate was again washed 5 times with PBST and once with PBS after which 100 µl of the HRP-labelled polyclonal anti-matrilysin antibody (HRP-pAb<sub>2</sub>) prepared in PBST at its optimal working dilution was added to each well and the plate incubated for 1 hr at 37°C. The plate was washed as before and then 100 µl of the substrate solution (OPDA prepared as described in section 2.2.6.1) was added to each well. Following incubation at 37°C for 30 mins, the reaction was stopped by the addition of 30 µl of 2 M HCl to each well and the absorbances read at 492 nm.

#### **2.2.6.4 One-step mAb / HRP-pAb<sub>2</sub> sandwich ELISA**

The wells of a 96-well ELISA plate were coated with 100µl of mAb prepared at its optimal working dilution in carbonate buffer and blocked with BSA as described in section 2.2.6.3. Following washing (5 times with PBST plus once with PBS), dilutions of standard recombinant matrilysin or test samples (100 µl/well) plus 50 µl of the HRP-pAb<sub>2</sub> prepared in PBST at its optimal working dilution were added to duplicate wells and the plate incubated for 1.5 hrs at 37°C. The plate was then washed 5 times with PBST and once with PBS prior to addition of 100 µl of the substrate (OPDA in citrate buffer as described in section 2.2.6.1) and further incubation for 30 mins at 37°C. The reaction was stopped by the addition of 30 µl 2 M HCl per well and the absorbances read at 492 nm.

#### **2.2.6.5 One-step mAb / RmAb sandwich ELISA**

The wells of a 96-well ELISA plate were coated with 100 µl mAb diluted to its optimal working concentration in carbonate buffer, pH 9.6 and the plate incubated for 2 hrs at 37°C. The plate was washed three times with PBST before being blocked by the addition of 200 µl of 2 % (w/v) BSA prepared in PBST to every well and the incubation of the plate for another 2 hrs at 37°C. Dilutions of standard recombinant matrilysin or test samples prepared in cell culture medium without serum (DMEM S<sub>0</sub>) were added to duplicate wells (100 µl/well) as well as 100 µl of the rat monoclonal anti-matrilysin antibody diluted in PBST to its optimal working dilution. The plate was

returned to the 37°C incubator for a further 1.5 hrs before being washed 5 times with PBST and once with PBS. 100 µl of the commercial HRP-labelled anti-rat antibody diluted to its working concentration in PBST was then added to each well and the plate incubated for 45 mins at 37°C. The plate was washed as before and 100 µl of the appropriate substrate for the enzyme (OPDA in citrate buffer as described in section 2.2.6.1) was added to each well and the plate incubated for a final 30 mins at 37°C. The colour reaction was stopped by the addition of 30 µl 2 M HCl to each well and the absorbances read at 492 nm.

## **2.2.7 BCA PROTEIN MICROASSAY**

In this assay,  $\text{Cu}^{++}$  reacts with the protein under alkaline conditions to give  $\text{Cu}^+$ , which in turn reacts with BCA to give a coloured product. Two separate reagents are supplied in the commercially available assay kit : A, an alkaline bicarbonate solution and B, a copper sulphate solution. Working solution is prepared by mixing 1 part reagent B with 50 parts reagent A. 200 µl of this working solution was added to 10 µl test sample or protein standard in wells of a microtitre plate. The plate was gently shaken to ensure thorough mixing and incubated at 37°C for 30 mins. The absorbance of each well was read at 560 nm using a Titertek Twinreader Plus microtitre plate reader. Protein concentrations were determined from a standard curve in the range 0 - 1 mg/ml.

## **2.2.8 DNA PREPARATION METHODS**

### **2.2.8.1 Preparation of competent cells**

Using a sterile inoculating loop, *Escherichia coli* DH5α were scraped from a frozen stock and inoculated into 5 ml of sterile SEB medium (1 % (w/v) tryptone; 0.5 % (w/v) yeast extract; 1 % (w/v) NaCl; 10 mM  $\text{MgCl}_2$ ; 10 mM  $\text{MgSO}_4$ ). This culture was grown overnight with agitation (200 rpm) at 37°C. The following day, 1 ml of the DH5α culture was inoculated into 100 ml of fresh, sterile SEB medium and the culture grown at 37°C at 200 rpm until the O.D. at 550 nm had reached 0.6 (2 - 3 hrs). The

culture was then centrifuged at 2500 rpm for 12 mins at 4°C. The pellet was resuspended in 33 ml of FSB, pH 6.4 (10 mM CH<sub>3</sub>COOK, 100 mM KCl, 45 mM MnCl<sub>2</sub>·4H<sub>2</sub>O, 10 mM CaCl<sub>2</sub>·2H<sub>2</sub>O, 3 mM HAcOCl<sub>3</sub>, 10 % (v/v) glycerol). The resuspended pellet was left on ice for 10 mins and then centrifuged at 2500 rpm for 10 mins at 4°C. The pellet was resuspended in 8 ml of cold (4°C) FSB. DMSO was then added dropwise until it reached a final concentration of 3.5 % (v/v). The mixture was left for 10 mins at 4°C after which the same quantity of DMSO was again added. The cells were quickly aliquoted into 200 µl amounts in pre-chilled eppendorfs and flash-frozen in liquid nitrogen prior to storage at -80°C.

#### **2.2.8.2 Transformation of competent cells**

10 ng of the required DNA was placed in a sterile microfuge tube. To this was added 100 µl of the competent cells which had been briefly thawed between the fingers. The tube was swirled gently and placed on ice for 30 mins. The cells were then heat-shocked by placing the tube in a waterbath at 42°C for 90 secs after which they were returned to ice for 2 mins. 1 ml of sterile LB medium, pH 7.5 (1 % (w/v) tryptone; 0.5 % (w/v) yeast extract; 1 % (w/v) NaCl) was added to the tube and the cells incubated at 37°C for 1 hr with gentle agitation (200 rpm). The cells were centrifuged at 6000 rpm for 1 min in an Hereaus Microfuge and the supernatant removed. The pellet was gently resuspended in 0.2 ml LB medium and then plated out on LB agar plus ampicillin (LB medium containing 1.5 % (w/v) agar and, after sterilising, 35 µg/ml ampicillin) plates which were incubated overnight at 37°C in an inverted position.

#### **2.2.8.3 Minipreparation of plasmid DNA**

Single colonies of bacteria from the transformed cells grown on the LB ampicillin plates were selected and each placed into sterile universal containers containing LB medium supplemented with 35 µg/ml ampicillin. These minicultures were grown overnight at 37°C and 150 rpm. The following day, 1.5 mls of each of the growing cultures were transferred to sterile microfuge tubes and centrifuged at 10000 rpm for 1 min after which the supernatants were aspirated. The pellets were resuspended in 100 µl of solution 1 (50 mM glucose, 25 mM Tris-HCl, pH 8.0; 10 mM EDTA, pH 8.0)



and vortexed. 200  $\mu$ l of solution 2 (0.2 M NaOH; 1% (w/v) SDS) was added to each tube and the tubes mixed by gentle inversion after which 150  $\mu$ l cold (4°C) solution 3 (3 M potassium acetate, pH 4.8) was added and the tubes were placed on ice for 5 mins. The tubes were then centrifuged for 5 mins at 10000 rpm after which the supernatants were carefully transferred to fresh microfuge tubes and the pellets discarded. 450  $\mu$ l of phenol : chloroform : 2 M Trizma (1 : 1 : 1, v/v/v) was then added to each tube and the mixtures vortexed and then centrifuged at 10000 rpm for 2 mins. The upper layer in each tube was carefully transferred into a fresh tube and 1 ml of 100 % ethanol added to it. The tubes were placed at -80°C for 5 mins to help precipitate the DNA. The suspensions were then centrifuged at 12000 rpm for 10 mins and the supernatants removed. 250  $\mu$ l of 0.3 M sodium acetate was added to the resultant pellets and mixed. 500  $\mu$ l of 100 % ethanol was added and the mixtures let sit at room temperature for 10 minutes prior to being centrifuged at 10000 rpm for 10 mins. Again the supernatants were removed and the pellets twice washed with 70 % (v/v) ethanol and then dried at 37°C before being resuspended in 25  $\mu$ l TE, pH 8.0 (10 mM Tris-HCl; 1 mM EDTA) and stored at 4°C until ready to digest.

#### **2.2.8.4 Maxipreparation of plasmid DNA**

Once it had been established by restriction digests (see section 2.2.8.6 below) that transformed bacteria did contain the correct plasmids, 500 ml LB medium supplemented with ampicillin (35  $\mu$ g/ml) was inoculated with a 5 ml miniculture prepared as for the minipreparation procedure (section 2.2.8.3) and the culture grown overnight at 37°C and 200 rpm. The procedure used to isolate the plasmid DNA was a modification of the alkaline lysis method and utilised pZ523 spin columns for final purification of the plasmid DNA.

The bacterial cells were pelleted from the 500 ml culture by centrifugation at 10000 rpm for 5 minutes at 4°C. The pellet was resuspended in 15 ml solution A (25 mM Tris-HCl; 10 mM EDTA, pH 8.0) and vortexed until fully in solution. 15 ml of solution B (0.2 M NaOH, 1% (w/v) SDS) was added and mixed by gentle swirling until the lysate appeared essentially uniform and translucent. To this was then added 15 ml ice-cold solution C (7.5M ammonium acetate) and the mixture thoroughly mixed

by gentle inversion. The suspension was then centrifuged at 10000 rpm for 30 mins at 4°C to pellet the protein precipitate. The supernatant was carefully transferred to a fresh centrifuge tube and room temperature isopropanol added to a final concentration (v/v) of 0.6X. This was carefully mixed by gentle inversion and centrifuged at 10000 rpm for 30 mins at 20°C and the resultant supernatant discarded. 12 ml of 70 % (v/v) ethanol was added to the pellet and the suspension briefly centrifuged for 1 - 2 mins at 10000 rpm to re-pellet the DNA. The supernatant was discarded and the pellet washed with 12 ml of 95 % (v/v) ethanol. After carefully aspirating the supernatant, the pellet was left to dry at 37°C for approximately 15 mins. The pellet was then dissolved in 1.5 ml of TE containing RNase A at a final concentration of 50 µg/ml and transferred to a PLG tube (provided with the pZ523 columns) prior to being incubated in a 37°C waterbath for 15 mins. The sample was extracted with 2 ml of phenol : chloroform : isoamyl alcohol (12.5 : 12 : 0.5, v/v/v) and centrifuged at 1500 rpm for 2 mins. The phenol : chloroform : isoamyl alcohol extraction and centrifugation was repeated after which the sample was extracted with chloroform : isoamyl alcohol (24 : 1, v/v). Following a further spin at 1500 rpm for 2 mins, 360 µl 5 M NaCl was added to the aqueous phase and the total sample volume adjusted to 1.8 ml with TE prior to loading onto the pZ523 spin column. The column was centrifuged in a swinging bucket rotor for 12 mins at 1100 rpm. The plasmid DNA was precipitated from the column eluant by addition of 1.2 ml room temperature 100 % isopropanol and centrifuging at 10000 rpm for 20 mins at 20°C. The pellet was washed 4 times with 4 ml of 70% (v/v) ethanol and then once with 95 % (v/v) ethanol prior to being dried briefly at 37°C. Finally, the plasmid DNA was resuspended in 300µl TE and stored at 4°C.

#### **2.2.8.5 Spectrophotometric analysis of nucleic acids**

DNA or RNA concentration was determined by measuring the absorbance at 260 nm, the wavelength at which nucleic acids absorb maximally ( $\lambda_{\text{max}}$ ). A 50 µg/ml preparation of pure DNA has an absorbance of 1 unit at 260 nm while 40 µg/ml of pure RNA also has an absorbance reading of 1 at this wavelength. The purity of an RNA or DNA preparation was determined by reading absorbances at 260 nm, the  $\lambda_{\text{max}}$  for nucleic acids, and at 280 nm, the  $\lambda_{\text{max}}$  for proteins and obtaining the ratio of these

absorbances. Pure DNA with no protein contamination has an  $Abs_{260} / Abs_{280}$  ratio of 1.8 while for pure RNA, the ratio is 2.0. Lower ratios indicate the presence of protein while higher ratios often indicate residues of organic reagents.

#### **2.2.8.6 Restriction digests**

DNA was digested with restriction endonucleases for identification purposes or for linearizing prior to probe manufacture. For mini-preparation DNA, the digest mixture contained 5  $\mu$ l DNA, 2.5 $\mu$ l of the 10x reaction buffer (supplied with the enzyme by the manufacturer), 16.5  $\mu$ l of a 25  $\mu$ g/ml solution of RNase A and 1  $\mu$ l (15 - 20 units, approximately) of the appropriate restriction enzyme. For maxipreparation DNA, 1  $\mu$ g of DNA was prepared in a reaction solution containing 10x reaction buffer and 1  $\mu$ l restriction enzyme. The digests were incubated at the appropriate temperature for the enzyme (usually 37°C) in a water bath for at least two hours.

#### **2.2.8.7 Agarose gel electrophoresis**

An agarose gel was prepared by boiling the appropriate quantity of agarose in 100 ml of 0.5X TBE buffer, pH 8.2 (0.04 M Tris; 0.02 M boric acid; 1 mM EDTA). The percentage gel used depended on the sizes of DNA being visualised with a lower percentage (0.6 - 1.2 % (w/v)) being used for visualising large sized DNA (*e.g.* over 2 kb) and higher percentage gels (2 - 3 % (w/v)) reserved for small DNA fragments (*e.g.* 200 - 500 bp). Once cooled to approximately 60°C, the gel was cast into the Hybaid Horizontal Gel Electrophoresis system.

Samples for electrophoresis were prepared by mixing them with a suitable quantity of 6X gel loading buffer (40 % (w/v) sucrose; 0.25 % (w/v) bromophenol blue). Typically 12.5  $\mu$ l of the digest mixtures were mixed with 2.5  $\mu$ l of the loading buffer and the samples loaded into the wells of the set gel. The gel was run at 120 V in 0.5X TBE. The electrophoresis was completed when the blue loading dye had run to within 0.5 cm of the bottom of the gel. The gel was then stained by submersion in a solution of 0.5  $\mu$ g/ml ethidium bromide for 20 mins, briefly rinsed in distilled water and visualised by placing on a UV transilluminator.

## **2.2.9 CELL CULTURE METHODS**

All cell culture techniques were performed in a sterile environment using a Holten laminar air flow cabinet. Cells were visualised with an Olympus CK2 inverted phase contrast microscope.

### **2.2.9.1 Culture of adherent cells**

SW480, SW620, WiDR, LoVo, A549, EJ and MCF-7 cells were maintained in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 5 % (v/v) foetal calf serum (FCS) [DMEM S<sub>5</sub>], 2 mM L-glutamine, 1 mM HEPES, penicillin (1 unit/ml) and streptomycin (1 µg/ml). All cultures were seeded into 25 cm<sup>2</sup> and 75 cm<sup>2</sup> tissue culture flasks. As these were all strongly adherent cells, trypsinisation was required for harvesting prior to sub-culturing. For trypsinisation, the growth medium was decanted and the flask rinsed with 2 mls of trypsin-EDTA (0.025 % (w/v) trypsin with 0.02 % (w/v) EDTA in 0.15 M PBS, pH 7.4) to remove any residual FCS which contains a trypsin-inhibitory activity ( $\alpha_2$ -macroglobulin). 2 ml of fresh trypsin-EDTA was then placed in each flask and the flask incubated at 37°C for 5 - 10 mins or until all the cells could be visualised as having detached from the flask. The cell suspension was decanted into a sterile universal container containing 5 ml DMEM S<sub>5</sub> and centrifuged at 2000 rpm for 4 mins. Cells were resuspended in culture medium at  $2 \times 10^5$  -  $1 \times 10^6$  cells/ml, using 20 ml of medium per 75 cm<sup>2</sup> culture flask and 10 ml per 25 cm<sup>2</sup> flask. All cell lines were incubated in a humid, 5 % (v/v) CO<sub>2</sub> atmosphere at 37°C in an Heraeus cell culture incubator.

### **2.2.9.2 Culture of cells in suspension**

K562 cells were maintained in DMEM supplemented with 10 % (v/v) FCS, 2 mM L-glutamine, 1 mM HEPES, 1 unit/ml penicillin and 1 µg/ml streptomycin. Cultures were seeded at densities of  $2 \times 10^5$  -  $1 \times 10^6$  cells/ml, using 20 ml per 75 cm<sup>2</sup> flask. Cells were incubated in culture flasks until approximately 90 % confluent. They were then harvested by flusing them with medium from the culture flask surface using a sterile pasteur pipette. After centrifuging at 800 rpm for 5 mins, the cell pellet was

resuspended in an appropriate amount of medium and placed into flasks. The cells were incubated in a humid 5 % CO<sub>2</sub> atmosphere at 37°C.

#### **2.2.9.3 Cell counts**

Cell counts were performed using an improved Neubauer haemocytometer slide. Trypan blue exclusion dye was routinely used to determine cell viabilities. To 100 µl cell suspension was added 20 µl trypan blue, and the mixture left to incubate for two mins. A sample of this mixture was added to the counting chamber of the haematocytometer and the cells visualised by light microscopy. Viable cells excluded the dye and remained clear while dead cells stained blue.

#### **2.2.9.4 Storage and recovery of cells**

Cells required for long term storage were frozen in liquid nitrogen and maintained in a cryofreezer (Cooper Cryoservice Ltd.). After centrifuging, the cell pellet was resuspended at a concentration of  $1 \times 10^6$  cells/ml in FCS containing the cryopreservative DMSO at a final concentration of 5 % (v/v). 1 ml aliquots were transferred to sterile cryotubes, lowered slowly into the gas phase and eventually immersed in the liquid nitrogen.

Cells were recovered from liquid nitrogen by thawing rapidly at 37°C and transferring to a sterile universal tube containing 5 ml DMEM S<sub>5</sub>. The cells were centrifuged at 2000 rpm for 4 mins resuspended in fresh medium, transferred to culture flasks and incubated at 37°C in 5% CO<sub>2</sub>.

#### **2.2.9.5 Mycoplasma detection**

Cells were screened periodically for contamination by mycoplasma using an indicator cell line (normal rat kidney - NRK) and the DNA-intercalating agent bisbenzimid (Hoechst 33258). A vial of mycoplasma-free NRK cells was grown in antibiotic-free medium at 37°C in 5 % CO<sub>2</sub> for at least 3 passages. Cells were removed from flasks by trypsinisation (as described in section 2.2.9.1) and resuspended at a concentration of  $1 \times 10^4$  cells/ml in antibiotic-free DMEM S<sub>5</sub>. 1 ml aliquots of this suspension were grown overnight at 37°C on sterile coverslips placed in petri dishes. The following day,

2 ml of culture medium from the cell line to be screened was added to the coverslips and the NRK cells returned to the incubator and grown for 2 further days. After incubation, the coverslips were removed and washed 3 times in 0.15 M PBS, pH 7.4. The cells were then fixed for 6 mins in a 1 : 1 (v/v) mixture of methanol : acetone at -20°C. The cells were again washed twice in PBS and stained with Hoechst 33258 prepared at a concentration of 0.05 µg/ml in PBS, for 10 mins. The coverslips were then washed 3 times with PBS, dried and mounted on slides. The cells were examined under a fluorescent microscope (Leitz Labrolux microscope) using a 100x oil immersion lens. Uncontaminated cells exhibited strong fluorescence in the nucleus only, while contaminated cells also had fluorescence distributed throughout the cytoplasm.

#### **2.2.9.6 Cytokine and TPA treatment of cells in culture**

Cell lines were routinely cultured for at least 2 passages prior to cytokine or TPA treatment. For cytokine experiments, cells were cultured until approximately 80 % confluent after which the growth medium was decanted and the cells rinsed with sterile 0.15 M PBS, pH 7.4. 8 ml of fresh serum-free DMEM was then added to each 75 cm<sup>2</sup> flask (4 ml to each 25 cm<sup>2</sup> flask and 1 ml to each 35 mm well of a 6 well plate). The cells were returned to the incubator overnight. The following day flasks were supplemented with one or other of the following cytokines or TPA (final concentrations in brackets) : IL-1β (25 ng/ml), IL-2 (1 unit/ml), IL-6 (100 units/ml), IGF-I (25 ng/ml), IGF-II (25 ng/ml), EGF (50 ng/ml), bFGF (25 ng/ml), TGF-β (1 µg/ml), IFN-γ (125 ng/ml) and TPA (6.2 ng/ml). The cells were then returned to the incubator for 8 hours after which the medium from each flask ('conditioned medium') was collected and the cells harvested for RNA isolation (section 2.2.11) or transfection analysis (section 2.2.10). Conditioned medium was clarified by centrifugation at 4000 rpm for 10 mins and stored at -80°C for further analysis.

#### **2.2.9.7 Calcium phosphate-mediated transfection**

This method was first described by Graham and Van der Eb (1973). 24 hrs prior to transfection, cells were trypsinised and seeded at a density of 3 x 10<sup>6</sup> cells per well of a

6-well tissue culture plate. Various amounts of DNA were aliquoted in sterile universal containers and the volume adjusted to 410  $\mu$ l with sterile water. 480  $\mu$ l of 2X HBS (HEPES buffered saline : 280 mM NaCl; 50 mM HEPES, 1.5 mM Na<sub>2</sub>HPO<sub>4</sub>), was aliquoted for each sample of DNA into separate tubes. 60  $\mu$ l of 2.5 M CaCl<sub>2</sub> was added into the tube containing the DNA with continual mixing using a vortex mixer. Immediately the DNA-CaCl<sub>2</sub> mixture was added into the 2X HBS, again dropwise and with continuous mixing using a vortex mixer. This mixture was then left standing at room temperature for exactly 30 mins after which it was added dropwise to the cells on the 6-well plate and the plate returned to the 37°C incubator for 6 hrs. To aid the entry of DNA into the cells, a glycerol shock was utilised. This involved removing the medium from the cells and placing 1 ml of 10 % (v/v) glycerol in 1X HBS in each of the wells and leaving for 3 mins. The glycerol was then removed, the cells rinsed in PBS and serum-free medium and then re-fed with 2 ml fresh DMEM S<sub>5</sub>. The cells were then returned to the incubator for 48 hrs before transfection analysis (section 2.2.10) or the the medium was replaced 24 hrs later with serum-free DMEM and the cells treated with cytokines as in section 2.2.9.6.

#### **2.2.9.8 DEAE-Dextran mediated transfection**

Cells to be transfected were prepared as described in section 2.2.9.7. Various amounts of the plasmid DNA (0.25 - 3.0  $\mu$ g) were mixed with 10 mM Trizma-HCl, pH 7.5 containing 0.05 % NaCl to a final volume of 100  $\mu$ l. This was then mixed with an equal volume of DEAE-dextran solution (1 mg/ml in 10mM Trizma-HCl, pH 7.5; 0.045 % (w/v) NaCl). Meanwhile, growth medium was removed from the cells and they were washed twice with 10mM Trizma, pH 7.5 containing 0.05 % NaCl. The DNA-DEAE dextran mixture was then dropped slowly into the centre of the cells. The cells were incubated for 15 mins at room temperature before the DNA-DEAE dextran mixture was carefully removed and the cells were rinsed once with the trizma solution. The cells were then fed normal growth medium and returned to the incubator. Transfections were analysed 48 hrs later (section 2.2.10).

### 2.2.9.9 DOTAP-mediated transfections

Cells were seeded at  $3 \times 10^6$  cells per 35 mm well of a 6 well tissue culture plate one day prior to transfection. Quantities of DNA were diluted to a concentration of  $0.1 \mu\text{g}/\mu\text{l}$  in 20 mM HEPES buffer, pH 7.5. In separate tubes, quantities of the DOTAP reagent were diluted to a final concentration of  $0.3 \mu\text{g}/\mu\text{l}$  with 20 mM HEPES. The DNA and DOTAP solutions were then mixed together and left standing at room temperature for 15 mins. The DNA-DOTAP mixture was then added to 1 ml of serum-free culture medium. Meanwhile, the cells were removed from the plate and centrifuged at 800 rpm for 4 mins. Each pellet was then gently resuspended in the 1 ml of serum-free culture medium containing the DNA-DOTAP mixture and the cells returned to the plate. The plate was returned to the incubator for 5 hrs after which the cells were again removed, centrifuged at 800 rpm for 4 mins and re-fed with 1 ml fresh serum-free culture medium. The cells were harvested after 48 hrs for transfection analysis (section 2.2.10) or treated with cytokines after 24 hrs (concentrations used as given in section 2.2.9.6), harvested and then analysed.

## 2.2.10 TRANSFECTION ANALYSIS

### 2.2.10.1 *In situ* assay of $\beta$ -galactosidase

In all transfections the plasmid pCH110, coding for  $\beta$ -galactosidase, was co-transfected with the matrixin promoter-containing plasmids as its presence could be easily detected and used as an assessment of transfection efficiency. The enzyme activity of  $\beta$ -galactosidase can be detected *in situ*, on the plate, using the synthetic substrate x-gal. 48 hrs following transfection, cells were washed twice with 0.15 M PBS, pH 7.4 and then fixed with 5 ml fix solution (0.2 % (v/v) glutaraldehyde; 0.02 M phosphate buffer, pH 7.3; 0.004 M EGTA; 0.002 M  $\text{MgCl}_2$ ) for 10 mins at room temperature. This step was repeated once. The cells were then washed twice for 10 mins each wash with 5 ml rinse solution (0.02 M phosphate buffer, pH 7.3; 0.002 M  $\text{MgCl}_2$ ; 0.1 mg/ml sodium deoxycholate; 0.2  $\mu\text{l}/\text{ml}$  NP-40) before 1 ml of stain solution (1 mg/ml X-gal; 1.65 mg/ml  $\text{K}_3\text{Fe}(\text{CN})_6$ ; 1.65 mg/ml  $\text{K}_4\text{Fe}(\text{CN})_6$  in rinse solution) was added to each well and the plates returned to the incubator overnight. The following day, the cells were



examined under the microscope. Cells that stained blue produced  $\beta$ -galactosidase and were therefore successfully transfected. Untransfected cells remained clear.

#### **2.2.10.2 Harvesting cells after transfection**

Cell lysates were prepared using the Reporter Lysis Buffer (Promega). Briefly, the medium was decanted from the cells and they were rinsed twice with 0.15 M PBS, pH 7.4. 250  $\mu$ l of the lysis reagent was then placed on the cells for 15 mins after which the lysate was scraped into a microfuge tube and centrifuged at 5000 rpm for 2 mins. The supernatants were transferred to fresh tubes and stored at -80°C until required.

#### **2.2.10.3 Assay of $\beta$ -galactosidase in cell lysates**

100  $\mu$ l of chlorophenyl red  $\beta$ -D galactoside (CPRG) solution (15 mg/ml CPRG prepared in buffer A : 100 mM NaH<sub>2</sub> PO<sub>4</sub>, pH 7.2; 10 mM KCl; 1 mM MgCl<sub>2</sub>) was placed in a microfuge tube. To this was added 400  $\mu$ l buffer A containing 10 mM  $\beta$ -mercaptoethanol and 25  $\mu$ l of the cell lysate prepared as described in section 2.2.10.2. Standards were prepared using a commercial stock of the enzyme  $\beta$ -galactosidase, in the 0.2 - 5.0 ng/ml range and these were also assayed. All tubes were incubated overnight in a 37°C waterbath. Absorbances were then read at 560 nm and the concentration of active enzyme in the transfected cells determined from a standard curve.

#### **2.2.10.4 CAT ELISA**

The activity of the chloramphenicol acetyl transferase (CAT) in the matrilysin-promoter CAT construct-transfected cells was analysed using a commercially available CAT ELISA kit (Boehringer Mannheim). Cell lysates were prepared as outlined in section 2.2.10.2. CAT enzyme standards for the ELISA were prepared as suggested by the manufacturers in the 0.0 - 0.91  $\mu$ g/ml range. The anti-CAT antibodies coated on the surface of the wells of the ELISA plate were rehydrated using the sample buffer provided. 200  $\mu$ l of each sample and standard was pipetted into appropriate wells of the plate (the samples actually contained 37.5  $\mu$ l of lysate made up to 200  $\mu$ l with PBS). The plate was covered and incubated at 37°C for 1 hr after which it was washed

5 times with washing buffer. 200  $\mu$ l of the digoxigenin-labelled anti-CAT antibody solution was then added to each well and the plate covered and again incubated for 1 hr at 37°C. The plate was washed as before and 200  $\mu$ l of an HRP-labelled anti-digoxigenin antibody solution added to each well. The plate was covered and incubated at 37°C for a further hour. Following another washing step, 200  $\mu$ l of the peroxidase substrate solution was applied to each well and the plate incubated at room temperature for 45 mins after which the absorbance at 405 nm was measured for each well.

#### **2.2.10.5 Luciferase assay**

The activity of the enzyme luciferase in the matrixin promoter-luciferase construct transfected cells was determined using the luciferase assay reagent from Promega and a Labsystems Lumiskan luminometer. 10  $\mu$ l of each lysate was pipetted into the wells of a white luminometer 96-well plate and the plate placed into the luminometer to equilibrate to assay temperature (23°C). Immediately prior to measuring luminescence, 100  $\mu$ l of the luciferase assay reagent was added to the lysate-containing well and the luminescence measured as an integral reading taken over 30 secs with a lag time of 10 secs to allow the machine to set up for measurement. The values obtained for luciferase activity were normalised using the  $\beta$ -galactosidase values (obtained as outlined in section 2.2.10.3) to correct for differences in transfection efficiency, and expressed as fold increase over control levels.

#### **2.2.11 RNA EXTRACTIONS**

RNA is easily degraded by the ubiquitous RNase enzymes. These enzymes are resistant to autoclaving but they can be deactivated by the chemical diethylpyro-carbonate (DEPC) when it is added to solutions at a final concentration of 0.1 %. Solutions containing amines such as Tris cannot be DEPC-treated directly as the DEPC is inactivated by these chemicals. These solutions were prepared in DEPC-treated water. All other solutions for RNA work were DEPC-treated and gloves and sterile plastics used at all times. The procedures must be carried out quickly and on ice to help prevent degradation of RNA by endogenous RNases.

### **2.2.11.1 RNA extraction from cultured cells**

The cells were detached from the surface of the flasks by trypsinisation, if necessary, and the pellets washed twice with cold (4°C) 0.15 M PBS, pH 7.4. The final pellets were resuspended in 1 ml of ice-cold Tris-saline, pH 7.2 (25 mM Tris; 0.13 M NaCl; 5 mM KCl) and transferred to sterile microfuge tubes. The cells were centrifuged at 2000 rpm for 1 min and the supernatants discarded. The cells were then resuspended in 250 µl Tris-saline and an equal volume of lysis buffer (Tris-saline, pH 7.2 containing 1 % (v/v) NP-40, 5 mg/ml sodium deoxycholate and 0.1 mg/ml dextran sulphate) added. The tubes were gently mixed by inversion and centrifuged at 2000 rpm for 1 minute. Supernatants were then carefully transferred (without disturbing pellets) to fresh microfuge tubes containing 0.5 ml phenol : chloroform : isoamyl alcohol [PCI] (25 : 24 : 1, v/v/v) and 25 µl of 20 % (w/v) SDS and 15 µl of 5 M NaCl added quickly to each tube. The tubes were vortex-mixed for 5 secs before being centrifuged at 13000 rpm for 5 mins. The upper aqueous phase was carefully transferred from each tube to a fresh tube containing PCI and the tubes vortexed and centrifuged as in the previous step. This was repeated until no protein (seen as a white precipitate) was visible at the aqueous / organic interphase in each tube. The extracts were then added to microfuge tubes containing 250 µl chloroform : isoamyl alcohol (24 : 1, v/v) to remove any residual phenol and the vortex and centrifugation repeated as before. The upper aqueous phase in each tube was then transferred into a tube containing 1 ml 100 % ethanol and the tubes placed at -20°C overnight to precipitate the RNA. The precipitate was pelleted by centrifugation at 10000 rpm for 20 mins and the final pellet resuspended in 50 µl DEPC-treated water. The concentration and purity of the RNA was assessed spectrophotometrically as outlined in section 2.2.8.5. Samples were also run on RNA gels (section 2.2.11.3) to check RNA integrity. The RNA isolates were stored at -80°C until required.

### **2.2.11.2 RNA isolation from human tissue samples**

A piece of frozen tumour tissue (approximately 100 mg wet weight) was ground in liquid nitrogen using a mortar and pestle to obtain a fine powder. The powdered tissue

was added to 1 ml denaturing solution ( 4 M guanidinium thiocyanate; 25 mM sodium citrate, pH 7.0; 0.5 % (w/v) N-lauroylsarcosine; 0.1 M  $\beta$ -mercaptoethanol) and mixed. To this was added 0.1 ml of 2 M sodium acetate, pH 4.0 and the suspension mixed by inversion. 1 ml of water-saturated phenol followed by 0.2 ml of chloroform : isoamyl alcohol (24 : 1, v/v) were then added and the suspension thoroughly mixed. The mixture was then left at 4°C for 15 mins prior to being centrifuged at 9000 rpm for 20 mins also at 4°C. The upper aqueous phase was transferred to a fresh tube and the RNA precipitated by the addition of 1 ml 100 % isopropanol and incubation at -20°C for 30 mins. This suspension was centrifuged at 10000 rpm for 10 mins and the supernatant discarded. The pellet was redissolved in 0.3 ml of denaturing solution and again precipitated by the addition of 0.3 ml 100 % isopropanol and incubated for 30 mins at -20°C. This was centrifuged at 10000 rpm for 10 mins and the pellet resuspended in 75 % (v/v) ethanol and vortexed. The tube was then incubated for 15 mins at room temperature before being centrifuged at 10000 rpm for 5 mins. The supernatant was discarded and the pellet dried briefly. The pellet was then re-suspended in 50  $\mu$ l DEPC-treated water and the RNA analysed spectrophotometrically as outlined in section 2.2.8.5. Samples were also run on RNA gels (2.2.11.3) to check for RNA integrity. The RNA isolate was stored at -80°C until required.

### **2.2.11.3 RNA analysis by gel electrophoresis**

To check that RNA isolated by either method (2.2.11.1 or 2.2.11.2) was intact and had not been degraded, samples were run on 1 % agarose gels. The gels were prepared as outlined in section 2.2.8.7. The RNA samples (5  $\mu$ l) were prepared for electrophoresis by mixing with 15  $\mu$ l sample buffer (50 % (v/v) deionized formamide; 8.3 % (v/v) formaldehyde; 0.027 M MOPS, pH 7.0; 6.7 mM sodium acetate; 0.67 mM EDTA) and 3  $\mu$ l loading buffer (50 % (v/v) glycerol; 1 mM EDTA; 0.4 % (w/v) bromophenol blue; 1  $\mu$ g/ $\mu$ l ethidium bromide) and the sample heated for 10 mins at 65°C prior to loading on the gel. The gel was run in 0.5X TBE as described in section 2.2.8.7. As ethidium bromide was included in the loading buffer, there was no need for further staining and the gel could be visualised directly on a UV transilluminator. The presence of 2

strongly staining bands, representing the 28 S and 18 S ribosomal subunits, signified intact RNA. Degradation could be seen as a smear running down the length of the gel.

### **2.2.12 REVERSE-TRANSCRIPTION PCR (RT-PCR)**

The polymerase chain reaction (PCR) has emerged as a powerful tool for amplifying small quantities of DNA for analysis. RT-PCR is a modification of the technique which allows analysis of small quantities of specific mRNA. Total RNA is first converted to cDNA using a reverse transcriptase enzyme. Specific cDNAs are then amplified in the PCR by inclusion of the appropriate primers. Details of the primers used are shown in Table 2.2. The PCR used was semi-quantitative as a constitutively expressed gene,  $\beta$ -actin, was always amplified in the same tube with the matrilysin or stromelysin target. This acted as an internal control and, by calculating ratios of matrilysin or stromelysin to  $\beta$ -actin, relative amounts of the targets could be determined from reaction to reaction.

#### **2.2.12.1 Reverse transcription**

1  $\mu$ g of total RNA (prepared as outlined in sections 2.2.11.1 or 2.2.11.2) was mixed with 0.5  $\mu$ g oligo dT primers and the mixture brought to a final volume of 5  $\mu$ l with sterile water. This priming reaction was incubated at 70°C for 10 mins. To this was then added 4  $\mu$ l 5X transcription buffer (supplied with the reverse transcriptase enzyme by the manufacturer), 2  $\mu$ l 100 mM dithiothreitol, 1  $\mu$ l RNasin, 1  $\mu$ l of a mix of dATP, dCTP, dGTP and dTTP each at a concentration of 10 mM, 6  $\mu$ l sterile water, and 1  $\mu$ l of Moloney murine leukaemia virus reverse transcriptase (MMLV-RT). The reaction mixture was incubated at 37°C for one hour before being heated to 95°C for 2 mins to inactivate the enzyme. The resulting cDNA was stored at 4°C until required for PCR.

#### **2.2.12.2 PCR**

A 45 $\mu$ l PCR mix was prepared by placing 5  $\mu$ l of the 10X reaction buffer supplied with the Taq polymerase enzyme, 1  $\mu$ l each of dATP, dTTP, dCTP and dGTP each at a concentration of 10mM, 1  $\mu$ l of each of the forward and reverse primers required in the

reaction, 0.5  $\mu$ l Taq DNA polymerase and sterile water to bring the volume to 45  $\mu$ l. To this reaction mixture was added 5  $\mu$ l of cDNA prepared as in section 2.2.12.1 and the total overlaid with 50  $\mu$ l mineral oil. The tube was placed on a Hybaid thermocycling machine and incubated for 3 mins at 95°C followed by 30 cycles of 1 min at 95°C, 1 min at 47°C and 3 mins at 72°C. The final step was a 7 minute incubation at 72°C. The PCR products were then removed from under the oil and placed in fresh tubes. 15  $\mu$ l amounts were run on 2 % agarose gels as described in section 2.2.8.7. The gels were viewed and saved on disc using a UHP Gel Documentation System. The relative intensity of each band was measured using a QUIPS image analysis package.

**Table 2.2 Primers for PCR**

Target	Primers	Fragment Size (bp)	Reference
Matrilysin	5' TGTATCCAACCTATGGAAATG 3' 3' CATTATTGACATCTACGCGC 5'	341 (cDNA)	Witty <i>et al.</i> , 1994
Stromelysin-1	5' CTTTCCAGGGATTGACTC 3' 3' AACATAAAAATGACCGGC 5'	324 (cDNA)	Matrisian <i>et al.</i> , 1985
$\beta$ -actin	5' CAACTTCATCCACGTTCAACC 3' 3' GAAGAGCCAAGGACAGGTAC 5'	225 (cDNA)	Takeshita <i>et al.</i> , 1994

The primers were chosen so that they amplified sequences specific to the particular gene. Also, primers were chosen to span an intron. This meant that any amplification of genomic rather than cDNA would result in larger products than those expected.

## 2.2.13 *IN SITU* HYBRIDISATION

### 2.2.13.1 Probe labelling

The plasmids pG7PumpEX and pG7Pump XE (shown in appendix A) were obtained from Prof. Lynn Matrisian, Vanderbilt University, Nashville, Tennessee, USA. These plasmids consisted of an 800 bp fragment of the matrilysin cDNA, encompassing an EcoRI site to XmnI site subcloned into pGem7Z(+) in both the forward (EX) and reverse (XE) orientations. The pGem7 vector has both SP6 RNA polymerase and T7 RNA polymerase initiation sites and, as the fragment was subcloned between them, either enzyme could be used to generate both sense and anti-sense RNA transcripts. For example, if pG7PumpEX was linearised with EcoRI, the enzyme SP6 could be used to generate an antisense transcript while a sense transcript could be generated using the T7 enzyme following linearising with Hind III.

Prior to labelling, 10 µg of plasmid was linearised using either of the restriction enzymes Hind III or EcoRI ( see section 2.2.8.6) and the digests cleaned by a phenol : chloroform extraction. This involved adding the digest mixture to an equal volume of phenol : chloroform (1 : 1, v/v), vortex-mixing, and centrifuging at 10000 rpm for 5 mins. The upper aqueous phase was transferred to a fresh tube and an equal volume of chloroform added. Again the mixture was vortexed and centrifuged at 10000 rpm for 5 mins. The linearised DNA was precipitated by placing the upper aqueous layer into a tube with 2.5 volumes of 100 % ethanol and 0.5 volume 7.5 M ammonium acetate and the tube placed at -80°C for 2 hrs. The precipitated DNA was pelleted by centrifugation at 10000 rpm for 15 mins and the pellet was washed with 70 % (v/v) ethanol to remove excess salt. The DNA was dried briefly at 37°C before being resuspended in 20 µl DEPC-treated water.

The probes were labelled with digoxigenin using the SP6/T7 DIG RNA labelling kit from Boehringer Mannheim. To a sterile microfuge tube on ice were added 2 µl 10X transcription buffer (kit component), 12 µl sterile DEPC-treated water, 2 µl NTP labelling mixture (kit component), 2 µl (1µg) linearised plasmid DNA and 2 µl SP6 or T7 polymerase (kit component). The tube was centrifuged briefly to ensure all components of the reaction were together at the bottom of the tube, and the tube

incubated for 2 hrs in a 37°C waterbath. To stop the reaction, 2 µl of 0.2 M EDTA, pH 8.0, was added. The labelled RNA was then precipitated with 2.5 µl of 4 M lithium chloride and 75 µl pre-chilled (-20°C) ethanol. The tube was incubated at 80°C for 2 hrs after which the RNA was pelleted by centrifugation at 12000 rpm for 20 mins. The pellet was washed once with 50µl cold 70 % (v/v) ethanol, dried and resuspended in 100µl DEPC-treated water.

### **2.2.13.2 Probe evaluation**

The sensitivity of the labelled probe could be estimated by dot blot experiments where various dilutions of denatured pG7PumpEX or pG7PumpXE plasmid were dotted onto a nylon membrane. The DNA was bound to the membrane by baking for 30 mins at 120°C. The blot was prehybridised by sealing it in a plastic bag with 10 ml hybridisation buffer (50 % (v/v) formamide; 5X SSC [0.75 M NaCl, 0.075 M sodium citrate, pH 7.0;]; 2 % (w/v) blocking reagent [Boehringer Mannheim]; 0.1 % (w/v) N-lauroylsarcosine; 0.02 % (w/v) SDS) and placing it in a 50°C incubator for 1 hour. For hybridisation, the labelled probe was diluted to a concentration of 50 ng/ml in 2 - 5 ml hybridisation buffer and this put into the plastic bag with the blot to replace the 10 ml of buffer used for pre-hybridisation. The hybridisation was performed for 16 hrs at 50°C. The blots were washed twice for 5 mins each wash in 2X SSC at room temperature, and twice with 0.1X SSC for 15 mins each wash at 68°C. After washing, the blots were rinsed in detection buffer 1 (100 mM Tris-HCl; 150 mM NaCl, pH 7.5) and blocked for 30 mins in detection buffer 2 (1 % (w/v) blocking reagent (Boehringer Mannheim) prepared in detection buffer 1). Once blocked, the blots were probed with alkaline phosphatase-labelled anti-digoxigenin antibody (1 : 5000 dilution in detection buffer 2) for 30 mins. The blots were then washed twice for 15 mins each wash with detection buffer 1 before being developed for 6 hrs with BCIP/NBT (175 µg/ml BCIP, 500 µg/ml NBT prepared in detection buffer 3 [100 mM Tris-HCl; 100 mM NaCl; 50 mM MgCl<sub>2</sub>, pH 9.5]). The colour reaction was stopped by rinsing the blots in distilled water.



### 2.2.13.3 *In situ* hybridisation

Cell culture growth slides (*i.e.* cells that had been grown on slides rather than the base of a cell culture flask) had been fixed in 3 % paraformaldehyde for 1 hr at 4 °C and stored at -20 °C. Cells were permeabilised with 0.1 M HCl for 10 mins followed by a proteinase K (10 µg/ml) digestion at 37°C for 10 mins. The digestion was stopped by placing the slides in DEPC-treated water for 5 mins followed by 0.2 % (w/v) glycine prepared in 0.15 M PBS, pH 7.4 for 10 mins. The cells were then post-fixed in 4 % (w/v) paraformaldehyde dissolved in 0.15 M PBS, pH 7.4 for 30 mins at 4°C. Slides were prehybridised for 1 hour at 50°C by covering with 50 µl of prehybridisation buffer ( 5 X SSC; 50 % (v/v) formamide; 250 µg/ml yeast tRNA; 250 µg/ml herring sperm DNA; 4 mM EDTA) and placing in a humid chamber in the incubator. Hybridisation buffer was identical to prehybridisation buffer except that the herring sperm DNA was omitted. 5 µl labelled probe was added to 35 µl hybridisation buffer and the mixture boiled for 10 mins and then placed directly on ice. After the prehybridisation, the slides were carefully dried and the probe/hybridisation buffer mixture placed on each section. The slides were then placed in the humid chamber and incubated for 16 hrs at 50°C. The slides were washed twice for 15 mins each wash at room temperature with 2X SSC. Any remaining unhybridised probe was then removed by a 30 minute digestion with RNase A (100 µg/ml RNase A in 2X SSC). The slides were washed more stringently for 20 mins at 42°C with 0.1 X SSC and for a further 10 mins at room temperature with 0.1X SSC. The slides were washed briefly with detection buffer 1, blocked for 30 mins with detection buffer 2 and probed with the alkaline phosphatase anti-DIG antibody (1 : 500 dilution in detection buffer 2) for 1 hr at room temperature. Following two 15 min washes with detection buffer 1, the slides were developed overnight with BCIP/NBT. The colour reaction was stopped by rinsing the slides in distilled water before they were mounted and photographed.

#### 2.2.14 IMMUNOHISTOCHEMISTRY

Matrilysin protein in sections cut from tumour samples or normal tissue was examined using the rat monoclonal anti-matrilysin antibody and a Vectastain ABC kit from Vector Laboratories. 5 µm formalin-fixed, paraffin-embedded tissue sections were cut and placed on poly-L-lysine coated slides. Before beginning the immunohistochemical procedure, these sections were dewaxed and rehydrated by placing in xylene baths for two 8 min incubations followed by a series of ethanol baths of decreasing concentration (100 %, 80 %, 60 % and 30 % (v/v)) for 2 mins in each. Any endogenous peroxidase activity that may have been present in the tissue was quenched by incubating the sections for 30 mins in 0.3 % (v/v) H<sub>2</sub>O<sub>2</sub> prepared in methanol. The sections were then washed for 5 mins in 0.15 M PBS, pH 7.4. The sections were blocked by incubating for 20 minutes with goat blocking serum (kit component). After removing excess serum, the sections were then incubated for 30 mins with the rat monoclonal anti-matrilysin antibody (RmAb) (1 : 200 dilution in 0.15 M PBS, pH 7.4). Following a 5 min wash in PBS, the sections were incubated with biotinylated anti-rat solution (kit component) for 30 mins. The slides were again washed for 5 mins in PBS. They were then incubated for 30 mins in VECTASTAIN ABC reagent (kit component) before again being washed for 5 mins in PBS. The slides were developed with peroxidase substrate solution (DAB) prepared as outlined in section 2.2.2. After suitable colour development, the sections were rinsed in tap water and counterstained with haematoxylin.

### **2.2.15 PROTEIN ISOLATION FROM TISSUE SAMPLES**

A piece of frozen tissue (wet weight approximately 100 mg) was ground to a fine powder using a mortar and pestle and liquid nitrogen. The powdered tissue was resuspended in 0.5 mls 50 mM Tris-HCl, pH 7.4 and gently shaken on ice for 5 mins. Triton-X100 was then added to a final volume of 1 % (v/v) and the mixture agitated on ice for 20 mins. Cellular debris was pelleted by centrifugation for 20 mins at 12000 rpm at 4°C. The supernatant was collected, analysed for protein content using a BCA microassay (section 2.2.7) and stored at -80°C until required.

## **Chapter Three**

### **Development of an ELISA for Matrilysin**

## Introduction

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In the early 1970s, the development of the principle of enzyme immunoassays and methods for the labelling of antibody or antigen with enzymes provided a whole new surge of research in immunoassay procedures (Engvall and Perlmann, 1971; Van Weeman and Schorrs, 1971). Enzyme-linked immunosorbent assays (ELISAs) are examples of heterogeneous assay systems *i.e.* the analyte-detector (antibody-antigen) complexes are separated from free analyte or detector using a solid phase. This approach has many of the properties of an ideal immunoassay : it is versatile, robust, simple to perform and uses stable reagents economically, qualities which make it ideal for the routine screening of large numbers of clinical samples. This chapter is concerned with the development of such an assay system for the measurement of the MMP matrilysin in conditioned media from cells in culture and, ideally, in tumour extracts from patients with breast tumours.

### 3.1 Antibody-based assays

Antibodies have a number of properties which make them suitable assay reagents. First, and principally, is their specificity, antibodies can distinguish between closely related molecules through binding a single different epitope and even between enantiomers of the same molecule (Carlsson and Glad, 1989). The binding strength of antibodies to their specific antigens is very strong and allows the use of detergent-based washing steps to disrupt weak, non-specific interactions. Antibodies can be prepared relatively easily, either as polyclonal mixtures obtained by immunisation of a suitable animal and collection of anti-serum (Hudson and Hay, 1980), or as monoclonal preparations. Monoclonal antibody production requires the fusion of splenocytes from an immunised animal with non-antibody producing myeloma cells resulting in specific antibody-producing hybridomas (Kohler and Milstein, 1975). Genetic methods of producing specific antibody fragments have only recently been developed (Nissim *et al.*, 1994) and the use of these recombinant fragments in routine immunoassays has yet

to be proved. Antibodies can generally be easily labelled with enzymes or other reporter molecules with minimal effect on either the antibody or label activity (Catty and Raykundlia, 1989) although significant losses of activity have been reported (Ishikawa *et al.*, 1983).

The specificity of any antibody is determined by the immunogen used in its production. Originally, only large protein molecules were used, although small molecules such as drugs can be used provided such 'haptens' are conjugated to carrier proteins. The antibodies produced must be carefully purified to remove those specific for the carrier or for the link region between hapten and carrier (Tijssen, 1985). More recently, peptide immunogens coding for specific epitopes have been used (Lerner, 1982). As they are such small molecules (typically 2 kDa or less), they too must be conjugated to carrier proteins to induce an immune response (Burrin and Newman, 1991). These have been beneficial in producing highly specific antibodies, although care must be taken in designing such peptides as often it is the tertiary structure of an epitope, not necessarily formed from a continuous amino acid sequence, that is recognised by an antibody. Also, the correctly folded epitope may only be produced from the primary peptide sequence in the context of a larger molecule (Burrin and Newman, 1991).

### **3.2 Types of ELISA**

ELISAs are generally classified into competitive and non-competitive assays. In a simple non-competitive ELISA, the antigen is bound to a solid surface (*e.g.* the wells of a polystyrene ELISA plate) and an enzyme-labelled specific antibody is added (Edwards, 1985). Following washing to remove unbound antibody, a suitable enzyme substrate is added. The amount of substrate converted (usually from a colourless substrate to a coloured product) by the enzyme is directly proportional to the amount of labelled antibody present which, in turn, is directly proportional to the amount of antigen bound to the surface. The concentration of coloured product is easily measured determining its optical density. A modification of this procedure, which can be used to increase sensitivity, involves the use of an unlabelled antigen-specific

antibody as the primary antibody, followed, after washing, by an enzyme-labelled anti-species antibody which will recognise and bind to the primary antibody. The increase in sensitivity or amplification occurs because potentially two or more labelled antibodies can bind to each primary antibody. A further advantage is that each specific primary antibody does not have to be labelled separately.

The use of a coating antibody to coat the solid phase surface can be used to further increase sensitivity of a non-competitive ELISA. This coating antibody 'captures' antigen and increases its local concentration. The antigen is then bound by the primary 'detecting' antibody followed by the enzyme-labelled secondary antibody. This sandwich or antigen-capture two-site immunoassay can theoretically achieve a sensitivity capable of detecting a single bound antigen molecule (Catty and Raykundlia, 1989) although, in general, the reported sensitivities are in the picogram range.

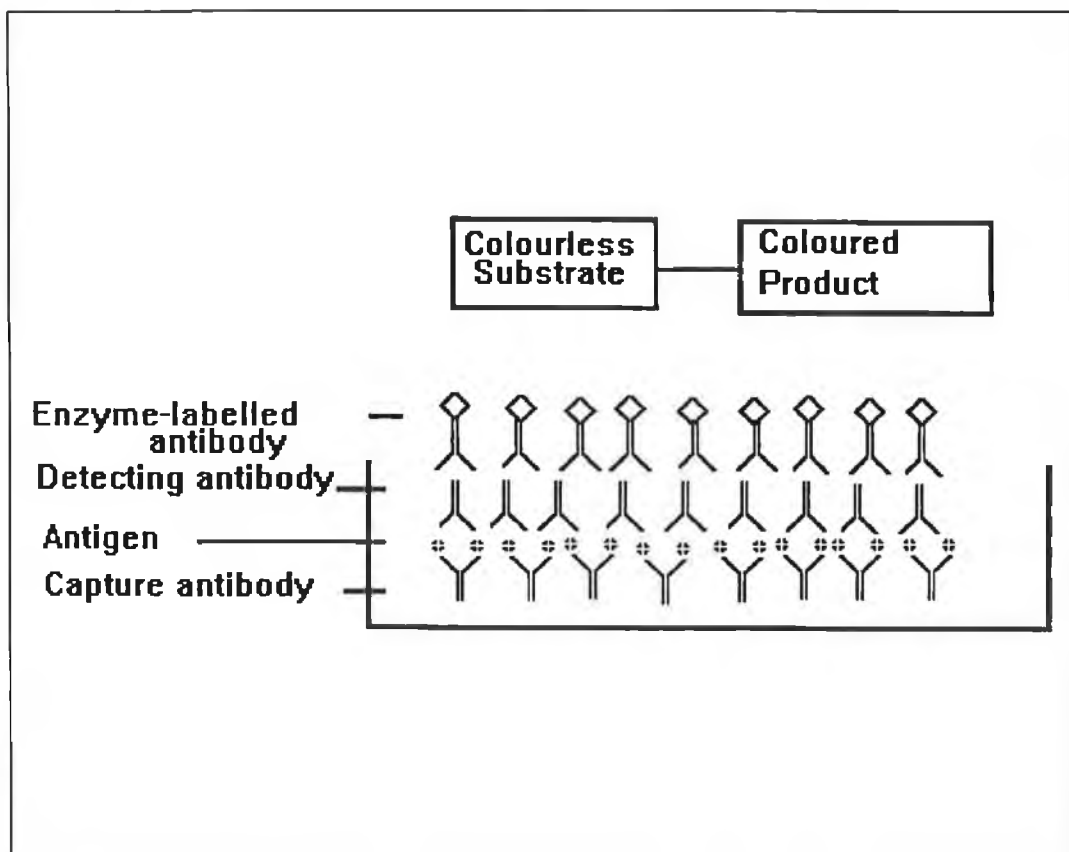


Fig. 3.1 : Diagram of sandwich ELISA

When designing such a system, a number of points must be borne in mind for optimal sensitivity :

- The antigen must possess multiple epitopes for antibody binding;
- The capture and primary (detecting) antibodies should recognise different epitopes;
- The capture antibody should preferably be a monoclonal of high affinity - the use of polyclonal antibodies which recognise a number of different epitopes can greatly reduce the number of binding sites available to the primary antibody. Also, it is generally the capture antibody which defines the sensitivity of the assay (Catty and Raykundlia, 1989);
- The primary antibody can be either monoclonal or polyclonal - monoclonals are often of higher affinity and are of defined specificity whereas polyclonals are more stable (Edwards, 1985);
- The capture and primary antibodies should be of different species so that the enzyme-labelled secondary antibody will only bind to a complex containing primary antibody and not any other component of the 'sandwich'.

Competitive ELISAs can be used to measure either antibody or antigen concentrations and are often used for antibody isotyping and serum antibody quantitations (Catty and Raykundlia, 1989). In these assays, a plate is coated with either standard antibody or antigen to which a standard labelled antigen or antibody can bind optimally. The optimal substrate conversion produced by the enzyme label is reduced when some of the binding sites on the coating layer are taken by unlabelled test antigen or antibody. In effect, the labelled standards are competed out by unlabelled molecules in the test sample and the resultant reduction in substrate conversion is easily measured. In this case, optical density readings decrease as concentration of analyte increases.

The principal advantages of ELISA format assays are speed, ease of use, low cost, quantitative ability and a high sample processing rate. Once developed, they can usually be modified for use in an automated environment further emphasising their advantages. They are, therefore, perfectly suitable for routine quantitative analysis of a



large number of samples. The simplest assays for routine use would utilise easily-obtainable clinical samples with little or no pre-processing required such as urine, saliva, whole blood or, most often, serum or plasma. Unfortunately detectable analyte levels in these fluids may not be representative so alternative samples such as tumour extracts may have to be used.

### 3.3 MMP ELISAs

ELISAs for matrix metalloproteinases 1 to 9 have been used for determination of their levels in serum, plasma and tumour extracts. Table 3.1 at the end of this section, summarises the various assays, their sensitivities and the types of samples with which they have been used. Almost all of these assays have used the sandwich format with two monoclonal antibodies raised against different epitopes of the specific proteinases.

The demonstration that these enzymes could be found in plasma (Johansson and Smedsrod, 1986; Vartio and Baumann, 1989; Moutsiakis *et al*, 1992) led to the development of a number of assay systems. The principal disadvantage of plasma is the presence of interfering proteins such as  $\alpha_2$ -macroglobulin, the non-specific protease inhibitor. Nevertheless, a number of researchers have demonstrated the effectiveness of their ELISA systems with plasma samples. Gelatinase A (MMP-2) in human plasma was found to be significantly increased in the second half of pregnancy (Zucker *et al*, 1992) due to the extensive connective tissue turnover associated with a growing foetus. The range of levels in a normal population was determined to be 61 - 828  $\mu\text{g/l}$  with an average value of  $509 \pm 178 \mu\text{g/l}$  for men and  $389 \pm 193 \mu\text{g/l}$  for women. This ELISA could detect latent and activated enzyme, TIMP-complexed enzyme and  $\alpha_2$ -macroglobulin-complexed gelatinase A although, in the latter case, the efficiency of the assay was reduced to 65%. An alternative immunoassay for gelatinase A uses a 'one-step' approach where the antigen is allowed to simultaneously react with both the capture and primary antibodies (Fujimoto *et al*, 1993). This assay has a sensitivity almost ten times greater than the Zucker assay. Progelatinase A and progelatinase A-TIMP complexes are recognised equally well. In a study of sera from patients with

various diseases, significantly increased progelatinase A levels were seen in patients with hyperthyroidism, primary biliary cirrhosis and hepatocellular carcinoma compared to normal controls. The normal serum level was calculated to be  $570 \pm 118 \mu\text{g/l}$  which is similar to the normal plasma level. Interestingly, this study also found conditions where significantly lower than normal progelatinase A levels were evident. These included rheumatoid and osteo-arthritis and stomach and pancreatic cancers. This may be because progelatinase A produced by cells at the sites of disease is quickly activated or degraded and not released into the bloodstream.

Interstitial collagenase (MMP-1) in human serum was found to have a much lower concentration in the normal population of  $8.5 \pm 5.2 \mu\text{g/l}$  using antibodies which could recognise both the zymogen and activated forms of the enzyme (Zhang *et al*, 1993). This ELISA could also detect, though with vastly decreased sensitivity, MMP-1-TIMP-1 and MMP-1-TIMP-2 complexes.

Using different combinations of monoclonal antibodies, a group from Celltech have created separate immunoassays for procollagenase (MMP-1) and collagenase-TIMP complexes with limits of detection of 1 ng/ml and 4.5 ng/ml, respectively (Cooksley *et al*, 1990). These ELISAs were then used with human serum samples from patients with rheumatoid arthritis or prostate cancer as well as healthy subjects. Significant elevations in collagenase levels were seen in both rheumatoid arthritis and prostate cancer patient sera compared to age-matched controls (Baker *et al*, 1994). Samples from patients with metastatic disease also showed significantly increased levels over non-metastatic samples. The ELISA was used to monitor treatment with levels returning to normal following a six-month therapeutic regimen.

In a study of progelatinase B (MMP-9) in plasma (Zucker *et al*, 1993), levels in breast cancer and colon cancer patients were shown to be significantly increased over normal controls. The ELISA used did not recognise activated enzyme and some reduction in detection due to progelatinase complexes with either  $\alpha_2$ -macroglobulin or TIMP-1 did occur. In contrast, assays developed by Tschesche and colleagues (Bergmann *et al*, 1989) could recognise both latent and active gelatinase B and neutrophil collagenase. Both sandwich and competitive assays were developed, however the sandwich format proved more sensitive for both enzymes. These

sandwich ELISAs were used for the measurement of gelatinase B and neutrophil collagenase in breast tumour extracts (Duffy *et al*, 1995) thus bypassing the possible inhibitory effect of  $\alpha_2$ -macroglobulin. Levels of the enzymes appeared to correlate with each other and with levels of TIMP.

So-called one-step sandwich assays have recently been developed for stromelysin-1 (MMP-3) (Obata *et al*, 1992) and matrilysin (MMP-7) (Ohuchi *et al*, 1996) by Prof. Okada's group in Japan. The stromelysin ELISA can detect latent and active enzyme as well as TIMP-complexed stromelysin. This assay was used to show significantly higher levels of the enzyme in sera of patients with rheumatoid arthritis ( $666 \pm 135$  ng/ml) when compared to normal controls ( $134 \pm 74$  ng/ml) or, interestingly, sera from osteoarthritis patients ( $139 \pm 76$  ng/ml). The assay is notable for its use of polystyrene balls as the solid phase coated with the capture antibody rather than the surface of an ELISA plate. After allowing the antibody-antigen-antibody complex to build up, these balls could then be placed in test tubes containing an appropriate substrate solution and the colour reaction allowed to proceed. Also noteworthy was the lack of interference from  $\alpha_2$ -macroglobulin seen in this study.

The matrilysin ELISA although sensitive (limit of detection is  $0.05\mu\text{g/l}$  in the presence of EDTA) has a major time disadvantage. The total assay time is over 72 hours with coating, blocking and antigen/primary antibody binding steps each requiring 24 hours. The researchers found a major reduction in sensitivity ( $32\ \mu\text{g/l}$  compared to  $0.05\ \mu\text{g/l}$ ) if the assay was not carried out in the presence of 20 mM EDTA. They suggested, based on gel filtration experiments, that matrilysin exists as large polymers in serum and it is these polymers, formed in the presence of EDTA, that are detected by the ELISA. Levels of pro-matrilysin in sera from healthy subjects was found to be in the range of  $10.7 \pm 18.8$  ng/ml.

Alternative approaches to classic MMP immunoassays involve the use of specific substrates as the capture layer. An assay for gelatinase A used gelatin to coat a microtitre plate prior to addition of sample (Wacher *et al*, 1990). A specific antibody to the gelatinase was then used and the assay proceeded as normal. The sensitivity is not very high when compared to the sandwich ELISAs with a limit of detection of

approximately 300 ng/ml. A non-specific assay used to determine overall ECM-degradative ability of a sample utilises an ELISA for the ECM component laminin released from a collagen-coated layer of matrigel (Ross and Laurenza, 1995). The amount of laminin measured using a straightforward immunoassay is directly proportional to the amounts of active, non-complexed proteases present in a sample. This assay has however only been used with cell lines to determine growth factor effects on cellular degradative ability.

**Table 3.1 : MMP ELISAs**

MMP	DETECTION LIMIT/ SENSITIVITY	ELISA FORMAT	SAMPLE TYPE	REFERENCE
Interstitial collagenase (MMP-1)	0.12 ng/ml	One-step sandwich	Serum	Zhang et al, 1993
Interstitial collagenase (MMP-1)	1 ng/ml	Sandwich	Serum	Cooksley et al, 1990
Interstitial collagenase - TIMP-1	4.5 ng/ml	Sandwich	Serum	Cooksley et al, 1990
Gelatinase A (MMP-2)	300 ng/ml	Substrate capture	Conditioned Medium	Wacher et al, 1990
Gelatinase A (MMP-2)	2 ng/ml	Sandwich	Plasma	Zucker et al, 1992
Gelatinase A (MMP-2)	0.24 ng/ml	One-step sandwich	Serum	Fujimoto et al, 1993
Stromelysin-1 (MMP-3)	20 ng/ml	One-step sandwich	Serum and synovial fluid	Obata et al, 1992
Matrilysin (MMP-7)	0.05 ng/ml (+EDTA) 32 ng/ml (-EDTA)	One-step sandwich	Serum	Ohuchi et al, 1996
Neutrophil collagenase (MMP-8)	2.5 ng/ml (Comp *) 0.25 ng/ml (Sand *)	Competitive and sandwich assays	Plasma Tumour extracts	Bergmann et al, 1989 Duffy et al, 1995
Gelatinase B (MMP-9)	0.25 ng/ml (Comp) 0.1 ng/ml (Sand)	Competitive and sandwich assays	Plasma Tumour extracts	Bergmann et al, 1989 Duffy et al, 1995
Gelatinase B (MMP-9)	0.8 ng/ml	Sandwich	Serum	Zucker et al, 1993

\* 'comp' indicates a competitive assay and 'sand' indicates a sandwich assay.

## RESULTS AND DISCUSSION

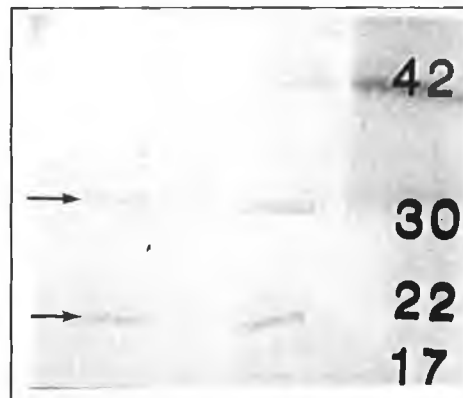
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Our aim was to develop a sensitive and specific assay for matrilysin using antibodies provided by our collaborators. A panel of three antibodies - a mouse monoclonal raised against recombinant matrilysin (mAb), a rabbit polyclonal raised against a matrilysin-specific peptide (pAb<sub>2</sub>) and a rat monoclonal antibody raised against the recombinant matrilysin (RmAb) - were used in various combinations and assay formats. An additional polyclonal antibody raised against a purified matrilysin preparation (pAb) was also used in preliminary experiments. The antibodies were first characterised as to their reactivity with matrilysin, purified and labelled as necessary, and assay formats optimised and, finally, validated.

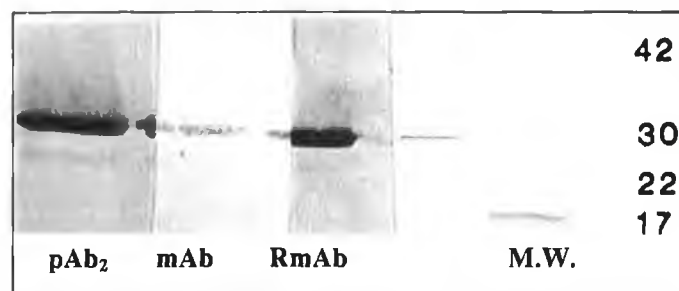
### 3.4. Western Blot to Demonstrate Specificity of Antibodies

The western blotting experiment was performed according to the method in section 2.2.2. 1 µg of recombinant matrilysin (includes both the 28 kDa zymogen and, due to autocatalysis, the 18.5 kDa active form of the enzyme) was loaded into each of three lanes of a 15 % polyacrylamide gel. An earlier blot demonstrated the presence of both forms of the matrilysin enzyme in approximately equal amounts using a polyclonal antibody, pAb, which recognised both active and latent matrilysin (Figure 3.4.1). After running the gel, blotting onto nitrocellulose and blocking, the blot was cut into three and the pieces probed separately with one of the antibodies [ mAb, pAb<sub>2</sub> or RmAb] at a dilution of 1 : 1000 in blocking solution. After washing, the probed blots were incubated with suitable commercially available enzyme-labelled antibodies : alkaline phosphatase (AP)-labelled anti-mouse at 1 : 2500 for the mAb-probed blot, AP-labelled anti-rabbit at 1 : 2500 for the pAb<sub>2</sub>-probed blot, and horseradish peroxidase (HRP)-labelled anti-rat at 1 : 5000 for the RmAb-probed section. The blots were subsequently developed with the appropriate substrates - DAB for the HRP-labelled antibody and BCIP/NBT for the AP-labelled antibodies (Figure 3.4.2).

All antibodies recognised the 28 kDa zymogen form of the enzyme. Only pAb<sub>2</sub> also recognised the 18.5 kDa active form although its affinity for this appears much less than for the higher molecular weight form as evidenced by the much fainter signal.



**Figure 3.4.1** Western blot demonstrating that the recombinant matrilysin contains both the 28 kDa and 18.5 kDa forms of the enzyme and these can be recognised by the rabbit polyclonal anti-matrilysin antibody pAb. For this blot, the pAb was used at a dilution of 1 : 1000. The two left hand lanes contained recombinant matrilysin and molecular weight markers were loaded in the right hand lane. The sizes (in kDa) are indicated. Two clear bands of roughly equal intensity (indicated with arrows) can be seen demonstrating the presence of both forms of the enzyme.



**Figure 3.4.2**

Western blot to demonstrate specificities of anti-matrilysin antibodies. The blot was prepared as described in the text with prestained molecular weight markers (M.W. - measured in kDa) loaded in the extreme right-hand lane. The arrow points to the intense band at 28 kDa indicating the zymogen form of matrilysin. A faint lower band can also be seen in lane 1 indicating that pAb<sub>2</sub> also recognises the 18.5 kDa active form of matrilysin.

### **3.5 Purification of pAb<sub>2</sub>**

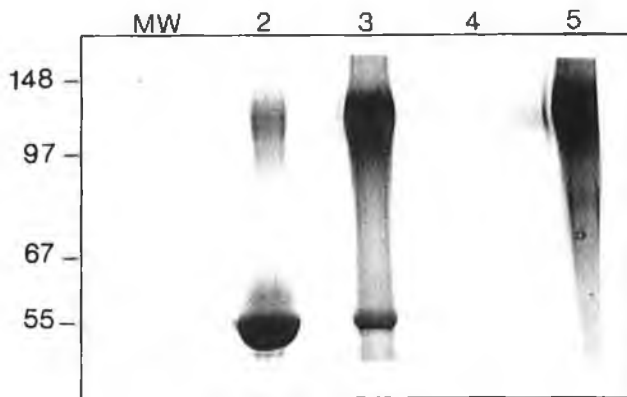
The pAb<sub>2</sub> was provided in the form of anti-serum and, in order to reduce possible non-specific binding, was purified by ammonium sulphate precipitation. This technique, also known as 'salting out', is a cheap and simple method of removing non-specific proteins while avoiding direct effects on the protein of interest. It also has the effect of concentrating the purified protein. Proteins precipitate at particular salt concentrations due to the exposure of hydrophobic residues facilitating hydrogen bonding between protein molecules. The salt concentration at which precipitation occurs is specific for different proteins although pH, temperature and total protein concentration can also influence the result. For immunoglobulin G (IgG), the recommended initial protein concentration is 3 % (w/v) while the salt concentration should be 40 % (Tijssen, 1982). The method used was as described in section 2.2.3.1.

Having completed the ammonium sulphate precipitation, an ELISA was attempted using a capture layer consisting of mouse mAb, pAb<sub>2</sub> as the secondary antibody with AP-labelled anti-rabbit as the detecting antibody. It was found that the detecting antibody bound not only to pAb<sub>2</sub> but also, non-specifically, to other components of the assay. For this reason, it was decided to directly label pAb<sub>2</sub> with the enzyme HRP. Prior to labelling, the antibody had to be pure as otherwise the labelling method would be very inefficient and the possible labelling of non-specific proteins could result in high background in an ELISA system. To achieve the recommended level of purity, an affinity-chromatography method was chosen. The 16 amino acid matrilysin peptide (MAP), against which pAb<sub>2</sub> had been raised, was coupled to cyanogen bromide-activated sepharose beads (2.2.3.2.1). The ammonium sulphate precipitated crude pAb<sub>2</sub> mixture was applied to these beads and, after allowing non-specific proteins to run off, was eluted with a low pH buffer (see section 2.2.3.2.2).

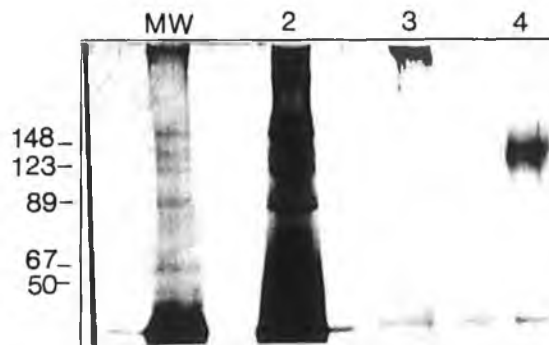
#### **3.5.1 Purification analysis by SDS-PAGE**

Appropriately diluted anti-serum, ammonium sulphate precipitated pAb<sub>2</sub> and affinity-purified pAb<sub>2</sub> as well as a sample of a commercial IgG were run on a 10 % SDS-

polyacrylamide gel under non-denaturing conditions (section 2.2.1). The enrichment of the ~130 kDa IgG band, as well as almost total removal of the large 60 kDa serum albumin band, can clearly be seen in the gel shown in figure 3.5.1. Unfortunately the affinity-purified pAb<sub>2</sub> is too dilute to be stained with Coomassie Blue so a second gel which was stained with a silver-staining technique is also shown (figure 3.5.2). Upon silver staining, the large amount of non-specific proteins actually present in the anti-serum can clearly be seen while a single band at approximately 120 kDa is seen in the sample of affinity-purified pAb<sub>2</sub>.



**Figure 3.5.1** SDS-PAGE analysis of pAb<sub>2</sub> purification. The molecular weight markers (measured in kDa) were loaded in the left-hand lane and their sizes are indicated. Lane 2 contains the anti-serum, lane 3 contains the ammonium sulphate precipitate, lane 4 the affinity-purified pAb<sub>2</sub> and lane 5 a commercial IgG for comparison. This gel was stained with Coomassie Blue .

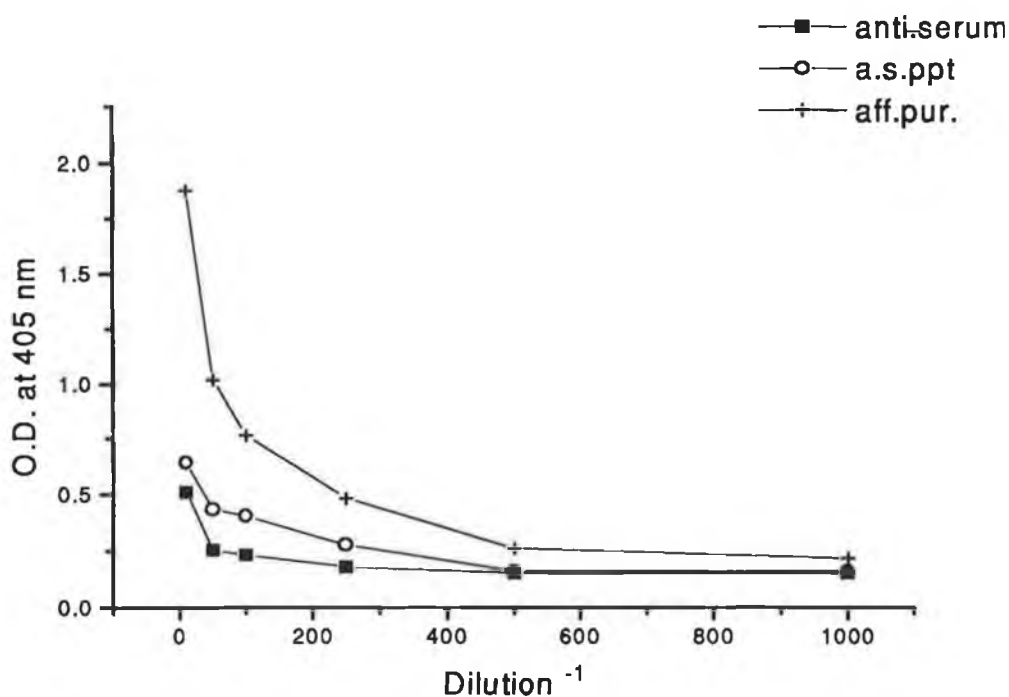


**Figure 3.5.2** SDS-PAGE analysis of pAb<sub>2</sub> purification. The molecular weight markers (measured in kDa) are loaded in lane 1 on the left (sizes as indicated), anti-serum in lane 2, ammonium sulphate precipitate (too dilute) in lane 3 and the affinity-purified pAb<sub>2</sub> in lane 4. This gel was stained using a silver staining technique which is more sensitive than Coomassie Blue staining.



### 3.5.2 Purification analysis by ELISA

Using a direct ELISA format (2.2.6.1), where 50 ng of recombinant matrilysin was coated directly onto the wells of an ELISA plate, an increase in titre after each purification step could be demonstrated. In this case, the titre was defined as that dilution at which an absorbance of approximately 0.5 could be obtained. The data is represented graphically in figure 3.5.3.



**Figure 3.5.3** Graph of absorbance at 405 nm vs. reciprocal dilution for a direct ELISA for matrilysin. The three lines represent the absorbances obtained for various dilutions of anti-serum, ammonium sulphate precipitated crude pAb<sub>2</sub> (a.s. ppt) and affinity purified pAb<sub>2</sub> (aff. pur.). The increased absorbance at each dilution indicates the success of the purification and resulting enrichment for specific pAb<sub>2</sub>.

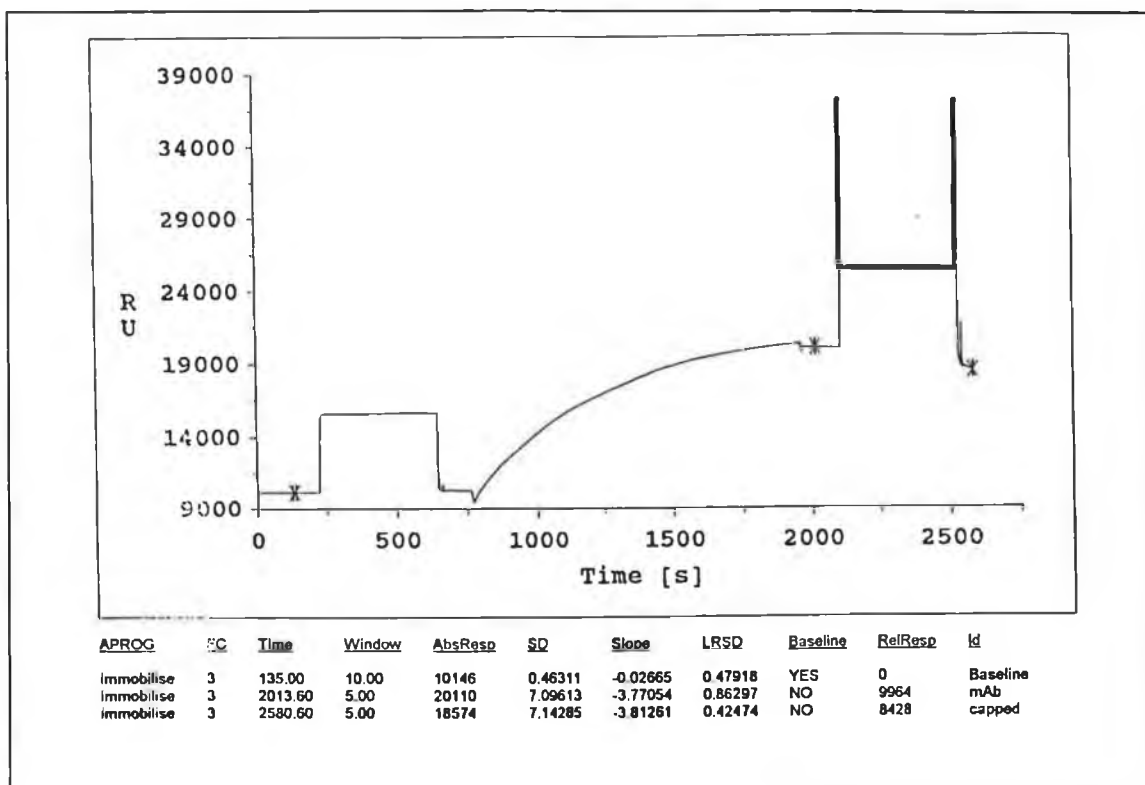
From the graph in figure 3.5.3, it can be seen that the titre for the anti-serum was approximately 1 : 10, for the ammonium sulphate precipitate it was approximately 1 : 25, while for the affinity purified pAb<sub>2</sub>, it had increased to 1 : 300.

### 3.6 Demonstration of suitability of sandwich ELISA using BIAcore

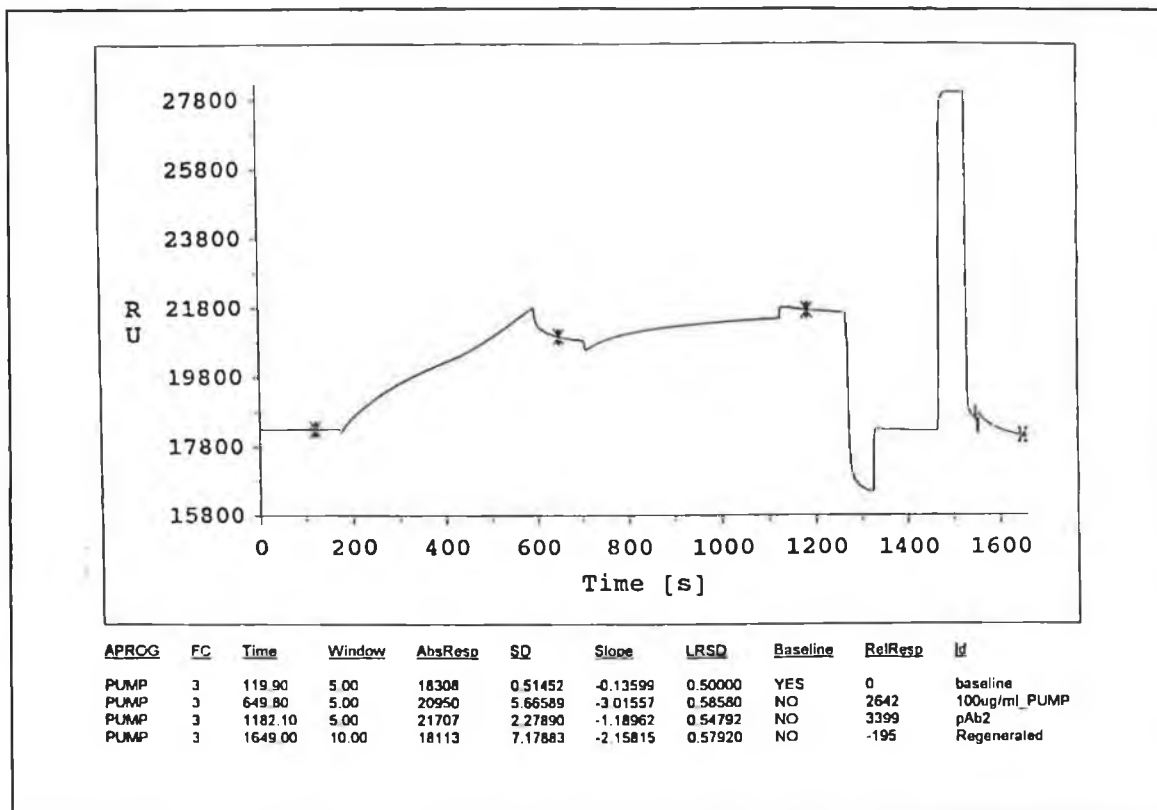
Real-time biospecific interaction analysis (BIA) is a general technique for observing the progress of biomolecular interactions as they occur. One of the interacting components is immobilised to a sensor chip surface, and the change in the surface concentration resulting from binding of the other component is measured using surface plasmon resonance (SPR). Measurements in BIAcore rely on the refractive index of the bound molecules which is directly related to the surface concentration. Values are given in arbitrary response units (RU).

The BIAcore system was used (as described in section 2.2.5) to demonstrate the specific binding of mAb to recombinant matrilysin (also known as rPump) and the binding of the purified pAb<sub>2</sub> to this complex. The antibody, mAb, was first immobilised to the surface of the sensor chip using EDC/NHS chemistry (figure 3.6.1). The increase in RU from baseline to 8428 after capping to remove any unbound molecules, indicates a satisfactory immobilisation. The baseline was then reset and a solution of recombinant matrilysin passed over the immobilised mAb. An increase in RU of 2642 indicates the binding of the matrilysin to mAb. A solution of pAb<sub>2</sub> was then passed over this complex and a further increase in RU obtained again indicating binding (figure 3.6.2). This suggests that antigenic determinants for both antibodies are present and available for binding simultaneously on the surface of the matrilysin molecule and, therefore, that the sandwich format is suitable. The absence of response when a non-specific ligand (BSA) is used is demonstrated in figure 3.6.3.

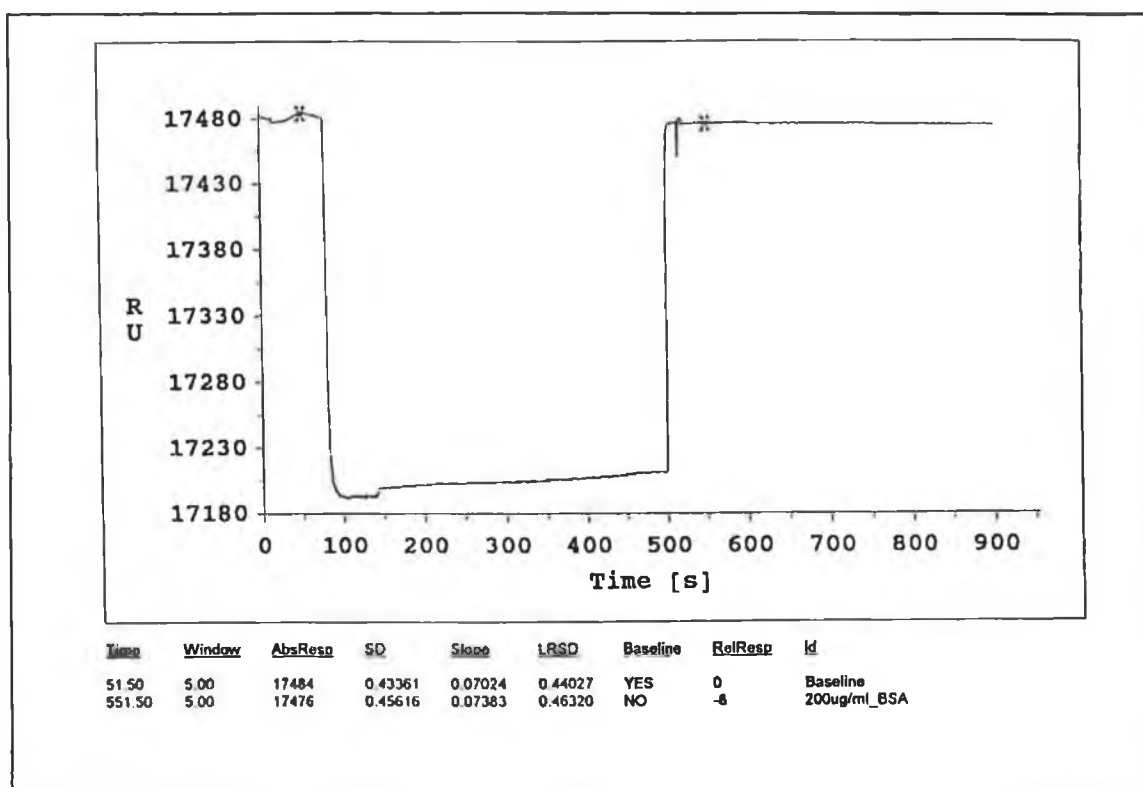
The possibility of using mAb as both capturing and secondary antibody is demonstrated in figure 3.6.4 where an increase in RU is seen when matrilysin binds to the immobilised mAb and a further increase occurs when this complex is bound by more mAb. This suggests that the recombinant matrilysin molecule has multiple recognition sites for this antibody.



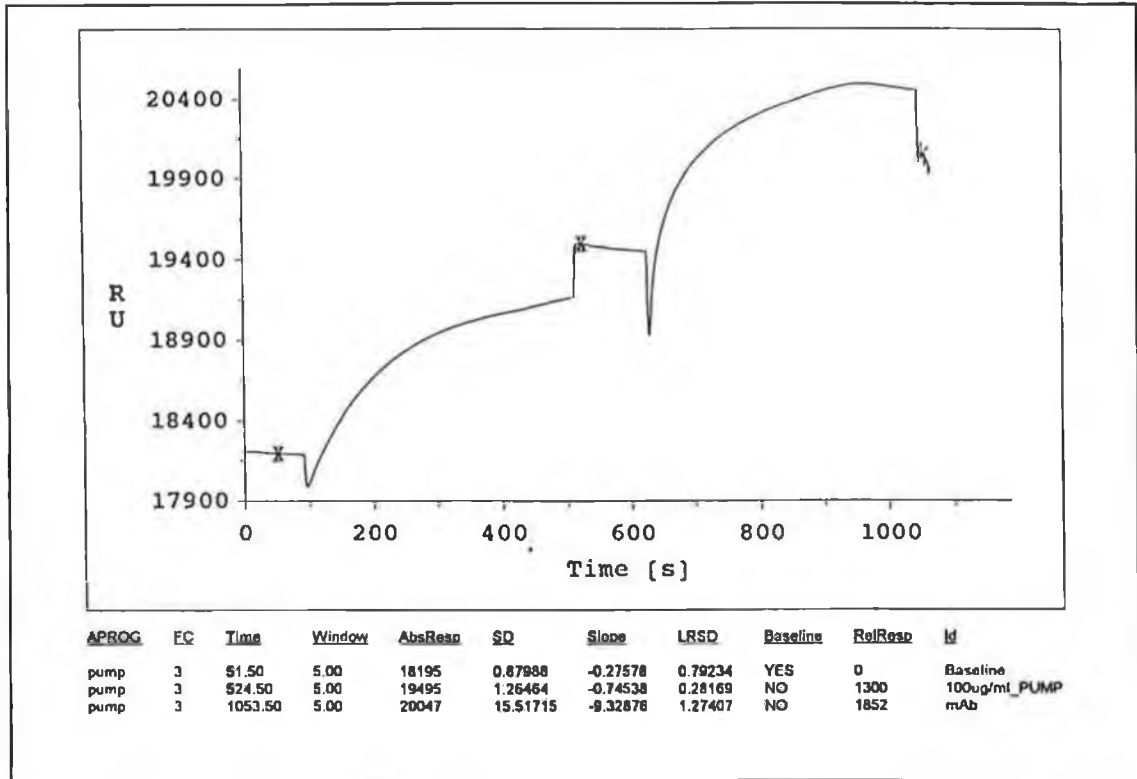
**Figure 3.6.1** Sensorgram obtained from the BIAcore system demonstrating successful immobilisation of mAb to the sensor chip surface. The plot indicates progressive interactions between the chip surface and the protein. The three stars indicated on the plot are the points at which the measurements shown below the graph, were taken. The relative response (RelResp), measured in arbitrary response units (RU), indicates changes in refractive index due to molecules adsorbing to the surface of the sensor chip.



**Figure 3.6.2** Sensorgram demonstrating the formation of a 'sandwich' complex. The baseline of 0 represents the immobilised mAb on the surface of the sensor chip. When a solution of rPump is passed over the chip, an increase in RelResp is seen. As before, the stars indicate the points at which measurements were taken. A further increase is seen when the purified pAb<sub>2</sub> is passed over the mAb-rPump complex. A pulse of acidic solution can then be used to remove the complex leaving the immobilised mAb only on the sensor chip ('Regenerated').



**Figure 3.6.3** Sensorgram demonstrating the lack of response when BSA is passed over the immobilised mAb. No significant change in RelResp is seen after a solution of BSA is passed over the mAb indicating a complete lack of interaction and, therefore, no non-specific binding.



**Figure 3.6.4** Sensorgram demonstrating a mAb-rPump-mAb sandwich. The increase in binding seen when a solution of mAb is passed over rPump bound to immobilised mAb indicates that a number of mAb binding sites are available on the rPump molecule.

### 3.7 Enzyme Labelling of pAb<sub>2</sub> and mAb

The results of the BIAcore analysis suggested that both mAb / pAb<sub>2</sub> and mAb / mAb sandwich ELISAs for matrilysin were possible. As mentioned previously, an attempt at a mAb / pAb<sub>2</sub> had been unsuccessful due to non-specific interactions between the enzyme-labelled anti-rabbit detecting antibody and various components of the assay. A mAb / mAb system was also, as expected, unsuccessful as the enzyme-labelled anti-mouse detecting antibody could bind to both the capture and secondary layers. Before proceeding with ELISAs therefore, both the mAb and pAb<sub>2</sub> were directly labelled with the enzyme HRP using a standard periodate method (section 2.2.4.1).

#### 3.7.1 Spectrophotometric Analysis of HRP-labelled conjugates

The wavelength at which IgG absorbs most strongly is 280 nm while HRP absorbs maximally at 403 nm. By measuring absorbances of the conjugates at these two wavelengths, an estimation of how successful the labelling procedure was could be made. The Abs<sub>403</sub>/Abs<sub>280</sub> ratio offers a guide to the amount of enzyme conjugated which is an important element in determining conjugate performance. Ratios in the range 0.25 - 0.5 offer high enzyme activity without prejudice to antibody titre, and have low background characteristics (Catty and Raykundalia, 1989).

ANTIBODY CONJUGATE	Abs at 280 nm	Abs at 403 nm	Abs <sub>403</sub> /Abs <sub>280</sub>
HRP - pAb <sub>2</sub>	0.159	0.038	0.240
HRP - mAb	0.241	0.014	0.058

**Table 3.7.1** Absorbance values at 280 nm and 430 nm for the mAb-HRP and pAb<sub>2</sub>-HRP conjugates. The ratio between the absorbance readings at the two different wavelengths is calculated.

The ratio for the HRP-pAb<sub>2</sub> conjugate is only just below the recommended value whilst the ratio for the mAb is very low indicating much fewer HRP molecules per antibody molecule. This conjugation experiment was repeated a number of times but the HRP-mAb ratio could never be substantially improved. Conjugation to another enzyme, alkaline phosphatase (AP) was also tried for this antibody using a one-step glutaraldehyde method (see section 2.2.4.2). Again, poor labelling was achieved so this approach was abandoned.

### **3.7.2 Direct ELISA to demonstrate antigen-binding activity of conjugates**

The wells of an ELISA plate were coated with 0.5 µg/ml rPump and, after blocking, various dilutions of the HRP-labelled antibodies added. After developing with the substrate OPDA, the absorbances at 405 nm were measured. Evidence that the conjugates have retained antigen-binding activity is given by the decrease in absorbance with increasing dilution and the lack of signal with no antigen present. These values are presented in Table 3.7.2 on the following page.

Taken together, the above results demonstrated that both the mAb-HRP and pAb<sub>2</sub>-HRP conjugates retained both enzyme and specific matrixin-binding activity. From the titres in Table 3.7.2 and the ratios in Table 3.7.1, it can be seen that the pAb<sub>2</sub> had been more successfully labelled than the mAb. Prior to using these conjugates in the development of sandwich ELISAs, they were concentrated ten-fold using ultra-filtration columns.



CONJUGATE	DILUTION	Abs <sub>405</sub> (Mean ± S.D.)
HRP - pAb <sub>2</sub>	1 : 10	0.385 ± 0.009
	1 : 20	0.226 ± 0.019
	1 : 50	0.123 ± 0.002
	1 : 100	0.099 ± 0.002
	No antibody / No antigen	0.071 ± 0.009
HRP - mAb	1 : 10	0.158 ± 0.006
	1 : 20	0.095 ± 0.002
	1 : 50	0.079 ± 0.001
	1 : 100	0.071 ± 0.003
	No antibody / No antigen	0.076 ± 0.011

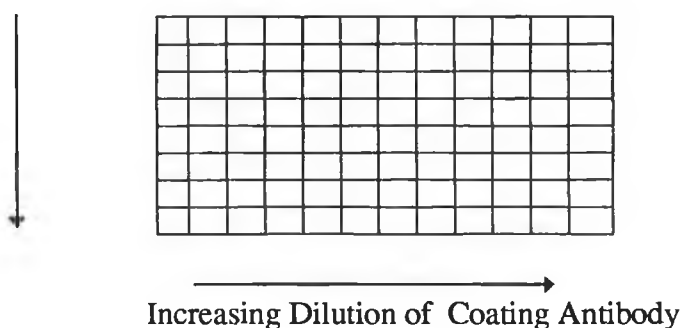
**Table 3.7.2** Results of direct ELISA to test HRP conjugates. Test wells which had been coated with BSA instead of rPump as a negative control gave similar readings as the 'no antibody' control wells. This indicated that the conjugates retained specific anti-matrilysin activity.

### 3.8 Optimisation of matrilysin ELISAs

To determine optimal working dilutions of coating antibody (mAb) and labelled secondary antibodies, a checkerboard sandwich ELISA system was used.

Concentrations of coating antibody decrease across the plate while concentrations of secondary antibody decrease down the plate. The amount of antigen (recombinant matrilysin) is the same in each well.

Increasing Dilution  
of secondary antibody



**Figure 3.8.1** Diagram of checkerboard ELISA plate used for determining optimal combination of antibody dilutions.

Using this system, a combination of 1 : 400 coating mAb with 1 : 100 HRP-pAb<sub>2</sub> secondary antibody was found to be most suitable as it gave a strong absorbance reading ( $0.634 \pm 0.011$ ) that was five times higher than background ( $0.139 \pm 0.012$ ) when an antigen concentration of 0.5  $\mu\text{g/ml}$  was used.

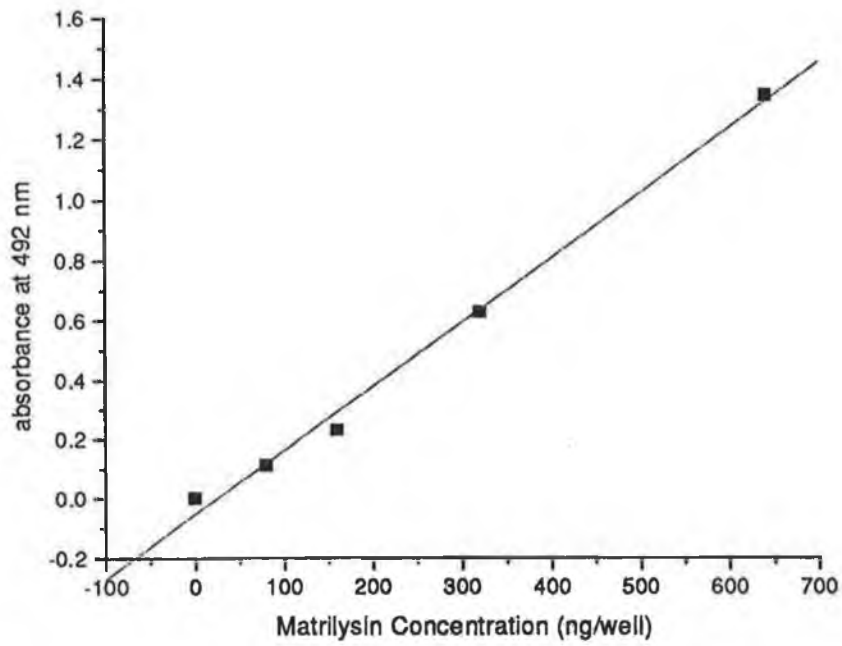
When this assay was attempted with the HRP-mAb conjugate as the secondary antibody, only the undiluted conjugate resulted in a strong signal with any dilution of coating antibody. Using only neat conjugate was not feasible so the mAb / mAb sandwich system was no longer investigated.

### 3.8.1 Determination of limit of detection and linear range

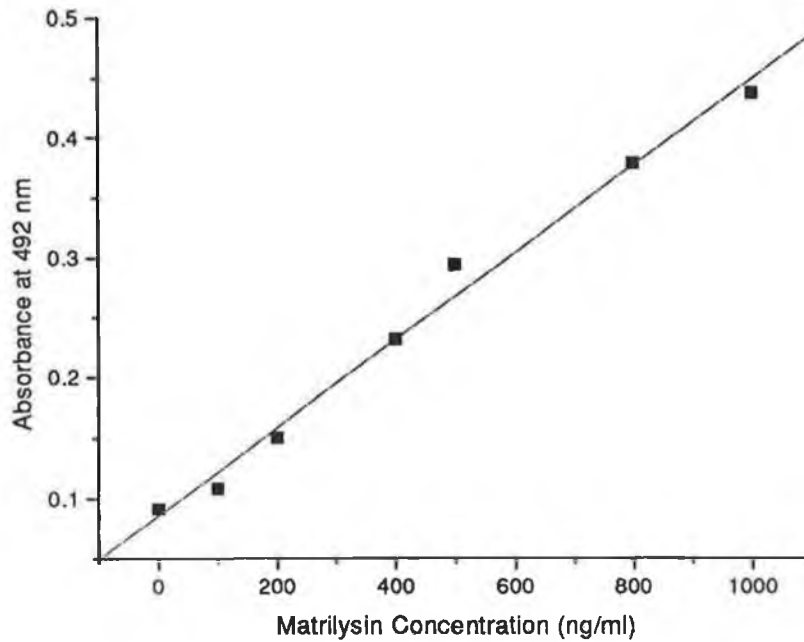
Using the dilutions of coating and secondary antibodies determined as most suitable from the previous experiment, a sandwich ELISA in which a range of concentrations of antigen was analysed was assembled. This assay showed linear quantitative binding in the range 20 - 640 ng/well (equivalent to 200 - 6400 ng/ml) with a detection limit of 15 ng (150 ng/ml) [Figure 3.8.2a]. Inspection of the figures for detection limits presented in Table 3.1 will reveal that the values obtained for this matrilysin ELISA do not compare favourably with other MMP ELISAs. By making this ELISA a 'one-step' system, in which the analyte (matrilysin) and secondary antibody (HRP-pAb<sub>2</sub>) are added simultaneously to the pre-coated ELISA plate, a small increase in sensitivity was achieved. The limit of detection was now 10 ng (100 ng/ml) with a linear range of 100 - 1000 ng/ml [Figure 3.8.2b]. No further increase in sensitivity could be achieved with this system. The use of a labelled anti-species antibody which could recognise the secondary antibody should, theoretically, amplify the signal and improve sensitivity. This, however, was not possible in this case because of non-specific binding effects.

At this point, a third anti-matrilysin antibody (RmAb) became available. As it was a monoclonal antibody and, therefore, secreted by a hybridoma cell line, limited quantity was not a factor that had to be considered. This fact automatically made it a more favourable option than pAb<sub>2</sub> of which only a limited amount was available, as the animal from which it had been obtained had been sacrificed.

(A)



(B)



**Figure 3.8.2** Standard curves obtained for mAb / HRP-pAb<sub>2</sub> sandwich ELISAs. (A) is for the traditional system where each component is added sequentially while (B) is that obtained when a one-step approach is used. The regression coefficients are (A) 0.997 and (B) 0.995. Although a wider linear range is seen in (A), the one-step method has a lower limit of detection making it more sensitive.

### 3.8.2 Checkerboard ELISAs for RmAb

As both mAb and RmAb are monoclonal antibodies, either should be suitable for the capture/coating layer in a sandwich ELISA system. When a RmAb coating and mAb detecting layer was used with a third enzyme-labelled anti-mouse antibody to provide the signal, high background signals were obtained. This appeared to be due to the labelled anti-mouse antibody also binding to the rat-derived coating layer. When the alternative system was used *i.e.* mAb coating, RmAb as detecting antibody and a labelled anti-rat as the secondary antibody, no such background was observed. This mAb / RmAb system was thus chosen for further optimisation.

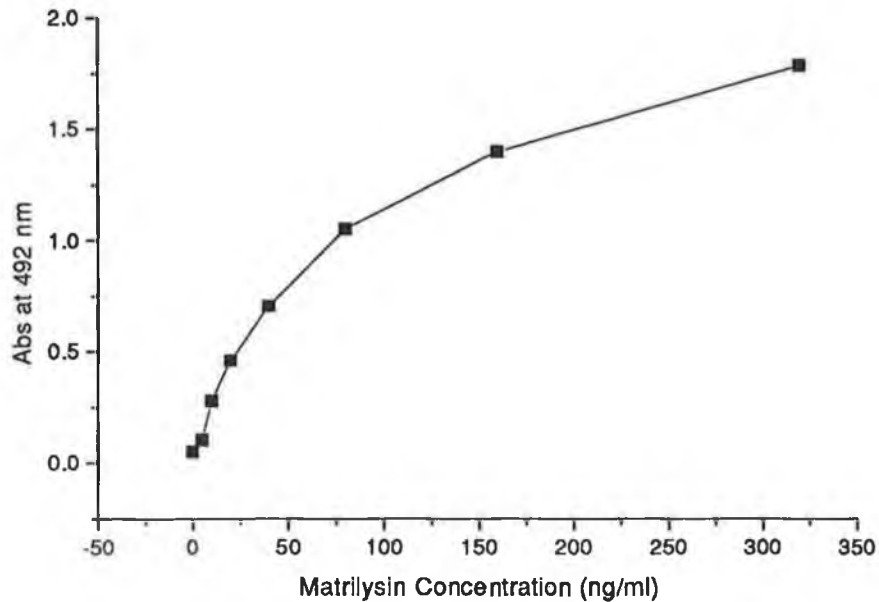
Using a checkerboard system similar to that described previously for the mAb / HRP-pAb<sub>2</sub> system, an optimal combination of 1 : 5000 mAb for the coating layer and 1 : 3000 RmAb for the detecting layer was selected. The secondary antibody (HRP-labelled anti-rat ) was used at the manufacturer's recommended dilution of 1 : 10000. As the one-step design had been observed to increase sensitivity in the mAb / HRP-pAb<sub>2</sub> system, it was the method used for all mAb / RmAb assays.

### 3.8.3 Determination of linear range and limit of detection

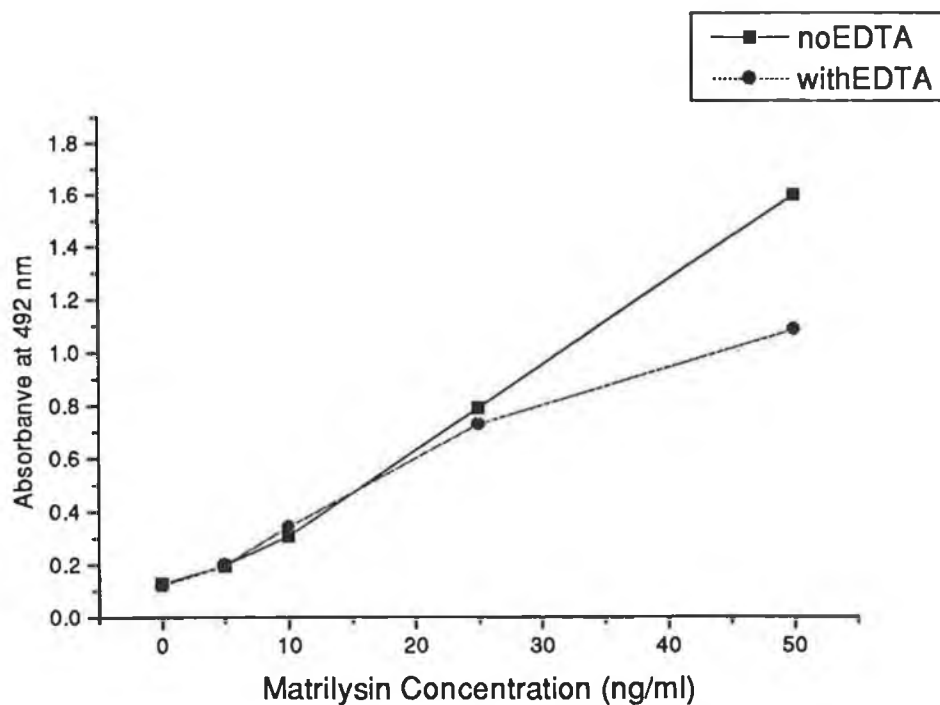
Using the optimal dilutions of coating, detecting and secondary antibodies and a range of matrilysin concentrations (0 - 320 ng/ml), a sandwich ELISA (2.2.6.5) was performed. The data obtained is presented in tabular form in Table 3.8.1 and graphically in Figure 3.8.3. The limit of detection was 0.45 ng/ml and the linear range was 5 - 50 ng/ml. The sensitivity of this system is over 200 times higher than the mAb / HRP-pAb<sub>2</sub> system which had a limit of detection of 100 ng/ml. It also compares favourably with figures obtained for other MMP ELISAs and is as sensitive as the recently published matrilysin ELISA, although the linear range is not as wide (Ohuchi *et al.*, 1996). The authors of that study found a vast difference in sensitivity of their assay when EDTA was used in the samples/ standards. EDTA, however, did not increase the sensitivity of our assay. In fact a reduction in the linear range was observed when EDTA was used. This is shown in Figure 3.8.4.

Matrilysin Concentration (ng/ml)	Absorbance at 492 nm (Mean $\pm$ S.D.) n = 4
0	0.047 $\pm$ 0.002
5	0.102 $\pm$ 0.003
10	0.276 $\pm$ 0.015
20	0.458 $\pm$ 0.016
40	0.697 $\pm$ 0.016
80	1.048 $\pm$ 0.112
160	1.396 $\pm$ 0.023
320	1.778 $\pm$ 0.042

**Table 3.8.1** Establishment of linear range and limit of detection for the mAb / RmAb sandwich ELISA system. The limit of detection is the lowest concentration value which gives an absorbance reading at least 2.5 standard deviations higher than the zero value.



**Figure 3.8.3** A plot of the data from Table 3.8.2. As can be seen the graph is only linear for the first five concentrations after which it begins to plateau.



**Figure 3.8.4**

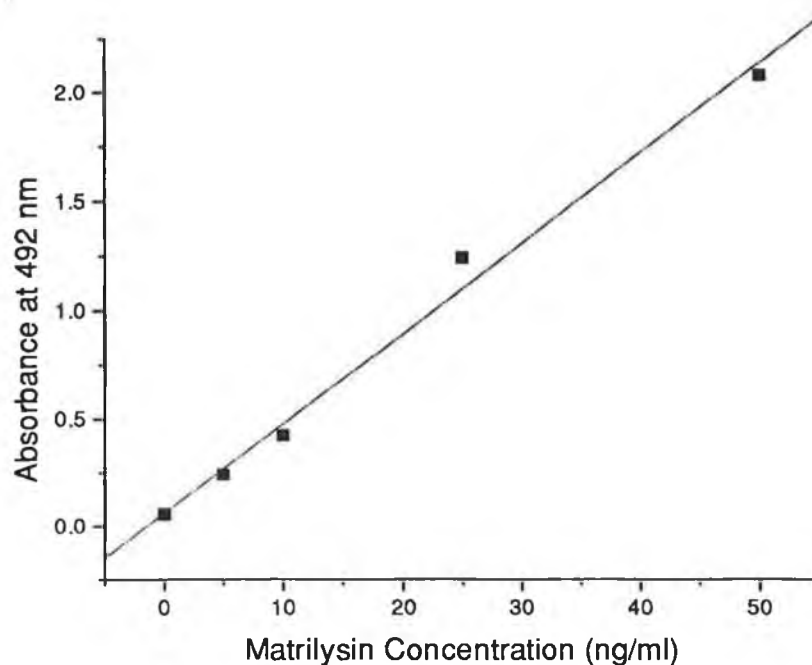
Effect of using EDTA in the preparation of standards for the mAb / RmAb sandwich ELISA for matrilysin. The absorbance values obtained for the lower concentrations of matrilysin (0 - 25 ng/ml) are approximately equal. When the matrilysin concentration is increased, however, the absorbance value for those samples prepared with EDTA is lower than when EDTA is omitted. The r value for the standards with no EDTA is 0.996. This was calculated across the linear range , 0 - 50 ng/ml. When EDTA is included, the r value is 0.997, however, this only applies to the linear part of the graph, 0 - 25 ng/ml.

### 3.9 Validation of Sandwich ELISA

To determine the intra-day accuracy and precision of the mAb / RmAb sandwich ELISA, five calibration sets of recombinant matrilysin were prepared in DMEM S<sub>0</sub> (serum-free cell culture growth medium) and assayed on the same day. The inter-day variability for samples in DMEM S<sub>0</sub> was determined by preparing a calibration set on five different days, with assay on the day of preparation.

#### 3.9.1 Intra-day accuracy and precision

For the intra-day variation analysis, absorbance values were interpolated at half-maximal signal to give mean concentration and coefficient of variation (CV). The mean was  $24.98 \pm 0.59$  ng/ml with a CV of 2.37 %. The standard curve from which this information was obtained is presented in Figure 3.9.1 below.



**Figure 3.9.1** Standard curve obtained for the mAb / RmAb sandwich ELISA for matrilysin. The linear range was 5 - 50 ng/ml ( $r = 0.995$ ).



### 3.9.2 Inter-day accuracy and precision

To calculate inter-assay variability on different days, samples (n = 5) were spiked with a known concentration of matrilysin and assayed along with the calibration sets. The results are presented in Table 3.9.1 below.

Day	Calculated Concentration (ng/ml) (Percentage recovery in brackets)
1	31.57 (105.2 %)
2	29.74 (99.1 %)
3	29.85 (99.5 %)
4	30.48 (101.6 %)
5	30.56 (101.9 %)
<b>Mean ± S.D. (C.V.)</b>	<b>30.44 ± 0.65 (2.14 %)</b>

**Table 3.9.1** Inter-assay validation. Samples were spiked with 30 ng/ml recombinant matrilysin and concentrations determined from calibration sets assayed at the same time. The low C.V. indicates an assay with excellent reproducibility.

## Summary

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Antibodies prepared against full recombinant matrilysin or a specific peptide sequence were kindly provided by Dr. Paul Cannon, Syntex Research, California and Prof. Lynn Matrisian, Vanderbilt University, Tennessee. These antibodies were first characterised as to their binding of the zymogen and/or active forms of the matrilysin enzyme. The polyclonal anti-serum was purified and enriched for specific anti-matrilysin antibodies by ammonium sulphate precipitation followed by affinity column chromatography. The feasibility of a sandwich-type immunoassay for the matrilysin molecule was demonstrated using BIAcore. Both a mouse monoclonal and the purified polyclonal antibody were labelled with the enzyme HRP. A sandwich ELISA system using the mouse monoclonal antibody as the coating layer and the HRP-labelled polyclonal antibody as the secondary antibody was developed. This assay, although linear in the range 100 - 1000 ng/ml, was not very sensitive having a lower limit of detection of 100 ng/ml. A second sandwich ELISA system using a coating layer of the mouse monoclonal antibody, a secondary layer of a rat monoclonal antibody and a detecting layer of HRP-labelled anti-rat antibodies was then developed. As the specificity of a sandwich ELISA is defined by the capture layer, all the assays developed recognised only the latent form of matrilysin. This second ELISA was sensitive, having a limit of detection of 0.45 ng/ml, and had a linear range of 5 - 50 ng/ml. The low CVs obtained for intra- and inter-day demonstrated excellent accuracy and precision. This assay was used to quantify matrilysin secretion by cells in culture and the data is presented in chapter 5.

## **Chapter Four**

### **Matrilysin Expression in Human Breast Tumours**

### 4.1 Tumourigenesis and metastasis

The critical difference between a (relatively) comforting diagnosis of a benign tumour compared to the fear-inspiring diagnosis of malignant cancer is the evidence of metastasis. This spread of malignant cells throughout the body that 'seeds' secondary tumours is the primary cause of death of most cancer patients (Vile, 1995).

The development of most cancers involves two distinct stages - transformation and metastasis - which may or may not be temporally separated (Vile, 1995). Cells of a primary tumour are said to be growth transformed when they no longer respond to normal cell division and growth controls. The usual controllers of cell proliferation are the products of proto-oncogenes (Knudson, 1985; Weinberg, 1985). Their protein products include growth factors, transmembrane receptors, inner membrane and cytoplasmic signal transduction proteins and nuclear proteins such as the transcription factors (Bishop, 1991). A mutation in a proto-oncogene giving rise to an oncogene may result in continuous cell division signals. In opposition to the division-promoting signals of the proto-oncogenes are the growth-inhibiting or differentiation-inducing tumour suppressor genes also known as anti-oncogenes. Mutations in or loss of these genes contributes to tumourigenesis by allowing continuous cell division and tumour growth (Sager, 1989). As cell division and differentiation are tightly controlled, the transformation process requires the accumulation of multiple genetic mutations (Vogelstein and Kinzler, 1993). In the case of colon cancer, a step-by-step progression from normal epithelium through benign adenoma to a fully malignant tumour has been defined (Fearon and Vogelstein, 1990). In this model, mutations in at least three tumour suppressor genes (p53, APC and DCC ) and one proto-oncogene (Ki-*ras*) are required.

Once transformed into tumours, cells can remain benign for a long time. In breast cancer for example, the period of transition from hyperproliferative but non-invasive disease to invasive cancer is estimated to average 6 years (Spratt *et al.*, 1986). However, as the cells are frequently dividing, various other genetic mutations can

accumulate. Many of these are irrelevant but some can push the tumour into the second phase of the disease, metastasis. One of these genetic events may result in mutation or loss of a putative metastasis suppressor gene, *nm23* (Liotta *et al.*, 1991a). This gene was identified by differential hybridisation of mRNAs from clones of a murine melanoma cell line exhibiting either high or low metastatic potential. This gene was consistently expressed in higher amounts in the low metastatic variants in four different metastasis models. The activity of the gene was also shown to be low in breast tumours from patients with positive lymph nodes and other signs of poor prognosis (Bevilacqua *et al.*, 1989). Proof that the anti-metastatic activity resulted directly from *nm23* expression was obtained by transfecting highly metastatic melanoma cells with the *nm23* gene and injecting these into nude mice (Leone *et al.*, 1991). Consistently lower numbers of metastases were found in mice injected with the transfected cells than in controls. The association of high *nm23* expression with low metastatic potential which apparently applies to ovarian (Mandei *et al.*, 1995) and gastric (Nakayama *et al.*, 1993) carcinomas as well as breast cancer, is not absolute. In other tumours, *e.g.* colorectal cancer (Zeng *et al.*, 1994), lung cancer (Higashiyama *et al.*, 1992), thyroid cancer (Zou *et al.*, 1993) and neuroblastoma (Leone *et al.*, 1993) it is instead advanced disease which is associated with high levels of *nm23*. The NM23 protein has been identified as a nucleoside diphosphate (NDP) kinase (Marx, 1993a). NDP kinases catalyze the transfer of the terminal phosphate group of 5' triphosphate nucleotides to 5' diphosphate nucleotides and may function in G protein regulation thereby suggesting a role for *nm23* in signal transduction (Liotta *et al.*, 1991b). A second *nm23* gene named *nm23-H2* has been cloned (Stahl *et al.*, 1991) and, although it shares approximately 90% homology with the first *nm23*, it has been suggested to function as a transcription factor (PuF) particularly associated with the proto-oncogene *c-myc* (Postel *et al.*, 1993).

In order to break away from a primary tumour and establish a secondary growth at a distant body site, a metastatic cell must express a number of biological functions (Liotta *et al.*, 1991a). Metastasis, like tumourigenesis, is also a multi-step event. Cells must detach from the primary tumour, invade locally, enter the circulation, arrest at a distant site, extravasate into the target organ tissue and proliferate as a

secondary colony all while escaping immune system interference. Only a very low percentage (less than 0.05%) of metastatic cells in the circulation survive to form secondary tumours (Liotta *et al.*, 1974; Nicolson, 1991). This may, in part, be due to the requirement for a suitable 'soil' for the metastatic cell 'seed' - specific target organs only are suitable for growth of secondary tumours from particular primary tumours (Fidler, 1993). For a tumour cell to invade, whether locally or through a blood vessel, basement membrane (the layer of collagens, fibronectins and proteoglycans separating epithelial, endothelial and parenchymal cells from underlying stroma) must be breached. This process can be divided into three stages (Stracke and Liotta, 1992) : (i) tumour cell adherence to basement membrane via cell surface receptors (*e.g.* integrins), (ii) degradation of membrane via local proteolysis, and (iii) migration of the cell through the lysed membrane. The local dissolution of the basement membrane barrier appears to be a critical turning point in the progression from benign to metastatic disease (Kohn and Liotta, 1995). Using the example of breast cancer, carcinoma *in situ*, an essentially benign lesion, remains surrounded by an intact basement membrane. In the transition to invasive carcinoma, however, tumour cells penetrate the epithelial basement membrane and interact with the stromal cells. The important molecules which appear to be differentially expressed in the invasion process are cell adhesion molecules, proteinases and motility factors (Van Roy and Mareel, 1992; Kemler, 1993; Schor, 1994; Dorudi, 1995; Kohn and Liotta, 1995). Although the proteinases are the principal subjects of this review, it should be remembered that the other factors are equally important. In fact, general unregulated proteolysis alone would be counter-productive as a tissue matrix with appropriate adhesion molecules is required for proper traction of an invading cell (Stracke and Liotta, 1992).

#### **4.2 Breast cancer and indicators of prognosis**

By the time many patients present with breast tumours, occult or detectable metastases may be present. The involvement of lymph nodes - a sign of metastasis - is the simplest and most reliable prognostic marker in patients with stage I or stage II disease (Hayes, 1996). Approximately one third of newly diagnosed breast cancers will have axillary node involvement (McGuire and Clark, 1992). In these cases, aggressive systemic

therapy is indicated. It is the remaining two-thirds of newly-diagnosed breast cancers for which other, accurate prognostic markers are required. Although node-negative patients have a better chance than their node-positive counterparts of remaining disease-free following surgery (to remove the primary tumour), there is still a 30% chance that the cancer will return (Marx, 1993b). Determining which patients fit into the 30% category and are thus suitable subjects for adjuvant therapy has been dependent on using the standard prognostic indicators of tumour size, histopathological classification, histological and nuclear grade, hormone receptor status and, more recently, DNA ploidy and S phase fraction determination (McGuire and Clark, 1992). A brief explanation of each of these established prognostic markers follows.

**Tumour Size** : Breast tumour staging is based on primary tumour size (T), lymphatic spread (N) and the presence or absence of distant metastases (M). Stage I disease, referred to as  $T_1N_0M_0$  is therefore defined as a small tumour of 2 cm diameter or less, with no evidence of lymphatic spread or more distant metastases (Hayes, 1996). Widespread use of mammography has led to the increased detection of small primary tumours allowing treatment of stage I disease before any progression to a more aggressive tumour can occur. A large American study has shown that patients with tumours less than 1 cm in diameter had a 5 year survival rate of almost 99% (Carter *et al.*, 1989). In a group of patients whose tumours were between 1 and 3 cm, the 5 year survival rate was 91 %. A second study showed a 20 year recurrence rate of just 14% in patients with tumours less than or equal to 1 cm in diameter (Rosen *et al.*, 1989). When the tumour size was between 1.1 and 2 cm, the 20 year recurrence rate increased to 31 % while patients with larger tumours had a recurrence rate of over 50%. The importance of tumour size is reflected in the advice from the American National Institutes of Health (NIH) Breast Cancer Consensus Conference that patients with tumours less than 1 cm in diameter should not usually receive adjuvant therapy (McGuire and Clark, 1992).

**Histopathological Classification** : There is increasing evidence that certain pathological features are helpful in anticipating the likely behaviour of an individual tumour. Ductal carcinoma *in situ*, pure tubular carcinoma, papillary carcinoma and

typical medullary carcinoma are subtypes of breast carcinoma with excellent prognosis in node-negative patients with long-term recurrence rates of less than 10% (Rosen *et al.*, 1989). By definition, carcinoma *in situ*, that is carcinoma confined by an intact basement membrane, will not metastasize as the tumour cells have no access to lymphatics or blood vessels and can therefore be removed leading to a complete cure (Leader *et al.*, 1995). It is, however, this complete removal of all cancerous cells that is difficult to achieve. Limited surgery with or without radio- or chemotherapy, mastectomy or even bilateral mastectomy may be necessary.

**Hormone Receptors :** Human breast cancer is dependent upon the ovarian hormone oestrogen for its onset and progression (Lippman and Dickson, 1989). The oestrogen initially acts as a growth factor for the tumour cells. As the cells proliferate and become more de-differentiated however, they may lose their dependence on oestrogen and grow without the external growth signals provided by the hormone (Marx, 1993b). The presence or lack of oestrogen receptors (ER+ or ER-, respectively) has been an important determinant of both prognosis and treatment, a poorer prognosis being associated with ER- tumours. Nevertheless, the use of hormone receptor status as a marker for malignant potential is now controversial. Studies have shown only an 8 - 10% difference in disease-free survival in node-negative patient groups divided on the basis of ER status (McGuire and Clark, 1992). Some of the controversy stems from the measurement methods used with a radioactive ligand binding assay and immunohistochemistry being the most usual (Hayes, 1996). Several researchers have shown discordance between values obtained by the two techniques (Mink *et al.*, 1995; Gaffney *et al.*, 1995). The disparity appears to result from the presence of oestrogen receptors on uninvolved normal tissue adjacent to the tumour in the biopsy samples used for ER determination. When using immunohistochemistry, this normal tissue and any positive staining associated with it can be ignored whereas the ligand binding assay does not allow for straightforward differentiation between tumour and normal tissue. Another cause for doubt of the prognostic value of ER status is the finding that in post-menopausal women, highest (ER++) as opposed to intermediate (ER+) levels indicated a poorer prognosis in terms of recurrence-free survival (Thorpe *et al.*, 1993). A second study, while confirming this finding, discovered a disparity between ER protein



and mRNA suggesting the high ER protein levels are the result of a modified ER gene product (Sancho-Garnier *et al.*, 1995). As a prognostic indicator, receptor status only appears to be helpful when combined with other information, however, it is useful in determining which patient's tumours may respond to anti-oestrogen *e.g.* tamoxifen therapy (Hayes, 1996).

**Nuclear and Histologic Grade :** The degree of differentiation of a tumour and the extent of mitosis evident in these cells can be evaluated by an experienced pathologist. It is, however, a subjective measure and valid results may not be obtained from a less experienced person (McGuire and Clark, 1992). A tumour classed as histological and nuclear grade I is well-differentiated and the nuclei show little variation in size and shape. Grade II nuclei are of variable size and shape and possess prominent nucleoli whereas grade III nuclei are large, irregular, contain multiple nucleoli and frequent mitoses (Leader *et al.*, 1995). Patients whose tumours are classified as grade I have a 5 year survival rate of over 90% (McGuire and Clark, 1992).

**S-phase Fraction and Ploidy :** The development of flow cytometry as a routine analytical method has allowed the determination of DNA content (ploidy) and proliferative activity (S-phase fraction) of tumour cells. The use of ploidy as a prognostic marker is somewhat controversial although there is evidence to suggest the more aneuploid (as opposed to diploid) a tumour, the worse the prognosis. Clark *et al.* (1989) evaluated the prognostic value of the combination of data on ploidy and S-phase fraction in node-negative patients and, after adjusting for other prognostic markers, found that ploidy was an independent prognostic factor, while in diploid tumours, S-phase fraction yielded additional prognostic information. Other researchers have found significant correlations between ploidy and tumour grade and ploidy and S-phase fraction (Rosanelli *et al.*, 1995), and between proliferative activity and histopathological grade (Schonborn *et al.*, 1995).

Despite the use of the prognostic factors outlined above, every year thousands of patients die whose progression to metastatic disease was not anticipated. Groups of these patients may fall into subsets for whom other, less well-characterized, markers

have a role. These could include *c-erbB2/neu*, p53, EGF-receptor, pS2, stress response proteins, topoisomerase 2, *nm23* and angiogenesis markers.

#### **4.3 Proteinases as prognostic markers in breast cancer**

A positive correlation between tumour aggressiveness and proteinase levels has been documented for all four classes of proteinases including serine, cysteine, aspartyl and metalloproteinases (Liotta *et al.*, 1980; Rochefort *et al.*, 1990; Matrisian *et al.*, 1991; Duffy, 1992; Blasi *et al.*, 1993; Nagle *et al.*, 1994; Yamashita *et al.*, 1994). All of these classes of proteinases have been investigated as possible prognostic markers in breast cancer.

**Aspartyl Proteinase - Cathepsin D** : The lysosomal proteinase, cathepsin D, is synthesised by both normal and tumour tissues, however, significant over-expression of the protein appears associated with increased disease recurrence in breast cancer patients (McGuire and Clark, 1992). A study using western blotting found an increased level of expression of a 31 kDa form of the protein in tissue from malignant breast tumours compared to that from benign tissue or normal controls (Schultz *et al.*, 1994). This 31 kDa form was not seen in sera from the same patients which, instead, contained only 52 kDa and 27 kDa forms of the protein. Using an enzyme assay, the same researchers found significantly increased amounts of activity in tissue from breast cancer patients compared to benign tissue or normal controls suggesting that the 31 kDa form is the proteolytically active form of the enzyme involved in breast cancer progression. The prognostic significance of cathepsin D expression was also analysed using immunohistochemistry in a series of 151 breast cancers (Aaltonen *et al.*, 1995). Strong expression was detected in 22% of cases and significantly associated with histological type and presence of metastases. Patients with cathepsin D over-expression had a significantly lower survival probability than patients with low expression. Using multivariate analysis, however, these investigators found cathepsin D to have no independent prognostic value over conventional prognostic factors. In contrast, an earlier study using a cathepsin D radio-immunoassay found the concentration of cathepsin D in tumour cytosol to be strongly related to both metastasis- and disease-free survival in a group of 122 patients (Spyratos *et al.*, 1989).

Using multivariate analysis, qualitative (*i.e.* high or low levels) but not quantitative cathepsin D analysis was associated with survival and was more important than lymph node status. Their most important finding was of a high cathepsin D concentration in those patients who later developed metastases but for whom conventional prognostic factors (tumour size, lymph node involvement, ER status and DNA ploidy) had indicated a good prognosis.

**Cysteine Proteinases - Cathepsins B and L :** The prognostic impact of these lysosomal proteinases for disease-free and overall survival was evaluated in a study of 167 breast cancer patients using ELISAs for each of the enzymes (Thomssen *et al.*, 1995). The median values for each of the enzymes in cancerous specimens was approximately 10 times higher than the corresponding values in normal samples. Multivariate analysis for disease-free survival showed cathepsin L as a strong and independent factor comparable to lymph node status and histopathological grade. Patients with high content of either cathepsin B or L had a statistically significant increased risk of disease recurrence than patients with low levels. Alterations in the trafficking of cathepsin B so that it is no longer confined to lysosomes has been suggested as the basis for its involvement in breast cancer malignancy (Sloane *et al.*, 1993).

**Serine Proteinase - Urokinase Plasminogen Activator :** Urokinase plasminogen activator (uPA) is synthesized as an inactive proenzyme (pro-uPA) which can be activated by plasmin or cathepsins B or L (Duffy, 1992). It is inhibited by two specific plasminogen activator inhibitors, PAI-1 and PAI-2, the second of which is a stronger and more specific uPA inhibitor (Bouchet *et al.*, 1994). Plasminogen activation by uPA occurs at the cell surface where the inactive pro-uPA is bound by its cellular receptor uPAR (Vile, 1995). When activated, uPA remains bound to uPAR where it is capable of converting plasminogen to the trypsin-like serine proteinase plasmin. Plasmin can itself degrade some ECM proteins and, more importantly, can convert other zymogens to their active forms by proteolysis (Wilhelm *et al.*, 1987; He *et al.*, 1989). In this way highly focalised centres exist where controlled areas of proteolysis can occur at the leading edge of an invading cell (Vile, 1995). Using an ELISA system, it has been

shown that high uPA levels correlate with reduced disease-free and overall survival in breast cancer patients (Duffy, 1990). As a predictor of disease-recurrence, uPA was better than axillary node involvement, tumour size or ER status when assessed by univariate analysis. In multivariate analysis, uPA was an independent risk factor for both disease-free and overall survival. Measurement of uPA as well as PAI-1 and PAI-2 levels was undertaken in another study (Bouchet *et al.*, 1994). A poor prognosis correlated with high levels of both uPA and PAI-1. Conversely, low levels of PAI-2 was also found to be a marker of poor prognosis. These researchers concluded, after subjecting all variables studied to statistical analysis, that PAI-1 provided the same prognostic information as uPA whereas PAI-2 increased the prognostic value of uPA.

**Matrix metalloproteinases** : Studies showing involvement of various members of the MMP family in breast cancer are numerous (Bae *et al.*, 1993; Wolf *et al.*, 1993; Zucker *et al.*, 1993; Soini *et al.*, 1994; Yu *et al.*, 1995; Patel and Schrey, 1995; Heppner *et al.*, 1996). These studies, however, were small in size and were performed with the intention of determining localisation and expression levels rather than prognostic impact.

As they were both initially isolated from breast tumour cDNA libraries both stromelysin-3 and collagenase-3 are especially associated with this cancer (Basset *et al.*, 1990; Freije *et al.*, 1994). Although no large scale studies assessing the true prognostic significance of either of these enzymes has yet taken place, there is some preliminary data to suggest they may be useful. In a study of stromelysin-3, Wolf *et al.* (1993) showed expression of the proteinase in all invasive carcinomas examined and in some *in situ* carcinomas where other factors indicated a high risk of the development of an invasive phenotype. Stromelysin-3 was not expressed in benign breast fibroadenomas or normal tissue. Similarly, collagenase-3 is not expressed in normal or benign breast tissue but was detected in all examined breast carcinoma samples (Freije *et al.*, 1994).

Gelatinase A, while present in both metastatic and non-metastatic breast tumour tissue, appears to be increasingly activated in metastatic disease. Quantitative zymography was used to demonstrate increased ratios of activated enzyme to total gelatinase A in breast carcinomas of increasing grade (Davies *et al.*, 1993c). A

different study found no marked correlation between gelatinase A expression and established staging and prognostic markers (Brown *et al.*, 1993) although the activation of gelatinase A did appear to be a more common event in invasive breast disease. It should be noted that both of the investigations just mentioned were small, pilot studies of approximately twenty patient samples. An earlier, larger study evaluated gelatinase A expression along with other basement-membrane associated markers (laminin receptors, type IV collagen and laminin) in 187 node-negative breast cancer patients (Daidone *et al.*, 1991) High levels of gelatinase A (or laminin receptors) did not influence disease-free or overall survival but were strong indicators of local-regional diffusion of the disease.

The assessment of the prognostic value of MMP levels in breast cancer should be easier as more sensitive and specific ELISAs become available. Already assays for interstitial collagenase and TIMP-1 have been used to assess metastatic potential in prostate cancer where they were found to be as valuable as prostate-specific antigen and alkaline phosphatase, established prognostic factors for this disease (Baker *et al.*, 1994). An assay for gelatinase B detected significantly raised levels of the enzyme in plasma from patients with breast cancer as compared to normal controls (Zucker *et al.*, 1993). No correlation was found between gelatinase B levels and breast cancer staging however the sample number was small and correlations with other prognostic markers were not analysed. A later study which investigated neutrophil collagenase, gelatinase B and TIMP-1 levels by ELISA showed that none of these correlated with tumour size or lymph node status but the collagenase and gelatinase levels inversely correlated with ER levels (Duffy *et al.*, 1995).

There are few reports of matrilysin expression in tumour tissue. In gastrointestinal carcinomas, it has been established that matrilysin expression is associated with tumour development as it is not seen in normal tissue but is expressed in carcinomas (McDonnell *et al.*, 1991). The presence of matrilysin in glandular tissue has also been demonstrated (Saarialho-Kere *et al.*, 1995). This investigation included normal mammary tissue in which matrilysin expression was localised to the secretory epithelium of ducts and ductules. A recent report demonstrated matrilysin expression in both normal and tumour samples of breast tissue (Heppner *et al.*, 1996).

In the work to be presented in this chapter, the aim was to examine a small number of breast tumour samples for expression of both matrilysin protein and mRNA using a number of different techniques. We hoped to determine the most suitable and informative detection method and to establish whether matrilysin expression has any potential as a prognostic marker of malignant breast disease.

## Results and Discussion

### 4.4 Tissue samples

This study included only a small number of samples as it was intended as a preliminary analysis of different detection methods. Breast tissue samples were obtained from the Pathology Dept., Mater Misericordiae Hospital, Dublin. Both frozen pieces of tissue and paraffin-embedded blocks were obtained. Ten samples were classified as infiltrating ductal carcinoma and were identified as samples T<sub>1</sub> to T<sub>10</sub>. Three paraffin-embedded blocks from histologically normal breast tissue were also obtained. These were identified as samples N<sub>1</sub> to N<sub>3</sub>. All tissue samples had been examined and classified by Prof. Peter Dervan, Consultant Pathologist. Details of the tumour tissue samples are presented in Table 4.1.

**Table 4.1 Breast Tumour Tissue Samples**

Sample Number	Patient Sex*	Patient Age	Diagnosis*	Tumour size
T <sub>1</sub>	F	72	Infiltrating ductal carcinoma	2 x 2 x 1.5 cm
T <sub>2</sub>	F	75	Infiltrating ductal carcinoma [P.D.]	5 x 3.5 x 6 cm
T <sub>3</sub>	F	53	Infiltrating ductal carcinoma	3 x 3 x 3 cm
T <sub>4</sub>	F	63	Infiltrating ductal carcinoma	6 x 4 x 3 cm
T <sub>5</sub>	F	54	Infiltrating ductal carcinoma [P.D.]	2 x 2 x 1.5 cm
T <sub>6</sub>	F	42	Infiltrating ductal carcinoma [M.D.]	1.7 x 1 x 1 cm
T <sub>7</sub>	F	37	Infiltrating ductal carcinoma	4 x 1 x 1 cm
T <sub>8</sub>	F	unknown	Infiltrating ductal carcinoma	4 x 3 x 3 cm
T <sub>9</sub>	F	unknown	Infiltrating ductal carcinoma	3 x 3 x 1 cm
T <sub>10</sub>	F	71	Infiltrating ductal carcinoma	3 x 3 x 1 cm

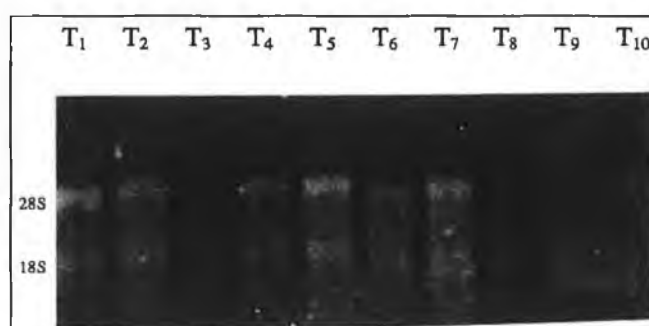
\* F = female.

+ P.D. indicates poorly differentiated and M.D. indicates moderately differentiated.

#### 4.5 RNA isolation

The RNA was isolated using a modification of the method of Chomczynski and Sacchi, (1987). The procedure is outlined in section 2.2.11.2. RNA could only be isolated from tumour samples as no frozen samples, just paraffin embedded blocks of the normal tissue samples were available. The integrity of the isolated RNA was checked using agarose gel electrophoresis in the presence of the reducing agent, formaldehyde (2.2.11.3). The gel obtained is shown in Figure 4.1. The presence of two bands representing the 28S and 18S ribosomal subunits is indicative of non-degraded RNA.

**Figure 4.1** Agarose gel electrophoresis showing intact RNA isolated from breast tissue samples T<sub>1</sub> - T<sub>10</sub>. The bands representing the 28S and 18S ribosomal subunits can be clearly seen. Some smearing, indicative of RNA degradation is also visible.

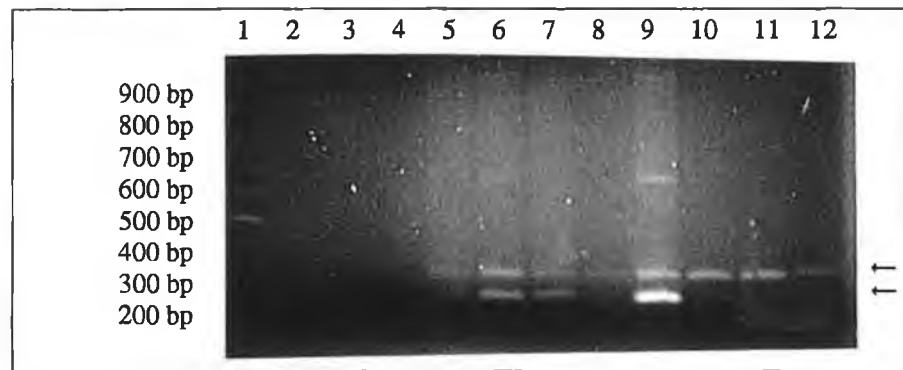


#### 4.6 Reverse Transcription - Polymerase Chain Reaction (RT-PCR)

The RNA isolated from the breast tumours was analysed for expression of matrixin-specific mRNA using RT-PCR. This technique is a modification of the polymerase chain reaction used for amplifying specific pieces of DNA. In RT-PCR, the mRNA was first converted to cDNA using a viral reverse transcriptase enzyme. Prior to reverse transcription, the mRNA was primed with oligo d(T) primers which bind to the polyadenylated tail associated with mature mRNA. Once cDNA was obtained, an aliquot can be amplified using PCR. The cDNA was mixed with Taq polymerase, nucleotides and a pair of primers specific for the target DNA. In all RT-PCR reactions described here, two sets of primers were included in every reaction. The first set recognised matrixin cDNA and amplified a fragment of 341 bp. As these primers



spanned an intron, any genomic DNA amplified would result in a larger product of 700 bp. The second set of primers recognised the constitutively expressed 'housekeeping' gene,  $\beta$ -actin and amplified a region of 225 bp. These primers were included as an internal control for each reaction. Figure 4.2 is a photograph of the products obtained after performing RT-PCR on the ten breast tumour samples.



**Figure 4.2** 3 % agarose gel showing products of RT-PCR for matrilysin and  $\beta$ -actin. Lane numbers are indicated. Lane 1 contains 100 bp size marker, Lane 2 contains a negative control (water used in place of RNA), Lanes 3 - 12 contain tumour samples T<sub>1</sub> - T<sub>10</sub>. The upper arrow indicates the matrilysin band at 341 bp. The second arrow indicates the lower  $\beta$ -actin band at 225 bp.

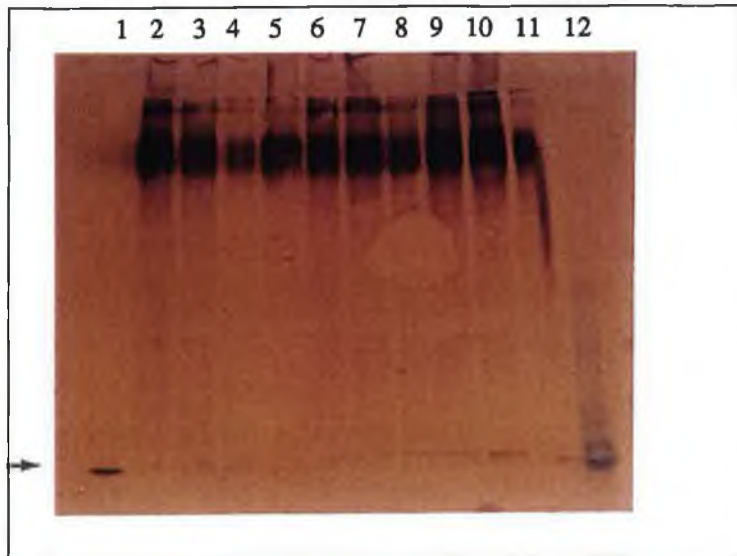
The presence of matrilysin-specific bands in 8 / 10 samples of infiltrating ductal carcinoma indicated that active transcription of matrilysin was occurring in a high proportion of tumour samples. Two samples were negative although this may have been a fault in the reaction as no  $\beta$ -actin product was obtained. Although equal amounts of total RNA were used in the original RT reaction, the intensities of the  $\beta$ -actin bands varies between samples. The concentration of total RNA isolated was assessed spectrophotometrically. This technique is accurate only when the sample being analysed is completely pure and undegraded as degraded RNA will contribute to an absorbance measurement but may not be reverse transcribed. It would be expected that isolates from tissue samples would contain different amounts of degraded RNA which would have interfered with the spectrophotometric measurement. RT-PCR is

reported as being a very sensitive technique able to detect as little one cell positively expressing a particular gene (Rappolee *et al.*, 1989). This means that the actual number of cells in any sample of tissue positively expressing matrilysin may be small. The possibility also exists that the positive mRNA is not arising from tumour cells but from normal cells within the tissue sample. The limitation of this technique, therefore, is that, unless an homogenous sample containing only one cell type is used, the source of a positive result is unknown.

#### **4.7 Western blotting**

Methods for detecting protein are often more suitable than mRNA detection methods as problems with RNA degradation do not arise. This is especially true when using archival material which may not have been collected and stored in RNase-free conditions. Protein methods, however, often depend on the availability of a suitable antibody raised against the protein of interest. A number of antibodies against matrilysin were available for use, however, none of these had previously been tested with tissue samples. For immunoblotting, a polyclonal antibody, pAb<sub>1</sub>, which recognised both the zymogen (28 kDa) and active (18 kDa) forms of the matrilysin enzyme was used. This was the only antibody of those available which could equally recognise both forms of the protein (see section 3.4, chapter three).

Total protein was isolated from the tumour samples using a simple detergent-based procedure (2.2.15). The protein content of the isolates was assessed using a BCA microassay (2.2.7), and an equal amount (200 µg) of each sample run on a 12 % SDS-PAGE gel (2.2.1). The blotting and probing procedure was performed exactly as outlined in section 2.2.2, with the primary antibody (pAb<sub>1</sub>) used at a dilution of 1 : 1000 and secondary antibody (AP-labelled anti-rabbit IgG) used at a dilution of 1 : 2500. The blot was developed with BCIP/NBT and photographed. It is shown in Figure 4.3.

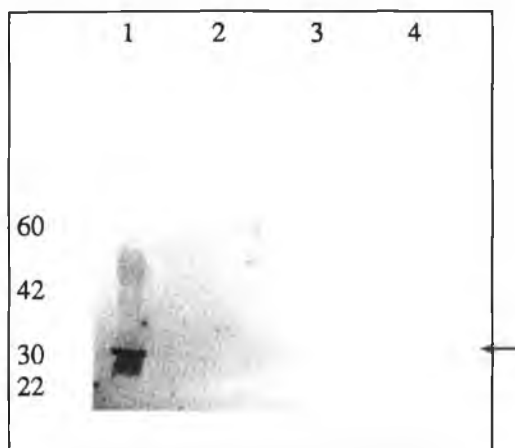


**Figure 4.3** Western blot demonstrating the presence of matrilysin in breast tissue samples T<sub>1</sub> - T<sub>10</sub>. Lane 1 contains a positive control (recombinant active [18 kDa] matrilysin), lane 12 contains molecular weight markers and the tissue samples are loaded in lanes 2 - 11. The sizes of the MW markers (measured in kDa) are indicated at the right of the figure. An arrow on the left points to the matrilysin bands.

As can be seen, non-specific staining is evident in all of the tissue samples. It is not known which component of the tissue isolates is responsible for the darkly staining high molecular weight band. Matrilysin bands of both sizes (28 and 18 kDa) are evident at the bottom of the blot. Almost all of the tissue samples appear to contain the zymogen form of matrilysin, while a number also contain the active form. This high proportion of positively-expressing samples is similar to that obtained with RT-PCR.

The degree of non-specific staining evident with this antibody makes it unsuitable for use in other procedures such as immunohistochemistry. A different anti-matrilysin antibody, RmAb which is a rat monoclonal antibody, was considered for use in this procedure. To ascertain whether any non-specific binding would result with this antibody, another western blotting experiment was performed. 200 µg of three of the tissue isolates were loaded on a 12 % SDS-PAGE gel and the immunoblotting

executed as before except that the primary antibody (RmAb) was used at a 1 : 200 dilution while the secondary antibody, an HRP-labelled anti-rat IgG, was used at a dilution of 1 : 5000. The blot was developed with DAB and is shown in Figure 4.4.



**Figure 4.4** Western blot demonstrating presence of matrilysin in breast tissue samples T<sub>1</sub>, T<sub>2</sub> and T<sub>3</sub>. A rat monoclonal anti-matrilysin antibody was used as the primary antibody. Lane 1 contains MW markers and the sizes (in kDa) are indicated. The tissue samples are loaded in lanes 2 - 4. An arrow on the right of the figure points to the matrilysin band.

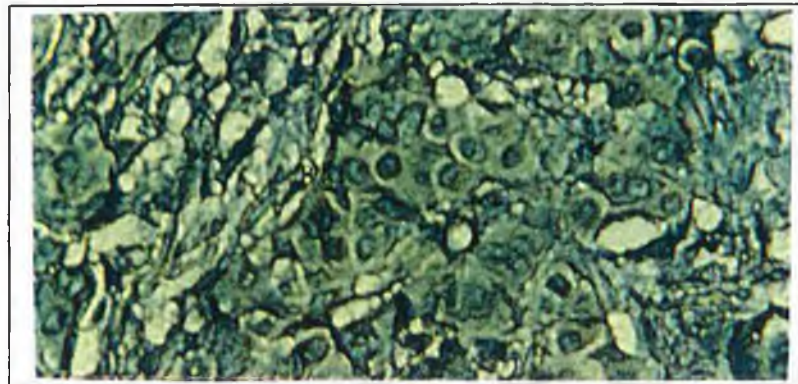
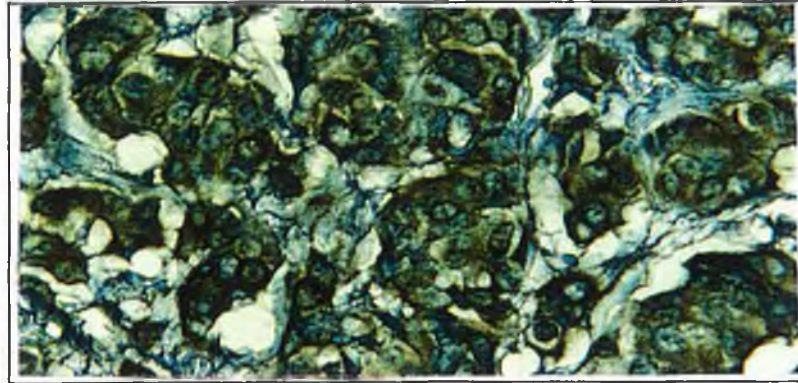
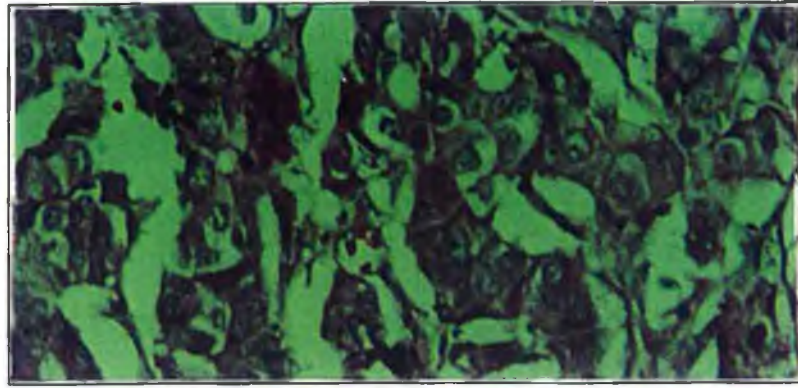
None of the non-specific staining seen with the polyclonal antibody used previously was evident in this blot. Faint bands at 28 kDa indicated positive staining for matrilysin. This antibody (RmAb) does not detect the active form of the enzyme so lower bands were not expected. The apparent specificity of this antibody made it a suitable candidate for immunohistochemistry.

#### 4.8 Immunohistochemistry

As with RT-PCR, positive results with western blotting give no indication of whether tumour or normal cells within a particular tissue sample express the protein of interest. The location of positively expressing cells within the tissue can be investigated using immunohistochemistry. Successful immunohistochemistry may be difficult to achieve as the suitability of the antibody used for detection is critical. The lack of non-

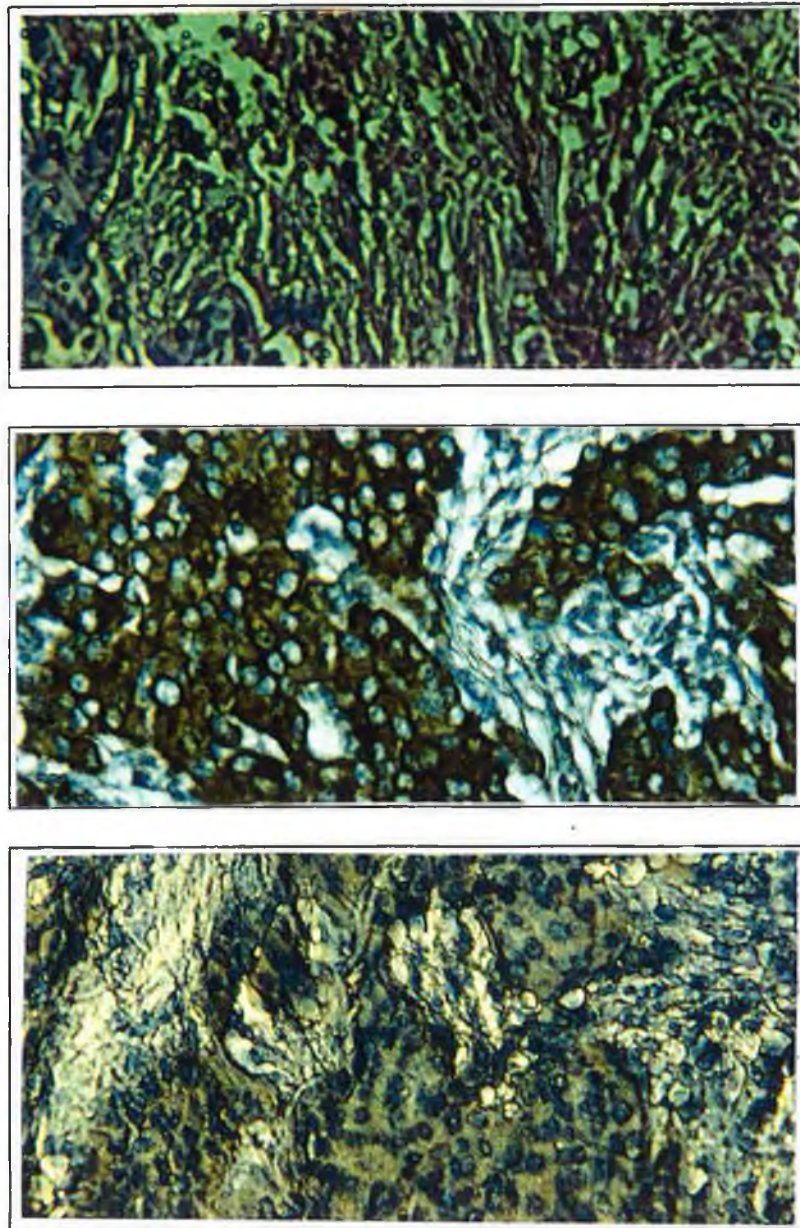
specific binding seen with RmAb in western blotting experiments suggested that this may be a suitable antibody to use, however, it was not initially developed for this purpose.

5  $\mu\text{m}$  sections were cut from the formalin-fixed, paraffin-embedded blocks of the tumour samples (T - T<sub>10</sub>) and the three samples of normal breast tissue (N<sub>1</sub> - N<sub>3</sub>). Prior to probing with the antibody, the sections had first to be dewaxed and rehydrated by passage through a series of graded alcohols. The actual immunohistochemistry procedure was performed exactly as outlined in section 2.2.14. Following development with DAB, the sections were counterstained with haematoxylin, mounted and viewed. Very little background staining was seen in any of the sections. All control sections, on which rat pre-immune serum was used in place of RmAb, showed no staining above background. Figures 4.5 to 4.8 are photographs of four of the sections (three tumour and one normal), which show examples of the staining patterns observed. The first two tumour sections, one poorly differentiated and one moderately differentiated, show intense staining for matrilysin protein in nests of tumour cells. No positive staining can be seen in the surrounding stromal cells. The last two sections, one tumour and one normal, show positive staining for matrilysin protein by normal ductal epithelial cells. This staining in normal ductal epithelium has previously been reported (Saarialho-Kere *et al.*, 1995; Heppner *et al.*, 1996). The intense staining associated with tumour cells has also been seen previously (Heppner *et al.*, 1996).

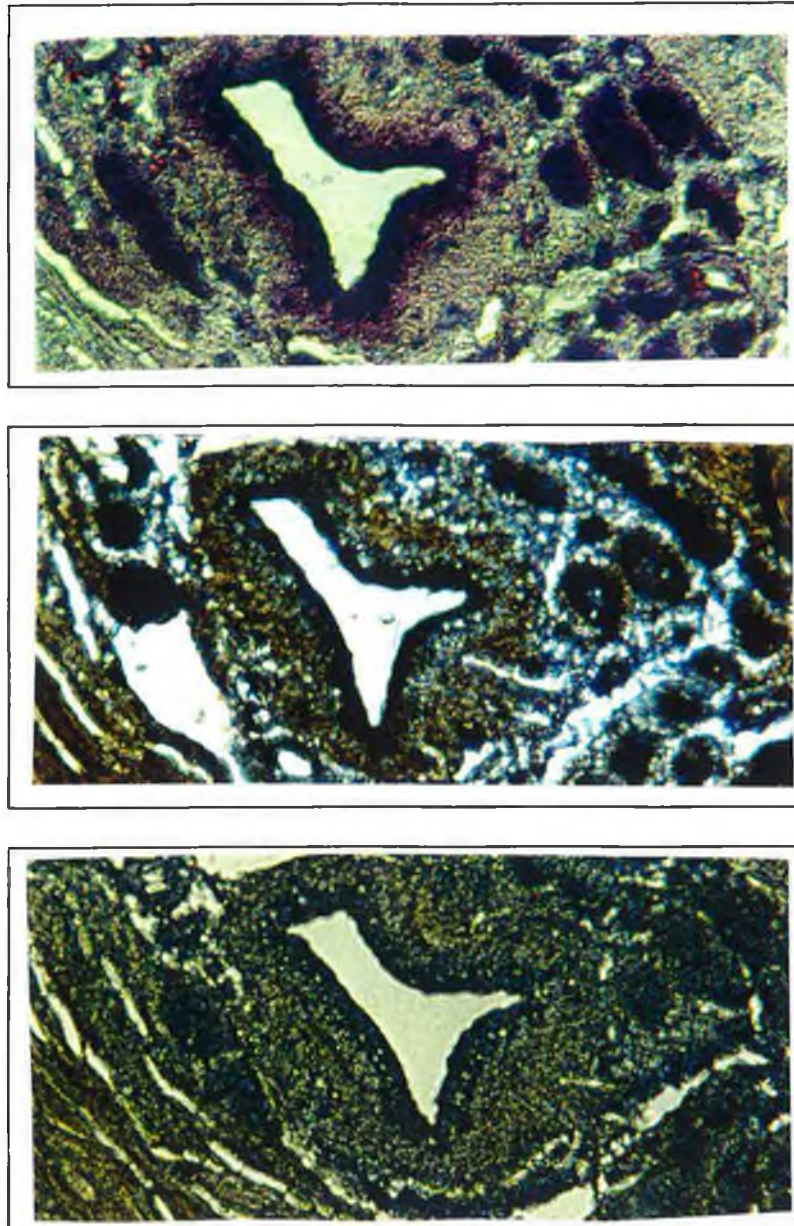


**Figure 4.5** Tissue section T<sub>8</sub>, a poorly differentiated infiltrating ductal carcinoma. The top panel shows this section stained with haematoxylin and eosin. The middle panel shows the results obtained when this section was probed with the rat anti-matrilysin antibody RmAb. The bottom panel shows the control reaction when pre-immune serum was used in place of the monoclonal antibody. Specific staining for matrilysin is visible in the nests of tumour cells. [Magnification 400X]



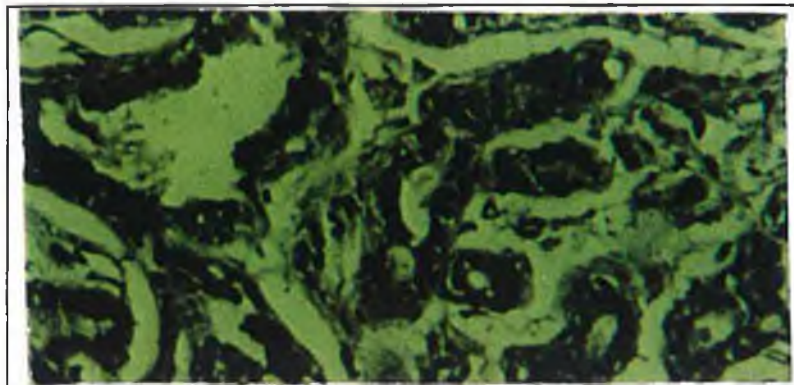
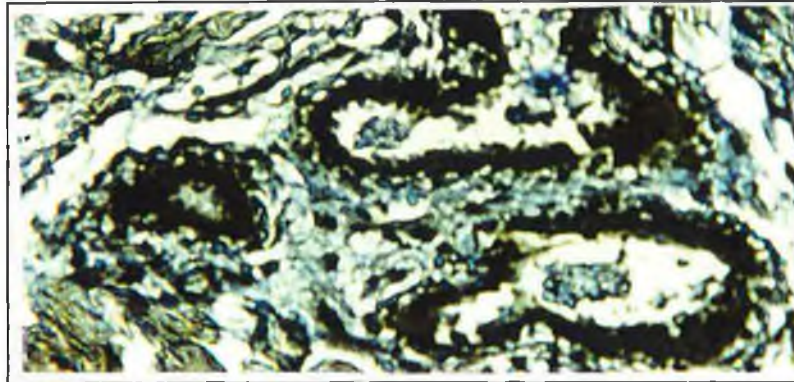
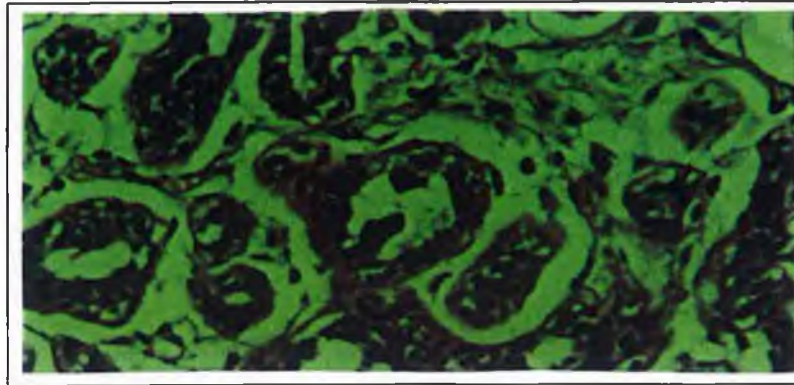


**Figure 4.6** Tissue sample T<sub>4</sub>, an infiltrating ductal carcinoma. The top panel shows this section stained with haematoxylin and eosin. The middle panel contains a view of the section probed with the rat monoclonal anti-matrilysin antibody, RmAb. The bottom panel is the control reaction when pre-immune rat serum was used in place of the RmAb. Once again, specific staining for matrilysin is observed in tumour epithelium and not surrounding stromal cells. [Magnification 400X]



**Figure 4.7** Tissue section T<sub>1</sub>, an infiltrating ductal carcinoma. The top panel shows this section stained with haematoxylin and eosin. The middle panel shows the results obtained when this section was probed with the rat monoclonal anti-matrilysin antibody, RmAb. The bottom panel shows the control reaction when pre-immune serum was used in place of the monoclonal antibody. Specific staining for matrilysin is observed in normal glandular epithelial cells present in this sample. [Magnification 400X]





**Figure 4.8** Tissue sample N<sub>1</sub>, normal breast tissue. The top panel shows this section with haematoxylin and eosin. The middle panel shows the results obtained when the section was probed with the rat anti-matrilysin antibody, RmAb. The bottom panel shows the control reaction when pre-immune serum was used in place of the monoclonal antibody. specific staining for matrilysin is observed in the glandular epithelium. [Magnification 400X]

The results of the immunohistochemical analysis of the tissue sections are striking. Matrilysin protein was detected in all sections. Immunoreactivity appeared as a cytoplasmic, granular or diffuse staining in all cases. It was associated with both invasive tumour cells (Figures 4.5 and 4.6) and with normal glandular epithelial cells (Figures 4.7 and 4.8). All sections showed areas without immunostaining. Table 4.2 summarises the results of the immunohistochemistry with all the sections examined.

**Table 4.2 Immunohistochemistry Results**

Specimen	Relative intensity of staining	
	TUMOUR	NORMAL
T <sub>1</sub>	+++	++
T <sub>2</sub>	+++	++
T <sub>3</sub>	+++	n.a.
T <sub>4</sub>	+++	+
T <sub>5</sub>	++	+
T <sub>6</sub>	++	++
T <sub>7</sub>	++	+
T <sub>8</sub>	+++	+++
T <sub>9</sub>	++	+
T <sub>10</sub>	++	++
N <sub>1</sub>	n.a.	+++
N <sub>2</sub>	n.a.	+
N <sub>3</sub>	n.a.	+

The relative intensity and amount of staining of either tumour cells ('TUMOUR') or normal epithelium ('NORMAL') in each section was assessed by eye and scored accordingly. '+' indicates low levels of staining, '++' indicates moderate staining and '+++' indicates a high level of intense staining. 'n.a.' indicates that a particular classification was not applicable as no example of that tissue was present in the section.

Similar expression of matrilysin mRNA in ductal epithelial cells of the mammary gland has been reported (Saarialho-Kere *et al.*, 1995). The authors of that study suggested that, as matrilysin is constitutively expressed by the glandular epithelium, it must serve a critical function. One possibility is that it is responsible for degrading proteinaceous debris thus maintaining the viability of the gland. The production of matrilysin mRNA by breast tumour cells has also been reported (Heppner *et al.*, 1996). It was localised to the epithelial components of tumours and to the epithelium of morphologically normal ducts, confirming exactly the results presented here. Therefore, matrilysin expression by normal and tumour cells of human breast tissue has now been confirmed at both mRNA and protein levels. An important consideration is that the antibody used recognises latent matrilysin only. It cannot be determined from these results if active enzyme is associated with either normal or tumour tissue. The activity of the enzyme could possibly be investigated using the technique of *in situ* zymography whereby a suitable substrate for the enzyme (*e.g.* casein) is incorporated into a photographic emulsion and placed on a section (Galis *et al.*, 1994). Alternatively an antibody which specifically recognises the active form of matrilysin could be utilised in immunohistochemistry. It would be interesting to ascertain if the enzyme produced by the normal ductal cells was actively degrading proteinaceous material thus supporting the gland cleaning function suggested by Saarialho-Kere *et al.* (1995). Active enzyme produced by tumour cells could be directly involved in invasion or indirectly through, for example, its possible role in activating urokinase (Marcotte *et al.*, 1992)

Table 4.3 indicates the results for each tissue sample obtained with the various methods of detecting matrilysin used. As can be seen, positive results were obtained for all tissue samples by at least one method and, in most cases, tissue samples were positive by all three methods.

SPECIMEN	ANALYSIS METHOD		
	RT-PCR	W. BLOTTING	IMMUNOHIST.
T <sub>1</sub>	neg	pos	pos
T <sub>2</sub>	neg	pos	pos
T <sub>3</sub>	pos	pos	pos
T <sub>4</sub>	pos	pos	pos
T <sub>5</sub>	pos	pos	pos
T <sub>6</sub>	pos	pos	pos
T <sub>7</sub>	pos	pos	pos
T <sub>8</sub>	pos	pos	pos
T <sub>9</sub>	pos	pos	pos
T <sub>10</sub>	pos	pos	pos
N <sub>1</sub>	N.D.	N.D.	pos
N <sub>2</sub>	N.D.	N.D.	pos
N <sub>3</sub>	N.D.	N.D.	pos

**Table 4.3** Results for each tissue section as determined by the three different methods for matrilysin detection. W. blotting = western blotting, and immunohis. = immunohistochemistry. The results are positive (pos) or negative (neg). N.D. indicates that a particular sample was not analysed by that method.

#### 4.9 Evaluation of Methods

RT-PCR as a technique is relatively easy to perform although prevention of RNA degradation can be difficult to achieve. False positive results are often associated with this technique due to its extreme sensitivity, however, appropriate preventative methods should insure against this. The inclusion of primers for a constitutively expressed gene such as  $\beta$ -actin acts as an internal control and allows for comparisons to be made between samples. This can be important as starting the method with equal amounts of RNA does not necessarily mean that direct comparisons of RT-PCR products can be made. Degradation of the RNA and also variations from tube to tube in the efficiency of the reactions can influence the amount of product obtained.

The two protein methods, western blotting and immunohistochemistry, indicated positive expression of matrilysin in all samples analysed. These techniques were relatively easy to perform, although the requirements for suitable antibodies may limit their usefulness. Immunohistochemistry was the most informative method used as identification of the cells responsible for protein expression could be made. The antibody used in this study recognised only latent matrilysin enzyme, however, so determination of matrilysin activity in these breast tissue samples has not been achieved.

It should be mentioned that the ELISA for matrilysin was intended for use with the breast tumour study, however, as the quantity of tissue available was extremely small, the total amount of protein isolated was not enough for both western blotting experiments and the ELISA. It would be expected that the ELISA method would be suitable for large-scale analysis of clinical samples and it is hoped to use it in the future for this purpose. However, as the antibodies used in the ELISA only recognise latent matrilysin, additional information regarding the presence of active enzyme would not result from this method.

#### **4.10 Matrilysin as a Prognostic Marker in Breast Cancer**

The finding of matrilysin expression in all tissue samples - both normal and tumour and, indeed, in both normal and tumour components of the samples - suggests that it would be of little use as an indicator of tumour presence or grade in breast tissue. Investigation of its expression in tumours of other tissues, however, may prove beneficial. It has previously been shown that matrilysin mRNA expression is associated with colon tumour development (McDonnell *et al.*, 1991; Witty *et al.*, 1994; Newell *et al.*, 1994) but, to date, few other tumours have been investigated for matrilysin expression.

## Summary

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The aim in this chapter, was to analyse a small number of breast tumour samples for matrilysin expression using a variety of techniques. Tissue samples were obtained from ten breast tumours. RNA and protein was extracted from these samples and used for RT-PCR and western blotting experiments, respectively. Matrilysin expression was found in eight of the RNA isolates and all ten of the protein isolates. The slight disparity may be a result of RNA degradation.

In order to determine tissue localisation of the positive expression, formalin-fixed, paraffin-embedded sections of the tumour samples were cut onto slides. A rat monoclonal anti-matrilysin antibody RmAb, appeared to be a suitable reagent for immunohistochemistry and this technique was tried. All sections showed positive immunoreactivity in distinct areas. Staining was, in all cases, localised to epithelial cells. These could be tumour-derived or normal glandular epithelium. Three tissue sections from histologically normal breast tissue were also examined by this technique. Positive staining was observed in ductal epithelial cells. The matrilysin protein expression seen in these samples, both in tumour and normal tissue, confirms results obtained by other groups for matrilysin mRNA expression (Saarialho-Kere *et al.*, 1995; Heppner *et al.*, 1996). Similar expression at the protein level has also been seen by at least one other group (Prof. Lynn Matrisian, pers. comm.) Determination of the activity of the matrilysin enzyme expressed by both normal and tumour cells would prove interesting and may help to confirm a suggested function for matrilysin in normal glands *i.e.* to clean the gland of proteinaceous debris.

Of the three techniques used for matrilysin detection, immunohistochemistry was considered the most informative. The presence of matrilysin in all sections, both tumour and normal, suggested it is of limited value as a tumour marker in breast tissue. Investigation of matrilysin expression in tumours of other tissues may, however, prove valuable.

## **Chapter Five**

### **Effects of Cytokines on Expression of Matrilysin and Stromelysin-1 in Human Tumour Cell Lines**



## Introduction

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The term 'cytokine' has generally been used to describe the signalling molecules associated with the immune system, separating these from the mitogenic molecules, the growth factors, which act on different cell types. As the molecules in question are better understood, this division has become redundant as many so-called cytokines have growth factor activity and vice versa. For this reason, the term cytokine, as used here, defines a soluble polypeptide which acts nonenzymatically in picomolar to nanomolar concentrations to regulate cellular function (Nathan and Sporn, 1991).

### 5.1 Historical Context

Cytokine research began in the 1930s when investigators demonstrated that macrophage migration was inhibited in cultures of antigen-treated lymphoid tissues. The molecule responsible for this antigen-induced inhibition of phagocyte migration was not identified for over thirty years when two groups independently demonstrated the presence of a soluble factor named migration inhibitory factor or MIF (Adelman *et al*, 1979). In the intervening period, other regulatory molecules such as nerve growth factor and interferon had been discovered (Nathan and Sporn, 1992). Over the following years, similar types of experiments where different immune system cells were isolated and treated with supernatants from other cells so modulating their function in some way, were performed (Mizel, 1989). In this way, numerous activities were described and named for the responses they had produced. As all the activities described had a connection with lymphocytes, the term 'lymphokine' was coined to describe them (Barth *et al*, 1987). A large number (over 100) of different activities were described with names such as antibody inhibitory material (AIM) or specific macrophage arming factor (SMAF) (Mizel, 1989). As some of the molecules responsible for these activities were purified, they were found to have a number of different effects *i.e.* exhibited pleiotropic behaviour. In truth, however, the

preparations were often impure so that some activities ascribed to certain molecules were due to the presence of contaminants. Only with the advent of recombinant DNA technology could the various activities be separated and definitely assigned to particular molecules (Hamblin, 1988). In 1979, the term 'interleukin' was adopted to describe molecules that served as communication links between leukocytes (Mizel, 1989). The first two interleukins, interleukin-1 and -2 (IL-1, IL-2) were designated to the various activities affecting T-cell proliferation and activation, IL-1 being derived from macrophages and IL-2 from T-cells themselves. This refinement of terminology and reclassification of these molecules began a period of intense research leading eventually to the use of recombinant IL-2 in a clinical setting for various cancers and AIDS (Kaplan *et al*, 1992). The actual number of cytokines characterised has also increased rapidly. There are now 15 known interleukins, each responsible for a specific set of activities and acting through a particular receptor.

The perception that other signalling molecules from the fields of virology (interferons), haematology (colony stimulating factors, CSFs) and cell biology (growth factors) were also important in immunology led to the gradual replacement of 'lymphokine' with 'cytokine'. It is only recently that the diversity of activity and cellular origin demonstrated by most cytokines has been realised (Nathan and Sporn, 1992) It is this diversity that has prompted us to examine the effects of what were previously thought of as immune system molecules on other processes.

## **5.2 Cytokine Functions and Mode of Action**

In the immune system, where their activities have been most studied, the various cytokines act differently and synergistically in a hierarchical manner (Sedlacek and Möröy, 1995). The concentration and combination of cytokines acting on a cell are decisive for its proliferation, differentiation and function. In order to provoke a specific response, cytokines have to bind to and activate specific receptors on the membranes of target cells. Due to the large number of cytokine molecules that have been characterised, it is impossible to describe them all in detail. The following is a brief

survey of the various cytokines used in the course of the investigations described later in this chapter, their receptors and activities.

**Interleukin-1** : IL-1 was first discovered in the 1940s and named endogenous pyrogen for its ability to cause fever. It has also been known under various other names such as leukocyte endogenous mediator, lymphocyte activating factor, catablin, osteoclast-activating factor and haematopoitin-1 (Dewhirst *et al*, 1985; Dinarello, 1988; Akira *et al*, 1990). There are two distinct IL-1 molecules, IL-1 $\alpha$  and IL-1 $\beta$ , coded for by separate genes. Although they only share 25% homology at the amino acid level, both forms bind to the same receptor (Barth *et al*, 1987) and are, therefore, usually referred to as the one protein, IL-1. Both proteins are synthesized as 31 kDa inactive precursor molecules which must be enzymatically cleaved to yield a 13 - 17.5 kDa mature protein (Mizel, 1989). This cleavage is achieved intracellularly by interleukin-converting enzyme (ICE) or extracellularly by plasmin or elastase (Sedlacek and Möröy, 1995). A proportion of activated IL-1 remains membrane-bound and may explain its ability to participate in autocrine and paracrine events without inducing the systemic effects that occur when mature IL-1 gains access to the circulation. Although initially described as a product of activated phagocytes, IL-1 has since been shown to be produced by a wide variety of cell types including synovial fibroblasts, keratinocytes, mesangial cells of the kidney, astrocytes and microglial cells of the brain, vascular endothelial and smooth muscle cells and corneal, gingival and thymic epithelial cells (Dinarello, 1988). This truly pleiotropic molecule has a myriad of effects both in specific cells *in vitro*, and systemically *in vivo*. Some of the more prominent activities are summarised in Table 5.1.

The induction of fever is due to the effects of IL-1 on the hypothalamic thermoregulatory centre. The release of several hypothalamic and pituitary peptides such as endorphins and adrenocorticotrophin (ACTH) can also be induced by this cytokine. As well as stimulating ACTH production, IL-1 also acts directly on the adrenal gland to upregulate steroid synthesis. The results of IL-1 treatment on the liver include the depression of liver cytochrome P450-dependent drug metabolism, reduction of hepatic albumin production with an increase of other hepatic proteins resulting in the

acute phase response (Abbas *et al*, 1991). The ability of IL-1 to initiate prostaglandin synthesis is perhaps one of its most important biological properties, accounting for many local and systemic effects (Dinarello, 1988). The induction, by IL-1, of prostaglandins, collagenase and collagen in synovial fibroblasts is responsible for the pain, degradation and scar formation leading to restricted movement in arthritic joints.

**Table 5.1 : Effects of IL-1**

Type of Effect	Examples
<i>In vitro</i> : Cell Growth	Fibroblast, keratinocyte, mesangial and glial cell proliferation
<i>In vitro</i> : Inflammation	Increased synthesis of collagen and procollagenase Bone resorption Prostaglandin E <sub>2</sub> synthesis in synovial fibroblasts
<i>In vitro</i> : Cytotoxic	Causes cell death in tumour cells , $\beta$ islet cells and erythrocytes
<i>In vitro</i> : Metabolic	Increases intestinal mucus production Decreases hepatic albumin transcription
<i>In vitro</i> : Leukocytes	Chemotaxis of B and T lymphocytes Basophil histamine release Neutrophil and monocyte thromboxane synthesis
Systemic : Central Nervous System	Fever Sleep Decreases appetite Stimulates ACTH release
Systemic : Metabolic	Increases hepatic proteins Decreases cytochrome P450 activity
Systemic : Vascular wall	Capillary leak syndrome Hypotension Increases prostaglandin synthesis
Systemic : Haematologic	Neutrophilia Lymphopenia Increases bone marrow growth factors

Compiled from Dinarello, 1988; Sedlacek and Möröy, 1995; Akira *et al*, 1990

Modulation of IL-1 levels is achieved physiologically by down-regulation of receptor levels by IL-1 itself and by the presence of a natural antagonist, interleukin receptor antagonist (IL-RA). IL-RA is a 17.5 kDa protein produced by monocytes, B lymphocytes, endothelial cells and keratinocytes which has no biological activity of its own (Sedlacek and Möröy, 1995). It binds to the same receptor as IL-1 although at a slower rate and must be present in 100-fold excess to interfere with IL-1 binding. As a pure antagonist, IL-RA has been investigated as a therapeutic agent and is in clinical development for use in septic shock, rheumatoid arthritis, graft versus host disease, asthma and inflammatory bowel diseases. Currently, the therapeutic modulators of IL-1 activity most often used in chronic inflammatory conditions are the corticosteroids (Sedlacek and Möröy, 1995).

A high-affinity IL-1 receptor has been cloned from an expression library and characterised (Akira *et al*, 1990). The receptor has three domains : an extracellular of 319 amino acids; transmembrane of 20 amino acids; and cytoplasmic of 213 amino acids. The extracellular portion possesses three immunoglobulin-like motifs showing that the IL-1 receptor belongs to the immunoglobulin superfamily. The intracellular portion has no significant homology with any known proteins although there is evidence for intrinsic G protein activity (Sedlacek and Möröy, 1995).

**Interleukin 2** : IL-2 is a 15 kDa protein exclusively produced by antigen-stimulated T lymphocytes (Sedlacek and Möröy, 1995). It would appear to be a true immune system molecule responsible for the proliferation and differentiation of lymphocytes via specific cell surface receptors. These receptors are constitutively expressed by natural killer (NK) cells whereas B and T cell receptor expression is dependent on antigenic and IL-1 stimulation (Barth *et al*, 1987). In addition to its cellular proliferative function, IL-2 stimulates the release of other cytokines, interferon (IFN)- $\gamma$ , GM-CSF and tumour necrosis factor (TNF) from NK cells and IL-1, IL-6, IL-8 and TNF from monocytes. It is also implicated in antibody production from activated B lymphocytes (Abbas *et al*, 1992). Due to its ability to stimulate cytotoxic T lymphocytes (CTLs) and NK precursors resulting in lymphokine-activated killer (LAK) cells, IL-2 has been used in tumour immunotherapy (Rosenberg *et al*, 1987) and AIDS treatment (Kaplan *et al*,

1992). When used alone in high doses, IL-2 appears associated with severe side-effects *e.g.* vascular leak syndrome, and few beneficial clinical responses. At lower doses and in combination with adoptive immunotherapy *i.e.* the administration of immune effector cells such as LAKs or CTLs, beneficial responses have been observed (Rosenberg *et al*, 1987; West *et al*, 1987) although some adverse reactions still occur. Current developments include the use of tumour infiltrating lymphocytes (TILs) genetically manipulated to deliver IL-2 and other cytokines directly to the tumour (Russell, 1990; Rosenberg, 1992). IL-2 has also been used to treat severe combined immunodeficiency (SCID) which may be the result of an IL-2 deficiency (Kaplan *et al*, 1992).

IL-2 interacts with target cells through binding of a specific receptor complex (Smith, 1989). This high affinity receptor is composed of at least two chains, one of 75 kDa molecular weight which has a large cytoplasmic domain of 286 amino acid residues and the other of 55 kDa molecular weight which has only a cytoplasmic 'anchor' of 13 amino acids (Kaplan *et al*, 1992). The two chains can function separately as low or intermediate receptors although only the 75 kDa chain has an intracellular signalling domain. This suggests that similar biochemical results are obtained when IL-2 binds to the p75/p55 dimer as when it binds to the p75 chain alone. The important difference is the affinity which is 100-fold higher in the case of the dimer (Kaplan *et al*, 1992). The constitutively-expressed IL-2 receptor on NK cells is composed of the p75 chain alone, the high affinity dimer resulting only from antigenic stimulation (Hamblin, 1988).

**Interleukin 6** : IL-6 is a glycoprotein with an  $M_r$  of 25 - 30 kDa, size differences being due to differential glycosylation (Mizel, 1988). It is a multipotent cytokine exerting numerous biological activities. These include the regulation of proliferation and differentiation of T and B lymphocytes, NK cells and normal haematopoietic progenitors as well as some epithelial and neural cells (Blay *et al*, 1992). One of its major functions is the induction of antibody production by B cells (Hutchins *et al*, 1990) and, because of this, it is often used as a medium supplement for hybridomas. Other activities include promotion of angiogenesis, particularly in the ovary and uterus, weak antiviral activity (hence the alternative name interferon  $\beta$ ) and,

synergistically with IL-1, induction of acute phase proteins from hepatocytes (Sedlacek and Möröy, 1995). It is produced principally by monocytes but also by a wide range of other cells such as lymphocytes, fibroblasts, bone marrow stromal cells, endothelial cells, microglia cells, chondrocytes and osteocytes (Mizel, 1989). Like IL-1 and TNF, IL-6 is found in the synovial fluid from joints of patients with rheumatoid arthritis (Akira *et al*, 1990). It is thought that IL-6 may participate with IL-1 in fine-tuning the catabolism of connective tissue components by modulating the balance between degradative enzymes and their inhibitors (Ito *et al*, 1992).

IL-6 is constitutively produced by a variety of solid tumours. For this reason it has been examined as a prognostic indicator of metastatic renal cell carcinoma (Blay *et al*, 1992). IL-6 levels were found to correlate with C reactive protein, an acute phase protein. Either or both of these factors were prognostic with high levels indicating poor survival and a poor response rate to IL-2, an experimental treatment for renal cell carcinoma. The same results were also obtained in a study of patients with metastatic melanoma (Tartour *et al*, 1994). A study of patients with various lung carcinomas indicated that high levels of IL-6 along with GM-CSF, G-CSF or M-CSF were indicative of poor survival (Katsumata *et al*, 1996). Studies of the effects of IL-6 on tumour growth in cell lines have been contradictory. Serve *et al* (1991) tested the effect of different levels of exogenous IL-6 on the growth of twenty six different human cell lines derived from solid tumours and found no effect. To test the possibility of autocrine growth stimulation by IL-6, neutralizing antibodies were used and, again, no effect on growth was observed. Another study suggested that in cell culture, IL-6 enhances, inhibits or has no effect on the proliferation of epithelial cells depending on the cell type examined (Krueger *et al*, 1991). The proliferation of keratinocytes was enhanced while breast carcinoma cell line growth was inhibited. The breast carcinoma cells exhibited a major change in phenotype for as long as IL-6 was present in the culture medium. These researchers observed that the phenotypic changes resulting from IL-6 exposure were similar to the changes that particular groups of epithelial cells undergo during embryogenesis and suggest a possible role for IL-6 in embryonic development. The contradictory observations on the effects of IL-6 on epithelial cell

growth may be due to the different stages of tumours examined. A study of melanoma lesions treated with IL-6 from dermal fibroblasts showed that early stage, metastasis-incompetent lesions were growth inhibited by the cytokine whereas later, metastatic lesions were unaffected or even stimulated by IL-6 (Lu *et al*, 1992). It is thought that as the tumour cells progress, they lose their dependence on IL-6 in a similar manner to the loss of oestrogen-dependence by breast cancer cells. Unlike the breast cancer cells however, the melanoma cells retain receptors and may exhibit proliferation in response to autocrine stimulation. Other studies have shown that breast cancer cells can also be inhibited by IL-6 (Danforth and Sgagias, 1993) perhaps due to down-modulation of ER. When transfected with *int-2*, a weakly transforming event, breast carcinoma cells can be stimulated by the cytokine, however this growth stimulatory effect is lost as the cancer progresses (Basolo *et al*, 1993). IL-6 upregulates expression of the adhesion molecule ICAM-1 (CD 54) on breast cancer cells (Hutchins and Steel, 1994). As this molecule appears to be a marker of well-differentiated cells (Kaiserlian *et al*, 1991), investigators have suggested that the inhibitory effects of IL-6 are mediated through it.

There is a network of cytokines whereby expression of a particular cytokine is influenced by the activity of others. IL-6 is potently induced by IL-1 and TNF, which are also pro-inflammatory cytokines. Production of IL-6, like IL-1 and TNF, can be super-induced by cycloheximide treatment suggesting that the regulation of cytokine production is under the control of labile repressor proteins (Akira *et al*, 1990). Dexamethasone and other glucocorticoids can suppress production of these three cytokines. Many cytokines, including IL-6, contain AU-rich sequences in the 3' untranslated regions of the transcribed genes which are thought to be involved in mRNA instability (Sen and Lengyel, 1992; Crawford and Matrisian, 1995). This ensures a short half-life which is important as cytokines are potent at extremely low concentrations.

The 80 kDa IL-6 receptor, like many other cytokine receptors, has four cysteine residues at the amino terminal region and a tryptophan-serine-X-tryptophan-serine motif in the region just external to the plasma membrane (Abbas *et al*, 1992). Although conserved in members of the cytokine receptor family, the functional



significance of these regions is unknown. The IL-6 receptor also has an immunoglobulin-like domain at the amino terminus, making it a member of the immunoglobulin superfamily. The relatively short, 82 amino acid, intracellular domain of the IL-6 receptor can be removed without affecting its activity (Akira *et al*, 1990). This has been found to be possible due to the presence of a 130 kDa membrane glycoprotein (gp130) which associates with the IL-6 receptor and mediates the signal transduction via cellular tyrosine kinase and protein kinase activities (Sedlacek and Möröy, 1995). The IL-6 receptor and gp130 do not form a dimer as such as they only become associated at their extracellular portions after the receptor is stimulated with IL-6 (Akira *et al*, 1990).

**Interferon -  $\gamma$** : Interferon- $\gamma$  (IFN- $\gamma$ ) is an antiviral and antiproliferative protein which also has potent immunoregulatory effects on a variety of cells (Hamblin, 1988). It was first isolated in 1957 as a protein that protected cells from the effects of viruses and was named 'interferon' because it interfered with the replication of the viruses by somehow changing the cells (Isaacs and Lindemann, 1957). Although the protein is only produced by activated T cells and NK cells, IFN- $\gamma$  receptors are present on almost every cell type except erythrocytes (Sedlacek and Möröy, 1995).

Mature IFN- $\gamma$  is a 166 amino acid protein with an  $M_r$  of 20 - 25 kDa depending on glycosylation (Hamblin, 1988). Although the detection of low levels in the absence of a specific inducer has been reported (Sen and Lengyel, 1992), *in vivo* IFN- $\gamma$  synthesis results from antigenic stimulation while *in vitro*, non-specific T cell inducers such as concanavalin A can be used. IL-2 is also implicated in the synthesis of IFN- $\gamma$  from NK cells (Barth *et al*, 1987). Double-stranded RNA is a potent inducer and is thought to be the IFN- $\gamma$ -inducing intermediate produced during virus replication (Sen and Langyel, 1992). IFN- $\gamma$ , like IL-6 and other cytokines, has destabilising sequences in its mRNA which ensure a short half-life.

IFN- $\gamma$  has also been known as 'immune interferon' due to its immunoregulatory functions. It is responsible for inducing the expression of major histocompatibility complex (MHC) class I and class II encoded proteins involved in antigen recognition

and presentation, increasing the cytotoxicity of NK cells and macrophages, enhancing TNF cytotoxicity and regulating immunoglobulin isotypes secreted during the humoral immune response (Sedlacek and Möröy, 1995). IFN- $\gamma$  can selectively inhibit the expression of several mitochondrial genes which may contribute to the anti-proliferative effects of this cytokine (Sen and Lengyel, 1992). The anti-viral, immunomodulatory and anti-proliferative functions of IFN- $\gamma$  and its sister molecule IFN- $\alpha$ , have led to their use as therapeutic agents in a range of diseases including hairy cell leukaemia, Kaposi's sarcoma, hepatitis B and C infections and chronic granulomatous disease (Henderson, 1993).

The IFN- $\gamma$  receptor is a 90 kDa membrane-bound high affinity receptor which contains two cysteine-rich domains (Hamblin, 1988). Upon binding, IFN- $\gamma$  induces phosphorylation of serine-threonine residues on the receptor which begins the signal transduction pathway (Sen and Lengyel, 1992). The ligand-receptor complex is rapidly internalised (Hamblin, 1988), dissociated, dephosphorylated and recycled ensuring a constant presence of IFN- $\gamma$  receptors on cells (Sedlacek and Möröy, 1995).

**Epidermal Growth Factor** : Epidermal growth factor (EGF) is a 6 kDa, 55 amino acid polypeptide produced by a wide range of cells (Sedlacek and Möröy, 1995). It is found in nearly all body fluids ( blood, milk, saliva, urine, sweat etc.) and in multiple organs and tissues. When first isolated from mouse submaxillary glands, it was shown to quicken the development of newborn mice and is now implicated in growth and differentiation during foetal development (Waterfield, 1985). It has a major role in angiogenesis and wound healing and has even been developed as an ointment for use with burns victims (Mutsaers and Laurent, 1995). EGF shares 40% homology with transforming growth factor (TGF)- $\alpha$  and both proteins bind to the same cellular receptor (Waterfield, 1985). Growth factors such as EGF cause quiescent cells in the resting or G<sub>0</sub> phase of the cell cycle to advance through the G<sub>1</sub> and DNA synthesis phases of the cycle resulting in mitosis and cell proliferation (Aaronson, 1991). Transition through the G<sub>1</sub> phase requires sustained growth factor stimulation over a period of several hours.

The EGF receptor (EGFr) is perhaps the best-characterised growth factor receptor due to the discovery that the A431 carcinoma cell line overexpresses large amounts of it making it easily available for study (Waterfield, 1985). It is a membrane-spanning glycosylated molecule of  $M_r$  170 kDa. Upon binding one molecule of EGF, the receptor dimerizes and an intrinsic receptor tyrosine kinase is activated resulting in autophosphorylation (Sedlacek and Möröy, 1995). A feedback mechanism which attenuates this tyrosine kinase activity results from the action of intracellular protein kinase C on a threonine residue of the EGFr (Hardie, 1991). This feedback mechanism is mediated by the second messenger diacylglycerol generated through EGFr activity. A second signal attenuation mechanism results from down-regulation of EGFr on the cell surface as a consequence of EGF binding (Gilligan *et al*, 1990). The EGFr is the product of the *c-erbB2* proto-oncogene (Hardie, 1991). A truncated form lacking an extracellular ligand-binding domain is the product of the *v-erb* oncogene and results in constitutive EGF-like responses in transformed cells. The *neu* proto-oncogene product appears very similar to the EGFr and its oncogenic version which carries a single point mutation in the transmembrane region results in enhanced cellular transformation (Aaronson, 1991). The EGFr or *c-erbB2/neu* gene product is frequently overexpressed in tumours and has been proposed as a prognostic marker in breast and ovarian cancers (Cerra *et al*, 1995; Scambia *et al*, 1995). Other EGR-related peptides which bind the EGFr include TGF- $\alpha$  and amphiregulin. A recent study showed that using antisense nucleotides directed against these peptides in conjunction with an EGFr-blocking antibody resulted in 80% growth inhibition of colon carcinoma cells (Normanno *et al*, 1996). Normally most epithelial cells express EGFr whereas cells of haematopoietic origin are EGFr-negative (Real *et al*, 1986), however, expression of EGFr is related to stage of cellular differentiation as well as cell lineage.

**Fibroblast Growth Factors** : The fibroblast growth factor (FGF) family presently consists of 7 members: acidic FGF (a-FGF) or FGF-1, basic FGF (bFGF) or FGF-2 and five additional recently-discovered FGF-like proteins, hst/ks, int-2, FGF-5, -6 and -7 (Mutsaers and Laurent, 1995). Acidic and basic FGF are very similar having the same  $M_r$  of 16-17 kDa and sharing 55% homology at the amino acid level (Sedlacek and

Möröy, 1995). Their names result from differences in isoelectric points. bFGF is an ubiquitous protein while aFGF is more specifically associated with brain, retina and bone matrix. Apart from proliferative signals to fibroblastic cells, the major function of a/bFGF is angiogenesis (Vile, 1995). This orderly migration and proliferation of blood vessels is important in embryonic development, wound healing and during the female reproductive cycle, all events where well-regulated neovascularization occurs. This is in contrast to the pathologic angiogenic activities associated with tumour development and other disorders. Both bFGF and aFGF are potent inducers of endothelial cell migration and proliferation and endothelial cells are the primary cell type involved in all forms of angiogenesis. *In vitro*, the FGFs are highly mitogenic for endothelial cells, while *in vivo*, picomolar concentrations only are required for angiogenesis (Vlodavsky *et al*, 1990). Despite bFGF being ubiquitous, endothelial cell proliferation is very low suggesting that bFGF is either inactive or sequestered. The latter suggestion appears to be the case with the ECM being the major storage depot for bFGF (Vlodavsky *et al*, 1990). This raises the possibility of invasive tumours being able to release bFGF as they degrade ECM and so constantly provide themselves with new sources of angiogenic factors as they continue to invade. bFGF also appears able to stimulate the release of plasminogen activators from endothelial cells (Sedlacek and Möröy, 1995). Plasminogen activators along with heparanase can cause the degradation of heparan sulphate, the ECM component specifically responsible for binding or sequestering bFGF (Vlodavsky *et al*, 1990). Therefore as more bFGF is released, more plasminogen activator is released resulting in further release of bFGF and greater angiogenic and proliferative potential.

FGFs can induce either proliferation or differentiation in endothelial cells depending on the binding of cells to ECM components such as fibronectin and collagen (Ingber and Folkman, 1989). The different results appear to stem from the activation of stretch-sensitive ion channels in the endothelial cells which are activated as cells adhere to matrix proteins.

There are two high affinity receptors for the FGFs, receptor I binds aFGF better than bFGF and receptor II binds bFGF more strongly (Sedlacek and Möröy, 1995).

Upon binding to either receptor, aFGF and bFGF are internalised and degraded in the lysosomal compartment. Both are slowly degraded to low molecular weight peptides which may persist in the cell for up to 24 hours. Like the EGFr, the FGF receptors are coded for by proto-oncogenes [*c-flg* and *c-bek*] and both have intrinsic tyrosine kinase activity (Aaronson, 1991).

**Insulin-like Growth Factors :** The insulin-like growth factors IGF-I and IGF-II are single chain polypeptides with structural homology to proinsulin (Mutsaers and Laurent, 1995). They have similar molecular weights (approximately 7.5 kDa) and share 70% homology. In serum these growth factors are bound to specific IGF binding proteins, the IGFBPs, of which there are six known so far (Sedlacek and Möröy, 1995). As IGFs are inactive when complexed, it is thought that these binding proteins are modulators of IGF activity (Mutsaers and Laurent, 1995). IGF-I and -II are also known as the somatomedins as they are secreted by the liver in response to the growth hormone somatotrophin (Hardie, 1991). The principal receptor for these factors, IGFr1 is widely distributed on hepatocytes, fibroblasts, lymphocytes, erythrocytes, chondrocytes, adipocytes, pituitary and neural cells. This distribution reflects the function of the IGFs as paracrine and autocrine growth factors for parenchymal cells (Sedlacek and Möröy, 1995). Physiologically, IGF-I is a regulator of cell proliferation while IGF-II appears to be important during foetal development (Macauley, 1992). IGF-I is apparently responsible for bone elongation during growth and serum levels of this cytokine in adolescent boys from pygmy races are only one third of the levels found in corresponding individuals from taller races (Hardie, 1991). IGF-II is widely expressed in mouse embryos but expression is progressively halted in virtually all tissues after birth (Christofori *et al*, 1994). In humans, however, rather high levels of IGF-II persist in adult serum but its function is unclear (Rogler *et al*, 1994). *Igf-II* is an example of an imprinted gene, that is one in which one allele is silenced so that all transcription takes place from the allele contributed by one parent only. In the case of *Igf-II*, the paternal allele only is active during development in mouse embryos (Macauley, 1992).

As they are potent mitogens for a wide variety of cell types, it is not surprising that the IGFs have been identified as major paracrine or autocrine growth factors in a number of cancers (Westley and May, 1995). There is also evidence that the IGFs have a role to play in the actual transformation process itself. Transgenic mice in which expression of IGF-II was targeted to the mammary gland by placing it under the control of the tissue specific  $\beta$ -lactoglobulin promoter, developed an excess of mammary tumours (Bates *et al*, 1995). Another group made transgenic mice in which IGF-II expression was targeted to the liver under the control of the major urinary protein promoter (Rogler *et al*, 1994). In this case, mice developed diverse tumours with a preponderance of hepatocellular carcinoma. A third study examined the oncogenic activity of the SV40 large T antigen under the control of the insulin promoter in transgenic mice (Christofori *et al*, 1994). At first no aberrant proliferation was seen but by 14 weeks of age, formation of highly vascularised solid tumours was observed in 1 - 2% of islets in the pancreas. The initial proliferative switch was seen to correlate with local activation of IGF-II. *In vitro*, transfection of tumour cells with antisense oligonucleotides directed against IGF-II mRNA interfered with tumour development while transgenic mice in which the IGF-II gene was ablated developed tumours with reduced malignancy. Similarly, in human breast tumours, expression of IGF-II in stromal fibroblasts appears to correlate with malignancy (Cullen *et al*, 1991). As a further test of the tumour developing function of the IGFs, Long *et al* (1995) directed antisense oligonucleotides against the IGF1r rather than the growth factors themselves. Tumour cells transfected with the antisense oligos, when injected into mice lost the ability to produce metastases compared to controls.

IGFs can bind to three different receptors : the insulin receptor, IGF1r and IGF2r. Of these IGF1r appears to be the receptor of highest affinity for these cytokines (Mutsaers and Laurent, 1995). Insulin can also bind to IGF1r although the affinity is 100-fold less than between the receptor and the IGFs (Sedlacek and Möröy, 1995). IGF1r is a tetrameric  $\beta\alpha\beta$  structure with two transmembrane  $\beta$  chains joined via disulphide bridges to two extracellular  $\alpha$  domains also joined to each other by a disulphide link (Hardie, 1991). The  $\beta$  chains have intrinsic tyrosine kinase activity.

although not identified as proto-oncogene products themselves, the  $\beta$  chains can act as substrates for the *src* oncogene product, an intracellular tyrosine kinase (Westley and May, 1995).

**Transforming Growth Factor  $\beta$**  : There are five known isoforms of TGF- $\beta$ , 3 of which have been found in mammalian tissues (TGF- $\beta_{1, 2, 3}$ ), one in avian tissues (TGF- $\beta_4$ ) and one in *Xenopus* (TGF- $\beta_5$ ) (Walker *et al*, 1994). The mammalian isoforms share approximately 80% homology. TGF- $\beta$  was initially isolated from platelets but was subsequently found to be expressed by almost all cells and is secreted in a latent form which is unable to bind to its receptor (Mutsaers and Laurent, 1995). Activation of pro-TGF- $\beta$  is achieved by proteinase [e.g. plasmin] action (Sedlacek and Möröy, 1995). TGF- $\beta$  is a potent growth inhibitor for most cell types although proliferative effects have been observed in osteoblasts and uterine lining epithelial cells (Mutsaers and Laurent, 1995). It can effect entry into the differentiation pathways in many cell types including fibroblasts, mesenchymal cells into bone cells and chondrocytes and bronchial epithelia into squamous cells (Sedlacek and Möröy, 1995). In contrast it can inhibit differentiation of mucoblasts and adipose cells. TGF- $\beta$  is a key regulator of the inflammation process, involved in both its initiation by stimulating monocyte migration and growth factor production, and its resolution by inhibiting immune cell adhesion to the vascular endothelium, down-regulating macrophage functions and antagonizing the actions of the pro-inflammatory cytokine TNF (Mauviel *et al*, 1993a). TGF- $\beta$  has a major role in wound healing being associated with increased tensile strength of a healing wound due primarily to its stimulating effect on ECM production (Mutsaers and Laurent, 1995). Apart from its potent stimulatory effects on fibronectin, procollagen I and III, elastin and tenascin, TGF- $\beta$  also induces the matrix proteoglycan decorin. Decorin can bind TGF- $\beta$  and neutralise its biological activity suggesting that it is a natural regulator. Apart from stimulating ECM formation directly, TGF- $\beta$  also has an inhibitory effect on ECM degradation by down-regulating proteinase production and upregulating proteinase inhibitor expression (Edwards *et al*, 1987).

TGF- $\beta$  was named for its ability to induce the phenotypic transformation of fibroblast cell lines (Derynck, 1994) so its role in cancer development should not be surprising. The relationship of TGF- $\beta$  to altered ECM expression and its effect on disease progression has been examined in invasive breast carcinoma using immunohistochemistry (Walker *et al*, 1994). Prominent immunoreactivity for TGF- $\beta$  was associated with lymph node metastasis, increased cellular fibronectin, more prominent tenascin and the presence of tumour associated macrophages and T lymphocytes indicating that TGF- $\beta$  may have a role in invasion and metastasis of breast carcinomas.

TGF- $\beta$  binds with high affinity to most cell types and to date at least nine receptor/binding activities have been identified although most of the activity appears to be mediated through type I and type II receptors (Mutsaers and Laurent, 1995). These receptors belong to a family of serine/threonine kinase receptors and have a short extracellular domain and a long cytoplasmic region that consists largely of the kinase domain (Derynck, 1994). An intrinsic tyrosine kinase activity has also been attributed to the type II receptor. Although very little is known about the intracellular events that follow TGF- $\beta$  treatment, it is thought the signalling mechanisms in cells stimulated to proliferate by this cytokine are different to those in cells that are growth arrested. In proliferating cells, TGF- $\beta$  appears to upregulate other growth factors such as platelet derived growth factor (PDGF) or receptors such as EGFr whereas in epithelial cells which are growth inhibited, TGF- $\beta$  appears to arrest the cell cycle in the G<sub>1</sub> phase (Derynck, 1994).

The preceding pages have served to introduce the general activities of a number of different cytokines. The specific effects of some of these molecules on MMP and TIMP expression will now be reviewed.



### 5.3 Cytokines and Metalloproteinases

As mentioned in chapter one, the expression of various members of the MMP and TIMP families can be modulated by a wide range of molecules. Table 1.2 listed the inhibitory or stimulatory effects of a number of these molecules on MMP expression. Amongst those molecules, the cytokines are perhaps the most important as they are physiologically relevant and their presence has been demonstrated in areas where MMP activity is manifested (Ito *et al*, 1992).

The proinflammatory cytokines IL-1, IL-6 and TNF are particularly associated with rheumatoid arthritis, an autoimmune disorder characterised by chronic inflammation (Abbas *et al*, 1991). It has been demonstrated that IL-1 in particular can upregulate interstitial collagenase and stromelysin-1, enzymes associated with this disease (MacNaul *et al*, 1990). The stimulatory effect of IL-1 could be further enhanced in a synergistic manner with TNF. The effects of IL-1 on stromelysin-1 and interstitial collagenase can also be improved by IL-6 which, on its own, has no effect on these MMPs in synovial fibroblasts (Ito *et al*, 1992). IL-1 and TNF appear to have no effect on TIMP-1 levels and are thus responsible purely for an increase in degradation in rheumatoid synovium (MacNaul *et al*, 1990). IL-6 on the other hand, does enhance the production of TIMP-1 by synovial fibroblasts (Ito *et al*, 1992). However the levels of TIMP-1 stimulated by IL-6 were far lower than the levels of interstitial collagenase and stromelysin-1 induced by IL-1 in conjunction with IL-6. The induction of TIMP-1 by IL-6 has been demonstrated in other cell types also. In human dermal fibroblasts, TIMP-1 production was enhanced by IL-6 whereas interstitial collagenase and stromelysin-1 were unaffected (Sato *et al*, 1990). The stimulation of TIMP-1 by IL-6 in hepatocytes both *in vitro* and *in vivo* has also been demonstrated (Kordula *et al*, 1992). As IL-6 is the principal mediator of the acute phase response which results in the enhanced synthesis of various liver proteins including the general anti-proteinase  $\alpha_2$ -macroglobulin, its effect on TIMP-1 should not be surprising. To determine whether the effect of IL-1 on the arthritis-associated MMPs was mediated through prostaglandins, the principal effectors of IL-1 activities, the direct effects of PGE<sub>2</sub> on

MMP production in fibroblasts has been examined (Mauviel *et al*, 1994). Unlike IL-1, PGE<sub>2</sub> did not affect stromelysin-1 expression although it did elevate levels of interstitial collagenase in a dose-dependent manner. The stimulatory action of PGE<sub>2</sub> on interstitial collagenase showed no additive or synergistic effects with IL-1. A previous study also showed that the IL-1-induced MMP expression could not be mediated through prostaglandins as the prostaglandin-inhibitory agent indomethacin had no effect on the stimulation attributed to IL-1 (MacNaul *et al*, 1990).

The effects of IL-1 and TNF as well as the potent tumour promoter 12-O-tetradecanoyl-phorbol-13-acetate (TPA) on MMP expression in a wide range of cell lines were comprehensively investigated by Mackay *et al* (1992). Gelatinase A and TIMP-2 activities were not affected by the cytokines or TPA in most cell lines. Stromelysin-1, gelatinase B and TIMP-1 stimulation in response to IL-1 or TNF treatment was observed in many tumour cell lines and in normal human umbilical vein endothelial cells (HUVECs). As expected a greater stimulation was produced by TPA. In the case of A549, a lung carcinoma cell line, TNF stimulated gelatinase B but not TIMP-1. This type of discoordinate induction is thought to cause the imbalance in proteinase and inhibitor activities which favours ECM degradation by invasive tumour cells. TNF has also been shown to upregulate secretion of gelatinase B by a promyelocytic cell line, HL-60 (Ries *et al*, 1994). Basal secretion of the proteinase was reduced after a TNF-neutralizing antibody was added to the cells suggesting that the constitutive production of gelatinase B exhibited by these cells can be attributed to autocrine stimulation by TNF. This type of activity may be important in regulating the invasive ability of leukaemia cells.

The effects of TGF- $\beta$  have previously been mentioned - it stimulates the synthesis of ECM components and appears to inhibit proteinases. This effect on proteinases has been demonstrated in cultures of human fibroblasts where levels of interstitial collagenase decreased over a number of days as the cultures were exposed to TGF- $\beta$  (Overall *et al*, 1989). In the same cultures, however, the synthesis of gelatinase A was increased almost two-fold. TIMP-1 was also stimulated in these cultures. The authors of this study suggest that the gelatinase A may be required for ECM

remodelling after increased matrix protein deposition caused by TGF- $\beta$ . TGF- $\beta$  also increases TIMP-1 in hepatocytes in a similar manner to IL-6 in these cells (Kordula *et al*, 1992). In cervical epithelial cell lines which are growth inhibited by TGF- $\beta$ , levels of both gelatinase A and B are upregulated in response to the cytokine (Agarwal *et al*, 1994).

The uterine endometrium is one of the few areas in human adults where extensive tissue remodelling regularly occurs. This remodelling has been found to be principally due to the MMPs stromelysin-1 and -2 and matrilysin (Osteen *et al*, 1994). While the stromally-produced stromelysins could be directly modulated by hormones, matrilysin, as an epithelial-specific MMP, was found to require an additional factor to mediate its suppression by the hormone progesterone. TGF- $\beta$  has been shown to be produced in response to progesterone and to inhibit matrilysin production by endometrial cells (Bruner *et al*, 1995). Matrilysin is also produced by developing phagocytes (Busiek *et al*, 1992). Its production by these cells does not appear to be affected by TGF- $\beta$  or by the pro-inflammatory cytokines IL-1, IL-6 and TNF, however inhibitory effects have been attributed to IL-4, IL-10 and IFN- $\gamma$ , all inflammation-modulating cytokines (Busiek *et al*, 1995). It should be noted that IL-4 and IFN- $\gamma$ , although both inflammation-modulating, usually have antagonistic effects. In contrast, matrilysin produced by the mesangial cells of the kidney does appear to be stimulated by IL-1 and TNF (Marti *et al*, 1992). This variation in responsiveness suggests the activity of tissue-specific factors within the various matrilysin-producing cell types.

EGF is stimulatory for a number of MMPs in various cell types. In fibroblasts, the induction of stromelysin-1 by EGF has been shown to occur via the intracellular second messenger protein kinase C and specific nuclear transcription proteins Fos and Jun (McDonnell *et al*, 1990). Stromelysin-1 and interstitial collagenase induction in fibroblasts by EGF is also due, in part, to increased stability of the MMP mRNA (Delaney and Brinckerhoff, 1992). The delineation of intra-cellular signalling pathways for MMPs responding to various other cytokines has not, in many cases, been carried out. This topic will be further discussed in the introduction to chapter six. Oesophageal cell lines have also been shown to respond to EGF treatment by upregulating

production of interstitial collagenase, stromelysin-1 and the gelatinases A and B (Shima *et al*, 1993).

IFN- $\gamma$  can stimulate the production of interstitial collagenase and stromelysin-1 but not TIMP-1 from cultured keratinocytes (Tamai *et al*, 1995).

From the above studies, it would appear that interstitial collagenase and stromelysin-1 are the most readily-inducible MMPs that can be produced by a range of different cell types. Matrilysin shows a very variable stimulation pattern depending on the cell type. While the effects of a number of different cytokines on a number of different MMPs in different cell types have been examined, there are not many studies where all these variables have been investigated together. The aim of the work presented in this chapter was to evaluate the effects of a range of different cytokines [IL-1, IL-2, IL-6, IGF-I, IGF-II, EGF, bFGF, TGF- $\beta$  and IFN- $\gamma$ ] on matrilysin and stromelysin-1 production in a range of different cell lines. Stromelysin-1 represents an apparently readily-inducible MMP while matrilysin represents a tissue-specific MMP. This also serves as an introduction to our work of dissecting the control elements of the matrilysin promoter which is discussed further in chapter six.

## Results and Discussion

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### 5.4 Cell Lines Used

A range of tumour cell lines representing different tissue types were used in this investigation. The cell lines used and their origins are outlined in Table 5.2.

Cell Line	Origin
SW480	Colon adenocarcinoma - primary tumour
SW620	Colon adenocarcinoma - lymph node metastasis
LoVo	Colon adenocarcinoma
A549	Lung carcinoma
K562	Chronic myeloid leukaemia
EJ	Bladder carcinoma
MCF-7	Breast carcinoma
Go-GCCM	Brain anaplastic astrocytoma

**Table 5.2** Cell lines used and the tumours from which they were initially derived.

### 5.5 Basal Expression levels of stromelysin-1 and matrilysin

Prior to examining the effects of cytokines on stromelysin-1 and matrilysin expression in these cell lines, their basal expression levels were determined. This was achieved by culturing the cell lines for at least two passages (see sections 2.2.9.1/2), before removing serum and culturing for 24 hrs in serum-free media. The cells were harvested and total RNA extracted as outlined in section 2.2.11.1. The expression of specific matrilysin and stromelysin-1 mRNAs was analysed using the Reverse Transcriptase -

Polymerase Chain Reaction (RT-PCR) (section 2.2.12). Briefly, the mRNA was primed with oligo d(T) primers which bound specifically to the polyadenylated 'tail' of mature mRNA. Using a viral reverse transcriptase enzyme, the primed mRNA was converted to cDNA. An aliquot of the cDNA was then mixed with the enzyme Taq polymerase, specific primers recognising target genes and nucleotides. The reaction mixture was then subjected to thirty cycles of different temperatures chosen for optimal amplification of the target DNA.

In all PCR assays described here, two sets of primers were included in each reaction. One set recognised a region of the specific target gene, matrilysin or stromelysin-1, while the second set recognised a region of the constitutively-expressed gene,  $\beta$ -actin. The  $\beta$ -actin acted as an internal control for the actual reaction and also allowed normalisation of results between reactions. The expected size of the  $\beta$ -actin product was 225 bp. The stromelysin-1 product was 324 bp while the matrilysin product was 341 bp. Both the matrilysin and stromelysin-1 primers spanned an intron so that if genomic DNA rather than cDNA from the RT reaction were amplified, larger products would result.

The data in Table 5.3 represents the results obtained when RNA from each of the cell lines mentioned was isolated and analysed for stromelysin-1 and matrilysin expression.

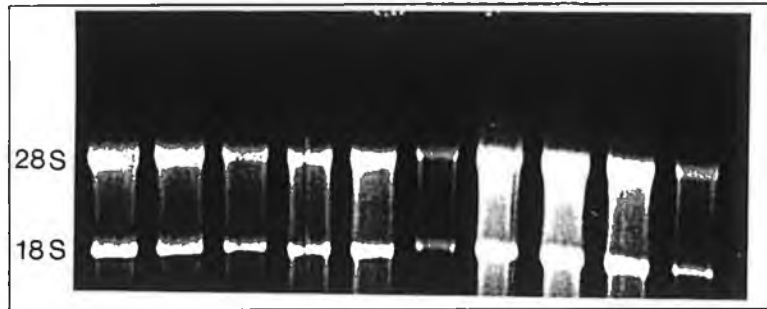
Cell Line	Matrilysin	Stromelysin-1
SW480	-	+
SW620	+	+
LoVo	+++	-
A549	++	+
K562	++	+
EJ	-	-
MCF-7	-	++
Go-GCCM	-	+

**Table 5.3** Expression of matrilysin and stromelysin-1 in human tumour cell lines. The relative levels of each of the proteinases, determined by inspection, are indicated using a simple scoring system where '-' indicates no expression and '+', '++' and '+++' indicate increasingly higher levels.

All cell lines used other than the leukaemia-derived K562 cell line, were epithelial in origin. Matrilysin is regarded as an epithelial-specific MMP (Crawford and Matrisian, 1995) while stromelysin, although usually associated with stromal cells, can also be produced by epithelial cells (Sato *et al.*, 1992). The production of these proteinases by the K562 cell line is more unusual. Kossakowska *et al.* (1993) examined 43 primary lymphomas for MMP expression and found matrilysin expressed in only a few cases while stromelysin-1 expression was not detected. Matrilysin is produced by developing monocytes (Busiek *et al.*, 1992) so its detection in a cell line derived from a blood cell lineage is, perhaps, not very surprising. It should also be borne in mind, as with all cell line work, that the cell lines may no longer fully resemble their cellular origins.

## 5.6 Cytokine Treatments

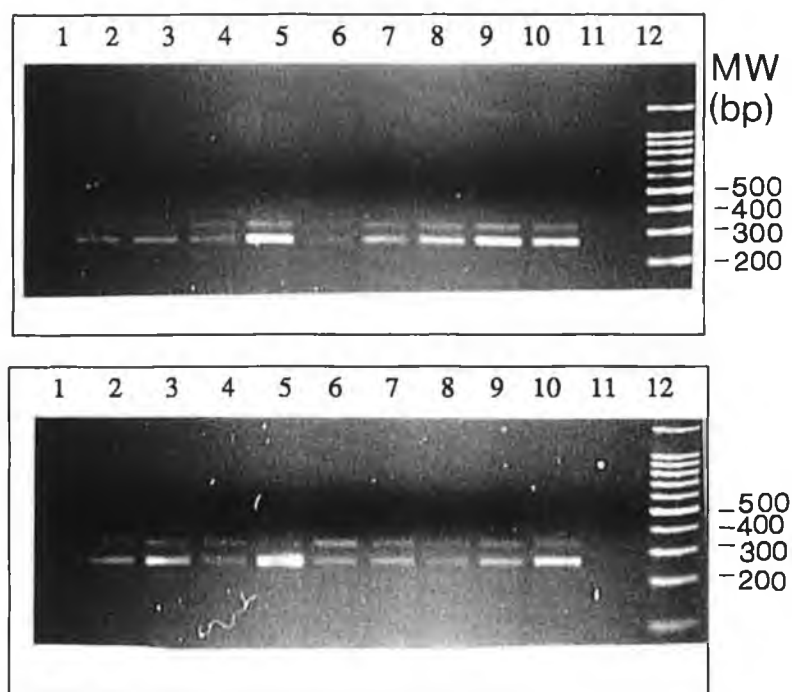
The cytokines used in this study represent a range of different activities including mediators of inflammation, classic growth factors, a growth-inhibitor and immunomodulators. To assess their effects on matrilysin and stromelysin-1 steady-state mRNA levels, cell lines were cultured overnight in serum-free media prior to treatment for 8 hrs with a specific cytokine. The choice of 8 hrs as the treatment time was suggested by literature (McDonnell *et al.*, 1990; Gaire *et al.*, 1994). The cells were then harvested, total RNA isolated and analysed as above. In order to demonstrate that the 'mini-preparation' RNA isolation method used was indeed suitable, aliquots of the isolated RNA were electrophoresed on agarose gels in the presence of the reducing agent, formaldehyde (section 2.2.11.3). The presence of bands representing the 28S and 18S ribosomal RNA subunits demonstrated successful RNA isolation with minimal degradation. The appearance of one of these gels is shown in Figure 5.1.



**Figure 5.1** Agarose gel electrophoresis of total RNA isolated from the A549 cell line following cytokine treatment. Each lane contains RNA isolated from a flask of A549 cells treated with a specific cytokine. The white bands represent the 28S (upper band) and 18S (lower band) ribosomal subunits. The almost complete lack of smearing indicates minimal degradation of the RNA.

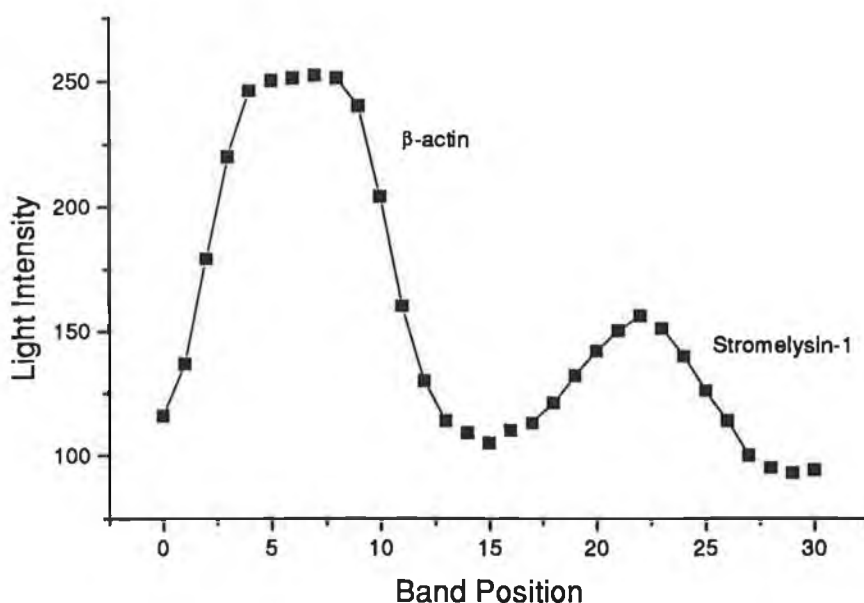


Satisfied that the RNA isolation procedure yielded RNA of high quality, the RNA from all 8 cell lines treated with the 9 different cytokines was analysed using RT-PCR. The DNA products for each cell line were run on 3 % agarose gels, stained with ethidium bromide and photographed. The gels obtained after RT-PCR analysis of one cell line (A549) are shown in Figure 5.2.



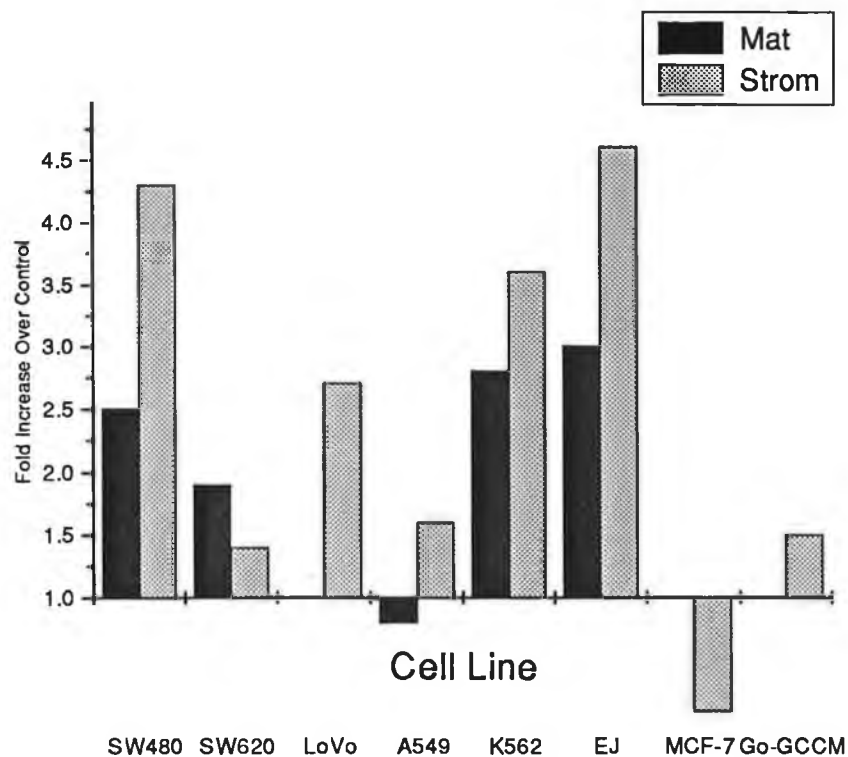
**Figure 5.2** Agarose gels of RT-PCR products from the total RNA isolates obtained from the cell line A549 following treatment with different cytokines. (a) shows bands representing  $\beta$ -actin (lower band at 225 bp) and stromelysin-1 (higher band at 324 bp), while (b) shows bands representing  $\beta$ -actin and matrilysin (341 bp) products. The right hand lane(12) in each gel contained a 100 bp ladder as size markers and the lane immediately to the left of it(11) contained the negative control reaction (RT-PCR with RNA template replaced by water). The other lanes represented (1) control, (2) IL-1 $\beta$ , (3) IL-2, (4) IL-6, (5) IGF-I, (6) IGF-II, (7) EGF, (8) bFGF, (9) TGF- $\beta$  and (10) IFN- $\gamma$ .

To assess the specific effect of each cytokine and to normalise for equivalent  $\beta$ -actin yields thus eliminating any variations between reactions, densitometric scanning of the gels was performed. Each pair of bands ( $\beta$ -actin and matrilysin or stromelysin-1) was analysed separately and a plot of light intensity versus band position was obtained. An example of one such plot is presented in Figure 5.3.

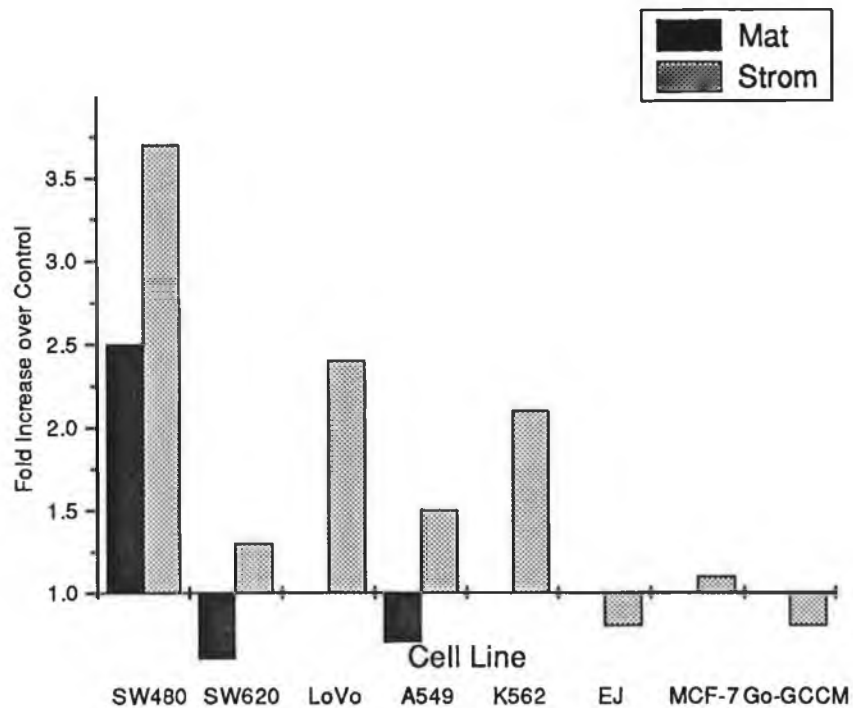


**Figure 5.3** Plot obtained from densitometric scanning of the ethidium bromide stained bands in a 3 % agarose gel. The pair of bands resulting from RT-PCR analysis of TGF- $\beta$  treated A549 cells were scanned and the data obtained plotted as above.

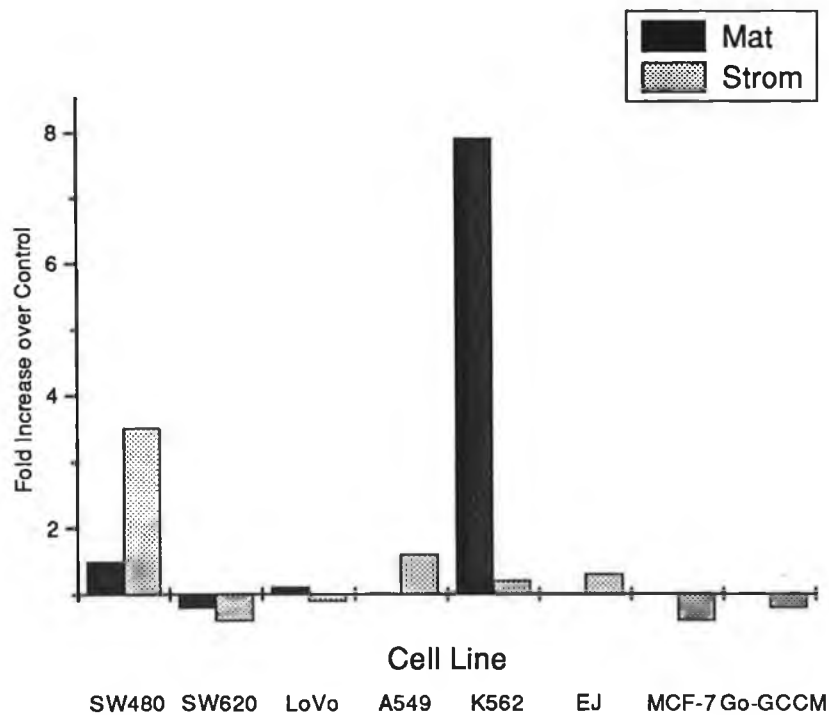
The area under the curve for each peak in these plots was then calculated. Ratios of matrilysin or stromelysin-1 to  $\beta$ -actin were obtained and compared for each cytokine in every cell line. Figures 5.4 - 5.12 indicate in graphical format the effect of each cytokine in a particular cell line as determined by this analysis. The plots are obtained from one set of experiments and are representative of the results obtained when these experiments were repeated.



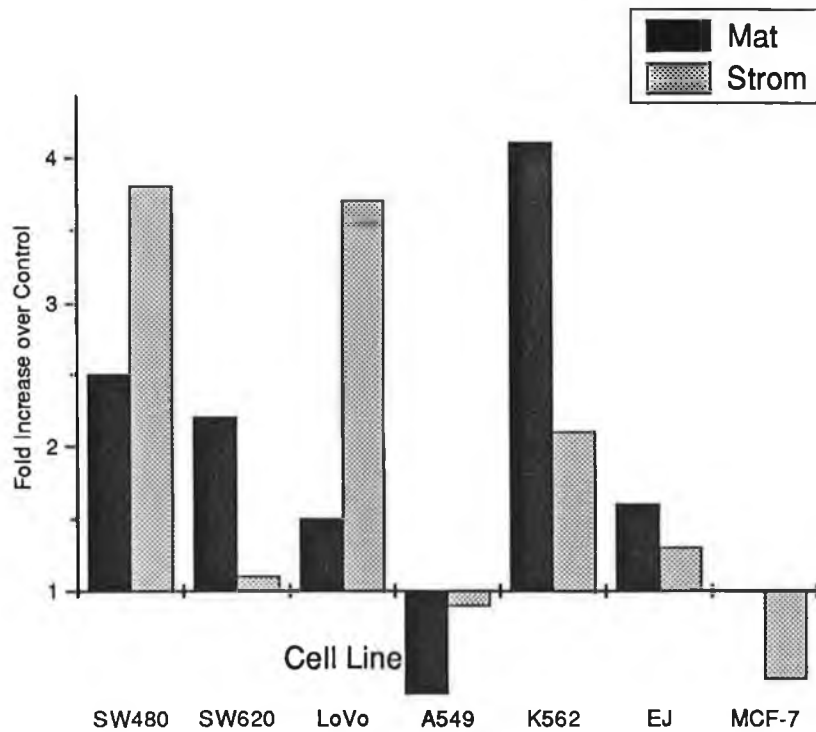
**Figure 5.4** Effects of IL-1 $\beta$  on matrilysin and stromelysin-1 expression by a range of cell lines. Levels of expression were determined by RT-PCR analysis and have been normalised relative to  $\beta$ -actin expression. The levels are represented as fold increase over control, with a baseline of 1 representing control (*i.e.* untreated) levels. Neither MCF-7 or Go-GCCM cells expressed matrilysin. The LoVo cell line expresses matrilysin at the same level as control following treatment with IL-1 $\beta$ .



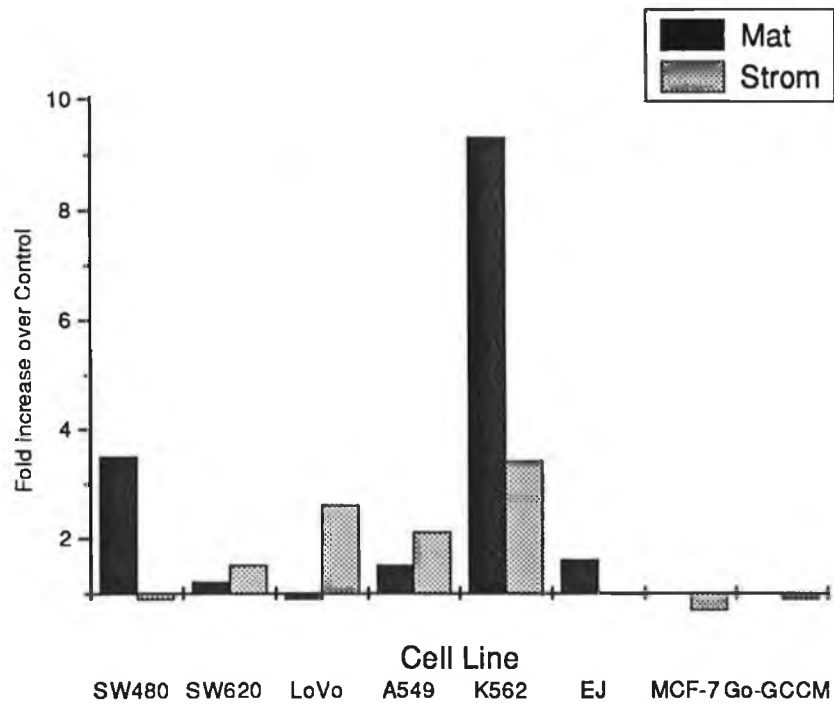
**Figure 5.5** Effects of IL-2 on matrilysin and stromelysin-1 expression in a range of cell lines. Levels of expression were analysed by RT-PCR and have been normalised relative to  $\beta$ -actin expression. The levels are represented as fold increase over control, with a baseline level of 1 representing control (*i.e.* untreated) levels. Matrilysin was not expressed by the MCF-7 or Go-GCCM cell lines. The level of matrilysin expressed by both the LoVo and EJ cells following treatment with IL-2 was similar to control.



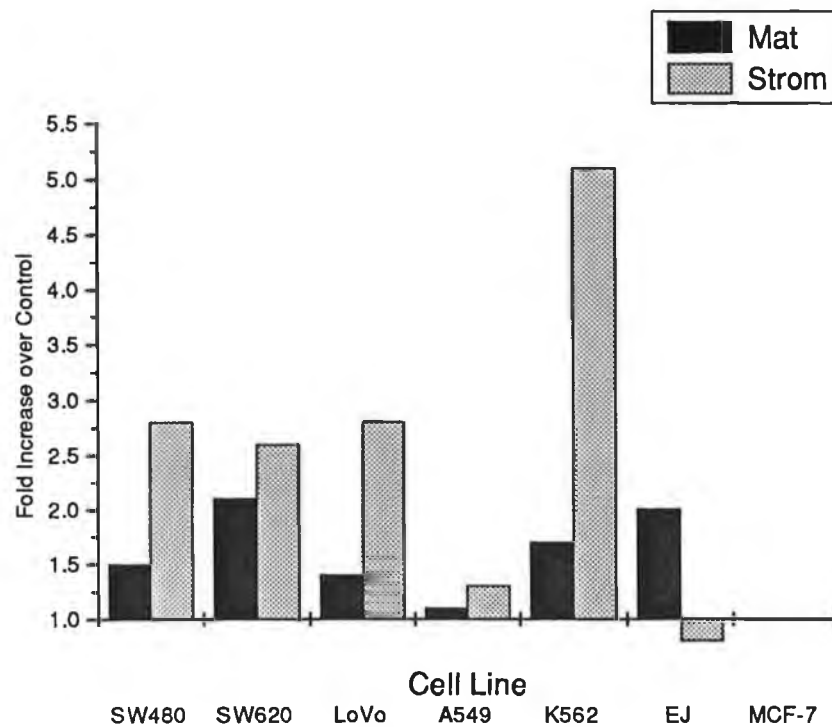
**Figure 5.6** Effects of IL-6 on matrilysin and stromelysin-1 expression in a range of cell lines. Levels of expression were analysed by RT-PCR and have been normalised relative to  $\beta$ -actin expression. The levels are represented as fold increase over control, with a baseline level of 1 representing control (*i.e.* untreated) levels. The MCF-7, Go-GCCM and EJ cell lines did not express matrilysin. The levels of matrilysin expressed by the A549 cells following treatment with IL-6 were similar to control.



**Figure 5.7** Effects of IGF-1 on matrilysin and stromelysin-1 expression in a range of cell lines. Levels of expression were analysed by RT-PCR and have been normalised relative to  $\beta$ -actin expression. The levels are represented as fold increase over control, with a baseline level of 1 representing control (*i.e.* untreated) levels. The MCF-7 cell line does not express matrilysin. Analysis of the effect of IGF-1 on the Go-GCCM cell line was not done.

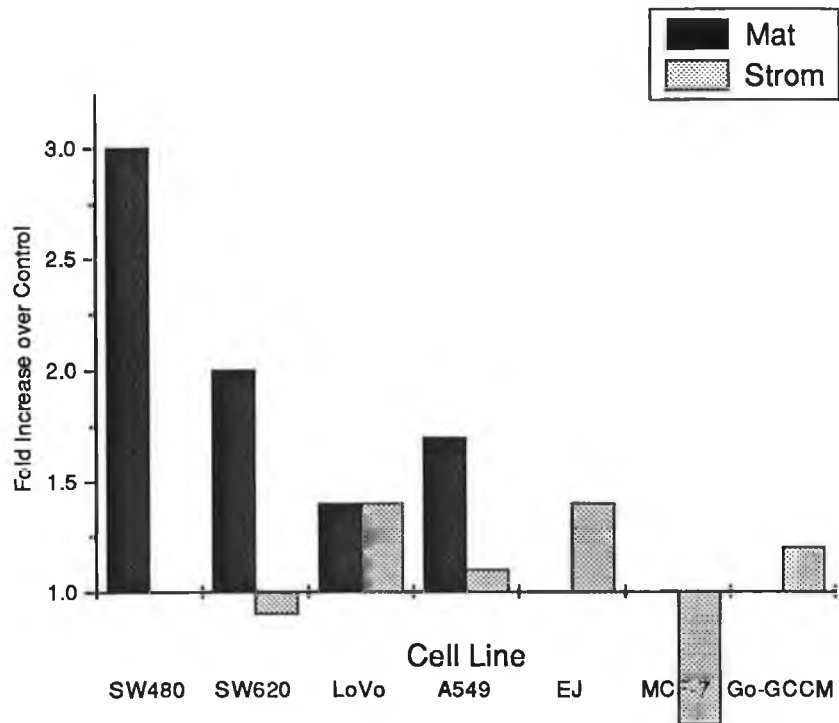


**Figure 5.8** Effects of IGF-II on matrilysin and stromelysin-1 expression in a range of cell lines. Levels of expression were analysed by RT-PCR and have been normalised relative to  $\beta$ -actin expression. The levels are represented as fold increase over control, with a baseline level of 1 representing control (*i.e.* untreated) levels. Neither MCF-7 or Go-GCCM cells expressed matrilysin. No result was obtained for stromelysin-1 expression by the EJ cell line.

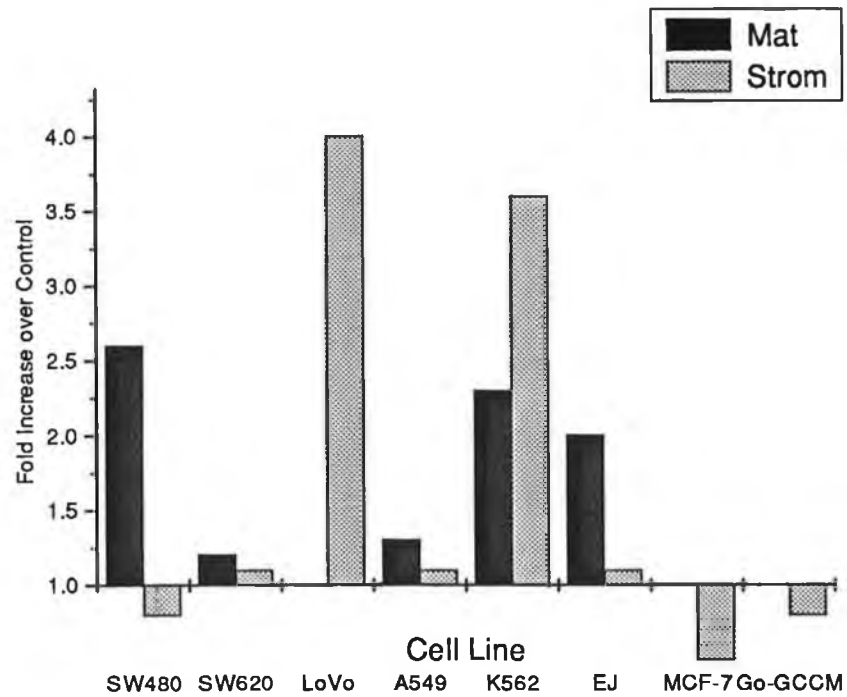


**Figure 5.9** Effects of EGF on matrilysin and stromelysin-1 expression in a range of cell lines. Levels of expression were analysed by RT-PCR and have been normalised relative to  $\beta$ -actin expression. The levels are represented as fold increase over control, with a baseline level of 1 representing control (*i.e.* untreated) levels. The MCF-7 cell line does not express matrilysin and its level of stromelysin-1 expression was unchanged from control levels following treatment with EGF. No results were obtained for the Go-GCCM cell line.

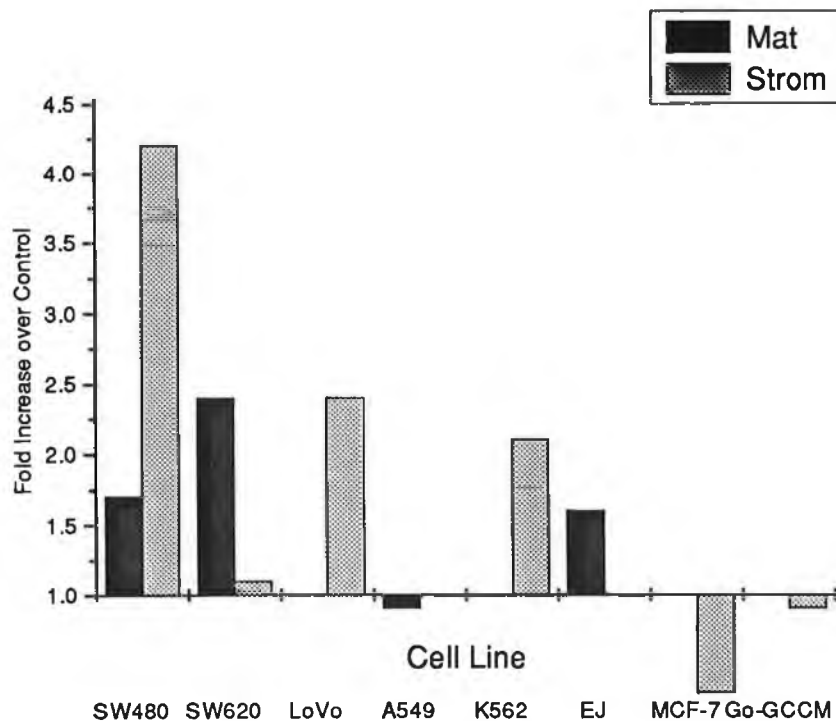




**Figure 5.10** Effect of bFGF on matrilysin and stromelysin-1 expression in a range of cell lines. Levels of expression were analysed by RT-PCR and have been normalised relative to  $\beta$ -actin expression. The levels are represented as fold increase over control, with a baseline level of 1 representing control (*i.e.* untreated) levels. Matrilysin was not expressed by the EJ, MCF-7 or Go-GCCM cell lines. bFGF had no effect on stromelysin-1 expression in the SW480 cell line as levels were similar to control. The effects of bFGF on K562 cells were not analysed.



**Figure 5.11** Effects of TGF- $\beta$  on matrilysin and stromelysin-1 expression in a range of cell lines. Levels of expression were analysed by RT-PCR and have been normalised relative to  $\beta$ -actin expression. The levels are represented as fold increase over control, with a baseline level of 1 representing control (*i.e.* untreated) levels. Matrilysin was not expressed by either the MCF-7 or Go-GCCM cell lines. LoVo matrilysin expression was unchanged from control levels.



**Figure 5.12** Effects of IFN- $\gamma$  on matrilysin and stromelysin-1 expression in a range of cell lines. Levels of expression were analysed by RT-PCR and have been normalised relative to  $\beta$ -actin expression. The levels are represented as fold increase over control, with a baseline level of 1 representing control (*i.e.* untreated) levels. Neither the MCF-7 or Go-GCCM cell lines expressed matrilysin. No result was obtained for matrilysin expression in K562 cells. IFN- $\gamma$  had no effect on matrilysin expression in the LoVo cell line, or stromelysin-1 expression by either the A549 or EJ cell lines, with levels similar to control.

## 5.6.1 Discussion of Cytokine Results

### Effects of IL-1 $\beta$

IL-1 $\beta$  is known as a pro-inflammatory cytokine responsible, amongst other functions, for the upregulation of interstitial collagenase and stromelysin-1 produced by synovial cells in rheumatoid arthritis (MacNaul *et al.*, 1990; Ito *et al.*, 1992). Its effects on MMP secretion by epithelial tumour cells are less well-characterised. Macakay *et al.*, (1992) reported that stromelysin-1 and gelatinase B as well as TIMP-1 could be stimulated in some tumour cell lines and normal human vein endothelial cells (HUVECs). Gelatinase A and TIMP-2 were refractory to IL-1 $\beta$  treatment in all cell lines tested. Their study did not include matrilysin and was limited to 4 tumour cell lines. The data presented in Figure 5.4 demonstrates that IL-1 $\beta$  has a stimulatory effect on stromelysin-1 production by almost all the cell lines tested ranging from a 4.5-fold increase in the bladder carcinoma line, EJ to a 1.3-fold increase in the colon cell line, SW620. Stromelysin-1 expression was inhibited by IL-1 $\beta$  in one cell line, the breast carcinoma MCF-7. Although not as dramatic as with stromelysin-1, the effects of IL-1 $\beta$  on matrilysin expression were also generally stimulatory. Two cell lines, MCF-7 and Go-GCCM, could not be induced to express matrilysin and the expression by the LoVo cell line remained unchanged from control. In 4 of the other cell lines examined, matrilysin expression was stimulated between 1.8- and 2.8-fold. There was a minor inhibitory effect on matrilysin expression by the A549 cell line. It is possible that matrilysin and stromelysin-1 could act synergistically to increase the invasive abilities of tumours if secreted by the one cell type. This may be of particular importance in tumours with a large amount of tumour-associated macrophages, as phagocytes are important producers of this cytokine.

### Effects of IL-2

IL-2 is generally regarded as an immune system-associated cytokine so it is quite surprising to find it has stimulatory effects on stromelysin-1 production in particular by tumour cells. The stimulatory effect of IL-2 on both matrilysin and stromelysin-1 is strongest in the colon adenocarcinoma-derived SW480 cell line. In

fact, stromelysin-1 expression is upregulated in all of the colon-derived cell lines following IL-2 treatment. It is possible that this *in vitro* effect is of little relevance *in vivo*, as it is thought that responses to cytokines are markedly affected by the ECM (Nathan and Sporn, 1991) which is lacking in cell cultures. It is also possible, however, that the influence of the ECM could be affected by MMP release from tumour cells in response to cytokines.

#### Effects of IL-6

IL-6, like IL-1 $\beta$ , is a proinflammatory cytokine, although its effects are not so widespread. The almost complete lack of response by the majority of the cell lines tested was expected as previous studies of IL-6 have shown it to be stimulatory for TIMP-1 but not MMP expression (Sato *et al.*, 1990; Kordula *et al.*, 1992; Ito *et al.*, 1992). The 8-fold increase in matrilysin expression by the promyelocytic K562 cell line following IL-6 treatment is quite dramatic. There are few reports of matrilysin expression by leukaemias (Kossakowska *et al.*, 1993) and none investigating cytokine effects on these types of cells. Matrilysin is produced by promonocytes (Busiek *et al.*, 1992) and there is a possibility that cytokines such as IL-6 are responsible for regulating that production.

#### Effects of IGF-I

There are no previous reports of IGF-I affecting MMP expression, however, it appears to be stimulatory for both matrilysin and stromelysin-1 in a range of cell lines. It has been reported that IGF-I can enhance proliferation of colon carcinoma cells (Lahm *et al.*, 1994) and matrilysin production by colon carcinomas has also been previously documented (McDonnell *et al.*, 1991; Newell *et al.*, 1994; Witty *et al.*, 1994). The role of matrilysin in colon tumour development is unclear but it is thought that it is linked to tumour cell proliferation. It is possible that the proliferative effects of IGF-I are mediated through matrilysin by an unknown mechanism. The greatest effect of IGF-I, however, is seen in the K562 cell line.

#### Effects of IGF-II

As is the case with IGF-I, there have been no previous reports of the effects of IGF-II on MMP expression. The strong effect of IGF-II on matrilysin expression by K562

cells is similar to that seen with IGF-I and, as both IGF-I and II can act through the same receptor, this is not very surprising. The fact that the effects of IGF-I in other cell lines are not repeated by IGF-II may be due to the apparently limited function of IGF-II in foetal development. Human tumour cells derived from adult tissues may not have the ability to respond to IGF-II whereas the blood cell precursor cell line, K562, may represent foetal cells.

#### Effects of EGF

The stimulatory effects of EGF on MMP production, particularly stromelysin-1 and interstitial collagenase, have been well-documented (Kerr *et al.*, 1988; McDonnell *et al.*, 1990; Delaney and Brinckerhoff, 1992; Gaire *et al.*, 1994). The similarity of MMP genes, almost all of which contain an AP-1 element in their promoter regions, is thought to be responsible for the widespread effects of EGF. There is, however, evidence for cell-type specific stimulation (Mauviel, 1993b) perhaps explaining the different levels of stimulation seen in the various cell lines used here. The lack of response in the MCF-7 cell line may be due to aberrant EGF receptors as changes in these receptors as a result of oncogenic transformation are often found in breast cancer cells (McGuire and Clark, 1992).

#### Effects of bFGF

The stimulatory effects of bFGF on matrilysin production are most evident in the colon carcinoma-derived cell lines. These effects have not previously been reported. The almost complete lack of effect on stromelysin-1 expression in these cell lines suggests bFGF is a matrilysin-specific cytokine. As matrilysin has been suggested to have a role in proliferation particularly of colon carcinomas (Witty *et al.*, 1994), interfering with bFGF-induced stimulation of matrilysin may provide a mechanism of reducing the tumorigenicity of these cells.

#### Effects of TGF- $\beta$

TGF- $\beta$  has previously been reported as having an inhibitory effect on stromelysin-1 and interstitial collagenase expression (Edwards *et al.*, 1987; Kerr *et al.*, 1990). These previous reports, however, examined stromelysin-1 expression by fibroblastic cells. It is interesting to see that in other cell types, TGF- $\beta$  appears to be stimulatory for

stromelysin-1 expression in 2 cell lines (LoVo and K562) and to have little or no effect on stromelysin-1 expression by most other epithelial cell lines. Matrilysin expression was not observed to be inhibited in any cell lines either and, was actually stimulated in 3 cell lines.

#### Effects of IFN- $\gamma$

The immunomodulatory cytokine IFN- $\gamma$  did not appear to have a large effect on matrilysin expression by any cell line, although some stimulation was seen in the SW480, SW620 and EJ cell lines. Stromelysin-1 expression has previously been shown to be down-regulated by IFN- $\gamma$  treatment (Mauviel, 1993b), although, as is the case with most previous studies, fibroblastic cells were studied. IFN- $\gamma$  appeared to upregulate stromelysin-1 expression in a number of tumour cell lines examined here, including K562, LoVo and SW480.

Taking all the results together, it may be seen that the various cell lines respond quite differently to the different cytokines. There are a number of possible explanations :

- Presence or absence of specific cytokine receptors;
- Different levels of specific receptors;
- Cell-type specific secondary and tertiary messengers for signal transduction;
- Differences in mRNA processing.

Information regarding expression of specific cytokine receptors on particular cell lines is scarce. An investigation into EGF receptor (EGFr) by Real *et al.* (1986) revealed the presence of moderate amounts of EGFr on both SW480 and MCF-7 cells. However, this study also found no EGFr expression by K562 cells which is contrary to the observed effects of EGF on both matrilysin and stromelysin-1 expression. This disparity may be due to an alternative form of the EGFr protein not recognised by the monoclonal antibody used in the receptor expression study. The IGF receptor, through which both IGF-I and IGF-II can act, has been found to be expressed on all colorectal carcinoma cell lines tested (Lahm *et al.*, 1994). The cell lines studied included SW480, SW620 and LoVo. Studies on how cytokines affect other cellular functions provide more information on receptor presence. The upregulation of the adhesion molecule,

ICAM-1, in a number of breast cancer cell lines following treatment with either IL-1 $\beta$  or IL-6 (Hutchins and Steel, 1994) suggests that receptors for these cytokines are present on MCF-7 cells. TGF- $\beta$ , IFN- $\gamma$  and the IGFs have also been shown to influence the growth and behaviour of breast cell lines (Lippman *et al.*, 1986; Cullen *et al.*, 1991). The lack of response by any cell line to a particular cytokine may, therefore, suggest a lack of receptor. For example EJ, Go-GCCM or MCF-7 cells show no response to IL-2 and this may be due to a lack of a receptor for IL-2. Although the expression of matrilysin is apparently not affected by IL-2 in LoVo cells, stromelysin-1 expression is enhanced suggesting a functioning receptor and signalling pathway.

Many gene-expression controlling elements are cell-type specific so that, although the regulatory message is transduced from the cell surface to the nucleus, gene activation cannot proceed. This may be the reason for the stimulatory effect of a particular cytokine in cell lines derived from certain tissues while no effect is seen in other cell lines. An example is the effect of EGF on stromelysin-1 expression. All the colon carcinoma cell lines (SW480, SW620 and LoVo) respond similarly while no response is seen in the lung (A549) or bladder (EJ) cell lines. Alternatively, the specific gene being investigated, may lack appropriate elements to respond to signals generated through the binding of a cytokine. bFGF, for example, has very little effect on stromelysin-1 expression in any cell line. The control elements of genes responsible for mediating signalling events are discussed further in chapter six.

A final possibility is that the effects seen are not actually due to interactions with matrilysin or stromelysin-1. As RT-PCR gives an indication of steady-state mRNA levels, increased levels may reflect mRNA of greater stability than usual. Some MMP genes, like some cytokine genes, appear to contain AU-rich destabilising sequences in the 3' untranslated region of the mRNA (Delaney and Brinckerhoff, 1992). It has been suggested that MMP-specific ribonucleases are required to degrade MMP mRNA (Brenner *et al.*, 1989). The regulation of these ribonucleases may be affected by cytokine treatment thus having an effect on MMP mRNA stability resulting in an apparent increase in steady-state levels.

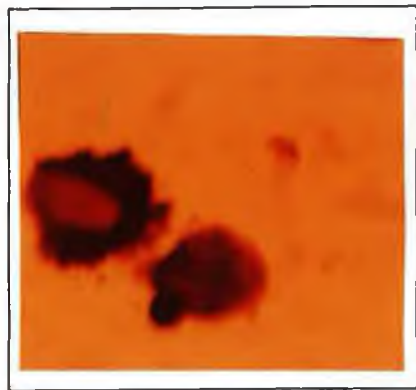


Overall, these results indicate that, depending on tissue type, many cytokines can influence expression of matrilysin and stromelysin-1 mRNA. Previous studies have tended to concentrate on the effects of cytokines on MMP levels in fibroblastic cells. This investigation demonstrates that MMP expression by epithelial cells, also, can be modulated by cytokines. Whether there are specific control elements within the matrilysin gene responsible for these effects will be further investigated in chapter six.

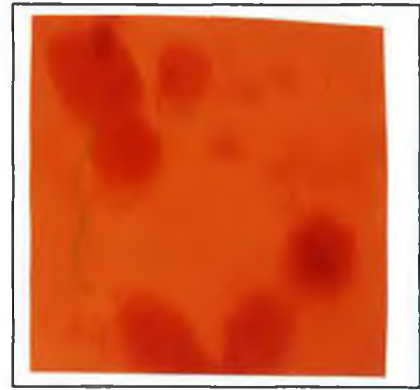
### **5.7 *In situ* hybridisation**

As further evidence of matrilysin expression in specific cell types, an alternative approach to demonstrating the presence of specific mRNA was used. *In situ* hybridisation shows specific mRNA localised within cells. The technique was used with the LoVo and K562 cell lines as these were to be further investigated for control of matrilysin expression (chapter six). A specific digoxigenin-labelled riboprobe for matrilysin was prepared and used as outline in section 2.2.13. The cells were grown in special chamber slides to allow ease of processing. Results obtained are shown in Figure 5.13.

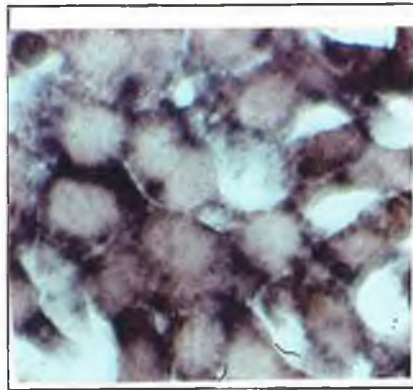
The presence of specific matrilysin mRNA can be clearly seen. The lack of staining within the nucleus demonstrates that the probe bound only to RNA and not DNA. It was attempted to show cytokine effects on matrilysin expression using this technique, however, no clear differences between treated and untreated cells could be discerned.



(a)



(b)



(c)



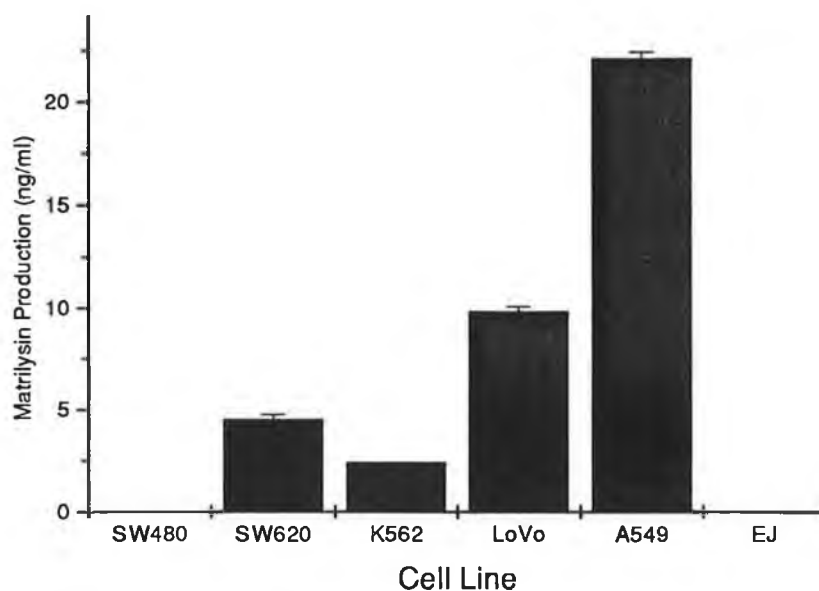
(d)

**Figure 5.13** *In situ* hybridisation of matrilysin mRNA in K562 [(a) & (b)] and LoVo [(c) & (d)] cells. Detection with the antisense probe is seen in (a) and (c) while the sense probe (negative control) was used in (b) and (d).

## 5.8 Effects of cytokines on levels of matrilysin protein

While specific mRNA is present only in cells actively expressing a gene, post-transcriptional and translational regulation may affect expression of the actual protein coded for by the gene. To examine the levels of matrilysin protein produced by cell lines, an ELISA for matrilysin was developed. The development and validation of this assay is outlined in chapter three.

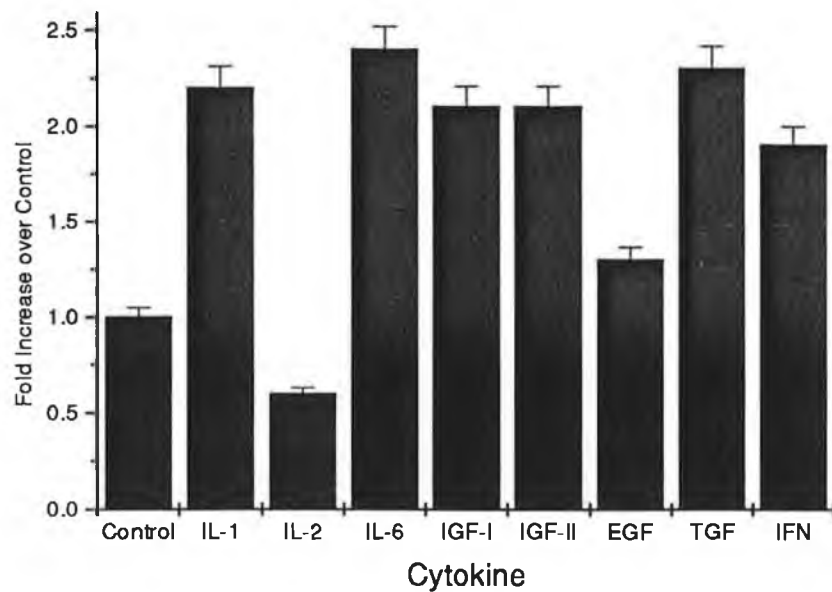
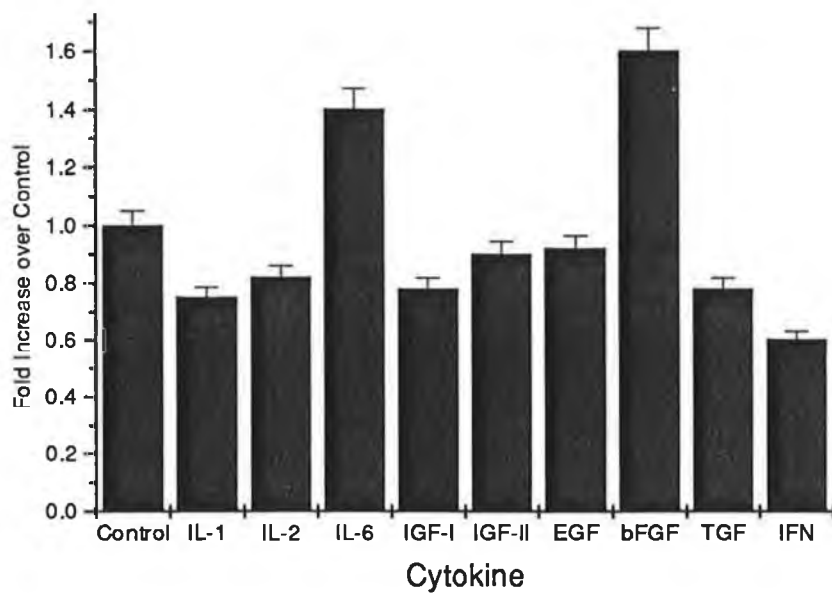
Conditioned media (CM) was collected from cell lines following 24 hr culturing in serum-free media. The medium was clarified by centrifugation and used directly in the ELISA. Results for matrilysin protein levels in different cell lines are shown in Figure 5.14.



**Figure 5.14** Matrilysin protein levels in CM from cell lines as determined by ELISA.

From the matrilysin mRNA analysis in different cell lines (Table 5.3), it would have been expected that the LoVo cell line would produce most protein with A549 and K562 lines producing moderate amounts and SW620 producing least. The actual values for protein production indicate that the A549 cell line is the most potent producer. This disparity may be due to the longer incubation time used for protein analysis (24hrs as compared to 8 hrs for mRNA). The extra time was considered necessary as both transcription and translation processes had to be completed for protein formation while only transcription was necessary for mRNA production. An alternative explanation relates to post-transcriptional processing of mRNA. A549 mRNA may have a longer half-life within the cell allowing more protein to be made from each transcript.

The ELISA was also used to assess the effects of cytokines on protein production by two cell lines, K562 and LoVo. The cells were treated with cytokines exactly as before but for a longer time period - 24 hrs rather than 8 hrs. The CM was collected from each flask, clarified by centrifugation and assayed for matrilysin using the sandwich ELISA. The ratio of cytokine-treated level to control (untreated) level was determined and the results plotted. The graphs obtained are shown in Figures 5.15 (A) and (B)



**Figure 5.15** Effects of cytokines on matrilysin protein production by (A) K562 and (B) LoVo cell lines. Results, determined by sandwich ELISA, are expressed as fold increase over control.

As with the data from different cell lines, the results for cytokine treatments do not correlate well with the RT-PCR results for mRNA. The stimulatory effect of IL-6 in K562 cells is seen both at the mRNA and protein level, however, the other strong stimulating agent at the mRNA level, IGF-II, does not appear to have the same effect on expression of the protein. Further investigations will be required to determine if this is due to differences in post-transcriptional processing of the mRNA. In the LoVo cells, stimulatory effects on protein production were seen with a wider range of agents than were seen at the mRNA level. IGF-I is stimulatory for both mRNA and protein, however, other cytokines such as TGF- $\beta$  and IL-6 also appear to have a strong up-regulating effect on protein production.

Another possible explanation for all the inconsistencies seen with the ELISA results may be due to its detection of the zymogen (28 kDa) form of matrilysin only. If this is rapidly being converted to the active (19 kDa) form in the culture medium from any of the cell lines, the actual levels assayed will be very low. This could be the case with the K562 cell line where the lowest levels of protein were detected despite the mRNA levels being relatively high.

## Summary

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A range of cell lines were assessed as to their ability to produce matrilysin at both the mRNA and protein levels. mRNA analysis was accomplished using RT-PCR and, in the case of the LoVo and K562 cell lines, *in situ* hybridisation. Protein levels were assayed using the sandwich ELISA for matrilysin developed and validated as described in chapter three. Matrilysin production was seen at both mRNA and protein levels in the SW620, LoVo, A549 and K562 cell lines. Some disparity between the mRNA and protein data was evident. This may have been due to post-transcriptional modifications of the mRNA within the cells, or the generation of active matrilysin protein which was not detected by the ELISA.

The cell lines were treated with a range of cytokines to determine the possible effects of these molecules on matrilysin and stromelysin-1 production by epithelial cells. The breast cell line MCF-7, and the brain cell line Go-GCCM did not express matrilysin under any circumstances. The colon cell line SW480 and the bladder cell line EJ, although not normally producers of the enzyme, could be induced to express matrilysin mRNA after treatment with certain cytokines. The cytokines IL-1 $\beta$ , IL-6, bFGF, IGF-I and IGF-II up-regulated matrilysin in a number of cell lines. Stromelysin-1 up-regulation resulted in many cases, from treatment with IL-1 $\beta$ , IL-2, EGF, TGF- $\beta$  and IFN- $\gamma$ . Some of the effects appeared to be tissue-specific. Further analysis of cytokine effects on the matrilysin promoter is presented in chapter six.

## **Chapter Six**

### **Matrilysin Promoter Studies**



This chapter is concerned with the analysis of the matrilysin promoter. The promoter regions for both the human and mouse genes have previously been cloned and sequenced (Gaire *et al.*, 1994; Prof. Lynn Matrisian, pers comm.). The direct effects of cytokine treatment on these regions were examined using the promoter sequences subcloned into reporter gene vectors. To do this, an appropriate cell line was selected and transfected (using a suitable method) with the promoter-reporter gene constructs, the cells were treated with the cytokines and the specific reporter gene assays used to quantify any effects. An introduction to control of gene expression is first presented.

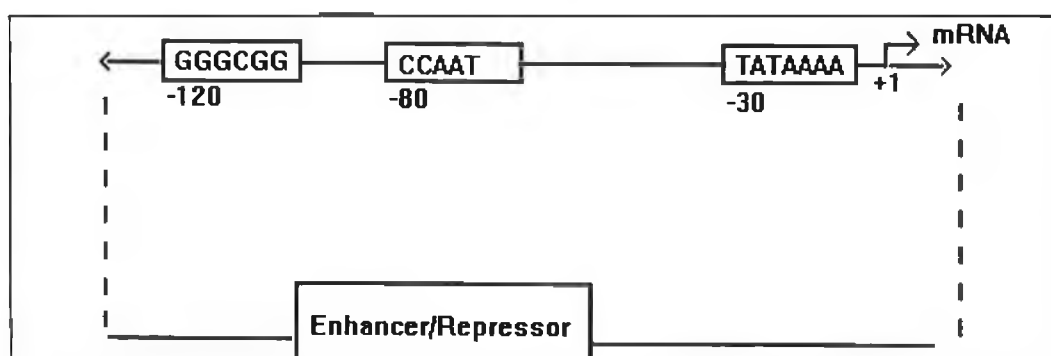
### 6.1 Control of gene expression

For a specific protein to be manufactured by a cell, the gene that encodes it must first be transcribed from DNA to messenger RNA (mRNA). This then travels from the cell nucleus to the cytoplasm where it acts as a template from which the protein can be generated. The act of transcribing is subject to regulation by factors within the nucleus. These factors are in turn controlled by second messengers, for example cAMP or protein kinase C, changes in the activity of which result from cell signalling events such as may occur when a cytokine binds to its receptor on the cell surface.

The enzyme responsible for transcription of structural (*i.e.* coding) genes is RNA polymerase II. For transcription to take place, the enzyme must be aligned at the correct position on the gene. RNA polymerase II attaches to promoter regions, however the enzyme itself appears to lack any inherent ability to recognise promoters (Dyana and Tjian, 1985; Rothwell, 1993). Short DNA sequence elements located near the transcriptional start site (+1), play an important role in regulating gene expression. Such sequences mediate transcriptional activation by binding specific proteins known as transcription factors (Latchman, 1990). Almost all promoters that interact with RNA polymerase II contain a conserved sequence known as the TATA box (consensus

sequence TATAAAA). It is located 20 - 30 bp upstream, *i.e.* 5', of the transcriptional start site. Up to six different transcription factors bind in the vicinity of the TATA box (Hawley, 1991). These core transcription factors are also known as RNA polymerase II - associated proteins or RAPs (Greenblatt, 1991). One of these, TFIID known as the TATA-binding protein, actually recognises the TATA box and must bind to it before any other molecule can do so. Once TFIID binds, it is then followed by TFIIA and TFIIB to which a complex composed of RNA polymerase II, TFIIE and TFIIIF (RAP30 / 74) can bind (Roeder, 1991). Another common promoter sequence is the CAT box (consensus sequence CCAAT) about 80 bp upstream from +1 (Raskó and Downes, 1995). TATA and CAT are promoter sequences for RNA polymerase II and are found in many, but not all, coding genes. Those genes lacking a TATA box do not all have fixed sites for the start of transcription and depend on certain transcription factors acting along with RNA polymerase II (Rothwell, 1993) In other promoters lacking a TATA box, a discrete element overlying the start site helps to fix the place of initiation (Dyanan, 1989). Apart from the transcription factors mentioned above that interact with the TATA box, there are many other nuclear proteins that bind other specific DNA sequences upstream of the +1 site *e.g.* the CAT box or GC elements (consensus sequence GGGCGG). Specific transcription factors and their recognition sequences will be discussed in section 6.2.

Some promoters are greatly influenced by DNA sequences named enhancers or activators. Figure 6.1 illustrates the possible arrangement of these elements.



**Figure 6.1** Arrangement of DNA sequence elements controlling transcription of a typical eucaryotic protein-coding gene. The enhancer may be located upstream or downstream of the site of transcriptional initiation.

Enhancers are not necessary for transcription and, by themselves, are not sufficient to initiate it, but they can greatly increase the efficiency of promoters. These sequences have a number of properties :

- (i) An enhancer element can activate a promoter when placed up to several thousand bases from the promoter;
- (ii) An enhancer can activate a promoter when placed in either orientation relative to the promoter;
- (iii) An enhancer can activate when placed upstream or downstream of the transcribed region, or within an intervening sequence which is removed from the mRNA by splicing (Latchman, 1990).

Enhancers can be general or tissue-specific. For example, the enhancer element of 8 nucleotides recognised by the DNA-binding protein OCT-2 is responsible for enhancing the transcription of immunoglobulin (Ig) genes. Its unique presence in B lymphocytes was shown by experiments in which the Ig gene promoter was placed in different cell types (Gillis *et al.*, 1983). Transcription above basal level could only be seen in this one cell type. Experiments in which enhancers are moved about also reveal that an enhancer will increase the rate of transcription of any promoter that is placed nearby, showing that a given enhancer is not limited in its effect to just one promoter of a given gene (Rothwell, 1993).

Elements that operate to decrease transcription also exist. These are termed silencers or repressors. Much like enhancers or upstream promoter elements, binding of specific proteins to these DNA sequences affects the way in which RNA polymerase II binds and functions. The complexes formed by enhancers/repressors and the basal transcriptional machinery are responsible for drawing RNA polymerase II to specific genes at specific rates.

## 6.2 Transcription factors

The promoters and enhancers that control the transcription of protein-coding genes are composed of multiple genetic elements or modules. Studies have shown that promoters are composed of discrete functional modules, each consisting of

approximately 7 - 20 bp DNA and containing one or more recognition sites for transcriptional activator proteins (Dynam, 1989). Enhancers and promoters appear to have a very similar modular organisation. In fact, there are instances such as the Ig octamer motif, where the same module can occur in the context of either a promoter or enhancer. This suggests that DNA-binding proteins bound to either of these types of regulatory element, may interact with the cellular transcriptional machinery in functionally the same way.

To activate or repress transcription, transcription factors must be located in the nucleus, bind DNA and interact with the basal (or core) transcriptional apparatus. Interactions between transcription factors or between a transcription factor and a DNA sequence are mediated through structural domains present in the transcription factors (Hill and Treisman, 1995). One of the most common of these structural motifs used for DNA binding is known as the zinc finger (Raskó and Downes, 1995). It consists of a sequence of 20 - 30 amino acids with a pair of cysteines near the amino end and a pair of histidines or a pair of cysteines or a histidine and a cysteine near the carboxy end of the sequence. These residues bind to a zinc atom which is essential for the activity of the protein. Between the zinc-bound elements, the protein forms a loop or finger which can enter the major groove of DNA and bind to approximately 5 bp. The zinc finger DNA-binding proteins characterised so far, exhibit a variety of DNA sequence specificities probably dictated by the particular amino acid residues which comprise the finger (Mitchell and Tjian, 1989).

The structural motif predominantly responsible for joining DNA-binding proteins to each other, is known as the leucine zipper (McKnight, 1991). This consists of an  $\alpha$ -helical region in which every seventh amino acid is a leucine. This 'heptad repeat' causes the leucines to be aligned in a plane along the length of the helix. It was first suggested that if two such helices lay side-by-side, their leucines would fit together like a zipper. More recently, it has been shown that, although the leucines are adjacent to each other, they do not 'zip' together (Raskó and Downes, 1995). The zippers do not form part of the DNA-binding domains; rather they allow pairs of DNA-binding proteins to join together to form a 'scissors-grip' structure. In this the two  $\alpha$ -helices of

the zipper domains lie together forming the blades of the scissors while the DNA-binding domains protrude at the end like handles, fitting into the major groove of DNA.

A different mode of DNA binding involves the helix-turn-helix or homeodomain motif. This domain encompasses approximately 60 amino acids with the most conserved positions between homeodomains of different proteins being occupied by basic and hydrophobic residues (Mitchell and Tjian, 1989).

Following is a brief introduction to some specific transcription factors or DNA-binding proteins which appear to play a role in the regulation of transcription of MMP genes :

### **SP-1**

Specificity protein-1 (SP-1) was the first transcription factor to be isolated (Dyner and Tjian, 1985). All SP-1 binding regions contain one or more perfect copies of the GC box hexanucleotide GGGCGG, which may be present in either orientation relative to the direction of transcription. However, not all GC boxes bind SP-1 equally well as the bases outside the core hexanucleotide sequence appear to modulate the efficiency of binding. The SP-1 protein molecule has 3 zinc finger motifs at one end which attach to the DNA sequences and, at the other end, a glutamine-rich domain which appears to be responsible for protein:protein interactions (Tjian, 1995). No shared regulatory feature is evident among the diverse promoters in which SP-1 binding sites have been found (Dyner and Tjian, 1985). It is possible that SP-1 provides a basal, constitutive level of transcription which can then be modulated by other positive or negative regulatory factors (Dyner, 1989).

### **The C/EBP family**

The CAT/Enhancer binding protein, C/EBP, was isolated by McKnight and colleagues from rat liver extracts (Landschulz *et al.*, 1988). This protein has affinity not only for the CAT motif in promoters, but also for a motif called the core homology, common to many enhancers. The protein is not found in all tissues and is, therefore, a possible contributor to tissue-specific gene expression. C/EBP is a 369-amino acid molecule which, in certain regions, has homology to the protein products of the proto-oncogenes *jun*, *fos* and *myc* (McKnight, 1991). These regions of homology have since been identified as leucine zipper domains and are primarily responsible for the dimer

formation associated with these proteins (known as basic zipper (bZIP) proteins). These proteins can only bind to DNA in the form of dimers and most can do so in the form of homo- or hetero-dimers (Jones, 1990)

Nuclear factor for IL6 (NF-IL6), also known as IL-6DBP (IL-6-dependent DNA binding protein) is a member of the C/EBP family of transcription factors (Akira *et al.*, 1990; Poli *et al.*, 1990). NF-IL6 is strongly homologous to C/EBP in the region of the basic (DNA binding) domain and in the leucine zipper region. The transcriptional activation of the IL-6 gene by IL-1 is dependent on a 15 bp palindromic sequence ACATTGCCACAATCT located at position -150 which can be bound by NF-IL6 (Akira *et al.*, 1990). NF-IL6 not only activates this gene but is also responsible for the regulation of genes encoding other inflammatory cytokines, acute phase proteins, serum albumin, c-Fos and some other genes. NF-IL6 and C/EBP can interact *in vitro* to form heterodimers that bind to DNA with the same specificity as the respective homodimers. NF-IL6 can also interact with the p50 subunit of another transcription factor NF- $\kappa$ B (LeClair *et al.*, 1992; Matsusaka *et al.*, 1993). Analysis of promoters of genes which respond to the inflammatory cytokines IL-6 and IL-1, reveals that binding sites for both NF-IL6 and NF- $\kappa$ B are frequently present. It is thought, therefore, that the interaction of subunits of these two transcription factors could be highly important in regulating gene expression.

Another member of the C/EBP family, NF-IL6 $\beta$ , also appears responsible for mediating effects of IL-6 on transcription. NF-IL6 $\beta$  is a 269-amino acid protein with a potential leucine zipper structure (Kinoshita *et al.*, 1992). It can, like C/EBP and NF-IL6, bind to the CAT box as well as the enhancer core sequence. The gene for NF-IL6 $\beta$  is expressed at an undetectable level in normal tissues but is induced by inflammatory cytokines such as IL-1 or IL-6. NF-IL6 $\beta$  easily forms a heterodimer with NF-IL6 and this heterodimer can bind the same DNA sequences as the respective homodimers. In fact, NF-IL6 $\beta$  is a stronger activator of transcription than NF-IL6 and appears to show a synergistic effect with it.

### **The *ets* family**

The first member of this family to be discovered was *v-ets*, which was named for the virus of its origin, E-twenty-six. It is quite a large family of related genes with 9

identified human genes (MacLeod *et al.*, 1992). In most of the proteins encoded by family members, a DNA binding domain (the ETS domain) is localised at the carboxyl terminus of the protein. This ETS domain, which covers approximately 85 amino acids, has no structural homology to other DNA-binding motifs such as the zinc finger, leucine zipper or homeodomain motifs. The p68<sup>c-ets-1</sup> protein binds to the PEA3 element of the polyoma virus enhancer which has the sequence AGCAGGAAGT (Wasylyk *et al.*, 1990). All *ets*-family members bind to DNA regions which contain the purine-rich core sequence GGAA/T (MacLeod *et al.*, 1992). *Trans*-activation by p68<sup>c-ets-1</sup> from the PEA3 site of the polyoma virus enhancer is cooperative with activation by the transcription factor AP-1 bound to an adjacent PEA1 site (Wasylyk *et al.*, 1990). This cooperativity is mediated by protein:protein interactions. The capacity of Ets proteins to interact with other transcriptional regulatory factors suggests a role in coordinating changes in gene expression in response to second messenger and extracellular signals.

### AP-1

The AP-1 DNA binding protein is actually a dimer of two proteins. It can be either a Jun-Jun homodimer or a Jun-Fos heterodimer. Both Jun and Fos are products of proto-oncogenes, *jun* and *fos* respectively (Chiu *et al.*, 1989). The DNA sequence to which AP-1 binds is known as the TPA (12-*O*-tetradecanoyl-phorbol-13-acetate) responsive element or TRE (consensus sequence TGAC/GTCA). The binding of AP-1 to the TRE is over 1000-fold more efficient when a heterocomplex of Jun and Fos, rather than the Jun-Jun homodimer, forms the AP-1 (Sassone-Corsi *et al.*, 1988). The differences in binding reflect differences in dimerisation stability with Jun-Fos forming at least 500-times more efficiently than Jun-Jun dimers (Jones, 1990). Fos proteins cannot form stable homodimers. Both Jun and Fos have leucine zipper motifs and this is how they interact with each other (Schütte *et al.*, 1989). Mutant versions of bZIP proteins containing altered basic domains can act as dominant/negative regulators of transcriptional activation (Jones, 1990). This is illustrated by the effects of JunB (a member of the Jun family which also contains c-Jun and JunD) on activation of AP-1 responsive genes. c-Jun is an efficient activator of genes containing a single TRE while JunB is not (Chiu *et al.*, 1989). In fact, JunB actually inhibits activation of these

promoters by c-Jun. JunB, c-Jun and JunD share significant sequence homology. They are coexpressed in many tissues, although at different levels (Schütte *et al.*, 1989). The transcription of *c-jun* and *junB* genes reveals different responses to some extracellular stimuli. These variations in the different Jun proteins may contribute to the regulatory effects of AP-1.

### The TIE

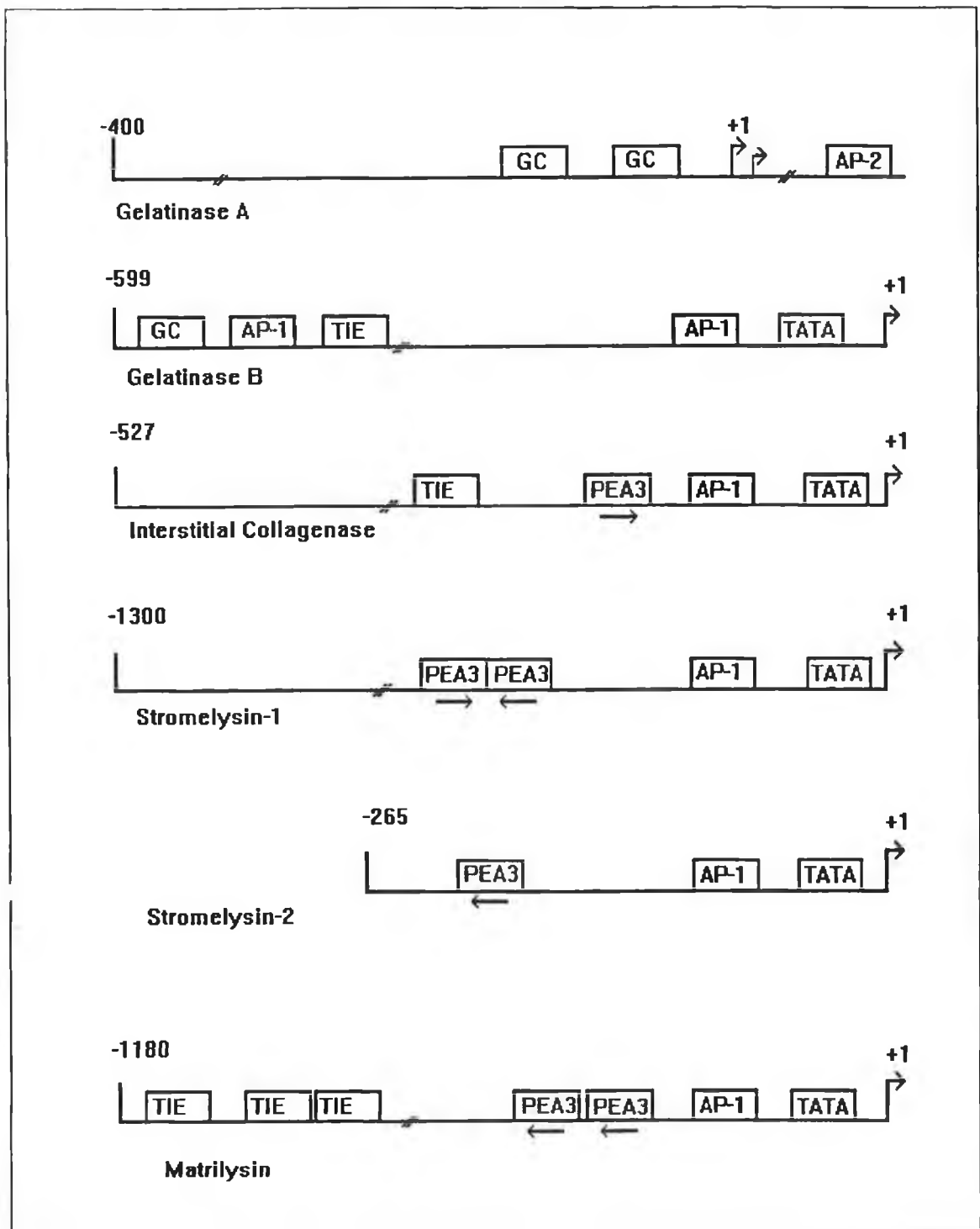
The existence of a TGF- $\beta$ 1 inhibitory element (TIE), consensus sequence GnnTTGGnGn, has been demonstrated in a number of promoters (Kerr *et al.*, 1990). Binding of the TIE appears to repress positive regulatory signals supplied by other transcription factors. The c-Fos protein has been demonstrated as the DNA binding element associated with the TIE.

### **6.3 Regulatory elements in MMP genes**

Sequencing of the promoter regions of MMP genes has yielded some surprising information. Gelatinase A has been shown to have no TATA or CAT boxes (Huhtala *et al.*, 1990). As mentioned previously, genes without TATA boxes can have variable transcriptional start sites. Gelatinase A appears to have two such sites, 9 bp apart. The lack of a TATA box is often associated with so-called 'housekeeping' or constitutively-expressed genes and the 72 kDa gelatinase A protein is, perhaps, the most widely expressed of the MMPs (Crawford and Matrisian, 1995). The gelatinase A promoter does have some regulatory elements; there are two GC boxes at positions -69 and -89 and an AP-2 potential binding site within the first exon at position +157 (Huhtala *et al.*, 1990). The AP-2 protein appears to mediate transcriptional activation through the second messengers protein kinase C and cAMP-dependent protein kinase A (Imagawa *et al.*, 1987).

Other MMPs, for which promoter sequences are available, have TATA boxes and AP-1 binding sites (TREs) (Angel *et al.*, 1987; Quinones *et al.*, 1989; Huhtala *et al.*, 1991; Gaire *et al.*, 1994). A comparison of the promoter sequences of the MMP genes is shown in figure 6.2.

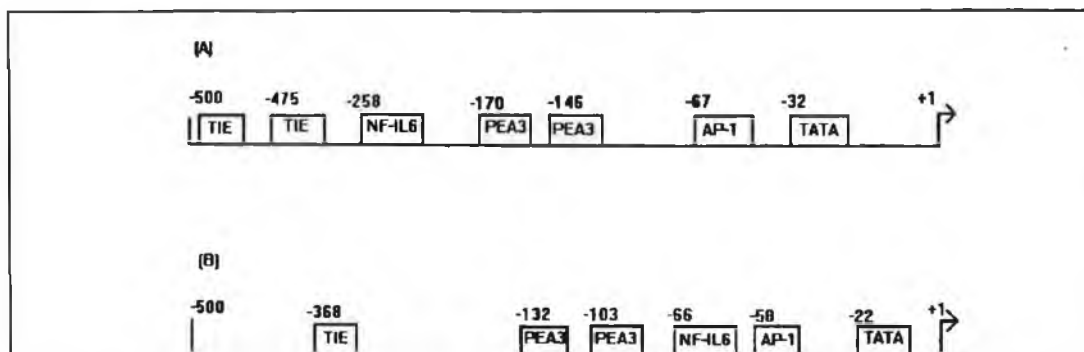




**Figure 6.2** A comparison of regulatory elements found in the promoters of MMP genes. Transcriptional start sites are indicated by +1. TATA boxes, AP-1 binding sites, PEA3 elements, AP-2 recognition sites, GC boxes and TIEs have been marked. The orientations of the PEA3 elements relative to transcription are indicated by arrows. The diagram is not to scale. (Taken from Gaire *et al.*, 1994).

Stromelysin-1, stromelysin-2, interstitial collagenase and matrilysin also contain PEA3 sites which bind members of the *c-ets* family of proteins. *C-ets* proteins can be induced by the activation of the proto-oncogenes *ras* and *src*, while AP-1 is composed of the protein products of the *fos* and *jun* proto-oncogenes. Such responsiveness to proto-oncogenes suggests that the MMP genes are linked to cellular growth and control (Crawford and Matrisian, 1995). It also suggests that the expression of these genes can be easily affected by cellular transformation. Inhibition of expression by TGF- $\beta$  would also appear to be a common feature of MMP genes given the frequency with which the TIE appears in MMP promoters. The matrilysin promoter, for example, has three apparent TIE regions upstream of other regulatory elements (Gaire *et al.*, 1994).

The fact that particular sequences are present in the promoter region of a given gene does not necessarily mean that the transcriptional regulatory elements they represent are functional. Figure 6.3 illustrates potential transcriptional elements present in the mouse and human matrilysin promoters based on sequence information. The work presented in this chapter is intended to demonstrate their potential activity. Proof that these elements are indeed functioning as thought will require further experimentation.



**Figure 6.3** Potential transcriptional elements within the human and mouse matrilysin promoters. This diagram represents approximately 500 bases of 5' sequence of the (A) human and (B) mouse matrilysin gene. +1 represents the transcription initiation site. TATA boxes, AP-1 binding sites, PEA3 elements TIE and NF-IL6 consensus binding motifs are indicated. The diagram is not drawn to scale.

## RESULTS AND DISCUSSION

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### 6.4 Preparation of Plasmids

A number of different plasmids were used in the course of this work. Restriction digests were used in order to identify particular plasmids (section 2.2.8.6). The expected sizes corresponding to each plasmid cut with a specific enzyme are presented in Table 6.1.

Plasmid	Restriction Enzyme	Expected Size of Fragments (bp)
pCH110	Pvu II	360, 700, 2600, 3500
pFLCAT	Eco R1 + Bam H1	751, 1382, 2400
pCMVCAT	Pvu II	333, 1727, 2054, 2567
p295HPCAT	Pvu II	535, 1728, 2567
p933HPCAT	Pvu II	1278, 1728, 2567
pGL <sub>2</sub> -Control	Bam H1 + Hind III	2900, 3146
pGL <sub>2</sub> -MMP	Pvu II	2500, 4000

**Table 6.1** The expected sizes (in bp) of fragments resulting from digesting specific plasmids with the enzymes mentioned. The different patterns obtained when these digest products were run on agarose gels were used to confirm identities of the plasmids.

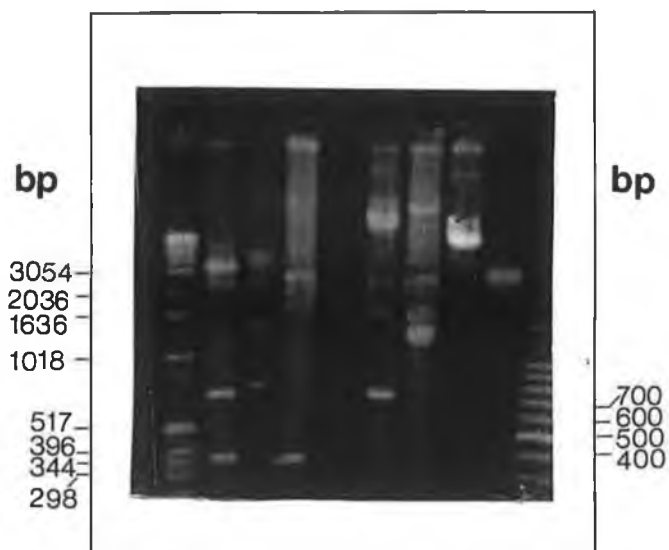
pCH110 carries the gene for the bacterial enzyme  $\beta$ -galactosidase. The gene is under the control of a promoter from the simian virus, SV40, so any cells which are successfully transfected with this plasmid should produce  $\beta$ -galactosidase. This enzyme is easily detected using either an *in situ* staining technique which utilises the synthetic substrate X-gal, or by assaying cell lysates with CPRG (see sections 2.2.10.1/3). The ease of detection makes this a suitable enzyme for assessing transfection efficiencies. The *in situ* staining technique was used in early experiments

when cell lines and transfection methods were being screened to find the most suitable combination. The CPRG assay was used in later transfections when the plasmid pCH110 was co-transfected with a matrilysin promoter construct. This allowed corrections to be made for variations in transfection efficiency observed among experiments.

The pCAT group of plasmids (kindly donated by Prof. Lynn Matrisian, Vanderbilt University) all contain the gene for the bacterial enzyme chloramphenicol acetyl transferase (CAT). The positive control plasmid, pCMVCAT, contains this gene under the control of a promoter from the cytomegalovirus (CMV). Any cells successfully transfected with this plasmid should express CAT protein. The negative control plasmid, pFLCAT, contains the CAT gene with an SV40 promoter attached to the 3' end. This orientation of promoter should not activate transcription of the CAT gene and so cells transfected with this plasmid will not express CAT protein. p295HPCAT and p933HPCAT are matrilysin promoter-reporter gene constructs. They contain various lengths of the human matrilysin promoter (designated by the figures in the plasmid names) attached to the CAT gene. By using different lengths of the promoter, various combinations of transcriptional elements could be examined as to their effect on transcriptional activation of the attached CAT gene.

The pGL<sub>2</sub> plasmids contain the gene for the firefly luciferase enzyme. The pGL<sub>2</sub>-control plasmid contains SV40 promoter and enhancer regions so that the luciferase enzyme should be efficiently produced by cells successfully transfected with this plasmid. pGL<sub>2</sub>-MMP (obtained from Prof. Lynn Matrisian, Vanderbilt University) contains the luciferase gene attached to 2.7 Kb of the mouse matrilysin promoter. No other promoter or enhancer sequences are present.

Figure 6.4 is an agarose gel showing the products obtained when these plasmids are digested with the appropriate restriction enzymes. On comparison with the data in Table 6.1, it may be seen that the plasmids, when digested, give the expected fragments thus confirming their identity.



**Figure 6.4** Plasmids cut with restriction digests (as detailed in Table 6.1) and run on a 1.2 % agarose gel. The sizes of the fragments obtained correspond to those expected.

- Lane 1 : 1 kb ladder - DNA size markers
- Lane 2 : pCH110 - Pvu II digest
- Lane 3 : pCMVCAT - Pvu II digest
- Lane 4 : pFLCAT - Bam H1 + Eco R1 digest
- Lane 5 :
- Lane 6 : p295HPCAT - Pvu II digest
- Lane 7 : p933HPCAT - Pvu II digest
- Lane 8 : pGL<sub>2</sub>-Control - Bam H1 + Hind III digest
- Lane 9 : pGL<sub>2</sub>-MMP - Pvu II digest
- Lane 10 : 100 bp ladder - DNA size markers

## 6.5 Optimisation of Transfections

For genes to be transcribed, control regions in their promoters must be bound by appropriate transcription factors. Some transcription factors are cell-type specific, therefore some genes may only be expressed in specific cell types under normal circumstances [aberrations may occur as a result of the transformation process]. To examine the transcription factors required for activation of a particular gene therefore, it is necessary that the study be carried out in cells where those transcription factors are present. For this reason, promoter studies of a given gene are usually undertaken in cells which normally express the gene as the necessary transcription factors are definitely present. Based on this reasoning, and using the results obtained by RT-PCR presented in chapter 5, the cell lines K562, SW620 and LoVo, all of which express basal levels of matrilysin, were chosen for transfection studies. A fourth cell line, SW480, which does not normally express matrilysin unless induced to do so by specific treatments, was also used in early experiments. This cell line had previously been shown to be transfectable and so was used as a positive control for some optimisation experiments.

Three different transfection methods were investigated. Calcium-phosphate mediated transfection was first described by Graham and Van der Eb (1973). The method is based on the formation of a crystalline precipitate produced when calcium ions interact with the negatively charged phosphate groups of DNA in the presence of soluble phosphate ions. The exact mechanism of this transfection technique is unclear, but it is thought that the precipitate attaches to the cell surface and is then taken into the cell by endocytosis.

DEAE-dextran-mediated transfection is quite similar. The positively charged DEAE-dextran binds to the negatively charged phosphate groups of the DNA. This complex then binds to the negatively charged cell surface. Uptake into the cells is also thought to be by endocytosis. Lipofection, that is transfection mediated by cationic liposomes, is a relatively new tool. Cationic lipids, such as DOTAP, form liposomes in aqueous solution. These can then interact spontaneously with negatively charged DNA to form

stable complexes. The complexes adhere to the cell surface, fuse with the phospholipid cell membrane and release the DNA into the cytoplasm of the cell.

The DEAE-dextran method is generally recommended for use with small amounts of DNA and is only suitable for transient transfections where the exogenous DNA is not incorporated into the target cell's genome. Calcium phosphate-mediated transfection and transfection with liposomes such as DOTAP, can be either transient or stable. In the case of stable transfections, exogenous DNA is incorporated into the genome of the target cell. This is usually achieved using a selective pressure such as antibiotic (*e.g.* neomycin) resistance by co-transfecting a gene coding for such resistance together with the desired gene.

All the transfection techniques investigated have a number of variables associated with them. Firstly, not all techniques are suitable for all cell lines. In fact there appear to be a number of cell lines which are extremely difficult to transfect for unknown reasons. Secondly, any cell line which can be transfected by a given technique will have an optimal DNA concentration suitable for transfection. With some cell lines, amounts of DNA above 10  $\mu\text{g}$  may prove toxic to the cells, whilst in other cell lines, 50  $\mu\text{g}$  of DNA may be required for transfection. Thirdly, in the case of lipofection reagents, the optimal ratio of liposome to DNA for efficient transfection of a given cell line must be determined empirically. Other points which should be considered include the amount of time following transfection for which the DNA precipitate is left in contact with the cells, the use of glycerol or other 'shocking' agents to stimulate cellular uptake of exogenous DNA, and the period of time following transfection for which the cells are left prior to harvesting for analysis. Varying the cell line, the amount of DNA and the ratio of transfection reagent to DNA were all investigated. The amount of time precipitates were left in contact with cells, the use of glycerol and the harvest time were fixed for all transfections. Precipitates of either calcium phosphate and DNA or DOTAP and DNA were left in contact with cells for 5 hours in all experiments. Glycerol was used in the calcium phosphate-mediated transfections following removal of the DNA precipitate to briefly shock the cells and stimulate uptake of DNA. In DEAE-dextran transfections, the drug chloroquine may be used for similar reasons, however, it was not used in these experiments. All transfected cells

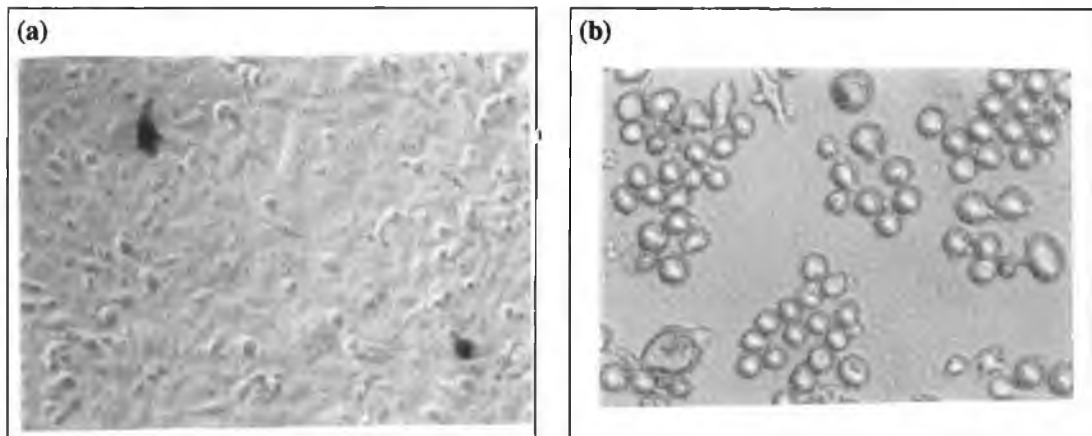
were left for 48 hours prior to harvesting. This was to allow the cells to recover and to give enough time for cytokine stimulations if they were used.

Using 6-well culture plates containing  $5 \times 10^5$  -  $5 \times 10^6$  cells per well (dependent on cell line used) and a range of concentrations of the plasmid pCH110, each of the transfection methods was tried with each of the cell lines. 48 hrs following transfection, the plates were fixed and stained for  $\beta$ -galactosidase activity (section 2.2.10.1). Blue cells, indicating the presence of the enzyme and hence successful transfection, were visible only in the SW480 and K562 cell lines following calcium phosphate or DOTAP-mediated transfections. No successfully transfected cells were observed in those plates transfected using DEAE-dextran. This information is presented in Table 6.2. The appearance of successfully transfected cells is shown in Figure 6.5. In SW480 cells, the enzyme appears to be produced in large amounts throughout the cell so that transfected cells are deep blue in colour. In contrast, the K562 cells that have been transfected only appear to produce small amounts of the enzyme in localised regions within the cells. The actual blue stain is quite difficult to observe.



Transfection Method	Cell Line	Positive X-Gal Conversion
Calcium Phosphate	SW480	+++
	K562	+++
	LoVo	-
	SW620	-
DEAE-dextran	SW480	-
	K562	-
	LoVo	-
	SW620	-
DOTAP	SW480	++
	K562	++++
	LoVo	-
	SW620	-

**Table 6.2** Results of various transfection methods on different cell lines. The presence of blue stain resulting from the action of the enzyme  $\beta$ -galactosidase (carried on the plasmid pCH110) on the substrate X-gal demonstrated the success of the transfection method. The degree of staining is indicated using a scoring system where '-' indicates no blue cells apparent, '+' indicates minimal staining, and '++', '+++ and '++++' indicate increasing levels of staining relative to each other.

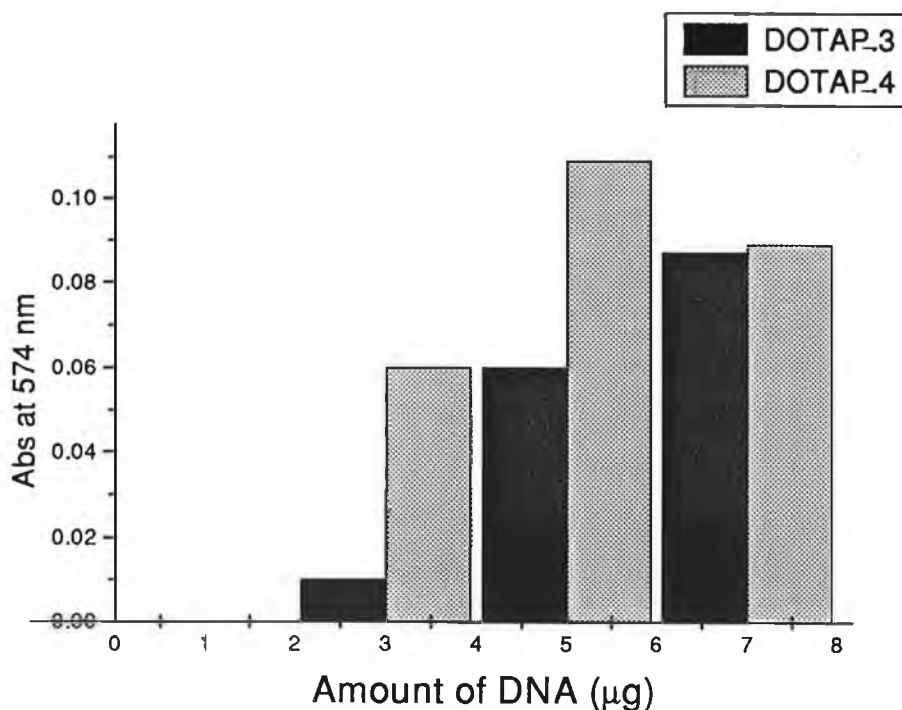


**Figure 6.5** *In situ* staining for production of the enzyme  $\beta$ -galactosidase. (a) SW480 cells magnified 200X. The positively staining cells are deep blue in colour and can be clearly seen. (b) K562 cells magnified 200X. The positively staining cells contain regions within them which are blue in colour.

At this stage, it was decided to concentrate with only one cell line, K562, and one transfection method, DOTAP-mediated transfection, as results obtained with other methods and suitable cell lines were disappointing.

### 6.5.1 Optimisation of DOTAP transfections in K562

As stated previously, the amount of DNA and the ratio of DNA to transfection reagent can affect the efficiency of a DOTAP-mediated transfection. To determine the optimal ratio of DOTAP to DNA and total amount of DNA, an experiment was conducted where various amounts of DNA were mixed in two different ratios with DOTAP. Following transfection and a 48 hr recovery period, the cells were harvested, lysed and the lysates assayed for  $\beta$ -galactosidase activity using a CPRG assay. The results are presented in Figure 6.6.



**Figure 6.6** Optimisation of DOTAP-mediated transfection of K562 cells. The effects of varying both the total amount of DNA and the DOTAP to DNA ratio were examined by assaying for  $\beta$ -galactosidase activity using a CPRG assay and measuring absorbances at 574 nm. The two DOTAP to DNA ratios examined were 3  $\mu\text{g}/\mu\text{g}$  DNA and 4  $\mu\text{g}/\mu\text{g}$  DNA. The three amounts of DNA examined at these two ratios, were 3  $\mu\text{g}$ , 5  $\mu\text{g}$  and 7  $\mu\text{g}$ .

The optimum DOTAP : DNA ratio was thus taken to be 4 µg DOTAP / µg DNA while a total amount of 5 µg of DNA was used in further experiments.

The actual efficiency of transfection when these amounts of DNA and DOTAP were used was then calculated. This was done by setting up a tissue culture plate with  $1 \times 10^6$  K562 cells and the following day, transfecting the cells with 5 µg of pCH110 DNA using DOTAP at a ratio of 4 µg / µg DNA. 48 hrs later, the cells were fixed and stained for β-galactosidase activity using the *in situ* staining technique. The positively staining (blue) cells were counted and the percentage transfected cells calculated. 26,988 blue cells were counted. This approximates to a transfection efficiency of 2.7% which is a suitable transfection efficiency for transient transfections.

## 6.6 Analysis of the human matrilysin promoter

Two different lengths of the human matrilysin promoter attached to the reporter gene, CAT, were available for study. The plasmid p295HPCAT contained 295 bp of the 5' flanking region of the human matrilysin gene. On examination of Figure 6.3, it may be seen that within this region there are two potential PEA3 elements, an NF-IL6 consensus sequence, an AP-1 binding sequence and a TATA box. The mitogenic tumour promoting agent, TPA, would be expected to activate transcription therefore through the AP-1 binding motif (otherwise known as the TPA responsive element) while the cytokines IL-1 and IL-6 could also potentially activate transcription through the NF-IL6 sequence. Other factors may activate transcription through the PEA3 elements. p933HPCAT contains 933 bp of the 5' flanking region of the human matrilysin gene. This length of promoter has all those elements present in the 295 bp region in addition to 2 TGF-β inhibitory elements or TIE sequences.

K562 cells were transfected with these plasmids and positive and negative controls - pCMVCAT and pFLCAT-using the DOTAP method as previously outlined. The plasmid pCH110 was cotransfected with each of the CAT plasmids to allow for correction of variations in transfection efficiencies. Following transfection, the cells were treated with various cytokines and TPA (see section 2.2.9.6). 48 hrs after transfection, the cells were harvested and lysed. A portion of each lysate was used for

the CPRG assay to determine levels of  $\beta$ -galactosidase resulting from transfection of pCH110. Significant values indicating enzyme activity were obtained with all samples of transfected cells indicating successful transfections. The presence of CAT protein was determined using a commercially available CAT ELISA (section 2.2.10.4). The amount of CAT protein in each sample was determined from a set of CAT standards also assayed using the ELISA. The results are presented in Table 6.3.

Treatment	Cells transfected with pFLCAT (pg protein)	Cells transfected with pCMVCAT (pg protein)	Cells transfected with 295HPCAT (pg protein)	Cells transfected with 293HPCAT (pg protein)
None	0	17	2	0
IL-1 $\beta$	2	21	0	0
IL-6	0	15	4	0
EGF	0	N.D.	0	0
TGF- $\beta$	0	N.D.	13	0
IGF-1	0	70	4	0
TPA	2	N.D.	0	4

**Table 6.3** Results of CAT ELISA following DOTAP-mediated transfection of K562 cells and cytokine/TPA treatment. The results given are pg of CAT protein in each lysate determined from a calibration set of CAT standards assayed using the CAT ELISA. The values have been corrected for equivalent  $\beta$ -galactosidase activity as determined from a CPRG assay. 'N.D.' signifies that a particular sample was not assayed.

All values are extremely low, possibly due to the lysate preparation technique used. Although the claims from the manufacturer are that the lysis buffer is suitable for use in assays for CAT,  $\beta$ -galactosidase and luciferase reporter genes, it has been optimised for use with luciferase assays. Also, the actual amount of lysate that was available for use in the assay was very small - equivalent to  $3 \times 10^4$  cells. The presence of CAT protein, albeit in small amounts, in the pCMVCAT transfected cells does indicate that transfections were successful. IGF-1 appears to stimulate the viral CMV promoter as the increase in levels of CAT over untreated pCMVCAT transfected cells is approximately 4-fold. The only other significant result is the slight stimulatory effect of

TGF- $\beta$  seen with the shorter promoter construct p295HPCAT. This length of promoter does not contain any TIE sequences and it is not known what elements are responsible for mediating a TGF- $\beta$  stimulatory effect.

Previous work carried out in this laboratory (O'Shea, M.Sc. Thesis, 1996) also demonstrated little activity with the human matrilysin promoter - CAT constructs. Recent results from our collaborators in Vanderbilt University corroborate these findings (Prof. Lynn Matrisian, pers. comm.). It is thought that the constructs may have been originally prepared with 5' flanking regions of the CAT gene included between the matrilysin promoter and the actual CAT coding gene. Another possibility is an error in the human matrilysin promoter sequence. The constructs are currently been remade with a different reporter gene and it is hoped to use these in the near future.

As another matrilysin promoter construct was available - 2.7 Kb of the mouse matrilysin promoter linked to the luciferase reporter gene - it was decided to conduct further transfection experiments with it.

## **6.7 Analysis of the mouse matrilysin promoter**

The plasmid pGL<sub>2</sub>-MMP contains 2.7 Kb of the 5' flanking region from the mouse matrilysin promoter. Sequence information is available for the first 500 bp only. From Figure 6.3, it can be seen that there are a number of potential regulatory elements within this region. These include a TATA box, an AP-1 binding site, two PEA3 elements, an NF-IL6 consensus sequence and a TIE sequence. From these sequences, it would be expected that cytokines such as EGF, IL-1, IL-6 and TGF- $\beta$  as well as the mitogen TPA, would have effects on transcriptional activation of the mouse matrilysin gene. In the pGL<sub>2</sub>-MMP plasmid, the matrilysin gene has been replaced with a luciferase gene and transcriptional activity can, therefore, be easily measured using luminescence.

Initially, the pGL<sub>2</sub>-control plasmid was used to check that luciferase activity could be detected. All cells were co-transfected with pCH110 and lysates analysed for both luciferase and  $\beta$ -galactosidase activity. Using increasing amounts of both

plasmids (up to a total of 6  $\mu\text{g}$  of DNA), K562 cells were transfected with pGL<sub>2</sub>-control and pCH110 and harvested 48 hrs later. The lysates were assayed as outlined in section 2.2.10.5 and the results obtained are presented in Table 6.4.

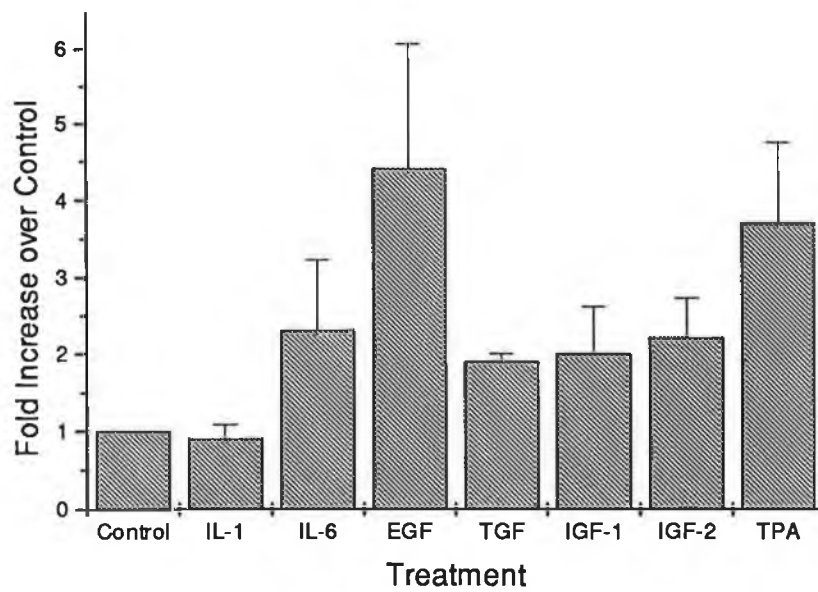
Amount of each plasmid ( $\mu\text{g}$ )	Luminometer Measurement		CPRG assay
			Absorbance at 574 nm
0		-0.016	0.000
1		0.102	0.155
2		0.288	0.178
3		0.407	0.276

**Table 6.4** Luminescence and absorbance values for K562 cell lysates following transfection with various amounts of the plasmids pCH110 and pGL<sub>2</sub>-control.

These results proved that the luciferase assay was valid for determining activity of a transfected luciferase gene. In all further transfections with luciferase constructs, 6  $\mu\text{g}$  total DNA was used which included equal amounts of pCH110 and the luciferase construct.

### 6.7.1 Effects of cytokines and TPA

Transfection experiments in which K562 cells were transfected using the reagent DOTAP, with plasmids encoding  $\beta$ -galactosidase (pCH110) and the mouse matrilysin promoter attached to the luciferase reporter gene (pGL<sub>2</sub>-MMP), were performed. 40 hrs after transfection, the cells were treated with various cytokines or TPA. After a further 8 hrs incubation, the cells were harvested, lysed and analysed for both luciferase and  $\beta$ -galactosidase activities using luminescence and CPRG assays, respectively. The luciferase values were corrected for equivalent amounts of  $\beta$ -galactosidase, and the results expressed as fold-increase over control. The data obtained from 3 such experiments was averaged and is presented in Figure 6.7.



**Figure 6.7** Transcriptional activity of mouse matrilysin promoter in K562 cells following treatment with cytokines and TPA. The data represented is averaged from three experiments.

EGF, TPA, IL-6, IGF-I, IGF-II and TGF- $\beta$  all appear to upregulate gene expression. TPA is a synthetic analogue of the cellular second messenger diacylglycerol which activates protein kinase C (Pohl *et al.*, 1988). Other molecules which use the same signal transduction pathway would be expected to have similar effects to TPA. Binding of the EGF receptor by EGF activates diacylglycerol (Hardie, 1991). The similarity of the effect seen with both EGF and TPA is, therefore, not surprising.

Stimulation of endogenous matrilysin mRNA in K562 cells by IL-6 and the IGF molecules was previously demonstrated (Figures 5.6 - 5.8, Chapter five). The promoter elements responsible for mediating these effects are apparently present within the first 2.7 Kb of the promoter. As these cytokines have not previously been shown to have an effect on matrilysin expression, analysis of the promoter regions responsible is certainly warranted. The stimulatory effect of IL-6 seen with this promoter construct is, however, less than the effect seen with matrilysin mRNA. It is possible that an additional NF-IL6 or similar element lies upstream of the promoter region examined here. In contrast, the lack of response to EGF seen with endogenous matrilysin mRNA could be due to repressor elements upstream of the promoter region. Another possible explanation for the lower levels of stimulation seen with the transfected promoter construct compared to endogenous matrilysin, is a difference in copy number. The transcription factors necessary to mediate a particular response may be present in limiting amounts so that when a gene is transfected with a high copy number per cell, lower transcriptional activation than usual can result (Frisch and Ruley, 1987).

The stimulatory effect of TGF- $\beta$  was not anticipated. Most previous studies have shown that, in fibroblasts, TGF- $\beta$  inhibits production of MMPs such as interstitial collagenase and stromelysin-1 (Overall *et al.*, 1989; Kordula *et al.*, 1992). A stimulatory effect on gelatinases A and B in uterine epithelial cells was observed in one study, however, stromelysin-1 was also down-regulated (Agarwal *et al.*, 1994). The mechanism by which TGF- $\beta$  exerts its inhibitory activity on levels of interstitial collagenase has been elaborated (Mauviel *et al.*, 1993). It upregulates production of the proto-oncogene product, JunB, which is a negative regulator of the AP-1 component, c-Jun. JunB has, however, been shown to be inhibitory only in promoters



containing a single AP-1 binding site (Chiu *et al.*, 1989). In promoters with multimeric TRE regions, JunB is an efficient transcriptional activator. It is possible that more TRE or similar elements are present as enhancer elements and this may explain the stimulatory effect of TGF- $\beta$ .

There is very little data available concerning the matrilysin promoter. The results obtained here greatly increase our knowledge of the factors which can transcriptionally activate the matrilysin gene. However, much more work is required. The next step in this analysis will be to determine minimal promoter lengths necessary to obtain particular responses. It would be expected that a short region of the promoter containing just the TATA and AP-1 boxes would be sufficient for the stimulatory effects of EGF and TPA. It will be more interesting to determine the regions responsible for mediating effects of the IGFs and IL-6, as these cytokines have not previously been linked with activation of an MMP gene. Once the regions responsible have been isolated to within 20 - 40 bp of DNA, site-directed mutagenesis and DNase I footprinting can be used to identify exactly the DNA sequences to which regulatory proteins can bind. The regulatory proteins themselves can then be purified from nuclear extracts using the DNA sequences as affinity isolation reagents. It is hoped eventually to define the signal transduction pathway by which binding of a particular cytokine such as IGF-I or -II results in upregulation of matrilysin gene expression. This could result in the design of matrilysin-specific inhibitors which, it is envisaged, could be therapeutic in certain tumours, particularly colorectal.

## Summary

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Four cell lines were investigated as to their suitability for transfection studies using three different transfection methods. One of these, SW480, did not express matrilysin and was used only as a control as it had previously been transfected by methods used in this study. Only the K562 cell line could be easily transfected by the methods examined. The DOTAP-mediated transfection method was demonstrated to be the most suitable method for this cell line. The optimal amounts of DNA used for transfection and the ratio of DOTAP to DNA were empirically determined prior to proceeding with promoter studies.

K562 cells were transfected with plasmids containing lengths of the human matrilysin promoter linked to the reporter gene CAT. The transfected cells were then treated with cytokines and TPA, harvested and lysed. Levels of CAT protein, as determined by a CAT ELISA, were very low and no discernable difference from control was found in the cytokine- or TPA-treated cells. This was thought to be due to problems with the manufacture of the promoter constructs. These promoter constructs are currently being remade and it is hoped to repeat the transfection experiments with them.

A 2.7 Kb length of mouse matrilysin promoter linked to the luciferase reporter gene was then transfected into K562 cells and the cells treated with cytokines and TPA. Stimulatory effects were observed in cells treated with TPA, EGF, IL-6, IGF-I, IGF-II and TGF- $\beta$  suggesting that the transcriptional control elements responsible for mediating effects of these molecules, lie within this region of the matrilysin promoter. This is the first report of transcriptional activation of the matrilysin gene by many of these factors. Only TPA and EGF had previously been investigated and shown to have an effect (Gaire *et al.*, 1994). Further investigations will be required to determine precisely which regions are linked with specific cytokines.

## **Chapter Seven**

### **Final Summary and Conclusion**

## Summary

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The research presented in this thesis is centred on the MMP, matrilysin. Four related but distinct areas were covered. Firstly, a sensitive and specific one-step, sandwich ELISA for matrilysin was developed. The monoclonal antibodies used in this assay were provided by our collaborators from Syntex Research, California and Vanderbilt University, Tennessee. The ELISA was validated for use with cell culture medium and was shown to have excellent reproducibility. The linear range was 5 - 50 ng/ml with a limit of detection of 0.45 ng/ml. Although this ELISA was not useful for the breast tumour study presented in this thesis, it is expected that it could be used for determination of matrilysin levels in other tumour types. Matrilysin expression has been particularly associated with colon tumour development (Witty *et al.*, 1994; Yamamoto *et al.*, 1995). Specific mRNA for matrilysin was not found in normal colon tissue but was seen in adenomas although at lower levels than in invasive carcinomas (Newell *et al.*, 1994). A sensitive and specific ELISA for matrilysin will be useful to determine if matrilysin protein levels can be correlated with stage of tumour development. The only previously described ELISA for matrilysin requires 3 days before results can be obtained (Ohuchi *et al.*, 1996). In order to assess possible correlations between tumour stage and matrilysin level, large numbers of patient samples should be analysed. Obviously, this can be achieved more easily with a faster assay. Our matrilysin ELISA is a significant improvement on that previously available as it requires only 7 hours before results can be obtained.

Matrilysin expression has previously been demonstrated in gastrointestinal carcinomas (McDonnell *et al.*, 1991). Its expression by tumour cells only and not in adjacent normal tissue or other non-tumour tissue samples, suggested a role for matrilysin in gastrointestinal tumour development. In order to analyse its expression in breast tissue, ten tissue samples from patients with infiltrating ductal carcinoma were studied using three different detection methods. One of these methods, RT-PCR, examined mRNA expression. Eight out of the ten samples were

positive for matrilysin mRNA expression using this method. The cell types responsible for the positive result were, however, unknown.

Two protein detection methods, western blotting and immunohistochemistry, were also used. It was not known if any of the available antibodies would be suitable for immunohistochemistry. The first antibody used for western blotting, pAb<sub>2</sub>, although the only available reagent for detecting both the zymogen and active forms of matrilysin, demonstrated non-specific binding to a high molecular weight component of the tumour tissue. All of the tissue samples were positive for expression of at least one of the two forms of the matrilysin enzyme when this antibody was used. A second antibody, RmAb, detected only the zymogen form of the enzyme but showed no non-specific binding. This antibody was then used for immunohistochemistry. The ten tumour samples as well as three samples of histologically-normal breast tissue, were analysed using this technique. Positive immunostaining was seen in all samples. The staining was associated only with epithelial cells, both tumour-derived and normal ductal cells. In a study of matrilysin expression in the normal mouse, the mammary gland was one of the few tissue types in which positive expression was demonstrated (Wilson *et al.*, 1995). It would appear that the human mammary tissue also expresses this protein. In breast tumours, therefore, the expression of matrilysin is not linked with tumour development. It would be interesting to investigate tumours of other tissues as constitutive expression of matrilysin by normal tissue would not appear to be widespread.

The regulation of MMP expression by cytokines is an area of great interest (Mauviel, 1993b). The demonstration that tumour-associated expression of these invasion-linked proteinases may be controlled by polypeptide factors released by tumour or adjacent stromal cells (Borchers *et al.*, 1994), suggests new possibilities in tumour immunotherapy. Information concerning cytokine regulation of matrilysin is limited. Of the cytokines investigated here, only EGF, TGF- $\beta$  and IFN- $\gamma$  have previously been shown to have any effect on matrilysin expression (Gaire *et al.*, 1994; Bruner *et al.*, 1995; Busiek *et al.*, 1995). Most previous studies of cytokines and MMPs have concentrated on fibroblastic cells which do not express matrilysin. In this study, eight tumour cell lines representing diverse tissue

origins, were analysed for matrilysin expression following treatment with a range of cytokines. Expression of another MMP, stromelysin-1, by these cytokine-treated cell lines was also examined. Matrilysin is apparently expressed in a tissue-specific manner although cytokines such as IL-1 $\beta$ , IL-6, bFGF, IGF-I and IGF-II can modulate levels, even inducing expression in cell lines which are normally negative such as SW480 and EJ. Stromelysin-1 was expressed in a wider range of cell lines but again, levels could be modulated by IL-1 $\beta$ , IL-2, EGF, TGF- $\beta$  and IFN- $\gamma$ . The demonstration that IL-6, in particular, is an inducer of matrilysin expression may serve to explain the levels of the enzyme present in normal breast tissue. A recent report shows that IL-6 is expressed in normal breast tissue but that levels decrease in invasive tumours (Basolo *et al.*, 1996). It has been previously demonstrated that IGF-I, IGF-II and bFGF are autocrine and paracrine growth factors for breast tumours (Lippman *et al.*, 1986; Cullen *et al.*, 1992). These factors may upregulate matrilysin expression by breast tumour cells. IGF-I and IGF-II are of particular interest as they have been shown to be important stimulators of tumour cell proliferation in colorectal carcinomas (Lahm *et al.*, 1994).

Transfection analysis of matrilysin promoter constructs in a suitable cell line was also performed. Human matrilysin promoter regions linked to the CAT reporter gene were shown to have little activity. Similar results have been reported by other groups (Prof. Lynn Matrisian, Dr T. Bowden, pers. comm.). It is thought that these results are due to an excess of 5' flanking sequence of the CAT gene in the constructs. The constructs are currently being remade and will be analysed as soon as they are available. A 2.7 Kb mouse matrilysin promoter region linked to the luciferase reporter gene was successfully transfected into the K562 cell line using a lipofection reagent, DOTAP. Following transfection, the cells were treated with cytokines and TPA. EGF and TPA were the strongest stimulatory agents and it is thought that they both act through the AP-1 binding site present in the matrilysin promoter. As similar results were not seen with EGF when endogenous matrilysin expression by K562 cells was examined, the presence of repressor regions upstream of the 2.7 Kb promoter region used in transfection analysis must be considered. Alternatively, differences between mouse and human matrilysin genes may explain the results. An investigation of rat matrilysin showed little

difference from the human form (Abramson *et al.*, 1995) so it is unlikely that the mouse version would deviate to a large degree. IL-6 and IGF-I and -II were shown, as in the previous section, to be stimulatory. The presence of an NF-IL6 element within the promoter may explain the IL-6 effect, however, it is not known which elements are responsible for effects of IGF-I and -II. These cytokines have not previously been shown to regulate MMP gene expression so a more detailed investigation of their effects would be recommended.

The data concerning the effects of cytokines on matrilysin expression particularly at the transcriptional level is very interesting. There are no previous studies of matrilysin transcriptional regulation using a mouse promoter and no reports of IL-6, IGF-I or IGF-II being linked to matrilysin expression. There is a growing body of evidence to suggest that matrilysin is especially important in the early development of colorectal cancer (McDonnell *et al.*, 1991; Witty *et al.*, 1994; Newell *et al.*, 1994; Yamamoto *et al.*, 1995). The most recent data links matrilysin expression with the APC gene which is implicated in colorectal tumourigenesis (Fearon and Vogelstein, 1990). Approximately 90 % of adenomas arising in mice carrying a mutation in the APC gene predisposing to multiple intestinal neoplasias (Min mice) express matrilysin. A cross between Min mice and mice in which the matrilysin gene has been ablated by homologous recombination results in progeny with a 60 % reduction in the number of intestinal neoplasias (Matrisian *et al.*, 1996). The significance of this link between matrilysin and early events in colorectal tumourigenesis remains to be elucidated. Some of the data presented in this thesis may contribute to our understanding of matrilysin in tumourigenesis. Of the three colon tumour cell lines examined, two, SW620 and LoVo, have been shown to produce and respond to IGF II in an autocrine manner (Lahm *et al.*, 1994). These cell lines constitutively produce matrilysin. The third colon cancer cell line, SW480, although expressing an IGF receptor, does not itself appear to produce IGF-I or II (Lahm *et al.*, 1994). SW480 cells do not produce matrilysin although they can be induced to do so by addition of exogenous IGF-I or II. This suggests that the matrilysin expression associated with colorectal tumourigenesis may be regulated by the IGFs. Investigation of the specific mechanism of IGF induction of matrilysin could lead to the development of a specific matrilysin

inhibitor which could then be used therapeutically for colorectal cancer. Our results also show that another cytokine, bFGF, is apparently associated specifically with matrilysin induction and should be further investigated.

The transcriptional activation of matrilysin by IL-6 has not previously been reported. Sequence analysis of the matrilysin promoter suggests the existence of possible IL-6 responsive elements but their activity has not been demonstrated. The next step in our work will be to determine minimal promoter lengths which respond to IL-6 and other specific cytokines and then to identify the specific DNA sequences involved. Ultimately, it is hoped to delineate the signal transduction pathways which result in matrilysin gene expression following cytokine treatment. This knowledge could increase our understanding of the tumour development process thus leading to the development of specific therapeutic agents, and could also be of benefit in understanding the regulatory effects of cytokines on many other genes.



## **Chapter Eight**

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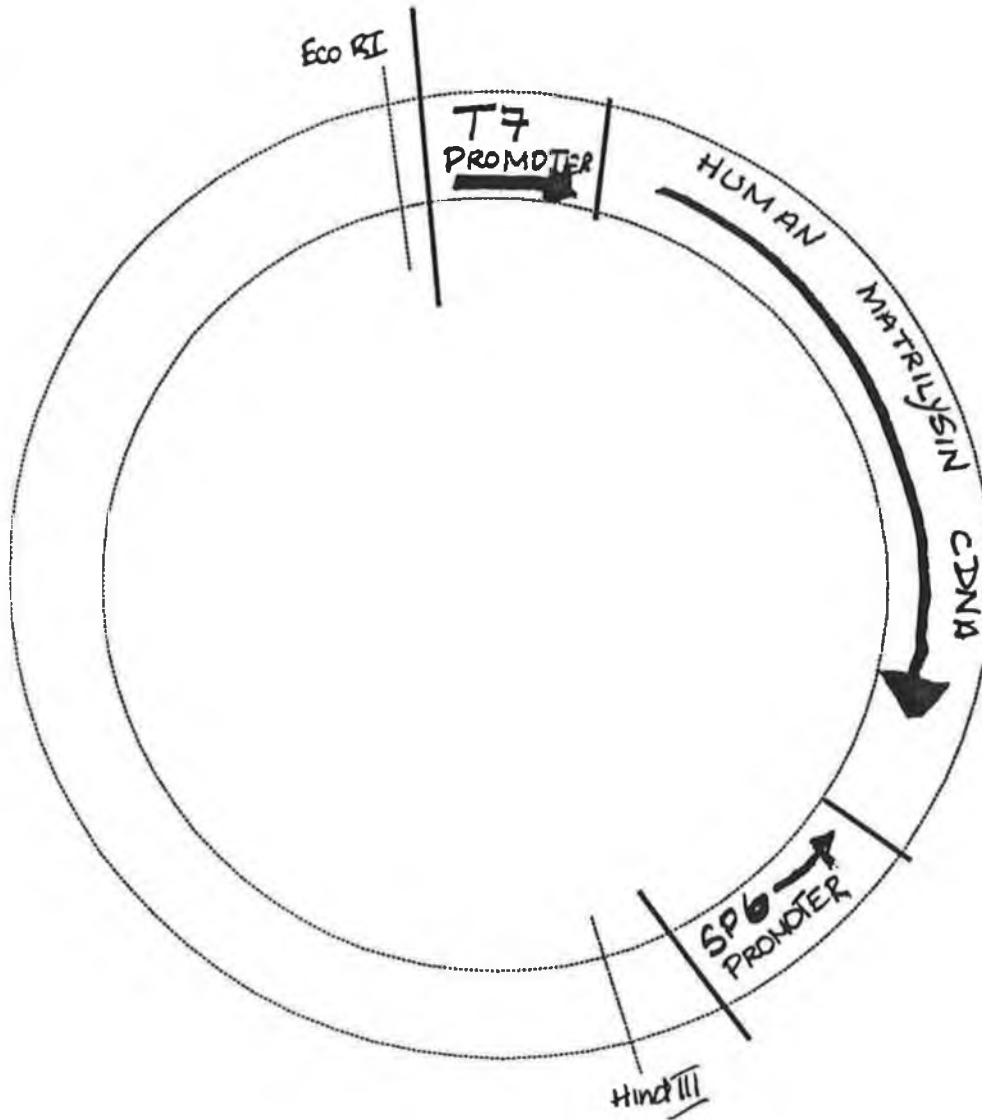
Zucker, S., Lysik, R. M., Zarrabi, M. H. and Moll, U. (1993) M<sub>r</sub> 92,000 type IV collagenase is increased in plasma of patients with colon cancer and breast cancer. *Cancer Res* 53 140 - 146.

## **APPENDIX A**

### **PLASMID MAPS**

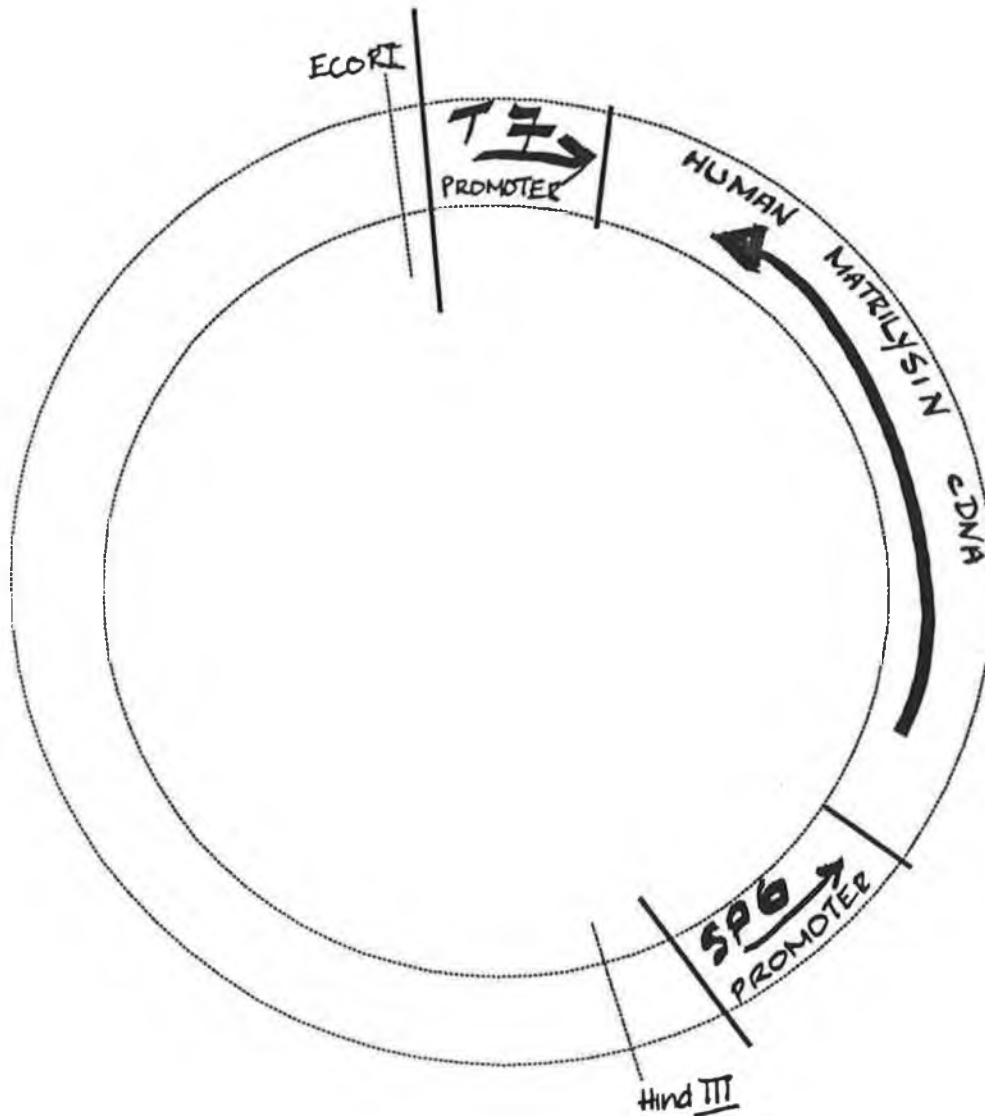


# pG7PumpEX



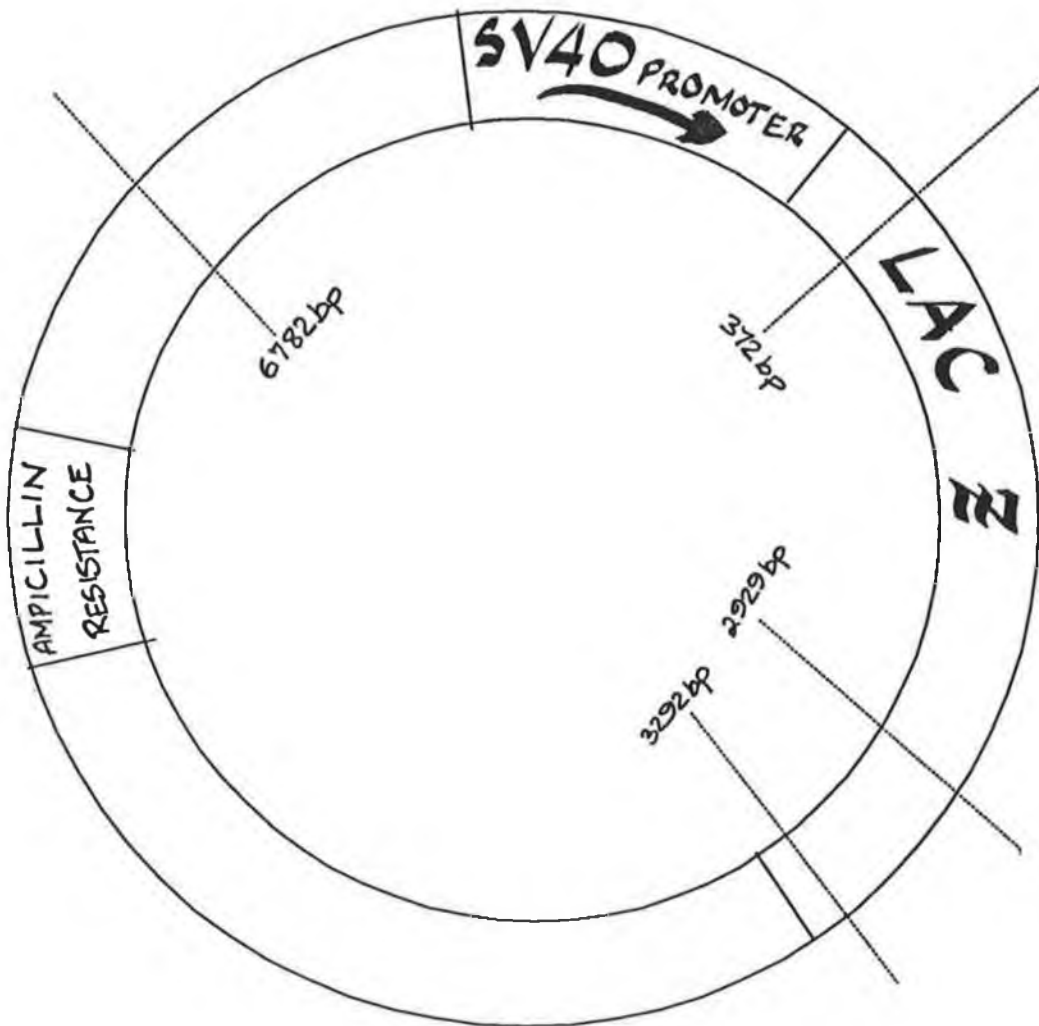
**The cDNA coding for the human matrilysin protein has been inserted between the T7 and SP6 promoters, The restriction sites used for linearising the plasmid are indicated by dashed lines.**

## pG7PumpXE



**The cDNA coding for the human matrilysin protein has been inserted between the T7 and SP6 promoters, The restriction sites used for linearising the plasmid are indicated by dashed lines.**

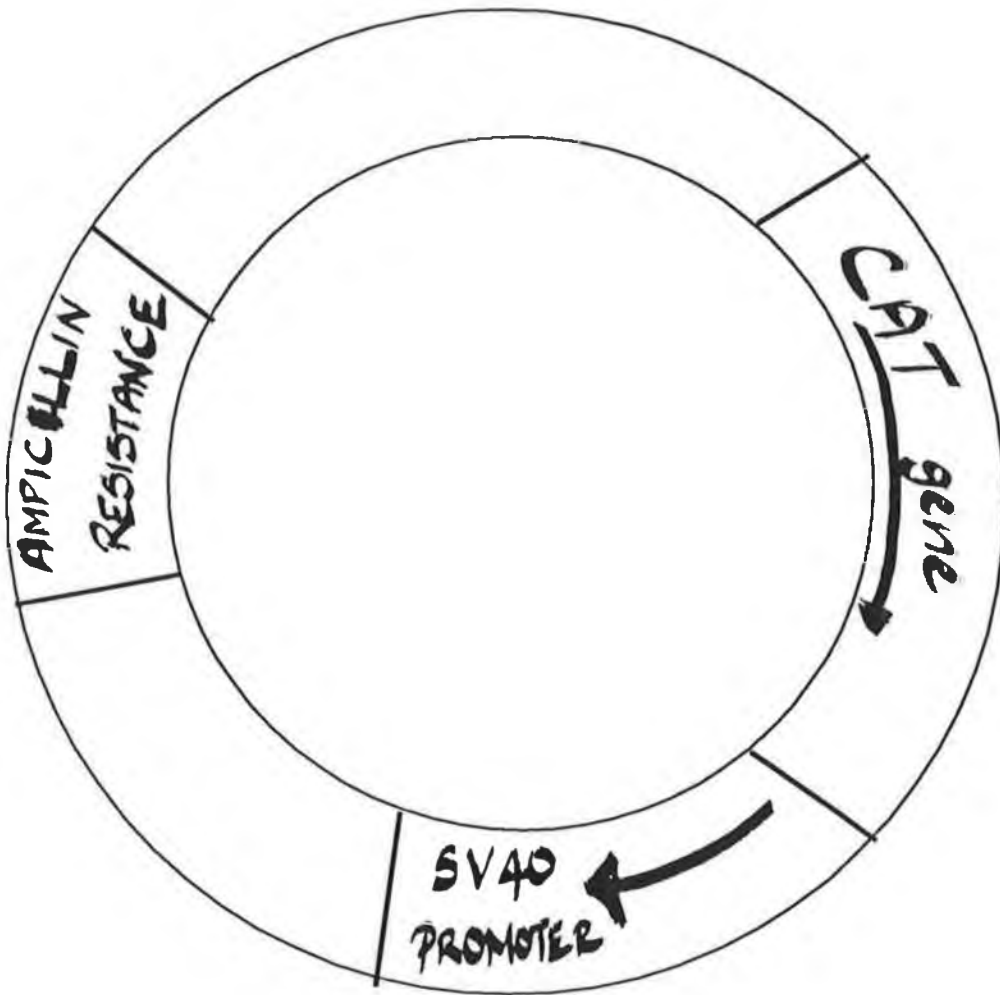
# pCH110



This plasmid contains a functional Lac Z gene, under the control of an SV40 promoter.

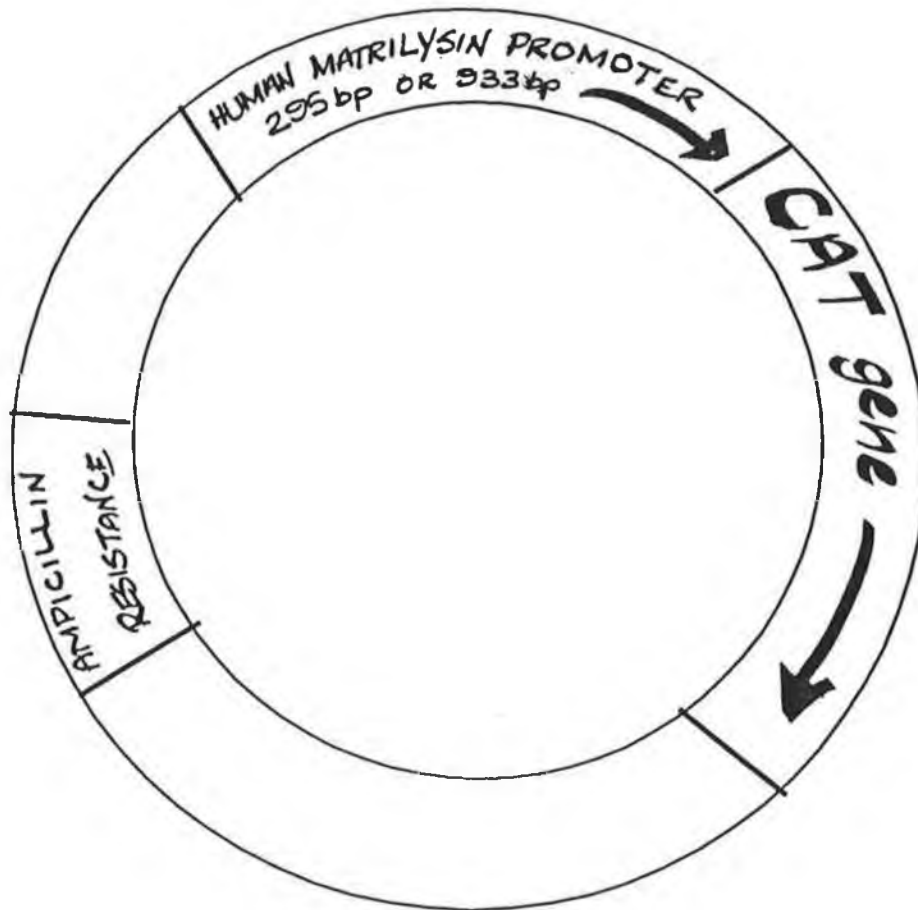
Pvu II cleavage sites are indicated by dashed lines.

## pFLCAT



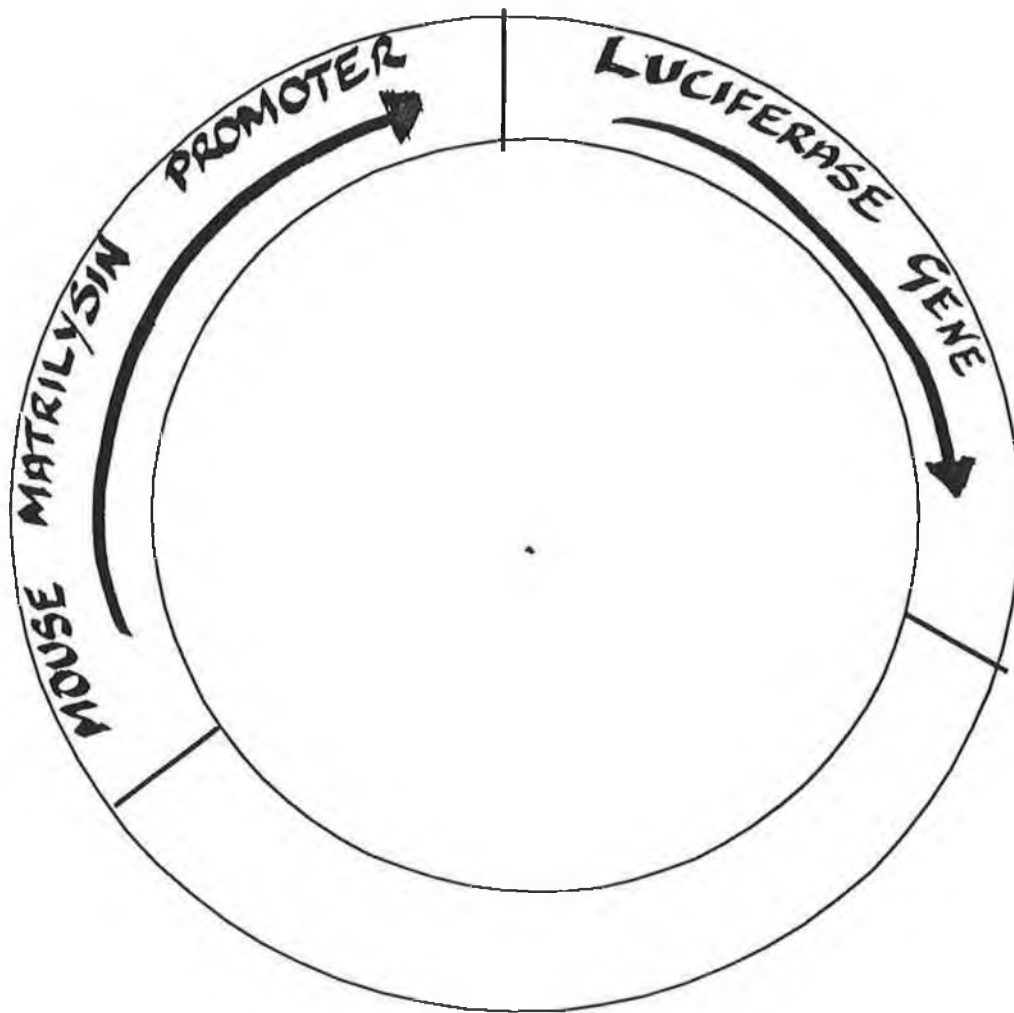
**The SV40 promoter has been placed after the gene encoding the CAT protein so that no transcription should occur.**

## **p295HPCAT & p933HPCAT**



**Two different lengths of the human matrilysin promoter (295 bp or 933 bp) have been inserted immediately in front of the gene encoding the CAT protein.**

## pGL2-MMP



**2.7 Kb of the mouse matrilysin promoter has been inserted ahead of the luciferase gene.**