Transcriptional regulation of matrilysin gene expression

A dissertation submitted for the degree of M.Sc.

By

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Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of M.Sc. 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.

Signed: Helen O'Shea. ID No.:93701071

Date: 27th January 1996

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Abbreviations

Abs	Absorbance
anti-CAT	antibody to CAT
anti-CAT-DIG	antibody to CAT conjugated to DIG
anti-DIG-POD	antibody to DIG conjugated to POD
ATP	Adenosine triphosphate
BCA	Bichinchoninic acid
Beta-gal	β-galactosidase
bp	base pairs
$CaCl_2$	Calcium chloride
$CaCl_2 2H_2O$	Calcium chloride dihydrate
CAP	Catabolite activator protein
CAT	Chloroamphenicol acetyltransferase
CH_3COOK	Potassium acetate
CMV	Cytomegalovirus
CPRG	Chlorophenyl red β-d galactoside
DEAE	diethylaminoethyl
DIG	digoxigenin
DMEM	Dulbecco's modification of Eagle's medium
$DMEMS_0$	DMEM with no supplement of FCS
$DMEMS_5$	DMEM supplemented with 5% (v/v) FCS
DMSO	dimethysulfoxide
DOPE	dioleoyl phosphatidylethanolamine
DOSPA	2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate
DOTAP	N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulphate
EDTA	Ethylenediaminetetra-acetic acid
EGF	Epidermal growth factor

EGTA	Ethylene glycol bis (β-aminoethyl ether) N,N,N',N'-tetra-acetic acid
ELISA	Enzyme-linked immunosorbant assay
FCS	Foetal calf serum
FSB	Frozen storage buffer
g	gram
$HACoCl_3$	Hexamine cobalt chloride
HBS	Hepes buffered saline
HCl	Hydrochloric acid
Hepes	(N-(2-Hydroxyethyl)piperazine-N-(2-ethane sulphonic acid)
hr	hour
KAc	Potassium acetate
Kbp	kilo base pairs
KCl	Potassium chloride
kDa	kilo Dalton
kV	kilo volts
$K_3Fe(CN)_6$	Potassium ferricyanide
$K_4Fe(CN)_6$	Poassium ferrocyanide
LB	liquid broth
M	Molar
mA	milli amps
mg	milligram
MgATP	Magnesium adenosine triphosphate
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
min	minute
ml	millilitre
mM	millimolar
MMP	matrix metalloproteinases

$MnCl_24H_2O$	Manganese chloride tetrahydrate
msec	millisecond
Na_2HPO_4	di-Sodium phosphate anhydrous
NaH_2PO_4	Sodium dihydrate phosphate
N.D.	Not determined
N.S.	Not suitable
NaAc	Sodium acetate
NaCl	Sodium chloride
NaOH	Sodium hydroxide
ng	nanogram
nM	nanomolar
O.D.	Optical Density
PBS	Phosphate buffered saline
pg	picrogram
POD	peroxidase
rpm	revolutions per minute
RT	room temperature
s	second
SBX	sucrose bromophenol blue xylene cyanol
SCID	severe combined immunodeficient
SDS	sodium dodecyl sulphate
SV-40	simian virus 40
TBE	Tris-boric acid-EDTA
TBS	Tris buffered saline
TE	Tris-EDTA
TEN	Tris-EDTA-NaCl
TF	Transcription factor
TFIIA	Trancription factor IIA
TFIIB	Transcription factor IIB
TFIID	Transcription factor IID

TFIIE	Transcription factor IIE
TGFβ	Transforming growth factor β
TIE	Transforming growth factor β inhibitory element
TIMP	Tissue inhibitors of metalloproteinases
TORU	TPA and oncogene responsive unit
TPA	12-O-tetradecanoyl-phorbol-13-acetate
TRE	TPA responsive element
TRIS	Tris(hydroxymethyl)methylamine
α_1PI	α_1 protease inhibitor
μF	micro Farrad
μg	microgram
μl	microlitre
v/v	volume per volume
w/w	weight per weight
X-gal	5-Bromo-4-chloro-3 indoyl-β-d galactoside

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Chapter 1

Introduction

1.1. An introduction to matrilysin and its functions

Matrilysin is a member of the family of matrix metalloproteinases (MMPs). The matrix metalloproteinase family of enzymes are involved in remodelling of the extracellular matrix (Gaire *et al.,* 1994). Remodelling of the extracellular matrix is a critical event in normal processes such as tissue morphogenesis, differentiation, and wound healing, and the matrix metalloproteinases are believed to be key role players in these events. In addition, these enzymes are also involved in pathological conditions such as tumour invasion, metastasis, and arthritis.

Matrilysin has also been shown to be involved in metabolic pathways that affect the activity of proteases that act on the extracellular matrix. Crabbe *et al.,* (1994) showed that human progelatinase A, the inactive form of the enzyme, can be activated by matrilysin. Matrilysin has also been shown to rapidly inactivate α_1-protease inhibitor (α_1PI), an inhibitor of elastase, by cleaving the Pro^{357}-Met^{358} peptide bond in its reactive centre. Matrilysin also plays an important role in the inactivation of serpins (serine proteinase inhibitors) in malignant tissues, where a cascade of metalloproteinases and serine proteinases are required for tissue degradation (Zhang *et al.*, 1994).

As a member of the MMP family, matrilysin plays a key role in metastasis and invasion. Malignancy, the potential of a tumour to metastasise, requires invasion. A benign tumour, which has no invasive properties, has a definite border. In contrast a malignant tumour has a poorly defined border called the invasive front, where individual tumour cells migrate away from the primary mass (Liotta *et al.*, 1992).

Metastasis is a multi-step process. Initially cells detach from the primary malignant tumour mass, and move into the blood vessels that nourish it. The first barrier that faces the migrating tumour cell, is the layer of endothelial cells that line the interior of blood vessels and lymph vessels. It has been demonstrated that tumour cells may possess special adhesive affinities for the endothelial surface. The binding of tumour cells to the endothelial layer causes it to retract and expose the tissue beneath it.

The barrier beneath the endothelial cells is the extracellular matrix. The matrix is a dense meshwork of diverse proteins and carbohydrate molecules. In mammalian organisms, fences of extracellular matrix divide tissues into a series of compartments.

One specialised form of the matrix is the basement membrane, which ensheaths the blood vessels, muscle cells and nervous system. Adjacent to the basement membrane is another type of matrix called the interstitial stroma, which holds other tissue cells and lymphatic vessels.

1.1.2. Properties and structure of matrilysin

So far in humans ten members of the MMP family have been identified and characterised, (Table 1.1). The members of the matrix metalloproteinase family that have been identified so far, can be divided into at least three subclasses by substrate specificity: the type I collagenases degrade fibrillar interstitial collagens; the gelatinases recognise basement membrane type IV collagen and denatured collagens; and the stromelysins, of which matrilysin is a member, hydrolyse proteoglycans and extracellular matrix glycoproteins. Another member has been added to the matrix metalloproteinase family. Sato *et al.*, (1994) identified an MMP that is an integral plasma membrane protein. It was the first membrane-type MMP (MT-MMP-1) discovered. MT-MMP-1 can activate pro-gelatinase A and so may be the upstream activator in the gelatinase A proteolytic cascade.

Matrilysin exhibits a number of functional and structural properties that are typical of the matrix metalloproteinase family. It contains a zinc ion and is secreted in a latent form that requires activation for proteolytic activity. Activation by autocatalysis does not occur unless the propeptide is perturbed and it is thought that *in vivo* activation may arise by propeptide clipping, by protease's such as plasmin, (Cockett *et al.*, 1993). Similar to other matrix metalloproteinases, matrilysin is inhibited by specific tissue inhibitors of metalloproteinases (TIMPs) (Matrisian 1990, McDonnell and Fingleton 1993).

The members of the matrix metalloproteinase family contain several distinct domains that are conserved, (Figure 1.1). Matrilysin has three domains, the pre, pro, and catalytic domain. A hemopexin domain is present in the other family members but is absent in matrilysin. The predomain is a leader sequence (~ 17 amino acids), which targets the molecule for secretion. It is subsequently removed and is therefore not present in the latent enzyme, (Wilhelm *et al.,* 1987). The propeptide domain, (~ 80

amino acids) is cleaved when the enzyme is activated. This domain contains the highly conserved sequence proline, arginine, cysteine, glycine, valine, asparagine, proline, aspartic acid (PRCGV/NPD), (Grant *et al.,* 1987; Stetler-Stevenson *et al.,* 1989a; Nagase *et al.*, 1990) which is present in all members of the MMP family. 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 proteolytic activation (Matrisian *et al.,* 1991).

In vitro this activation can be achieved by organomercurials, oxidants, sulfhydryl alkylating agents and, in some cases, proteolytic cleavage by trypsin or plasmin (Wilhelm *et al.,* 1987; Stetler-Stevenson *et al.,* 1989a; He *et al.,* 1989; Springman *et al.,* 1990). The third domain, the catalytic domain contains the zinc molecule. Activation is thought to occur by an unfolding of the latent enzyme freeing the zinc molecule from its binding with the cysteine residue in the conserved PRCGV/NPD region, and allowing the zinc atom to be bound by water, (Springman *et al.,* 1990).

Matrix-degrading metalloproteinases			
Name	**Latent (kDa)**	**Active (kDa)**	**Degrades**
Subclass 1			
a. Interstitial collagenase	55	45	Fibrillar collagens Gelatin Proteoglycan
b. Neutrophil collagenase	75	58	As interstitial collagenase
c. Collagenase-3	55	54	As 1(b)
Subclass 2			
a. Gelatinase-A	72	66	Denatured collagens Collagen IV, V, VII, X Elastin
b. Gelatinase-B	92	86	As gelatinase-A
Subclass 3.			
a. Stromelysin-1	57	45	Proteoglycan Collagen II, IV, XI Gelatins, laminin, Fibronectin
b. Stomelysin-2	57	44	As stromelysin-1
c. Matrilysin	28	19	As stromelysin-1
d. Stromelysin-3	51	44	Unknown
e. Elastase	53	22	Elastin

Table 1.1. Properties of human matrix metalloproteinases.

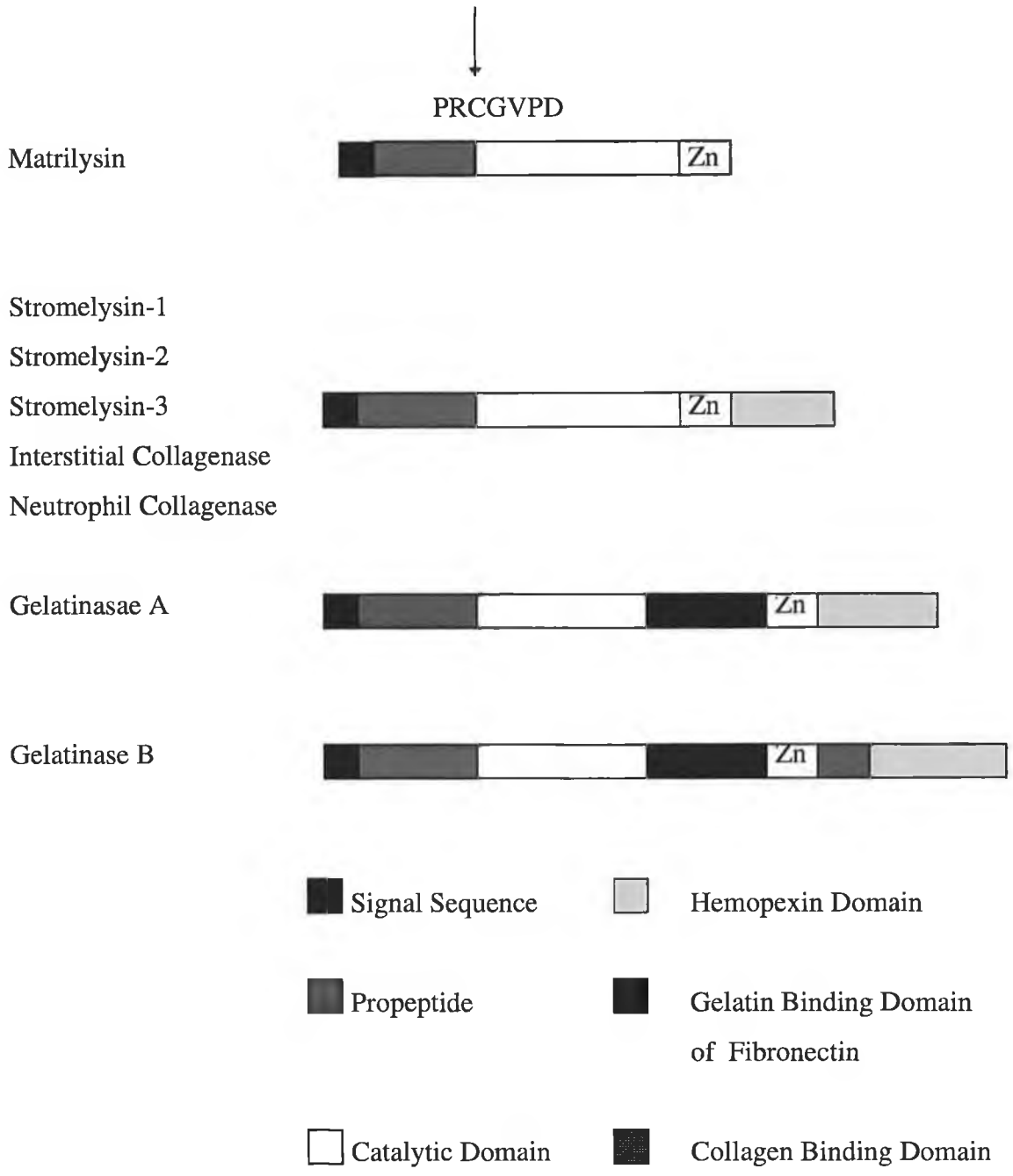


Figure 1.1 Domain structure of the MMP family members. All members of the MMP family contain at least three protein domains: a predomain encoding the leader sequence that targets the enzymes for secretion, a prodomain which is removed when the enzyme becomes activated, and the catalytic domain which contains the zinc binding region.

1.1.3. Tissue inhibitors of metalloproteinases (TIMPs)

TIMPs are a multigene family which at present consists of three members, TIMP-1, TIMP-2 and TIMP-3 (Stetler-Stevensin *et al.,* 1990, Uria *et al.*, 1994). Both TIMP-1 and TIMP-2 contain 12 cysteine residues at virtually identical positions clearly indicating that these two proteins are from the same family. These two proteins bind noncovalently to active metalloproteinases in a 1:1 molar ratio and specifically inhibit their enzymatic activity. TIMP-1 is a glycoprotein (M_r 30 kDa) and its cDNA and amino acid sequence has been reported (Docherty *et al.,* 1985; Carmichael *et al.,* 1986), although purification and characterisation of the protein from rabbit bone tissue had earlier been achieved by Cawston (1981), and others (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 cells as compared to the level in normal or poorly invasive cells (Hicks *et al.,* 1984). The second member of the TIMP family, TIMP-2 is a nonglycosylated protein (M_r 23 kDa) which was isolated and cloned more recently 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 characterised. It reacts stoichiometrically with active interstitial collagenase and also prevents the activation of this enzyme from its 52 kDa proform to 42 kDa active form (DeClerck *et al.,* 1991). It is thought that the TIMP-2 prevents autocatalytic activation of gelatinase-A (Howard *et al.,*1991), and probably works in a similar fashion with matrilysin. A more recent article has a different opinion on matrilysin being inhibited by TIMPs, and suggests, that matrilysin resists inhibition by TIMP *in vivo,* as it lacks the C-terminal domain which contributes to the binding of TIMP, (Baragi et al., 1994).

TIMP-3 displays low sequence similarity to the TIMP-1 and TIMP-2 but shows a high degree of of similarity with chicken inhibitor of metalloproteinase 3. The latter is a recently described metalloproteinase inhibitor stimulated during oncogenic transformation of chicken fibroblasts and with the ability to promote some phenotypic properties of transformed cells (Uria *et al.,* 1994).

1.1.4. Expression of matrilysin

Matrilysin mRNA and protein has been observed in a number of human carcinoma samples from a variety of tissue types. Initial experiments using Northern Blot analysis showed that matrilysin was expressed in 8 of 10 gastric carcinomas and in 6 of 8 colon carcinomas, (McDonnell *et al.,* 1991). Matrilysin was not detectable in normal adjacent tissue from any of these patients. In this study, matrilysin was localised to the cancerous tissue by *in situ* hybridisation and immunohistochemistry.

A series of colon lesions ranging from small adenomas to carcinomas, using *in situ* hybridisation and immunohistochemistry were examined in an attempt to establish the role of matrilysin in colorectal tumourigenesis. The initial results suggested that matrilysin expression arises in areas of benign adenoma polyps destined to become malignant carcinomas, (Newell *et al.,* 1993). These results supported the hypothesis that matrilysin expression is a late event in colon carcinogenesis associated with the conversion of a benign tumour to a malignant tumour.

As mentioned above, in colorectal cancers matrilysin is produced by cancer cells themselves, while other MMPs, are produced by stromal cells (Pyke *et al.,* 1993). This suggests that matrilysin may directly contribute to invasion of colon cancer cells. Yamamoto *et al.*, (1995), modulated the expression of matrilysin in colon cancer cells by introducing both sense and anti-sense matrilysin and tested the effects of this modulation on the ability of colon cancer cells to migrate across an artificial membrane *in vitro*. They found that the introduction of matrilysin into a colon cancer cell line that did not express matrilysin caused these cells to become more invasive as assessed by an *in vitro* invasion assay.

Matrilysin has also been shown to play a role in prostate cancer. Pajouh (1991) showed that 14 of 18 prostate adenocarcinomas and 3 of 11 normal prostate tissue samples express matrilysin. A human prostate cancer cell line, which does not normally express matrilysin was transfected with a plasmid containing the full-length matrilysin cDNA. After intraperitoneal injection into severe combined immunodeficient (SCID) mice, the matrilysin transfected cell lines were observed to invade through the diaphragm more effectively than control transfected cell lines,

(Powell *et al.*, 1993). Matrilysin expression has also been detected in both benign and malignant breast tumour samples (Basset *et al.*, 1990).

Muller *et al.*, (1991) investigated the presence of matrilysin mRNA in head and neck tumours and in lung tumours. High levels of matrilysin mRNA were detected in 45% of head and neck squamous cell carcinoma samples. Matrilysin mRNA could be detected in nearly all types of lung cancer except for in a fibrosarcoma sample. 60% of normal bronchial mucosa samples analysed displayed detectable levels of matrilysin mRNA. High levels of matrilysin mRNA were detected in 62% of the lung squamous-cell carcinoma samples analysed. Also matrilysin mRNA overexpression was detected in primary lung adenocarcinomas.

Despite this wealth of data documenting matrilysin expression in numerous processes, little is known of the mechanisms regulating its expression. The aim of this project was to examine the regulation of matrilysin expression at the transcriptional level. This information could lead to the design of more effective therapeutic agents to reduce the incidence of metastatic cancer and other tissue degenerative disorders like rheumatoid arthritis.

1.2. Regulation of transcription

Genes primarily have two functions; to be duplicated and to be transcribed. Duplication of the genome is necessary if the genetic information of a species is to pass from one generation to the next. In order though, for this genetic information to perform its task it has to be interpreted. Transcription is the name given to the interpretation of the genetic code. During transcription, RNA that is complimentary to a segment of DNA is formed. As with DNA, RNA has a definite orientation, a 5' end and a 3' end, due to the ester linkages between the phosphate and the sugar. The polarity of the RNA formed in transcription is just the opposite of that of the DNA template on which it was assembled. It is the 3'-5' chain of the DNA double helix that is transcribed to form the messenger RNA. Thus the mRNA that is formed during transcription holds identical information to that in the 5'-3' chain of the DNA double helix.

The ultimate aim of this project was to elucidate the mechanisms which control the transcription of the matrilysin gene. All genes have promoters. This is the control element of a gene. The promoter is regulated by a variety of factors that determine whether or not a gene is switched on or transcribed. Transcription takes place with the aid of RNA polymerases. In eukaryotes, three distinct types of RNA polymerase are found in the nucleus of the cell. One of these, RNA polymerase 1, is confined to the nucleolus and is concerned exclusively with the synthesis of the major classes of RNA of the ribosome. The other two species of the enzyme occur in the nuclear cytoplasm. RNA polymerase 2, is responsible for the formation of mRNA transcripts from all the various kinds of structural genes coding for polypeptides. RNA polymerase 3, is reserved for certain other small RNA molecules, such as the smallest RNA molecule of the ribosome.

These three polymerases are unable by themselves to recognise and bind to specific base sequences in promoters. The initiation of transcription depends entirely on molecules known as transcription factors. These factors recognise specific sequences in promoters that act to initiate transcription. Transcription factors are diffusible proteins that affect expression of unlinked genes, thus they are frequently referred to as trans-acting factors. Regulatory sequences, on the other hand, that control

transcription of adjacent genes are referred to as *cis*-acting elements. The *cis*-acting regulatory sequences of a number of genes have been identified and were found to contain binding sites for factors that activate transcription.

Almost all promoters that interact with RNA polymerase 2, contain a conserved sequence know as the Hogness box, or more commonly, the TATA box. This sequence, found about 15-30 bases upstream from +1 , the start site of transcription, has the characteristic consensus sequence TATAAAA. Investigations have revealed that four different transcription factors bind in the vicinity of the TATA box. One of these transcription factors 11D (TF11D) , which is known as the TATA protein, actually recognises the TATA sequence and must bind to the DNA before any other molecule can do so. Once TF11D binds, it is then followed in a specific order by the binding of, transcription factors TFIIA, then TFIIB, the RNA polymerase, and finally TF11E. TF11D is thought to be an essential part of the general mechanism that stimulates transcription in eukaryotic cells, since the TATA sequence is found in most RNA polymerase 2 promoters.

The TATA sequence often works in conjunction with another sequence known as the CAAT box, which has the characteristic consensus GGCCAATCT, and is usually located further upstream in the promoter.

Other sequences have also been identified that affect eukaryotic transcription. These include two GC boxes, each of which contain the sequence GGGCGG. One such box is usually found upstream and one downstream from the CAAT box in most polymerase 2 promoters.

The transcription factors which affect these regulatory sequences in the promoter, can either have a repressive or a facilitative nature depending on the transcription factor.

DNA sequences known as enhancers can greatly influence transcription. An enhancer can increase the rate of transcription from some promoters as much as 200 times. The enhancer element is unusual in that it can exert its effect on a initiation site even 10 kb upstream or downstream and may even be found within the coding region of the gene itself.

The promoter region of the gene is not transcribed to form part of the mRNA transcript. Transcription begins past the promoter region designated as +1. All sequences in the 5'-flanking region of the start site that is in the promoter, are

designated a minus number. It is in this region that the various transcription factors regulating the transcriptional process bind. Figure 1.2.1. further elucidates this process.

The final product of transcription of a gene is the mRNA for that gene. It is from the mRNA that the protein for which the gene/DNA coded for, is made at a site on the ribosome. The mRNA contains a segment at its 5' end called the leader. The leader is not translated by the ribosome, as it does not code for information that designates the amino acid sequence in a polypeptide. The leader sequence is involved in the binding of the mRNA to the ribosome so that translation of the nucleotide sequences that do code for amino acids can take place properly. The codon in the mRNA that usually designates where translation is to begin is AUG. The sequence of the transcript that codes for the amino acid sequence of a polypeptide is found in that part of the mRNA between the leader and trailer. This trailer sequence like the leader is not translated. A chain terminating codon (UAG, UGA or UAA) signals the end of translation. The trailer is any sequence at the 3' end of the mRNA that follows a chain terminating codon, (Rothwell 1993).

Thus, with these basic fundamentals of transcription discussed a closer look at how transcription can be induced to take place, and the role that growth factors have in this process, will be examined.

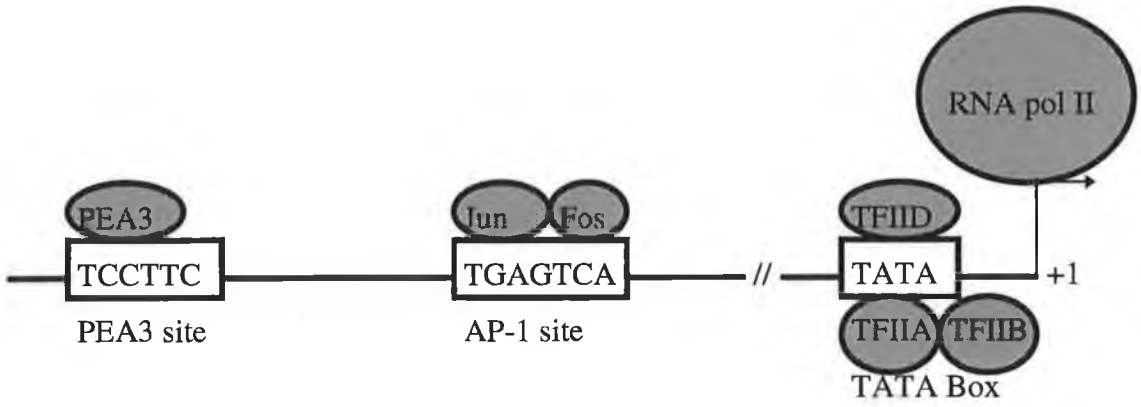


Figure 1.2.1. Features of the transcriptional control region for a mammalian protein-coding gene. This is a hypothetical array of elements that constitute a promoter of a gene transcribed by pol II. Proteins that assemble at these control regions are symbolically represented and include pol II, TFIIA, TFIIB, and TFIID, of the general transcriptional machinery and various DNA binding proteins (Jun, Fos and PEA3), that activate through specific elements. The figure is not meant to imply that all the DNA binding factors must be bound simultaneously as depicted here in order to initiate transcription.

1.2.1. Growth factors and the nuclear response

Growth factors exert their biological effects on cells by interaction with specific cell surface receptors leading to the activation of a number of possible signal transduction pathways. The endpoint of growth factor action resides in the regulation of gene expression. Activation of gene expression by growth factor-mediated signals has a number of characteristic features. A specific subset of genes are activated or repressed and the exact set (or combination) of genes responsive to any growth factor is highly influenced by the identity of the responding cell.

There are now a considerable number of genes which are known to be transcriptionally activated in quiescent cells after exposure to a growth factor signal. Table 1.2.1. lists a small proportion of these. Whilst some genes listed in this table can be clearly implicated in the process of DNA synthesis (such as nucleotide metabolism enzymes or histones), a considerable proportion of these genes appear to have a biological function unrelated to the processes of mitogenesis. For example, represented amongst the set of growth-factor-inducible genes are several secreted extracellular proteases and extracellular matrix components. This illustrates the point that growth factors have many important biological effects on cell function aside from the induction of DNA synthesis. This type of response, (i.e. induction of genes coding for extracellular protease and proteins), to stimulation by growth factors has been shown to be cell type specific. Many growth-factor inducible genes can be induced in responsive cells by a variety of different growth factors as well as pharmacological mitogens such as phorbol esters, e.g. 12-O-tetradecanoyl-phorbol 13-acetate (TPA).

It can be concluded that the induction of gene expression by growth factors involves a select group of genes, representing a wide variety of biochemical functions whose expression is often subject to some form of cell-type specific programming.

Gene expression is regulated in a cascade like fashion. In this cascade of gene activation as a result of stimulation with a growth factor, some genes are expressed rapidly in response to receptor-mediated signalling. Some of these genes are involved in the transcriptional activation of the intermediate class of genes activated. A number of the genes activated in this intermediate step are involved in the late class of gene activation.

Early genes	Function
c-fos	Transcription factors
Krox-20	
Krox-24	
Fra-1	
c-myc	
Intermediate genes	
collagenase	Metalloproteinase
JE	Cytokine
Cathepsin L	Protease
Osteopontin	Extracellular matrix protein
Fibronectin	Extracellular matrix protein
TIMP	Protease inhibitor
Late genes	
Dihydrofolate reductase	Nucleotide metabolism
Histone H4	Chromatin structure
Thymidine kinase	Nucleotide metabolism

Table 1.2.1. Some examples of growth-factor inducible genes

This model of cascade regulation has been investigated in several ways. Amongst the most fruitful approaches has been the analysis of *cis*-acting transcriptional regulatory

elements required for intermediate gene expression. An example of an intermediate gene, is the secreted metalloproteinase collagenase, whose expression can be induced in quiescent fibroblasts by a variety of growth factors as well as phorbol esters such as TPA. Analysis of sequences present in the collagenase promoter, required for transcriptional activation by TPA, lead to the discovery of an 8 base pair (bp) segment of DNA (termed the TRE or TPA responsive element), which could confer induction, (with intermediate timing characteristics), by TPA (or growth factors such as epidermal growth factor -EGF) on a heterologous gene. In other words the TRE behaves as an inducible enhancer (Angel *et al.*, 1987). Enhancers are DNA sequences that can greatly increase the rate of transcription of genes and whose influence can be felt far upstream of far downstream from the promoters they stimulate.

Many growth-factor inducible genes with intermediate timing characteristics contain identical or related sequences within their genetic regulatory elements. This suggests that activation of many intermediate genes involves a molecular species which interacts, in a sequence-specific manner, with the TRE (or related sequences), whose function is to induce transcription of the target gene. A variety of different technical approaches led to the identification and characterisation of this molecular species (or transcriptional activator) responsible for TRE-dependent growth-factor-mediated gene expression. This proved to be a complex of two proteins Fos and Jun, the products of the oncogenes *c-fos* and *c-jun*, which act by binding directly to the TRE and closely related sequences, (Curran *et al.*, 1989, Rauscher *et al.*, 1988).

Fos and Jun interact to form a transcription complex by means of a protein structural motif termed a "leucine zipper". This is created by an α-helical domain containing a regular spacing of leucine residues on one face of the helix. The leucine residues, in association with specific surrounding amino acids, permit the two molecules to dimerize creating the sequence-specific DNA-binding domain (Koudarides *et al.*, 1988). A variety of evidence demonstrates that sequence-specific binding of the fos/jun dimer initiates gene transcription and perhaps the most compelling evidence is that Fos/Jun-mediated TRE-dependent transcription is abolished upon mutation of the leucine zipper domain of either partner.

This discussion of the effect that growth factors have on the nuclear response is not a complete one as the whole picture of every pathway in this complex series of events

has not yet been fully elucidated. Having discussed the basic fundamentals of transcription and how the transcriptional process can be induced by growth factors the following section examines the matrilysin promoter.

1.3. Sequence analysis of the matrilysin promoter

4.2 Kbp of the matrilysin promoter has been isolated and up to 933 bp has been sequenced (Gaire *et al.,* 1994). Figure 1.3.1. outlines a schematic representation of the matrilysin promoter.

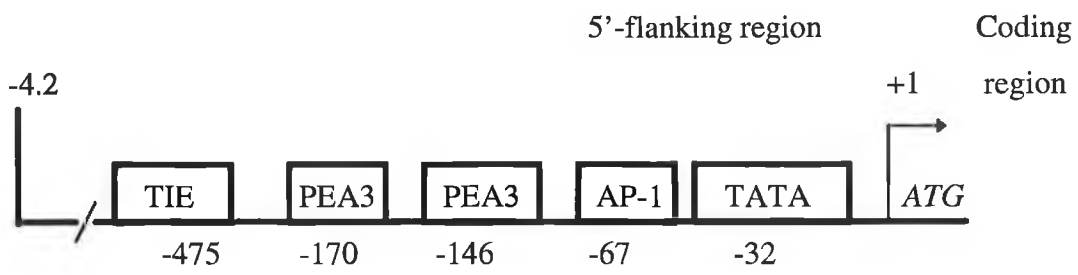


Figure 1.3.1. Regulatory sequences present in the matrilysin promoter. This diagram illustrates the regulatory sequences present in the matrilysin promoter. As can be seen a TATA box, an AP-1 motif, two PEA3 motifs and a TIE motif are present. There are also two more TIE elements located further upstream at positions -500 and -820. The sequence ATG is the codon that designates where transcription begins and +1 signifies the start site of transcription.

Analysis of the 5'-flanking region revealed a TATA box at positions -32 to -25, centred at position -30. This finding supported the identification of the nucleotide indicated at +1 as the catabolite activator protein (CAP) site. It has been found that for full transcription of certain genes to occur in *E.coli* , two factors are necessary. One is a simple molecule, cyclic adenosine monophosphate (cAMP) formed from ATP. This small cAMP molecule has been designated the second messenger because of its interactions, with hormones in their control of cell activity. cAMP does not stimulate transcription directly. It must activate a specific cell protein, the CAP.

When CAP is activated by cAMP the CAP-cAMP complex binds directly to the DNA. The action of the CAP-cAMP complex is not completely known. The binding of the complex is thought to destabilise the DNA in the TATA box region, causing it to denature and open up. In promoters that require CAP-cAMP, the RNA polymerase is unable to achieve this denaturation and cannot proceed with transcription without the CAP-cAMP complex.

The matrilysin promoter contains an AP-1 motif between positions -67 and -61. The positions of the TATA box and AP- 1 site in the matrilysin promoter are very similar to those seen in human interstitial collagenase, stromelysin, stromelysin-2 and rat stromelysin and stromelysin-2, (Gaire *et al.*, 1994). The AP-1 motif is known to confer responsiveness to a variety of oncogenes, growth factors and tumour promoters and is recognised by a transcriptional complex composed of members of the *c-fos* and *c-jun* families. The AP-1 motif, is also known as the TRE (TPA responsive element).

The promoter also contains two inverted PEA3 elements, upstream to the AP-1 motif, relative to the consensus sequence (C/G) AGGAAG (T/C). The sequences that are observed are CTCCTTCA and GTCCTTCG. The PEA3 motif, first recognised in the polyoma virus enhancer, is also an oncogene, growth factor and phorbol ester-responsive element. The PEA3 motif can also serve as a binding site for the products of the *ets* oncogene family. PEA3 binds to the PEA3 motif. PEA3 is a transcription factor which binds to the polyoma virus enhancer and whose activity is regulated by the expression of a number of oncogenes..

Gutman *et al.*, (1990) carried out investigations to establish whether PEA3 played a role in the induction of the collagenase promoter by oncogenes. A point mutation in the PEA3 motif was found to reduce the level of transcription by TPA and oncogenes in the PEA3 and AP-1 area, by 15-27 % of the wildtype. This suggested that the PEA3 element in the collagenase promoter played a role in the induction of this promoter by oncogenes.

Mutations in the AP-1 motif of the human collagenase promoter completely abolished the inducibility by TPA of the minimal collagenase promoter, which encompassed the AP-1 and PEA3 motifs. These results led to the definition of a TPA and oncogene responsive (TORU) unit in the collagenase promoter composed of the two different but interacting elements PEA3 and AP-1. It is possible that the PEA3 motif acts

synergistically with the AP-1 motif in human promoters, to achieve maximal levels of transcriptional activation, (Gutman *et al.*, 1990).

The matrilysin promoter contains sequences at positions -475, -500 and -820 with a high homology to the transforming growth-β1 (TGF-β1), inhibitory element, (TIE), which was originally identified in the rat stromelysin-1 promoter, (Kerr *et al.*, 1990). A suppressive effect of TGF-β1 on matrilysin gene expression in the cycling human endometrium has been observed (Bruner *et al.*, 1995). TGF-β1 inhibits the EGF, *v-src* and *H-ras* induction of stromelysin at the level of transcription. It was found that a 10 bp element in the stromelysin promoter was required for the TGF-β1 inhibitory effects. The TIE specifically binds a nuclear protein complex from TGF-β1 stimulated rat fibroblasts. This complex contained the *c-fos* protooncogene product, Fos, and induction of Fos expression was required for the inhibitory effect of TGF-β1 on stromelysin gene expression. These results led to the postulation that TGF-β1 inhibition of gene expression is mediated by the binding of a Fos-containing protein complex to the TIE promoter sequences.

The binding of Fos to the TIE raised the possibility that the TIE may contain AP-1 binding site-like sequences, like those found in the TRE. Although there are similarities in the proteins that bind to the TIE and TRE, it is thought that it is unlikely that the TIE directly mimics the TRE (Kerr *et al.*, 1990).

These experiments carried out on other matrix metalloproteinase promoters would serve as an experimental model that investigates the role of the regulatory elements in the matrilysin promoter.

1.3.1. Identification of functional elements within promoters

Although sequence analysis has shown the presence of various transcription regulatory elements within the matrilysin promoter, as yet, no studies have demonstrated functional activity for any of these elements. The identification of functional transcription-regulatory sequences is most easily achieved by attaching these pieces of DNA to a marker gene whose gene product is easily assayable following its introduction into the cells of interest by transfection. Transfection is the ability of cells in culture to take up exogenous DNA. Several marker genes have been

successfully used for this purpose, including the chloramphenicol acetyltransferase (CAT) gene, the human growth hormone gene and the luciferase gene. Various lengths of the human matrilysin promoter had already been linked to the CAT gene (Gaire *et al.*, 1994). These are illustrated in figure 1.3.1

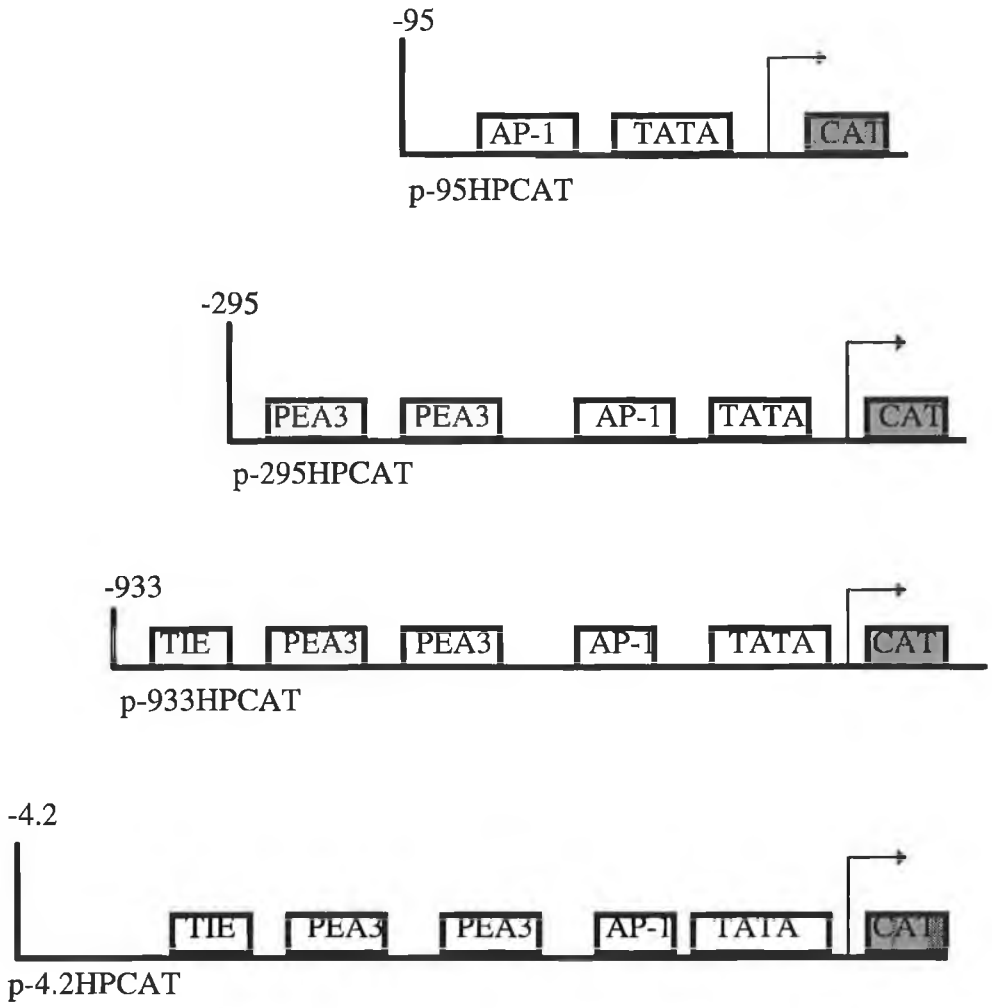


Figure 1.3.1. The human matrilysin promoter constructs, containing various lengths of the human matrilysin promoter linked to the CAT gene. CAT sequences are indicated by shaded boxes; the transcription start site is depicted by bent arrows; and the relative positions of the TATA, AP-1, PEA3, and TGF-β1 inhibitory (TIE) elements are boxed. The most 5'-nucleotide of the promoter sequence contained in the construct is indicated on the diagram and in the name of the construct.

1.4. Transfection methods

A variety of different transfection methods are available, each of them targeting a different means for the DNA to traverse the cellular membrane. Cells can undergo a transfection treatment so that they transiently express the foreign gene. This expression of the gene usually lasts 2-3 days. In order to transform a cell line stably, or permanently, it is necessary to transfect in a gene that codes for resistance to a antibiotic or chemical, along with the gene of interest. Once the medium is supplemented with the antibiotic to which resistance has been conferred, the cell line will stably integrate the exogenous DNA randomly into its genome.

1.4.1. DEAE-dextran method

This technique was first developed for assaying the infectivity of SV40 (Kressman *et al.*, 1978), and subsequently was extended to assay the infectivity of polyoma DNA (Melton *et al.*, 1979) and poliovirus RNA (Wickens *et al.*, 1980).

The diethylaminoethyl (DEAE)-dextran-DNA mixture is soluble and no precipitate is involved. DEAE-dextran is polycationic, and is thought to act by mediating, the productive interaction between negatively charged DNA and components of the cell surface in endocytosis. Reeves *et al.,* (1985) demonstrated that once plasmid DNA enters the DEAE-dextran transfected cells, it is rapidly assembled into nucleosome-containing minichromosomes.

There are a number of parameters to be optimised when using this method with a particular cell line. Of these, the number of cells, the concentration of DNA, and the concentration of DEAE-dextran added to the dish are the most important to optimise. Cells that are 30-50% confluent on a dish are most suitable for transfection. Several investigators have shown that most cell types that can be transfected using DEAE-dextran, will have a preference for 1-10 μg DNA/10 cm dish and for 100 to 400 μg DEAE-dextran per ml of media.

Other factors that need to be optimised include the duration of the incubation of the transfection mixture with the cells, the use of a dimethysulfoxide (DMSO) or glycerol shock to aid entry of the bound DNA to the cells and the use of chloroquine in the

post-transfection media. Treatment with chloroquine is thought to reduce degradation of the endocytosed plasmid DNA, (Mortlock *et al.,* 1993). Chloroquine is extremely toxic to cells in culture so its toxic effect has to be closely monitored. DEAE-dextran is most suitable for small amounts of DNA. It is not useful for production of stably transfected cell lines. DEAE-dextran has been used to reproducibly infect 25% of BSC-1 monkey cells with Simian virus 40 (SV40) DNA. This result was obtained after an 8 hour exposure to 200 μg/ml of DEAE-dextran.

1.4.2. Glass bead method of transfection

The initial work on the use of glass beads was carried out by McNeil *et. al.,* (1987). In this article they described the use of glass beads to load macromolecules into living cells in culture. In brief, the culture medium of the cell monolayer is replaced by a small volume of the macromolecule to be loaded. Glass beads (75-500 μm diameter) are then sprinkled onto the cells, agitated and the cells are washed free of beads and exogenous macromolecules, and "bead -loading" is completed. The glass beads are thought to load macromolecules by causing a "stress" on the cells, producing a momentary rupture in the cell membrane allowing the macromolecules to enter.
Mathews *et al.,* (1993) carried out experiments that investigated the use of glass beads to transfect cells. Firstly the glass beads that they used were pretreated before use. As purchased, the glass beads have been washed in acid (Hydrochloric acid, HCl). These acid-washed beads were found to adhere strongly to cells. To alter bead surface properties so as to render them less adherent to cells, the beads were washed in alkali (4M Sodium hydroxide, NaOH). They also investigated a number of other parameters that needed to be optimised. These included the effect of DNA concentration, the conformation of the plasmid DNA (supercoiled or linear), and bead size among others. The experimental results led them to conclude that transfection efficiency increases with DNA concentration but reaches a plateau at 50 μg/ml. The topology of the DNA had no significant effect. Beads of the largest diameter tested (425 μm) were found to be the most effective. The smaller beads were found to adhere too strongly to the cells, and it proved difficult to remove them with washing. They found the transient transfection efficiency of this method to be approximately 3%.

1.4.3 Transfection using the $CaPO_4$ method

Most applications of the calcium phosphate method of DNA transfection into mammalian cells utilise the basic procedure of Graham and Van der Eb (1973). The basic features of this procedure include a mixture of DNA with $CaCl_2$ and sodium phosphate in buffered saline, formation of a calcium phosphate ($CaPO_4$)-DNA precipitate, and incubation with cultured cells. After removal of the DNA-containing medium, a brief incubation with dimethyl sulfoxide or glycerol is usually employed to enhance DNA uptake.

Loyter *et al.*, (1982) have shown that the DNA-calcium phosphate complex enters the cells via two routes, namely, by endocytosis and direct penetration. Within a cell population most cells endocytose the complex but only a minor fraction of them take up the DNA in a free form. It may be that the free DNA, but not the endocytosed DNA, is mainly responsible for the biological activity of the transfected molecules.

Unfortunately, there is a wide variability in the receptivity of a given cell type to transfection by this technique. Reported transient transfection efficiencies using the calcium phosphate method with plasmid DNA range from <0.1-50% of cells expressing the encoded proteins, (Wilson *et al.*, 1995).

The primary factors that influence efficiency of $CaPO_4$ transfection are firstly, the amount of DNA in the precipitate, secondly the length of time the precipitate is left on the cells, and finally the use and duration of glycerol or DMSO shock. Generally high concentrations of DNA are used, 10-50 μg. Total DNA concentration can have a dramatic effect on efficiency of uptake of DNA with $CaPO_4$-mediated transfection. With some cell lines, more than 10 to 15 μg of DNA added to a 10-cm dish, results in excessive cell death and very little uptake of DNA. With other cell lines it is necessary to use up to 50 μg of DNA. The optimal length of time that the precipitate is left on cells varies with cell type and has to be optimised for each cell type. This can vary from 4 to 24 hr. Also the effect of using a glycerol or DMSO shock has to be investigated. For some cells this shock causes cell death, whereas, for others it greatly improves the level of transfection.

1.4.4. The electoporation method

Electroporation, utilises the transient application of a brief, high voltage electric pulse to induce pore-formation on the cell surface, (Neumann *et al.,* 1982, Potter *et al.,* 1984 and 1988). Otherwise nonpermeant molecules diffuse, or are electrophoresed, into the cells before the pores re-seal and the cell returns to its resting state. These pores are artificially made and it is in this way that electroporation differs from the other types of transfection methods which rely on natural cellular pathways such as endocytosis (e.g. $CaPO_4$ or DEAE-dextran mediated transfection) and lipofection which depends on membrane fusion.

Electroporation is most dependent on the applied electric field, and the field (E) is related to the voltage (V) and the distance (d) by the equation E=V/d. Other factors critical to efficient transfection are the biological parameters for each reagent used (cells, DNA) and the duration of the electric pulse, (An *et al.,* 1982, Anderson *et al.,* 1991, Andreason *et al.,* 1988). These factors must be further considered and optimised in terms of the practical aspects of an experiment. DNA amounts in the range of 10 to 40 μg per 10^6 cell upwards work well.

Field strength (voltage/ distance) is related to (a) the interelectrode distance and the applied voltage with voltage being related, by Ohm's law, V=IR, (b) the current generated and (c) the conductivity of the media in which cells are treated. Thus, anything which influences either the voltage (the set voltage, internal resistances of the electroporation device, the electroporation media,) or the distance (cuvette conformation, size of the gap between electrodes) will affect the electric field, (Anderson *et al.,* 1991, Andreason *et al.,* 1988, Glogauer *et al.,* 1992).

The duration of the pulse is related to the capacitance of the electroporation device and the conductivity of the media in which cells are treated.

The field strength is the most important variable to optimise. It is important to optimise this because, in some cell lines, the curve for efficiency versus. field strength is a very sharp peak with changes of 50 to 70 V/cm decreasing efficiency from 50 to 90%, (Anderson *et al.,* 1991, Andreason *et al.,* 1989, Potter *et al.,* 1988). Most mammalian cells have been efficiently electroporated in culture media at field

strengths of 500 to 1000 V/cm at capacitances of 500 or 960 micro Farrads (μF). However the optimum for some cell lines will fall outside these points.

Some investigators have been able to increase efficiency of transfection at one capacitance over another (Chu *et al.,* 1987 Jiang *et al.,* 1991). In this case one requires a series of voltage optimisation curves for each capacitance tested. Capacitance and voltage are not inverse-linearly related: if one uses a capacitance of 25 μF, an approximately two-fold higher electric field will be required relative to transfection at 500 or 960 μF, (Holliday *et al.,* 1991).

In conclusion, an approach to optimisation of electroporation for a particular cell line involves keeping the amount of DNA, the number of cells and the electroporation medium constant. The applied voltage and the capacitance can then be varied, thus, resulting in differential field strength and pulse.

1.4.5. Lipofection as a method of transfection

Lipofection is the term used to describe the use of cationic lipid-mediated transfection, applicable for the functional delivery of plasmid DNA into cultured cells. Cationic vesicles interact spontaneously and rapidly with polyanions such as DNA, resulting in liposome/polynucleotide complexes that capture practically 100% of the polynucleotide. The resulting polycationic complexes are taken up by the anionic surfaces of tissue culture cells. The aqueous cationic lipid reagent is mixed with nucleic acid molecules and the complexes are introduced into culture cells.

The cationic lipid and polynucleotide aggregates that form are sticky, and can be seen to stick to glassware and plastic. Polypropylene and glass attract these aggregates more than polystyrene. For this reason polystyrene mixing containers are preferred.

Optimum transfection activity occurs under conditions in which the net negative charge on the polynucleotide is substantially reduced. The optimum transfection activity for DNA occurs when the ratio of positively charged molar equivalents (contributed by the cationic lipid) is nearly equivalent to the number of molar equivalents of negative charge contributed by the polynucleotide, (Felgner *et al.,* 1993).

A number of cationic lipids, including Polybrene, Lipofectamine and DOTAP were investigated. Polybrene is a commercially lipid available from Aldrich. The lipofectamine reagent used was a 3:1 (w/w) liposome formulation of the polycationic lipid-2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,N-dimethyl-1 propanamin--ium trifluoroacetate (DOSPA), and the neutral lipid dioleoyl phosphatidylethanolamine (DOPE) in membrane filtered water, from Gibco/BRL. N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulphate (DOTAP) is a liposome formulation available from Boehringer Mannheim. Of the three lipids used DOTAP. Of the three lipids used DOTAP is the more easily metabolised by the cells than the other formulations and therefore, less toxic to the cells. It also has an added advantage in that it can be used with serum if necessary whereas components present in serum can affect transfection of cells using most other lipid formulations (Walker *et al.*, 1992). The lipofection procedure is noted for its simplicity and reproducibility. The optimisation procedure is quite simple. The critical factors for optimisation are the amount of DNA and lipid formulation used.

1.5. Experimental aims

EGF and TPA have been shown to stimulate matrilysin mRNA in two colon adenocarcinoma cell lines, WiDr and SW620, which endogenously express matrilysin (Gaire *et al.*, 1994). These cell lines were chosen to carry out experiments to investigate if EGF and TPA are transcativated through the AP-1 and PEA3 elements located in the matrilysin promoter. The approach adopted was to transfect the human matrilysin promoter constructs illustrated in figure 1.3.1. into these cell lines and stimulate with EGF and TPA. The cells once transfected and harvested would be stimulated, and CAT activity analysed using the CAT ELISA. If the factor being investigated had a stimulatory effect on the matrilysin promoter, it will activate the promoter causing the 3'-flanking CAT gene to be transcribed. The amount of CAT protein produced is directly proportional to the regulatory effect that the particular factor has on the promoter. Whichever construct showed the greatest stimulation of the promoter, that is the largest amount of CAT production, would tell us that it is in that region of the promoter that EGF or TPA is exerting its effect.

Before these transfection experiments investigating the regulation of matrilysin gene expression could be carried out in the SW620 or WiDr cell line, the transfection conditions for these cell lines had to be optimised. A number of methods were investigated, the details of which were discussed earlier in sections 1.4.-1.4.5. The plasmid pCH110, which codes for the bacterial protein β-galactosidase, was used in these transfection optimisation experiments. This plasmid can be transfected into cells and its activity assayed either colourimetrically using the synthetic substrate chlorophenyl red β-d galactoside (CPRG) or *in situ* by staining with 5-Bromo-4-chloro-3 indoyl-β-d galactoside (X-gal). In this way, the efficiency of various transfection methods can be estimated. It was hoped to find an efficient means of transfecting the SW620 or WiDr cell line, and then transfect in the matrilysin CAT promoter constructs and analyse the activity of the matrilysin promoter following treatment with EGF and TPA.

Chapter 2

Materials and Methods

2.1 Materials

Matrilysin promoter CAT reporter constructs were supplied by Prof. Lynn Matrisian, Vanderbilt University, Nashville Tennessee USA. All growth factors and cytokines used were obtained from Boehringer Mannheim, Hannover, Germany. All restriction endonucleases were bought from Promega, Southampton, UK. L-glutamine, penicillin-streptomycin solutions were purchased from Sigma Chemical Co., Poole Dorset, England. Foetal calf serum was obtained from Biowhittaker, Verviers Belgium. Cell culture media was purchased from Gibco BRL Uxbridge, Middlesex, England. Cell culture plastics and vessels were obtained from Costar, Cambridge, MA, 02140, USA. Chemicals were of Analar grade and were purchased from Sigma Chemical Co., BDH Chemicals Ltd., and Merck, Poole, Dorset, England.

Cell lines used in the course of this work are listed in Table 2.1.1.
Details of specific kits are listed in Tables 2.1.2.
Details of plasmids used are outlined in table 2.1.3.

Cell Line	Cell type	Cat. No.	Source
SW480	Human colon adenocarcinoma	CCL 228	European Collection of Animal Cell Cultures
SW620	Human colon adenocarcinoma Lymph node metastasis	CCL 227	CAMR, Porton Down Salisbury, Wiltshire SP4 OJK England
WiDR	Human colon adenocarcinoma	CCL 218	
A549	Human lung adenocarcinoma	CCL 185	

Table 2.1.1 Cell lines used during the project.

Materials	Supplier
PZ523 DNA purification kit	5'⟶ 3' CP Laboratories, England.
CAT ELISA kit	Boehringer Mannheim Hannover, Germany.
Bio-Rad protein assay kit	Bio-Rad Laboratories Munich, Germany
BCA protein assay kit	Pierce & Warriner, England
Hoechst 33258	Calbiochem, Behring Diagnostics, La Jolla, CA 92037 USA.
DOTAP	Boehringer Mannheim Hannover Germany
Lipofectamine	Gibco/BRL England

Table 2.1.2. Specific kits and miscellaneous reagents used in the course of the work.

Plasmid	Description
pCH110	codes for β-galactosidase
p-95HPCAT	95 bp of the human matrilysin promoter linked to CAT
p-295HPCAT	295 bp of the human matrilysin promoter linked to CAT
p-933HPCAT	933 bp of the human matrilysin promoter linked to CAT
p-4.2HPCAT	4.2 Kbp of the human matrilysin promoter linked to CAT
pFLCAT	contains the SV40 promoter linked to the 3'end of the CAT gene, therefore the CAT gene is not transcribed.
pCMVCAT	contains the CMV promoter linked to the 5' end of the CAT gene, therefore the CAT gene is transcribed.

Figure 2.1.3. Plasmids used during the course of this work.

2.2 Equipment

2.2.1. Centifugation

A Heraeus Christ Labofuge was used for centrifugation of universal tubes (1-25 ml) and centifugation tubes (1-50 ml). Smaller volumes were centrifuged on a Heraeus Biofuge 13 using eppendorf containers (1-1.5 ml). For centrifugation of large volumes (up to 200 ml), at higher speeds a Sorvall centifuge was used.

2.2.2. Cell Culture

Aseptic cell culture techniques were undertaken in a Holten Laminar Air flow cabinet, HB 2448. Cells were incubated in a humid 5% CO_2 atmosphere at 37°C in a Heraus incubator.

Long term storage cells were cryopreserved in a cryocontainer, (Cooper Cryoservice Ltd.)

Cells were visualised on an Olympus CK2 microscope.

2.2.3. Electrophoresis

Electrophoresis was performed on a Hybaid horizontal electrophoresis system.

2.3 DNA Preparation methods

2.3.1. Preparation of competent cells

Using a sterile platinum wire, *E.coli DH5*α were inoculated by scraping directly from a frozen stock, (stored at -70°C in freezing medium), into 5 ml of SOB, (Liquid broth (LB), 10g typtone, 10g Sodium chloride (NaCl), 5g yeast extract, with 10 mM Magnesium chloride ($MgCl_2$) and 10 mM Magnesium sulphate ($MgSO_4$)). A streak was also made from the stock onto SOB and ampicillin plates (35 μg ampicillin/ml SEB), to check for contamination of stock. There should be no growth on SOB and

ampicillin plates. Both plates and liquid cultures were grown overnight at 37^0C, (150-250 rpm).

1 ml of the overnight culture of the *DH5α*, was seeded into 100 ml of SOB. The culture was inoculated at 37^0C and agitated at 200 rpm until the O.D. at 550 nm read 0.45-0.6. O.D. Samples were taken every 20 to 30 minutes (min). The culture was grown for approximately 2-3 hours (hr).

For efficient transformation, it is essential that the number of viable cells should not exceed 10^8 cells/ml. To correlate cell viability / number to O.D. at 550 nm, samples were taken from a growing culture of *E.coli* at different times in its growth cycle, the O.D. at 550nm read, and dilutions of these samples were then plated out on LB agar plates in the absence of antibiotics.

The culture was then centrifuged at 2,500 rpm for 12 min at 4^0C. The pellet was resuspended in 1/3 of the volume, 33 ml of Frozen storage buffer (FSB) pH 6.4, (10 mM Potassium acetate (CH_3COOK), 100 mM Potassium chloride (KCl), 45 mM Manganese chloride tetrahydrate ($MnCl_24H_2O$), 10 mM Calcium chloride dihydrate ($CaCl_22H_2O$), 3 mM Hexamine cobalt chloride ($HACoCl_3$), 10 % Glycerol).

The resuspended pellet was left on ice for 10 min at 4^0C and then centrifuged at 2,500 rpm for 10 min at 4^0C. The pellet was resuspended in 1/12.5 of the volume, 8 ml of FSB at 4^0C. DMSO was added to 3.5 % of the final volume at 4°C. This was left for 10 min at 4^0C and then the same quantity of DMSO was added. 200 µl aliquots were prepared in pre-chilled eppendorfs. These were flash frozen in liquid N_2. The competent cells were subsequently stored at -80^0C for up to 5 months.

2.3.2. Transformation of competent cells

10 ng of the required DNA was aliquoted in a tube. 100 µl of competent cells were thawed quickly, and added to the DNA. The tube was swirled gently and placed on ice for 30 min. The tube was then transferred to a heating block at 42^0C for 90 seconds (s). The sample was then placed on ice for 2 min. 0.8ml of LB was then added to the transformation tube. The sample was incubated at 37^0C for 1 hr, (225 rpm). The transformed cells were then plated out on ampicillin plates, (35 µg/ml), which were incubated at 37^0C overnight.

2.3.3. Minipreparation of plasmid DNA

From the transformed cells plated on the ampicillin plate, a single colony of bacteria was selected and placed into a universal containing 5 ml of LB containing ampicillin (35 µg/ml). This was grown overnight at 37^{0}C, and agitated at 150 rpm. Approximately 16 hr later, 1.5 ml of the culture was transferred to a sterile microfuge tube. This was microfuged for 1 min and the resultant supernatant aspirated. The pellet was resuspended in 100 µl of solution 1, (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM Ethylenediaminetetra-acetic acid (EDTA) pH 8.0), vortexed and left at room temperature (RT) for 5 min 200µl of solution 2, (1% Sodium dodecyl sulphate (SDS) 0.2 M NaOH), was added to the mixture, which was inverted a number of times and then placed on ice for 5 min. 150 µl of ice-cold solution 3, (3 M Potassium Acetate (KAc), 5 M Acetic acid pH 4.8), was added to the lysate which was then placed on ice for 10 min. This was then microfuged for 5 min at 10,000 rpm. The supernatant was removed and the heavy white protein precipitate discarded. 150 µl of 2 M Trizma base, chloroform, and phenol (pH 7.8) were added to the supernatant. This was vortexed and microfuged for 2 min. The upper aqueous layer was removed, avoiding the interface, as this contains protein. 1 ml of 100% ethanol was added to this aqueous phase and incubated at -80°C for 5 min. After this incubation, the minipreparation was microfuged for 10 min. The supernatant was removed. At this point, a white pellet was observed. 250µl of 0.3M Sodium acetate (NaAc) was combined with the pellet and mixed. 500 µl of 100% ethanol was added and the mixture was left at RT for 10 min, and then microfuged at 10,000 rpm for 10 min. The supernatant was removed and the pellet was washed with 70% ethanol and dried at 37^{0}C. The pellet was resuspended in 25 µl Tris-EDTA-TE, (10 mM Tris-HCl pH 8.0, 1 mM EDTA), and stored at 4^{0}C prior to digestion.

2.3.4. Restriction digest and electrophoresis of miniprep plasmid DNA

The DNA that was obtained from the minipreparation was digested as follows. 5 µl of DNA was added to the digest mixture which contained 1 µl of restriction enzyme, 2.5 µl of 10X buffer, (supplied with commercial enzyme), and 16.5 µl of sterile water and

RNase, (2 µl of RNase A in TE, 50 µg/ml, and 14.5 µl of water). This was incubated at 37^{0}C for at least 2 hr. 12.5 µl of the digest was removed,and 2.5 µl of gel loading buffer, 6X sucrose, bromophenol blue, xylene cyanol (SBX), (40% w/v sucrose, 0.25% w/v bromophenol blue, 0.2% xylene cyanol), was added. 15 µl was loaded onto a 1.2 % agarose gel. The gel was run at 100 V and 200 mA in 0.5X Tris-boric-acid EDTA (TBE) buffer, (108g Tris base, 55g of Boric acid, 9.3g EDTA-1L 10X stock, pH 8.2-8.4). Following electrophoresis, the gel was stained in ethidium bromide, (0.5 µg/ml), and viewed using the UV transilluminator.

2.3.5. Maxipreparation of plasmid DNA

Once it had been established that the minipreparation cultures were transformed with the correct plasmid DNA these cultures were used to inoculate 800 ml of LB with ampicillin (35 µg/ml). The procedure used was a modification of the alkaline lysis technique (Zervos *et al.,* 1988) which was performed with reagents made in the laboratory, and also using the PZ523 spin columns.

The bacteria were pelleted from the culture at 10,000 rpm for 5 min at 2-4^{0}C. An aliquot of this culture was used to prepare glycerol stocks. 500 µl of the culture was mixed with 500 µl of sterile 50% glycerol. These glycerol stocks were then stored at -80^{0}C until they were required for setting up miniprep. cultures. The bacterial pellet was resuspended in a total of 24 ml of Solution A, (25 mM Tris-Cl, 10 mM EDTA, pH 8.0). It was important that the pellet was fully resuspended. 24 ml of room temperature Solution B, (0.2 M NaOH, 1.0% SDS), was added to the suspension. This was thoroughly mixed by repeated gentle inversion. The lysis was allowed to occur until the lysate became essentially uniform and translucent. 24ml of ice-cold Solution C, (7.5 M Ammonium Acetate), was added to the lysate. A flocculent precipitate appeared on thorough mixing. The protein and cellular debris was pelleted from the mixture by centrifugation at 10,000 rpm for 30 min at 2-4^{0}C. 43 ml of 100% isopropanol was added to the resultant supernatant. The DNA was pelleted from the mixture by centrifugation at 10,000 rpm for 30 min at 20^{0}C. The pellet was washed sequentially in 70% and 95% ethanol, and the pellet was dried at 37^{0}C for 10-15 min.

The pellet was dissolved in 1.5 ml of TE containing 15 µl RNase (50 µg/ml). The sample was consolidated by centrifugation for 1-2 min at 10,000 rpm. The sample was incubated at 37^0C in a water bath for 15 min. The sample was then extracted with 2 ml of phenol (pH 7.8)-Chloroform-Isoamyl Alcohol (PCI), mixed and then centrifuged at 13,000 rpm for 2 min. This step was repeated, followed by a Chloroform-Isoamyl extraction. The resultant aqueous phase was transferred to a fresh tube and 360 µl of 5 M NaCl was added to the sample. The sample volume was adjusted to 1.8 ml with TE, pH 8.

The 1.8 ml sample was added into the reservoir portion of the PZ523 spin column and centrifuged at 1,100 rpm for 12 min. The plasmid was precipitated by adding 0.6 ml of isopropanol and 0.6 ml of 100% ethanol and incubating at -80^0C for 2 hr. This was then centrifuged for 20 min at 20^0C. The pellet was washed with 4 ml of 70% ethanol and 4 ml of 95% ethanol 4 times. The pellet was dried at 37^0C for 10 min and dissolved in TE.

2.3.6. Restriction digest, electrophoresis, and quantitation of maxiprep. plasmid DNA

The DNA obtained from the maxipreparation was digested as described for miniprep DNA, except that only 1µl of DNA was digested, and RNase was not included in the reaction mixture. Electrophoresis was also carried out as described previously. The DNA concentration was determined by measuring the O.D. at 260 nm. A 1/100 dilution of the DNA was made and the O.D. of this dilution was read. The reading obtained was multiplied by 100, to take into consideration the dilution, and then multiplied by 50, as 1 absorbance unit signifies a DNA concentration of 50 µg/ml. The purity of the preparation was determined by measuring the O.D. at 280 nm and calculating the ratio of $O.D._{260}/O.D._{280}$. A clean preparation of DNA has a ratio of 1.8. Lower ratios indicate the presence of excess protein and higher ratios indicate that chromosomal DNA could be present.

2.4. Tissue Culture Methods

2.4.1. Adherent cell cultures

SW620, SW480, WiDr and A549 were cultured in Dulbecco's modification of Eagle's medium (DMEM) containing 5% foetal calf serum (FCS) (v/v), L-glutamine (2 mM), Hepes (1 mM), penicillin (1 unit/ml), and streptomycin (1 µg/ml). All cultures were seeded into 25 cm^3 and 75 cm^3 culture flasks. For passaging adherent cell lines it is necessary to trypsinise the cells. Firstly the culture medium is decanted. The culture vessel is then flushed out with phosphate buffered saline (PBS). This has a twofold effect in that it ensures all FCS, which could potentially interfere with trypsin activity, is removed and secondly, any non-adherent dead cells are removed. A 2.5 ml aliquot of the trypsin-EDTA solution, (trypsin 0.25%, with 0.02% EDTA in sterile PBS), was added to the flasks. The culture was incubated at 37^0C for 10 min. The cell suspension was added to a universal container containing 5 ml of complete culture medium, 100 µl was removed for a cell count, and the remaining cell suspension was centrifuged at 2000 rpm for 5 min. Cells were resuspended in the appropriate amount of culture medium and seeded into culture flasks or plates. All cell lines were incubated in a humid, 5% CO_2 atmosphere at 37^0C.

2.4.2. Cell Counts

Cell counts were performed on a Neubauer Haemocytometer slide using the Trypan blue stain. 20 µl of trypan blue was added to the 100 µl cell sample taken after trypsinisation. This was examined after 5 min by light microscopy. Live cells exclude the stain and maintain their integrity while dead cells stain blue. The number of cells in the five squares was counted. This figure was divided by 5, multiplied by 1.2, to take into consideration the dilution of the cells, and then multiplied by 1×10^4 to take into account the dimensions of the haemocytometer. The resultant figure is the number of cells/ml, in the cell sample the aliquot for counting was taken.

2.4.3. Storage of cell lines

Stocks of cells were maintained in the liquid phase of liquid nitrogen in Cryofreezer, (Cooper Cryoservice Ltd.). Washed cells were resuspended dropwise in 1ml of ice-cold FCS supplemented with 5% (v/v) (DMSO) and transferred to sterile cryotubes. The cryotubes were lowered slowly one level at a time over 2 hr, into the gas phase and eventually into the liquid phase of liquid nitrogen.

Cells were recovered from liquid nitrogen by thawing them rapidly at 37^0C and then washing in complete medium. The cells were centrifuged at 1000 rpm for 5 min and resuspended in complete medium.

2.4.4. Mycoplasma detection - Hoechst 33258 fluorescent assay

Mycoplasma was detected using the Hoechst stain. The method used was that first described by Chen, (1977) and modified by Carroll *et al.*, (1988). Bisbenzimid, (Hoechst 33258) is a DNA interchelator which detects mycoplasma in cellular cytoplasm. 5 x 10^3 mycoplasma free-NRK cells were cultured overnight in 1ml of complete medium on coverslips. Culture supernatant was removed from the test cell line and added to the NRK cells which were incubated for a further 3-4 days. The coverslips were then washed 3 times in PBS and fixed for 6 min in methanol:acetone (1:1) solution at -20^0C. The coverslips were then washed 3 times in PBS and incubated for 10 min with the Hoechst stain. The coverslips were washed again to remove unused stain and mounted on fresh slides. Samples were stored in the dark until viewed under UV light on a Nikon fluorescence microscope at 100X magnification with oil immersion and a B2 combination filter.

2.5 Optimisation of transfection

In order to optimise transfection and compare the efficiency of transfection of the different methods, the plasmid pCH110 which codes for β-galactosidase was used in these transfection optimisation experiments. The product of this plasmid can be

assayed either colourimetrically using a synthetic substrate (2.6.3) of by direct *in situ* staining of the cells (2.6.4).

2.5.1. DEAE-dextran-Method A

The following method was adapted from a paper by Mortlock *et al.* (1993). 24 hr before transfection, cells were trypsinised and seeded into 100 mm plates at a density of 1 x 10^6, in 10 ml of medium. A stock solution of DEAE-dextran, 10 mg/ml in PBS was prepared and filter sterilised. Various amounts of DNA ranging from 1-10 µg were placed in a polystyrene tube. The volumes were adjusted to 560 µl with sterile PBS, without Ca^{++} and Mg^{++}. Finally 12 µl of the DEAE-dextran stock solution, (10 mg/ml) was added to the transfection mixture. The final volume was 572 µl and the final concentration of DEAE-dextran was 200 µg/ml.

The medium was aspirated, and the plates were rinsed in PBS. The transfection solution, 572 µl was added to the cells and the dish was tilted to ensure even spreading of the transfection solution. The cells were then incubated at 37^0C for 1 hr. Several min before the end of the 1 hr incubation, chloroquine was added to the required volume of DMEM to a final concentration of 100 µM. The transfection solution was removed from the cells and 6 ml of the chloroquine medium was added. The dishes were transferred to the 37^0C incubator, and incubated for up to 4 hr. The chloroquine medium was then removed and replaced with 10 ml of DMEM. The cells were returned to the incubator for 48 hr and then harvested and assayed for activity of the transfected gene.

2.5.2. DEAE-dextran Method B

This method was adapted from a paper by Lopata *et al.,* (1984). 1 x 10^6 cells were plated out on 100 mm plates and allowed to grow for 24 hr. Various amounts of the DNA were placed in a polystyrene tube and the volumes were adjusted to 40µl with Tris buffered saline (TBS), (154 mM NaCl, 50 mM Tris pH7.5 and 1 mM $MgCl_2$). This was then added to various volumes of warm (37^0C), 10 mg/ml DEAE-dextran in TBS. The concentrations of DEAE-dextran investigated ranged from 100-400 µg/ml.

The medium was aspirated from the plates, which were then rinsed with PBS and 4 ml of fresh medium was then added to the plates. The DNA/DEAE-dextran mixture was added dropwise to each plate, swirling between each drop. The plates were incubated at 37^0C for 4 hr in a tissue culture incubator. The DNA/DEAE-dextran mixture was then aspirated from the plates. The cells were then shocked by adding 5 ml of 10% DMSO in PBS for 1 min at RT. The DMSO was aspirated and the cells were then washed with PBS. 10 ml of complete medium was then added to the cells. The cells were returned to the incubator for 48 hr and then harvested and assayed for activity of the transfected gene.

2.5.3. Bead transfection

24 hr before transfection cells were trypsinised and seeded at a density of 1 x 10^6 in 100 mm plates. Glass beads, 425 μm in diameter, were obtained from Sigma and treated according to the method of MacNeil and Warder, (1987), with the following modifications. The beads were first acid-washed in 2 volumes of 5 M HCl for 12 hr and rinsed 5 times with sterile distilled water. The beads were alkali-washed with 2 volumes of 4 M NaOH for 12 hr and rinsed in distilled water until the pH of the wash water was 7.0. The beads were soaked in 70% isopropanol for 2 hr and rinsed with a physiologic buffer (buffer A: 140 mM KCl, 10 mM Hepes [pH 7.25], 1mM EDTA, 0.193 mM Calcium chloride ($CaCl_2$), 10 mM glucose, 1mM $MgCl_2$ and 1 mM Magnesium adenosine triphosphate (MgATP)).

The cells were washed twice with PBS and further washed with buffer A (without MgATP). Various amounts of DNA, (25, 50 and 100 μg/ml), were added to 2 ml of buffer A (with MgATP) together with 0.4 ml (approximate volume) of glass beads to the cells. The beads were distributed over the cells by tilting the plate at an angle of 45^0 in a backward and forward motion up to 4 times. The beads and the DNA-containing solution were washed off with 5 ml of buffer A with MgATP and then 10 ml of appropriate media was added and the plates were returned to 37^0C. The cells were returned to the incubator for 48 hr and then harvested and assayed for activity of the transfected gene.

2.5.4. Transfection with the lipopolyamine polybrene

Polybrene was prepared in sterile distilled water at a concentration of 3 mg/ml and stored at -20^{0}C in small aliquots (50 µl). Transfection was performed according to the method of Dollard *et al.,* (1993) with minor modifications. Briefly, various amounts of DNA, (2.5, 5, 10 µg), were added to 10 ml of DMEM medium containing Polybrene, (concentrations examined 30, 10, 5 µg/ml), and allowed to stand at RT for 15 min. The complexes were then added to cells that had been seeded one day earlier at 1 x 10^6 in 100 mm plates. After a 3 hr incubation in the 5% CO_2 incubator, the transfection medium was removed and the cells refed with DMEM. The transfection mixture was removed after 3 hr of contact and the cells were refed with DMEM. The effect of a glycerol shock was also investigated. 3 ml of a 10% glycerol solution in Hepes buffered saline (HBS-280 mM NaCl, 50 mM Hepes, 1.5 mM Disodium hydrogen phosphate anhydrous (Na_2HPO_4)), was added to the cells for 3 min at R.T. The glycerol solution was removed and the cells refed. The cells were returned to the incubator for 48 hr and then harvested and assayed for activity of the transfected gene.

2.5.5. $CaPO_4$ method of transfection

This method was first described by Graham and Van der Eb *et al.,* (1973). 24 hr before transfection cells were trypsinised and seeded at a density of 1 x 10^6 in 100 mm plates. Various amounts of DNA were aliquoted in sterile polystyrene tubes and the volume was adjusted to 410 µl with sterile H_2O. 480 µl of 2X HBS was aliquoted for each volume of DNA. 60 µl of 2.5 M $CaCl_2$ was added dropwise into the tube containing the DNA with continual mixing using a vortex mixer. Immediately the DNA-$CaCl_2$ mixture was added dropwise into the 2X HBS, with continous mixing using the vortex mixer. This was then left in the laminar flow at RT for 30 min exactly. The cells were removed from the incubator, and the DNA-$CaPO_4$ mixture was added dropwise to the cells. The plate was swirled gently to ensure even mixing. The plates were returned to the incubator for various lengths of time, ranging from 4 hr to 24 hr. To aid the entry of DNA a glycerol shock was carried out. The media and the precipitate was removed from the cells. 3ml of 10% glycerol in 1X HBS was

added for 3 min. The glycerol was removed, the cells were rinsed in PBS and serum-free medium and then refed with fresh growth medium. The cells were returned to the incubator for 48 hr and then harvested and assayed for activity of the transfected gene.

2.5.6. Transfection by electroporation

This method involves the application of brief, high-voltage electric pulses which results in the formation of nanometer sized pores in the plasma membrane, (Neumann *et al.* 1982; Zimmermann *et al.,* 1982).

Cells that were 50%-70% confluent were used in this experiment. One or two days before electroporation, the cells were passaged to ensure that the cells were in optimal, log-phase growth conditions prior to harvesting for the experiment.

The media was changed 4 hr before harvesting. The cells were then trypsinised at 4^0C. The pellet was resuspended in 5 ml of media. An aliquot for a cell count was taken. The cells were pelleted by centrifuging at 2,000 rpm for 5 min. These were left on ice until the cell counts had been carried out. 10 µg of DNA was added to the electroporation cuvette. The cells were resuspended at a final concentration of 5×10^6 to 1×10^7 cells / 800 µl of DMEM at 0^0C. The cells were added to the electroporation cuvette. The DNA and the cells were mixed and the cuvettes were left on ice for 5 min or longer. The resistance was set at ∞. The capacitance was set at 25, 500, and 960 µF. The voltage was varied from 0.2 to 1.7 kilo volts (kV). The cuvettes were then returned to ice for 10 min. The transfected cells were diluted 20-fold in non-selective media, in 100 mm plates. The cuvette was rinsed with the same media so as to transfer all transfected cells. The cells were returned to the incubator for 48 hr and then harvested and assayed for activity of the transfected gene.

2.5.7. Transfection using Lipofectamine reagent

Cells were seeded at 5×10^5 in 60 mm dishes one day prior to transfection. Various amounts of DNA, (0.5, 1.0, 3.0, 5.0, and 10 µg), were placed in sterile tubes and the volume was adjusted to 100 µl using serum-free media. For each quantity of DNA 20µl of lipofectamine was placed in a sterile tube and 80 µl of serum-free media

added. These mixtures were combined and allowed to sit at RT for 30 min. Then, 800 µl of media was added to the micelle mixture and layered over a plate of cells that had been rinsed three times in serum-free media. The plates were incubated for 5 hr at 37^0C. The medium was then changed to serum-containing media. The cells were returned to the incubator for 48 hr and then harvested and assayed for activity of the transfected gene.

2.5.8. Transfection using DOTAP reagent

Cells were seeded at 2.5 x 10^5 in 60 mm dishes one day prior to transfection. Quantities of DNA were diluted to a concentration of 0.1 µg/µl in 20 mM Hepes buffer. In a separate tube quantities of DOTAP were mixed with 20 mM Hepes, so that the final concentration of DOTAP was 0.3 µg/µl. The DNA and DOTAP were mixed and this transfection mixture was incubated at RT for 10-15 min. Subsequently, the DOTAP/DNA mixture was added directly to the 5-6 ml of culture medium and gently mixed by rocking the culture dish to ensure an equal distribution of the transfection mixture. The cells were incubated with the DOTAP/DNA mixture for approximately 6 hr. The medium was replaced with fresh culture medium. The cells were returned to the incubator for 48 hr and then harvested and assayed for activity of the transfected gene.

Variations were carried out in the amount of DNA and the ratio of DNA to DOTAP used. Initially the DNA concentration was kept constant at 5 µg and the DNA:DOTAP ratio was varied from 1:1 to 1:6. The optimum ratio was then picked to optimise the concentration of DNA in the range of 2.5 to 20 µg of DNA.

2.6. Assaying for β-galactosidase activity

The plasmid pCH110, which codes for β-galactosidase, was used in these optimisation experiments. The assays used to report transfection efficiency detected levels of β-galactosidase.

2.6.1. Harvesting cells after transfection

The cells were rinsed twice with PBS, before they were harvested. The cells were harvested by scraping in 1 ml of TBS, and centrifuged at 4^0C for 5 min. The pellet was resuspended in 60 µl of TBS. This was freeze / thawed four times, (5 min at -80^0C, followed by 5 min at 37^0C). The resulting lysate was minifuged at top speed for 5 min at 4^0C. The supernatant was removed and analyzed for protein and β-galactosidase content.

2.6.2. BCA protein microassay

The bichinchoninic acid (BCA) protein assay reagent used was supplied by Pierce, (table 2.1.2). The working reagent was made by combining 50 parts reagent A with 1 part reagent B. A set of protein standards was prepared using BSA in the range 0.1-2.0 mg/ml. 10 µl of each standard, blank or unknown sample was pipetted into the appropriate microtiter plate wells. 200 µl of working reagent was added to each well. The samples were mixed for 30 s on a microtiter plate shaker. The microtiter plate was covered and incubated at 37^0C for 30 min. The absorbance was read at 560 nm, on a microtitre plate reader.

2.6.3. Assay of β-galactosidase in cell extracts

0.1 ml of a 15 mg / ml CPRG, solution,(chlorophenyl red β-d galactoside) prepared in buffer A (100 mM sodium dihydrogen phosphate (NaH_2PO_4) pH 7.2, 10 mM KCl, 1 mM $MgCl_2$ and 10 mM β-mercaptoethanol) without β-mercaptoethanol, was placed in a 1.5 ml eppendorf for each sample. Equal quantities of protein were loaded for each sample and the volume was adjusted to 510 µl with buffer A. Standards were prepared using a commercial stock of the enzyme β-galactosidase, in the 0.1 to 2.5 ng (0.2 to 5.0 ng / ml) range. 0.1 ml of the 15 mg / ml CPRG solution was added to each standard and the volume was adjusted to 510 µl with buffer A. These were incubated at 37^0C overnight. A colour change from orange to red indicates a positive result. The absorbance of these samples was read at 560 nm on a microtitre plate reader.

2.6.4. Assay of β-galactosidase using an *in-situ* stain

The cells were washed twice with PBS. The cells were fixed with 10 ml, (10 ml for 100 mm plates, 5 ml for 60 mm plates), of fix solution, (for 50 ml of fix solution- 0.4 ml of 25 % glutaraldehyde, 10 ml of 0.1 M phosphate buffer pH 7.3, 2.5 ml of 0.1 M Ethylene glycol-bis (β-aminoethyl ether) N,N,N',N'-tetra-acetic acid (EGTA) pH 8.0, 0.1 ml of 1.0 M $MgCl_2$ and 37 ml of distilled water), for 10 min at R.T. This step was repeated. The cells were then washed twice for 10 min with 10 ml of rinse solution, (for 200 ml of rinse solution- 40 ml of 0.1 M phosphate buffer pH7.3, 160 ml of distilled water, 0.4 ml of 1 M $MgCl_2$, 20 mg of sodium deoxycholate and 40 µl of NP-40). The cells were then stained with 2.5 ml, (2.5 ml for 100 mm plates, 1.5 for 60 mm plates), of stain solution, (10 ml of rinse solution: 0.4 ml of X-gal(5-Bromo-4-chloro-3 indoyl-β D-galactoside, 25 mg /ml solution in dimethyl formamide), 16.5 mg of Potassium ferricyanide ($K_3Fe(CN)_6$) and 16.5 mg of Potassium ferrocyanide ($K_4Fe(CN)_6$)).

The plates were incubated overnight at 37^0C. The next day the cells were observed under a microscope. Positive cells stain blue while the negative cells remained clear. The number of blue cells in the whole plate were counted. The stain was removed from the plates, and 5 ml of rinse solution was added. The plate was covered with parafilm and stored indefinitely at 4^0C.

2.7 Investigating the transcriptional regulation of matrilysin gene expresssion

2.7.1. Stimulating the A549 transfected cells with EGF and TPA

The A549 cells which were transfected with the matrilysin promoter reporter constructs, were stimulated with EGF and TPA, to establish the regulatory effect that they had on matrilysin gene expression. EGF was used at a concentration of 5 ng/ml, and TPA at 100 ng/ml. The appropriate volumes of these factors were added to the A549 transfected cells after they had been incubated in serum-free media for 8 hr. CAT protein analysis was carried out 24 hr later.

2.7.2. Analysis of CAT protein using a CAT enzyme linked immunosorbant assay (ELISA)

Cells were harvested for analysis by aspirating the medium and washing the cells three times in PBS. A 750µl aliquot of Tris-EDTA-NaCl-TEN (40 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, pH 7.8) was added to the cells. The cells were removed from the plate by scraping and transferring into a microfuge tube. The plate was rinsed with 400 µl of TEN and added to the microfuge tube. The cells were centrifuged for 1 min and the supernatant was aspirated. The pellet was resuspended in 150 µl of Tris buffer, pH 7.8. This was freeze / thawed as previously described in 2.6.1.

Protein determination of the cell extracts was performed using the biorad protein microassay procedure. Standards were made in the 1-20 µg / ml range. Equal amounts of protein were loaded for each cell extract in the CAT ELISA. CAT enzyme standards for the ELISA were prepared in the 20 - 200 pg / 200 µg range.

The CAT antibodies (anti-CAT) coated to the surface of the wells of the commercial plate were rehydrated using sample buffer (all buffers and reagents were supplied in the kit). 200 µl of each standard and sample was pipetted into the wells. All CAT contained in cell extracts and standards binds specifically to the anti-CAT antibodies bound to the surface of the wells. The plate was covered and incubated at 37^0C for 1 hr. The samples and standards were discarded after 1 hr and the wells were washed 4 times with washing buffer and tapped on absorbent paper. 200 µl of the digoxigenin-labeled antibody to CAT (anti-CAT-DIG) working dilution was added to each well. The anti-CAT-DIG antibody binds to the CAT bound from the samples and standards. The plate was covered and incubated at 37^0C for 1 hr. After the hr the solution was discarded and washed as described above. 200 µl of the antibody to digoxigenin conjugated to peroxidase (anti -DIG-POD) working dilution was added to the wells. This antibody binds to the digoxigenin. The plate was covered and incubated at 37^0C for 1 hr. The plate was then washed as described. Subsequently, 200 µl of the peroxidase substrate was added to each well. The peroxidase enzymes catalyses the cleavage of the substrate, producing a coloured reaction product. The plate was incubated at R.T. until positive samples showed a green colour. An incubation time of 30 to 60 min was routinely used. The absorbance of the samples was measured at

405 nm, with a reference wavelength at approximately 490 nm using a microtiter plate reader.

Chapter 3

Results

&

Discussion

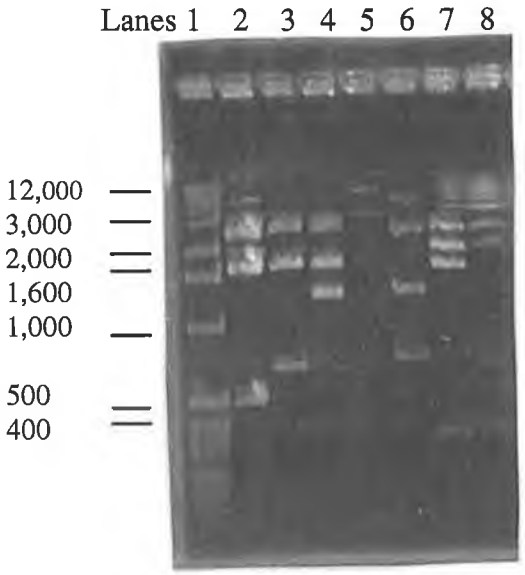
3.1 DNA preparation methods

A number of plasmids were used in the course of this work. Four of these were matrilysin promoter CAT constructs, which were obtained from our collaborators in Nashville, see figure 2.1 for details. These constructs are named p-95HPCAT, p-295HPCAT, p-933HPCAT and p-4.2HPCAT. A negative and positive control plasmid also had to be prepared; pFLCAT (obtained from Nashville) and pCMVCAT (obtained from Dr. Walls D.C.U.). The pFLCAT plasmid is a negative control plasmid as it contains the SV40 promoter linked to the 3' end of the CAT gene, and therefore the CAT gene is not transcribed. The pCMVCAT plasmid contains the CMV promoter linked to the 5' end of the CAT gene, enabling the CAT gene to be transcribed. These plasmids were used to investigate the regulation of matrilysin gene expression. The final plasmid to mention was the plasmid pCH110 that codes for β-galactosidase. This was used in the transfection optimisation experiments.

Sections 2.3.1 through to 2.3.6. outline the procedures employed to prepare these plasmids. The maxipreparation method used is described in detail in section 2.3.5. and was an alkaline lysis method based on work by Zervos *et al.*, (1987). The yield and purity of plasmid DNA was obtained by measuring the absorbance at 260 nm and 280 nm as described in section 2.3.6. All the plasmids were purified in mg amounts. All the $O.D._{260}/O.D._{280}$ ratios were in the acceptable range of 1.8-2.0. To check that they were the correct plasmids, restriction enzyme digests and electrophoresis of the digestion products were carried out. Table 3.1.1. outlines the restriction enzymes used to cut each plasmid and the expected fragment sizes obtained as a result of cutting with these enzymes, calculated from restriction enzyme sites in each plasmid map. Figure 3.1.1. illustrates the digestion products for each plasmid electrophoreised on a 1.2 % gel and stained with ethidium bromide.

Plasmid	Restriction Enzymes	Diagnostic Cut (bp)
pCH110	*Pvu II*	360, 700, 2,600, 3,500
p-95HPCAT	*Pvu II*	425, 1,728, 2,567
p-295HPCAT	*Pvu II*	535, 1,728, 2,567
p-933HPCAT	*Pvu II*	1,278, 1,728, 2,567
p-4.2HPCAT	*Xho I, Bam HI*	3,000, 6,000
p-FLCAT	*Ecor I, Bam HI*	751, 1,382, 2,400
pCMVCAT	*Pvu II*	333, 1,727, 2,054, 2,567

Table 3.1.1. Restriction analysis of plasmids. This table lists the plasmids used during the course of this work, the restriction enzymes used to cut them, and the resultant fragments.



Markers 1 kbp ladder

Lanes	1	1 kbp markers
	2	p-95HPCAT
	3	p-295HPCAT
	4	p-933HPCAT
	5	p-4.2HPCAT
	6	pFLCAT
	7	pCMVCAT
	8	pCH110

Figure 3.1.1. Restriction analysis of plasmids. This diagram illustrates the restriction digest pattern for each of the plasmids.

3.1.2. Discussion

Figure 3.1.1., shows the products of the restriction digest of each of the plasmids following electrophoresis on a 1.2 % gel. Lane 2 shows the diagnostic cut for the plasmid p-95HPCAT. From table 3.1.1., it can be seen that the correct bands obtained after cutting this plasmid with *Pvu II*, are 425, 1,728 and 2,567. Comparing the bands in lane 2 to the marker bands it can be seen that these bands are present. The plasmids p-295HPCAT and p-933HPCAT in lanes 3 and 4 respectively, also contain the 1,728 and 2,567 bp bands. The characteristic 535 bp band obtained when p-295HPCAT is cut with *Pvu II* can also be seen in lane 3 and correspondingly the 1,278 bp band of the diagnostic *Pvu II* cut for p-933HPCAT can be seen in lane 4.

Lane 5 shows the 3,000 bp and 6,000 bp bands obtained when the plasmid p-4.2HPCAT is cut with *Xho I* and *Bam HI*. The 6,000 bp marker on this gel is not resolved from the other higher bp markers as this is a 1.2 % gel and so comparison of the 6,000 bp band in lane 5 and the corresponding 6,000 bp band in the marker lane is difficult. A lower percentage gel (0.6%) would resolve these higher bp bands but the lower bp markers would then have to be run off the gel which are needed for comparison of the bands in all the other diagnostic cuts. For the purposes of this experiment which was to show all the diagnostic cuts of all the plasmids on the one gel the 1.2 % gel was used.

Lane 6 shows the bands 751, 1,382, and 2,400 bp, which are obtained when pFLCAT is cut with *Ecor I* and *Bam HI*. Lane 7 and 8 illustrate the correct diagnostic *Pvu II* cuts for the pCMVCAT (333, 1,727, 2,054 and 2,567 bp) and pCH110 (360, 700, 2,600, and 3,500 bp), plasmids. Any higher bp bands in any of the lanes, represent the linearised and nicked plasmid.

The plasmid DNA preparations were now completed. Experiments optimising the transfection conditions in the SW620 or WiDr cell lines could now be carried out. These cell lines were chosen because they endogenously express matrilysin and also matrilysin mRNA expression has been shown to be enhanced by EGF and TPA (Gaire *et al.,* 1994). Once transfection conditions were optimised, experiments investigating the transcriptional regulation of matrilysin gene expression could then be carried out

by transfecting the matrilysin promoter constructs into either of these cell lines. The plasmid pCH110 was used in these transfection optimisation experiments. This plasmid codes for the bacterial protein β-galactosidase which can be assayed for using the chlorophenyl red β-d galactoside (CPRG) assay (section 2.6.3.) or the *in-situ* stain (section 2.6.4.). Figure 3.1.2.1. illustrates a standard curve for the CPRG assay. This curve intercepts the y axis at 0.11 (corresponding to 0 ng of β-galactosidase) thus all absorbance readings in the optimisation experiments ≤ 0.11 correspond to negative β-galactosidase values. Absorbance readings ≥ 0.3 (corresponding to 0.15 ng of β-galactosidase) are significant readings as they denote that the cells were transfected sufficiently with pCH110, for transient transfection assays (Wilson *et al.*, 1995).

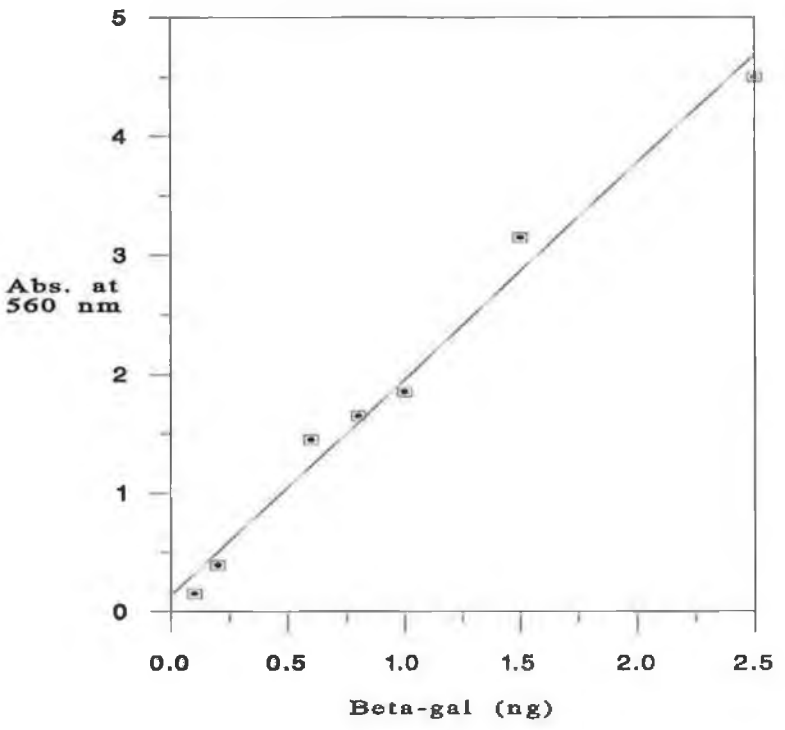


Figure 3.1.2.1. β-galactosidase standard curve

As can be seen this standard curve is linear in the 0-2.5 ng range of β-galactosidase activity. This curve intercepts the y axis at 0.11 (corresponding to 0 ng of β-galactosidase) thus all absorbance readings in the optimisation experiments ≤ 0.11 correspond to negative β-galactosidase values. Absorbance readings ≥ 0.3 (corresponding to 0.15 ng of β-galactosidase) are significant readings as they denote that the cells were transfected sufficiently with pCH110, for transient transfection assays (Wilson *et al.*, 1995).

3.2. Optimisation of the DEAE-dextran methods A and B

The theoretical basis of transfecting with DEAE-dextran was discussed in detail in section 1.4.1. DEAE-dextran is a polycationic molecule which binds the negatively charged DNA. This DNA-DEAE-dextran mixture which is used to transfect the cells is a soluble mixture with no visible precipitate. This mixture enters the cells by endocytosis. The main features to optimise when using this method include the following. The first parameter is the plated number of cells. It is important that 24 hr after seeding the cells are 30-50% confluent as this is the cell density at which efficient transfection can occur (Reeves *et al.*, 1985). Secondly the concentration of DNA and the DEAE-dextran used needs to be optimised. Finally the length of the incubation time of the transfection mixture with the cells, the use of a glycerol shock and the use of chloroquine also need to be considered.

Two variations of this method were investigated. The first method investigated is referred to as method A. For experimental details see section 2.5.1. Optimisation experiments of the DEAE-dextran method were carried out in the SW620 cell line. To achieve a cell density of 30-50% 24 hr after seeding, it was necessary to seed the plates with 1 x 10^6 cells in 10 ml of DMEM containing 5% FCS. A range of DNA concentrations from 1-10 μg per plate in a final volume of 572 μl were added to each plate. DEAE-dextran was also included in this volume at a concentration of 200 μg/ml. This was incubated with the cells for 1 hr after which time it was removed and 6ml of medium containing chloroquine (100 μM) was added to each plate. This was left on for 4 hr after which time it was replaced with DMEM plus 5% FCS. Duplicate plates were also set up in which no chloroquine was added. 48 hr later the cells were harvested and assayed for protein, (sections 2.6.1. and 2.6.2. for details), before proceeding with the CPRG assay. The results of this experiment are outlined in table 3.2.1.

Method B differs from method A in that firstly, no chloroquine was used. Secondly the DNA-DEAE-dextran mixture was incubated for a total of 4 hr in a volume of 4 ml and a shock using 10% DMSO for 1 min was investigated. The results of this experiment are outlined in table 3.2.2.

Sample	DNA concentration (μg)	Chloroquine	CPRG Assay Abs. At 560 nm
1	0	+	0.012
2	1	+	0.004
3	2	+	0.002
4	4	+	0.000
5	6	+	0.003
6	8	+	0.005
7	10	+	0.005
8	1	-	0.006
9	2	-	0.003
10	4	-	0.002
11	6	-	0.000
12	8	-	0.006
13	10	-	0.004
14	water blank		0.000
15	Positive Control		→ 2.345

Table 3.2.1. Optimisation of DEAE-dextran transfection method A in SW620 cells. This table outlines the results obtained from optimisation experiments involving method A on the SW620 cells. Sample 1, the negative control for the transfection procedure, was a sample of SW620 cells which were not transfected. Sample 14, the water blank was a negative control for the CPRG assay. Sample 15, the positive control involved incubating 1.25 ng of a commercial preparation of the enzyme β-galactosidase in the reaction mixture. Significant absorbance readings (≥0.3) are designated by an arrow.

Sample	DNA (μg) conc.	DEAE-dextran (μg/ml)	CPRG Assay Abs. at 560nm
1	1	400	-0.041
2	1	200	-0.047
3	1	100	-0.034
4	4	400	-0.015
5	4	200	-0.032
6	4	100	-0.030
7	10	400	-0.029
8	10	200	-0.027
9	10	100	-0.027
10	0	200	-0.029
11	water blank	-	0.000
12	Positive Control	-	→ 2.541

Table 3.2.2. Effect of DEAE-dextran concentration on SW620 cells. This table outlines the results obtained when method B was used for the DEAE-dextran optimisation on the SW620 cells. These results were obtained when a DMSO shock was used. The experiment was repeated without the use of a DMSO shock and the same results were obtained. Sample 10, the negative control for the transfection procedure, was a sample of SW620 cells which were transfected with 0 μg of DNA. Sample 11, the water blank was a negative control for the CPRG assay. Sample 12, the positive control involved incubating 1.25 ng of a commercial preparation of the enzyme β-galactosidase in the reaction mixture. Significant absorbance readings (≥0.3) are designated by an arrow.

3.2.1. Discussion

As can be seen from table 3.2.1. samples 1-14 resulted in absorbance readings that corresponded to negative β-galactosidase values, as all readings were <0.11 (the β-galactosidase standard curve intercepts the y axis at 0.11, corresponding to 0 ng of β-galactosidase). In this experiment two conditions were varied, the use of chloroquine and the amount of DNA. The amount of DEAE-dextran used was kept constant at 200 μg/ml. According to the literature the DEAE-dextran method is suitable for small amounts of DNA. The ranges of DNA concentration investigated, 1-10 μg, adequately covered this criteria, so we did not deem this to be the problem area.

The use of chloroquine in the incubation media was investigated as it has been shown to reduce degradation of endocytosed plasmid DNA, (Mortlock *et al.,* 1993). In this experiment chloroquine was shown to have no effect. As chloroquine is meant to improve transfection efficiency it was surmised that its lack of effect in this experiment pointed to a lack of transfection, due to the concentration of DEAE-dextran used. As mentioned above the concentration of DEAE-dextran was kept constant at 200 μg/ml. Before drawing the conclusion that the DEAE-dextran method was not suitable for the SW620 cells we decided to examine a range of DEAE-dextran concentrations and that lead us to method B.

In this experiment we examined a series of DEAE-dextran concentrations using the same range of DNA concentrations (1-10 μg) as for method A. To improve uptake of the DNA by the cells, a DMSO shock after the incubation of the transfection mixture with the cells, was employed. The incubation time of the transfection mixture with the cells was increased from 1 hr to 4 hr. The effect of chloroquine in this experiment was not investigated.

The results of this experiment are outlined in table 3.2.2. As can be seen from the results no variation resulted in transfected cells. It was concluded that neither DEAE-dextran method was suitable for transfection of the SW620 cells, as no variation employed with either method resulted in transfected cells.

3.3. Optimisation of Bead transfection

This technique involves removing the culture medium that the cells have been growing in, and replacing it with a small volume of the DNA to be transfected. Glass beads are sprinkled onto the cells, agitated and the cells are washed free of beads and exogenous macromolecules and "bead-loading" is completed.

The beads are thought to cause a momentary "stress" to the cell membrane allowing perforations to form through which the DNA can enter. The precise method can be found in section 2.5.3. and background information in section 1.4.2.

The main variable in this method is DNA concentration. Previous work carried out by Mathews *et al.,* (1993), recommended using the 0.4 mm beads as any smaller size beads stick to the cells, and any larger, cause permanent cell damage. Three concentrations of DNA, (25 µg/ml, 50 µg/ml and 100 µg/ml), in a 2 ml volume, with the SW620 cells were examined. Mathews *et al.*, (1993), recommended using these concentrations in optimisation experiments. The results of this experiment can be seen in table 3.3.1.

3.3.1. Discussion

As can be seen from table 3.3.1., none of the SW620 cells were transfected using the glass bead method. This set of transfections was carried out in two lots. After the initial set of plates (samples 1-3) had been incubated 48 hr post-transfection, it was noticed that up to 70 % of the cells had floated off after the transfection procedure. The bead transfection was repeated but this time the SW620 cells were seeded in plates that had been coated in poly-L-lysine. Poly-L-lysine is a substance that is used to facilitate stronger binding of the cells to the tissue culture plates.

Our aim was to see if using the poly-L-lysine would prevent so many of the cells from detaching after the glass bead treatment. After the 48 hr post-transfection incubation the cells were observed and it was noted that the poly-L-lysine had marginally reduced the detachment of the cells. Although this treatment improved cell adherence, (judged by observing the cells under magnification), the results from the CPRG assay show that none of the SW620 cells had been transfected, as samples 1-6 resulted in

absorbance readings that corresponded to negative β-galactosidase values, as all readings were <0.11 (the β-galactosidase standard curve intercepts the y axis at 0.11, corresponding to 0 ng of β-galactosidase)..

It was concluded that this method was not suitable for the transfection of the SW620 cells, primarily because the glass bead treatment caused excessive cell death and any of the remaining cells did not take up significant amounts of the pCHIIO plasmid.

Sample	DNA (μg/ml) conc.	Poly-L-lysine	CPRG Assay Abs. at 560 nm
1	25	-	-0.064
2	50	-	-0.068
3	100	-	-0.093
4	25	+	-0.056
5	50	+	-0.061
6	100	+	-0.059
7	-	-	0.000
8	water blank	-	0.000
9	Positive Control	-	→ 2.612

Table 3.3.1. Optimisation of bead transfection on the SW620 cells. This table shows the effect of varying DNA concentration and also the effect of using poly-L-lysine. Sample 7, the negative control for the transfection procedure, was a sample of SW620 cells which were not transfected. Sample 8, the water blank was a negative control for the CPRG assay. Sample 9, the positive control involved incubating 1.25ng of a commercial preparation of the enzyme β-galactosidase in the reaction mixture. Significant absorbance readings (≥0.3) are designated by an arrow.

3.4. Transfection with the lipopolyamine Polybrene

The process of using cationic lipids to transfect cells is known as lipofection. Cationic vesicles interact spontaneously and rapidly with polyanions such as DNA, resulting in liposome/polynucleotide complexes that capture practically 100 % of the polynucleotide. The resulting polycationic complexes are taken up by the anionic surfaces of tissue culture cells. Polybrene is a cationic lipid available from Aldrich. Consult section 1.4.5. for information on the process of lipofection and section 2.5.4. for the precise methods involved in using polybrene to transfect cells.

The critical factors for optimisation of the lipofection procedure are the amount of DNA and lipid formulation used.

Polybrene was used in transfection experiments with the SW620 cells. Three concentrations of DNA, 2.5, 5, and 10 μg/ml, and three concentrations of polybrene were investigated according to the method of Dollard *et al.,* (1993). The effect of a glycerol shock was also examined. The results of these experiments can be seen in tables 3.4.1.-3.4.3.

Sample	DNA (μg) conc.	Polybrene (μg/ml) conc.	Glycerol shock	CPRG Assay at 560 nm
1	2.5	30	-	-0.007
2	5	30	-	-0.001
3	10	30	-	0.012
4	2.5	30	+	0.000
5	5	30	+	-0.001
6	10	30	+	-0.036
7	-	-	-	0.000
8	water blank	-	-	0.000
9	positive control	-	-	→ 2.372

Table 3.4.1. Optimisation of transfection using the polybrene method in the SW620 cells. This table shows the effect of varying the DNA concentration and the use of a glycerol shock while keeping the concentration of polybrene constant at 30 μg/ml. Sample 7, the negative control for the transfection procedure, was a sample of SW620 cells which were not transfected. Sample 8, the water blank was a negative control for the CPRG assay. Sample 9, the positive control involved incubating 1.25ng of a commercial preparation of the enzyme β-galactosidase in the reaction mixture. Significant absorbance readings (≥0.3) are designated by an arrow.

Sample	DNA (μg) conc.	Polybrene (μg/ml) conc.	Glycerol shock	CPRG Assay at 560 nm
1	2.5	10	-	0.043
2	5	10	-	0.054
3	10	10	-	0.017
4	2.5	10	+	0.034
5	5	10	+	0.021
6	10	10	+	0.006
7	-	-	-	0.000
8	water blank	-	-	0.000
9	positive control	-	-	→ 2.372

Table 3.4.2. Optimisation of transfection with polybrene. This table shows the effect of varying the DNA concentration and the use of a glycerol shock while keeping the concentration of polybrene constant at 10 μg/ml. Sample 7, the negative control for the transfection procedure, was a sample of SW620 cells which were not transfected. Sample 8, the water blank was a negative control for the CPRG assay. Sample 9, the positive control involved incubating 1.25ng of a commercial preparation of the enzyme β-galactosidase in the reaction mixture. Significant absorbance readings (≥0.3) are designated by an arrow.

Sample	DNA (μg) conc.	Polybrene (μg/ml) conc.	Glycerol shock	CPRG Assay at 560 nm
1	2.5	5	-	0.028
2	5	5	-	0.032
3	10	5	-	0.041
4	2.5	5	+	0.023
5	5	5	+	0.005
6	10	5	+	0.058
7	-	-	-	0.000
8	water blank	-	-	0.000
9	positive control	-	-	→ 2.372

Table 3.4.3. Optimisation of transfection with polybrene. This table shows the effect of varying the DNA concentration and the use of a glycerol shock while keeping the concentration of polybrene constant at 5 μg/ml. Sample 7, the negative control for the transfection procedure, was a sample of SW620 cells which were not transfected. Sample 8, the water blank was a negative control for the CPRG assay. Sample 9, the positive control involved incubating 1.25ng of a commercial preparation of the enzyme β-galactosidase in the reaction mixture. Significant absorbance readings (≥0.3) are designated by an arrow.

3.4.1. Discussion

As can be seen from tables 3.4.1.-3.4.3. three concentrations of polybrene were investigated, 30, 10 and 5 μg/ml. It can be observed in tables 3.4.1.-3.4.3. that samples 1-6 in each table, resulted in absorbance readings that corresponded to negative β-galactosidase values, as all readings were <0.11 (the β-galactosidase standard curve intercepts the y axis at 0.11, corresponding to 0 ng of β-galactosidase). Absorbance readings ≥ 0.3 (corresponding to 0.15 ng of β-galactosidase) are significant readings as they denote that the cells were transfected sufficiently with pCH110, for transient transfection assays (Wilson *et al.*, 1995).

It was concluded that using polybrene to transfect the SW620 cells was not suitable. At this stage it was becoming apparent that the SW620 cells were proving to be very resistant to transfection and therefore, two other cell lines were investigated. These were the WiDr and the SW480, colon carcinoma cell lines. As mentioned in the introduction (section 1.5.), both the WiDr and the SW620 cell lines express matrilysin and so are suitable cell lines in which to carry out transcriptional control experiments. The SW480 cell line does not express matrilysin and so would not be a suitable cell line to carry out the transcriptional control experiments. This cell line was included in the transfection optimisation experiments, as literature was available documenting the use of the $CaPO_4$ method, to transfect the SW480 cell line. Thus the SW480 cell line could be used as a positive control cell line for the $CaPO_4$ method.

3.5. The $CaPO_4$ transfection method

Optimisation experiments using this method were carried out in the SW620, WiDr and the SW480 cell lines for reasons mentioned in section 3.4.1. This method is based on the original work of Graham and Van der Eb (1973), and involves mixing a Hepes buffered saline, DNA and $CaCl_2$ together under constant stirring. Section 2.5.5. covers the experimental details and section 1.4.3. an overall look at the technique. A $CaPO_4$-DNA precipitate is formed and is taken up by the cells by endocytosis.

There is a wide variability in the receptivity of a given cell type to transfection by this technique. For some cell types, this method causes a high percentage of transformed cells, whereas, for others it has a negligible effect. The main variables to optimise include the amount of DNA, the length of time the precipitate is left on the cells, and whether or not a glycerol shock is used to aid entry of the DNA into the cells. The results of the $CaPO_4$ optimisation experiments involving the SW620, the WiDr, and the SW480 cell line can be observed in table 3.5.1., 3.5.2, and 3.5.3 respectively.

Sample	DNA (μg) conc.	Incubation Time (hr)	Glycerol Shock	CPRG Assay Abs. at 560 nm
1	10	4	-	-0.001
2	20	4	-	-0.005
3	30	4	-	-0.001
4	40	4	-	0.000
5	50	4	-	0.002
6	60	4	-	0.007
7	10	8	-	0.003
8	20	8	-	0.000
9	30	8	-	-0.002
10	40	8	-	-0.004
11	50	8	-	-0.001
12	60	8	-	-0.005
13	10	16	-	-0.002
14	20	16	-	-0.001
15	30	16	-	0.000
16	40	16	-	0.002
17	50	16	-	0.006
18	60	16	-	0.008
19	10	24	-	0.003
20	20	24	-	0.000
21	30	24	-	0.001
22	40	24	-	-0.000
23	50	24	-	-0.002
24	60	24	-	0.005
25	-	-	-	0.000
26	water blank	-	-	0.000
27	positive control	-	-	2.612 ←

Table 3.5.1. Optimisation of the $CaPO_4$ method in the SW620 cells. This table shows the effect of varying the DNA concentration and also the amount of time the precipitate was left on the cells. The above was also repeated with a glycerol shock and the same results were obtained. Sample 25, the negative control for the transfection procedure, was a sample of SW620 cells which were not transfected. Sample 26, the water blank was a negative control for the CPRG assay. Sample 27, the positive control involved incubating 1.25ng of a commercial preparation of the enzyme β-galactosidase in the reaction mixture. Significant absorbance readings (≥0.3) are designated by an arrow.

Sample	DNA (μg) conc.	Incubation Time (hr)	Glycerol Shock	CPRG Assay Abs.at 560 nm
1	2	4	-	-0.008
2	5	4	-	-0.007
3	10	4	-	-0.008
4	15	4	-	-0.003
5	20	4	-	-0.003
6	40	4	-	-0.008
7	2	4	+	-0.003
8	5	4	+	0.001
9	10	4	+	0.002
10	15	4	+	0.004
11	20	4	+	0.000
12	40	4	+	-0.005
13	2	16	-	-0.027
14	5	16	-	0.007
15	10	16	-	-0.003
16	15	16	-	0.009
17	20	16	-	0.015
18	40	16	-	0.006
19	2	16	+	0.007
20	5	16	+	0.003
21	10	16	+	0.004
22	15	16	+	0.002
23	20	16	+	-0.012
24	40	16	+	-0.009
25	-	-	-	0.000
26	water blank	-	-	0.000
27	positive control	-	-	2.612 ←

Table 3.5.2. Optimisation of the $CaPO_4$ method in the WiDr cells. This table shows the effect of varying the DNA concentration, the use of a glycerol shock and also the amount of time the precipitate was left on the cells. Sample 25, the negative control for the transfection procedure, was a sample of WiDr cells which were not transfected. Sample 26, the water blank was a negative control for the CPRG assay. Sample 27, the positive control involved incubating 1.25ng of a commercial preparation of the enzyme β-galactosidase in the reaction mixture. Significant absorbance readings (≥0.3) are designated by an arrow.

Sample	DNA (μg) conc.	Incubation Time (hr)	Glycerol Shock	CPRG Assay Abs.at 560 nm
1	2	4	-	-0.010
2	5	4	-	0.005
3	10	4	-	0.006
4	15	4	-	0.144
5	20	4	-	0.214
6	40	4	-	1.299 ←
7	2	4	+	-0.016
8	5	4	+	0.018
9	10	4	+	0.307 ←
10	15	4	+	0.406 ←
11	20	4	+	0.960 ←
12	40	4	+	2.322 ←
13	2	16	-	-0.003
14	5	16	-	-0.006
15	10	16	-	0.028
16	15	16	-	0.038
17	20	16	-	0.078
18	40	16	-	0.194
19	2	16	+	0.107
20	5	16	+	0.156
21	10	16	+	0.067
22	15	16	+	0.015
23	20	16	+	0.166
24	40	16	+	0.194
25	-	-	-	0.000
26	water blank	-	-	0.000
27	positive control	-	-	2.612 ←

Table 3.5.3. Optimisation of the $CaPO_4$ method in the SW480 cells. This table shows the effect of varying the DNA concentration, the use of a glycerol shock and also the amount of time the precipitate was left on the cells. Sample 25, the negative control for the transfection procedure, was a sample of SW480 cells which were not transfected. Sample 26, the water blank was a negative control for the CPRG assay. Sample 27, the positive control involved incubating 1.25ng of a commercial preparation of the enzyme β-galactosidase in the reaction mixture. Significant absorbance readings (≥0.3) are designated by an arrow.

3.5.1. Discussion

A wide range of DNA concentrations were used in the $CaPO_4$ transfection optimisation experiments. Table 3.5.1. shows the results obtained when 4, 8, 16, and 24 hr incubations were used without a glycerol shock, on the SW620 cell line. As can be seen from the data samples 1-24, resulted in absorbance readings that corresponded to negative β-galactosidase values, as all readings were <0.11 (the β-galactosidase standard curve intercepts the y axis at 0.11, corresponding to 0 ng of β-galactosidase). Absorbance readings ≥ 0.3 (corresponding to 0.15 ng of β-galactosidase) are significant readings as they denote that the cells were transfected sufficiently with pCH110, for transient transfection assays (Wilson *et al.*, 1995).

This experiment was also repeated with a glycerol shock. Again, the absorbance readings that were obtained corresponded to negative β-galactosidase values, as all readings were <0.11 (the β-galactosidase standard curve intercepts the y axis at 0.11, corresponding to 0 ng of β-galactosidase). We concluded from this that the $CaPO_4$ transfection technique could not be used to transfect the SW620 cell line.

Table 3.5.2. illustrates the results obtained when the $CaPO_4$ transfection optimisation was carried out on the WiDr cell line. As can be seen from the table the results are similar to those obtained for the SW620 cell line. The table shows the results obtained when a 4 hr incubation was carried out with and without a glycerol schock and when a 16 hr incubation was used with and without a glycerol shock. The 8 and 24 hr incubations with and without glycerol shocking were also carried out but these variations did not result in any transfected cells, as all the absorbance readings obtained corresponded to negative β-galactosidase values, as all the readings were <0.11.

Table 3.5.3. illustrates the results obtained when a range of DNA amounts were transfected into the SW480 cell line using a 4 and 16 hr incubation with and without a glycerol shock. The lower concentrations for the 4 hr incubation without a glycerol shock did not result in any transfected cells as all the absorbance readings obtained were <0.11 (samples 1-3). β-galactosidase expression was detected in sample 4, with the use of 15 μg of DNA (0.144 O.D., using a β-galactosidase standard curve illustrated on p52, this absorbance reading corresponded to 0.018 ng of β-

galacosidase). When 20 μg and 40 μg of DNA were used under these conditions the cells were transfected resulting in a β-galacosidase expression of 0.056 (0.214 O.D.) and 0.644 (1.299 O.D.) ng respectively (samples 5 and 6). Sample 6 was the only sample from the 4 hr incubation without a glycerol shock group, that resulted in significant absorbance readings. Absorbance readings ≥ 0.3 (corresponding to 0.15 ng of β-galactosidase) are significant readings as they denote that the cells were transfected sufficiently with pCH110, for transient transfection assays (Wilson *et al.*, 1995).

If the results are compared to those obtained when a 4 hr incubation with a glycerol shock was used, a dramatic change is observed. This is due to the effect of the glycerol shock used. Samples 9-12 all resulted in significant absorbance readings (readings all ≥0.3) denoting that the cells were sufficiently transfected with pCH110, for transient transfection assays.

As can be seen from table 3.5.3. the use of a 16 hr incubation without a glycerol shock was not very effective. A low level of transfection was observed, (0.045 ng of β-galactosidase, 0.194 O.D.) with 40 μg of pCH110 (sample 18). In comparison, glycerol shocking the cells after a 16 hr incubation marginally improved transfection, but it did not have the dramatic effect that was observed when the cells were glycerol shocked after a 4 hr incubation.

The effect of an 8 and 24 hr incubation were also investigated. The results obtained from the 8 hr incubation were similar to those obtained with the 16 hr incubation. The 24 hr incubation did not yield any transfected cells. It can be concluded from these results that a 4 hr incubation with a glycerol shock and 20 μg of DNA are the optimum conditions for the $CaPO_4$ transfection of the SW480 cell line. Even though 40 μg of DNA resulted in a higher level of transfection, 20 μg would be more than sufficient to use. Figure 3.8.4. (b) illustrates a photograph of the SW480 cells transfected under these optimum conditions and stained using the *in-situ* stain described in section 2.6.4.

These transfection experiments carried out in the SW480 cell line clearly established that the transfection protocol was correctly carried out. Even though this method was effective in transfecting the SW480 cell line, this cell line could not be used in transcriptional regulation experiments, since it does not express matrilysin nor can it

be stimulated with growth factors to overexpress matrilysin (Gaire *et al.,* 1994). The next transfection method investigated was the electroporation method.

3.6. Optimisation of the electroporation method

Experiments investigating the use of electroporation were carried out on the SW620, WiDr and SW480 cell lines. Transfection by electroporation is based on the administration of a brief high voltage electric pulse which causes momentary pores in the cell membrane through which DNA can enter, (Neumann *et al.,* 1982, Zimmermann *et al.,* 1982).

Electroporation is most dependent on the applied electric field (E), and the field is related to the voltage (V) and the distance (d) by the equation E=v/d. The most important factors to optimise are the field strength and the duration of the electric pulse. These two factors can be varied by adjusting the voltage and the capacitance respectively. For more background details consult section 1.4.4. and for the precise method section 2.5.6. Three capacitance values were investigated in these experiments, namely 25, 500 and 960 μF. When the higher capacitances (500 and 960 μF) were used, the equipment did not allow any voltages higher than 0.46 kV to be applied.

The results of the electroporation experiments on the SW620 cell line can be seen in table 3.6.1., on the WiDr cell line in table 3.6.2. and the results on the SW480 cell line in table 3.6.3.

Sample	Voltage (kV)	Capacitance (μF)	CPRG Assay Abs at 560 nm
1	0.260	25	0.006
2	0.460	25	0.018
3	0.660	25	0.007
4	0.860	25	0.004
5	1.060	25	0.003
6	1.260	25	0.003
7	1.470	25	0.000
8	0.210	500	-0.002
9	0.260	500	-0.001
10	0.310	500	0.003
11	0.360	500	0.003
12	0.410	500	0.006
13	0.430	500	0.005
14	0.460	500	0.004
15	0.210	960	0.002
16	0.260	960	0.001
17	0.310	960	0.001
18	0.360	960	-0.003
19	0.410	960	0.004
20	0.430	960	0.002
21	0.460	960	-0.002
22	water blank	-	0.000
23	Neg. Con.	-	0.000
24	Pos. Con.	-	1.947 ←

Table 3.6.1. Optimisation of the electroporation transfection method with the SW620 cells. This table shows the effect of varying the voltage and the pulse applied to the cells. The amount of DNA used was kept constant at 10 μg. Sample 22, the the water blank was a negative control for the CPRG assay. Sample 23 the negative control for the transfection procedure, was a sample of SW620 cells which were not transfected. Sample 24, the positive control involved incubating 1.25ng of a commercial preparation of the enzyme β-galactosidase in the reaction mixture. Significant absorbance readings (≥0.3) are designated by an arrow.

Sample	Voltage (kV)	Capacitance (μF)	CPRG Assay Abs at 560 nm
1	0.260	25	-0.002
2	0.460	25	-0.001
3	0.660	25	0.003
4	0.860	25	0.003
5	1.060	25	0.006
6	1.260	25	0.005
7	1.470	25	0.004
8	0.210	500	0.002
9	0.260	500	0.006
10	0.310	500	0.018
11	0.360	500	0.007
12	0.410	500	0.004
13	0.430	500	0.003
14	0.460	500	0.003
15	0.210	960	0.000
16	0.260	960	0.003
17	0.310	960	-0.001
18	0.360	960	-0.003
19	0.410	960	-0.004
20	0.430	960	-0.001
21	0.460	960	0.006
22	water blank	-	0.000
23	Neg. Con.	-	0.000
24	Pos. Con.	-	1.857 ←

Table 3.6.2. Optimisation of the electroporation transfection method with the WiDr cells. This table shows the effect of varying the voltage and the pulse applied to the cells. The amount of DNA used was kept constant at 10 μg. Sample 22, the the water blank was a negative control for the CPRG assay. Sample 23 the negative control for the transfection procedure, was a sample of WiDr cells which were not transfected. Sample 24, the positive control involved incubating 1.25ng of a commercial preparation of the enzyme β-galactosidase in the reaction mixture. Significant absorbance readings (≥0.3) are designated by an arrow.

Sample	Voltage (kV)	Capacitance (μF)	CPRG Assay Abs at560 nm
1	0.260	25	0.075
2	0.460	25	-0.039
3	0.660	25	0.039
4	0.860	25	-0.027
5	1.060	25	0.189
6	1.260	25	-0.009
7	1.470	25	0.018
8	0.210	500	-0.002
9	0.260	500	0.009
10	0.310	500	-0.003
11	0.360	500	-0.021
12	0.410	500	0.018
13	0.430	500	0.003
14	0.460	500	0.003
15	0.210	960	0.009
16	0.260	960	-0.003
17	0.310	960	-0.021
18	0.360	960	0.018
19	0.410	960	0.003
20	0.430	960	0.000
21	0.460	960	0.003
22	water blank	-	0.000
23	Neg. Con.	-	0.000
24	Pos. Con.	-	2.020 ←

Table 3.6.3. Optimisation of the electroporation transfection method with the SW480 cells. This table shows the effect of varying the voltage and the pulse applied to the cells. The amount of DNA used was kept constant at 10 μg. Sample 22, the the water blank was a negative control for the CPRG assay. Sample 23 the negative control for the transfection procedure, was a sample of SW480 cells which were not transfected. Sample 24, the positive control involved incubating 1.25ng of a commercial preparation of the enzyme β-galactosidase in the reaction mixture. Significant absorbance readings (≥0.3) are designated by an arrow.

3.6.1. Discussion

These electroporation experiments were carried out using 10 μg of DNA and 5 x 10^6 cells. Three capacitance values were investigated in these experiments, namely 25, 500 and 960 μF. These affected the duration of the pulse applied. The time of each of the pulses was also recorded. A 1.1 msec pulse was applied when a capacitance of 25 μF was used. A 19 and a 38 msec pulse were applied when capacitances of 500 and 960 μF were used.

As can be seen from table 3.6.1. the different pulses applied to the SW620 cells did not result in any transfected cells as all the absorbance readings corresponded to negative β-galactosidase values, as all readings were <0.11 (the β-galactosidase standard curve intercepts the y axis at 0.11, corresponding to 0 ng of β-galactosidase). Absorbance readings ≥ 0.3 (corresponding to 0.15 ng of β-galactosidase) are significant readings as they denote that the cells were transfected sufficiently with pCH110, for transient transfection assays (Wilson *et al.*, 1995).

This was also the case for the WiDr cell line. One of the pulses applied to the SW480 cells resulted in a low level of transfection, which was not sufficient for transient transfection assays as the O.D. reading was <0.3. This was sample 5 which had an absorbance reading of 0.189. This reading corresponds to a 0.04 ng expression of β-galactosidase, in 10 μl of this sample cell extract, (total volume of cell extract is 60 μl). This level of β-galactosidase expression was obtained by using the β-galactosidase standard curve. If this level of β-galactosidase expression, 0.04 ng in sample 5 from table 3.6.3., is compared to that obtained when the SW480 cells are transfected using the $CaPO_4$ method, (0.4 ng of β-galactosidase), under optimum conditions, sample 20 from table 3.5.3., it can be seen that the $CaPO_4$ method is 10 times more effective at transfecting the SW480 cells than the electroporation method.

It can be concluded from these results that the electroporation method is not suitable for transfecting the SW620 and WiDr cell lines and also this method results in a low level of transfection of the SW480 cell line when a capacitance of 25 μF and a voltage of 1.060 kV is used. The next set of experiments dealt with investigating the use of lipofection to transfect a suitable cell line.

3.7. Transfection using Lipofectamine

The use of a cationic lipid such as lipofectamine to transfect cells is known as lipofection. Optimum transfection efficiency occurs when the ratio of positively charged molar equivalents (contributed by the cationic lipid), is nearly equivalent to the number of molar equivalents of negative charge contributed by the polynucleotide, (Felgner *et al.*, 1993). The lipofectamine reagent used was a 3:1 (w/w) liposome formulation of the polycationic lipid DOSPA and the neutral lipid DOPE in membrane filtered water from Gibco / BRL. More information on lipofection can be found in section 1.4.5. and the method used in section 2.5.7.

The effectiveness of lipofectamine to transfect the WiDR, SW620, and the SW480 cell line was investigated. The results of these experiments can be found in tables 3.7.1., 3.7.3. respectively.

Sample	**DNA (μg) conc.**	**lipofectamine (μl)**	**CPRG Assay Abs. At 560 nm**
1	0.5	20	0.012
2	1.0	20	0.003
3	2.5	20	-0.002
4	3.0	20	-0.005
5	5.0	20	0.008
6	10.0	20	0.006
7	water blank	-	0.000
8	Neg. Con.	-	0.000
9	Pos. Con.	-	2.300 ⟵

Table 3.7.1. Optimisation of transfection using the lipofectamine method in the WiDr cells. This table shows the effect of varying DNA concentration, using the lipofectamine method. Sample 7, the the water blank was a negative control for the CPRG assay. Sample 8 the negative control for the transfection procedure, was a

sample of WiDr cells which were not transfected. Sample 9, the positive control involved incubating 1.25ng of a commercial preparation of the enzyme β-galactosidase in the reaction mixture. Significant absorbance readings (≥0.3) are designated by an arrow.

Sample	DNA (μg) conc.	lipofectamine (μl)	CPRG Assay Abs. At 560 nm
1	0.5	20	0.010
2	1.0	20	0.003
3	2.5	20	-0.006
4	3.0	20	-0.005
5	5.0	20	0.016
6	10.0	20	0.016
7	water blank	-	0.000
8	Neg. Con.	-	0.000
9	Pos. Con.	-	1.875 ←

Table 3.7.2. Optimisation of transfection using the lipofectamine method in the SW620 cells. This table shows the effect of varying DNA concentration, using the lipofectamine method. Sample 7, the the water blank was a negative control for the CPRG assay. Sample 8 the negative control for the transfection procedure, was a sample of SW620 cells which were not transfected. Sample 9, the positive control involved incubating 1.25ng of a commercial preparation of the enzyme β-galactosidase in the reaction mixture. Significant absorbance readings (≥0.3) are designated by an arrow.

Sample	DNA (μg) conc.	lipofectamine (μl)	CPRG Assay Abs. At 560 nm
1	0.5	20	0.003
2	1.0	20	0.006
3	2.5	20	-0.010
4	3.0	20	-0.004
5	5.0	20	0.016
6	10.0	20	0.336 ←
7	water blank	-	0.000
8	Neg. Con.	-	0.000
9	Pos. Con.	-	1.855 ←

Table 3.7.3. Optimisation of transfection using the lipofectamine method in the SW480 cells. This table shows the effect of varying DNA concentration, using the lipofectamine method. Sample 7, the the water blank was a negative control for the CPRG assay. Sample 8 the negative control for the transfection procedure, was a sample of SW480 cells which were not transfected. Sample 9, the positive control involved incubating 1.25ng of a commercial preparation of the enzyme β-galactosidase in the reaction mixture. Significant absorbance readings (≥0.3) are designated by an arrow.

3.7.1. Discussion

When using the lipofectamine procedure to transfect cells, it is recommended that a fixed volume of 20 μl of lipofectamine is used while a range of DNA concentrations are tested. The incubation of the transfection mixture with the cells is kept constant at 5 hr. This procedure is prohibitively expensive (approximately £12 per transfection plate) and can only be used in serum-free media.

As can be seen from tables 3.7.1. and 3.7.2. the lipofectamine procedure was not successful in transfecting the SW620 or the WiDr cell line. It was however suitable

for transfecting the SW480 cell line, (see table 3.7.3.). A 20 µl volume of lipofectamine and 10 µg of DNA resulted in an absorbance reading of 0.336 (this corresponded to a 0.122 ng level of β-galactosidase expression, calculated from a β-galactosidase standard curve) in a 10 µl of cell extract, (total cell extract volume 30 µl). This was not further optimised as we were only using the SW480 cell line as a positive control cell line as it can be readily transfected. This result was repeated and the plate was stained using the *in-situ* stain, of which a photograph can be seen in figure 3.8.4. (c).

It was concluded that the lipofectamine method was not suitable for transfecting the SW620 and the WiDr cell lines. It was also concluded that these two cell lines were proving impossible to transfect. It was decided to take a look at transfecting another cell line the A549 cell line which is not a colon carcinoma cell line but a lung adenocarcinoma cell line. This cell line has been shown to endogenously express matrilysin mRNA (Fingleton 1995). Unpublished results from this laboratory had demonstrated that matrilysin mRNA expression could be stimulated in this cell line by EGF and cytokines and therefore would be suitable to investigate the transcriptional regulation of matrilysin gene expression.

3.8. The use of DOTAP in transfection experiments

This liposome formulation is available from Boehringer Mannheim. As a lipid formulation it is very easily metabolised by cells in culture than other formulations and therefore less toxic to the cells. It also has an added advantage in that it can be used with serum if necessary whereas components present in serum can affect transfection of cells using most other lipid formulations (Walker *et al.*, 1992).
Optimisation experiments were carried out in the A549 cell line, (see section 2.5.8. for details on method). The results of the experiments carried out in the A549 cell line in figures can be found in figures 3.8.1-3.8.3.

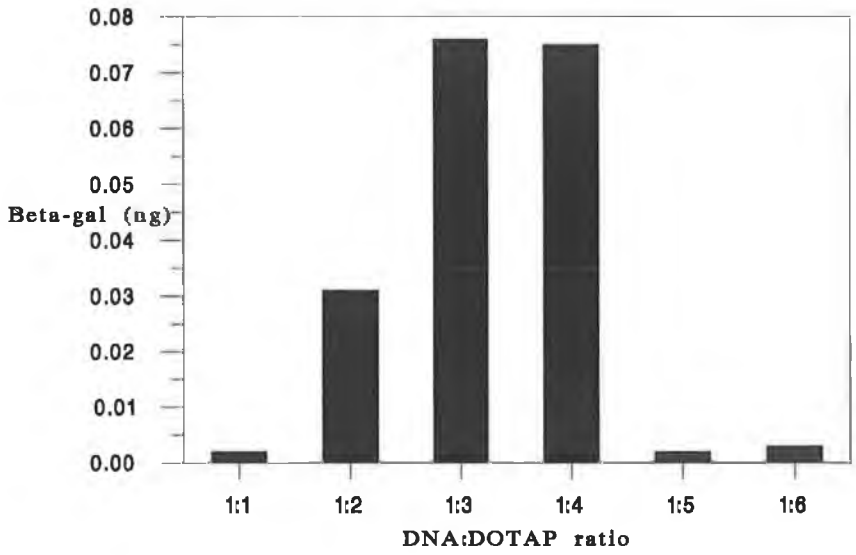


Figure 3.8.1. Optimisation of the DNA:DOTAP ratio for the A549 cell line. This figure shows the effect of varying the DNA:DOTAP ratio. The quantity of DNA was kept constant at 5 μg. Levels of β-galactosidase >0ng represent transfected cells. Levels of β-galactosidase ≥0.15 ng are significant, as they denote an adequate level of transfection for transient transfection assays.

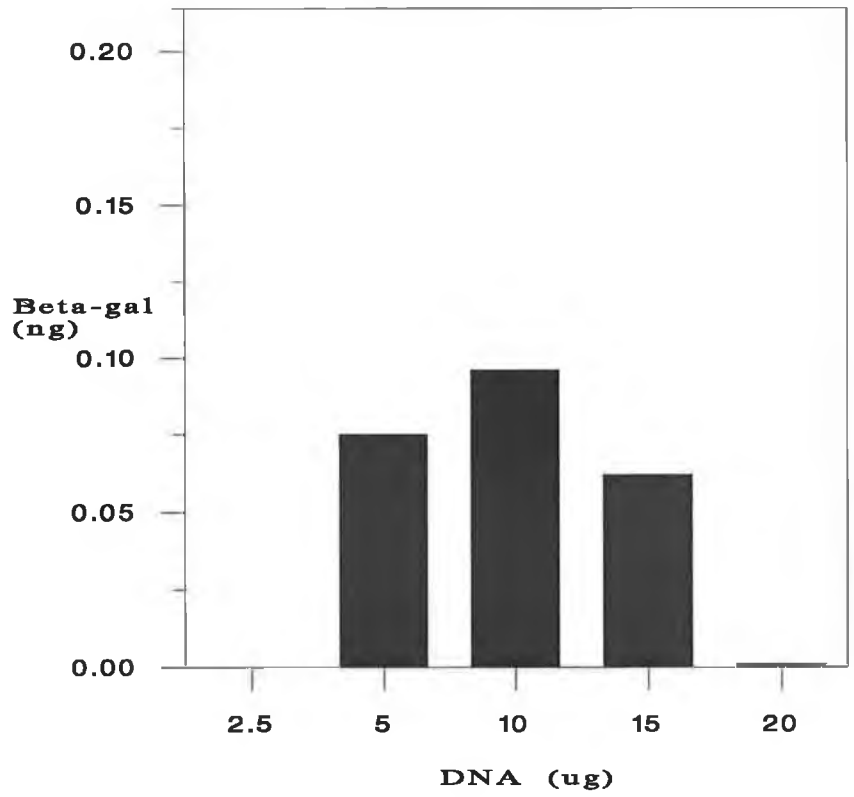


Figure 3.8.2. Optimisation of DNA concentration for the A549 cell line. This figure shows the effect of varying the DNA concentration. The ratio of DNA:DOTAP was kept constant at 1:3. Levels of β-galactosidase >0ng represent transfected cells. Levels of β-galactosidase ≥0.15 ng are significant, as they denote an adequate level of transfection for transient transfection assays.

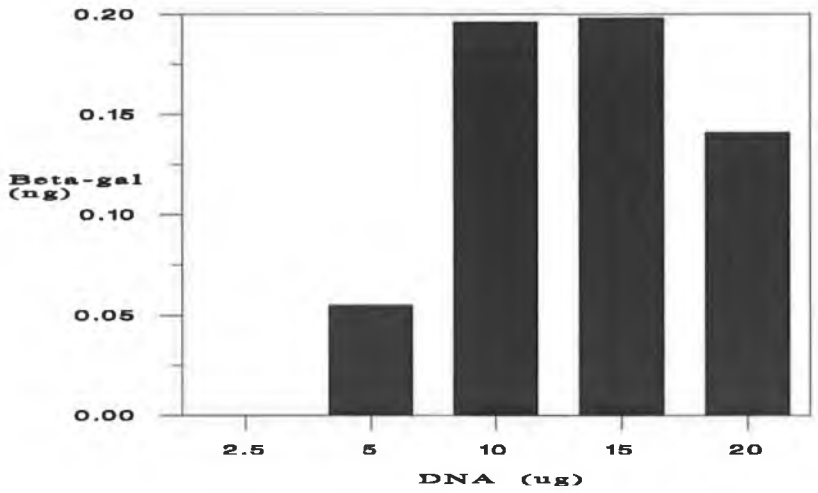


Figure 3.8.3. Optimisation of DNA concentration for the A549 cell line. This figure shows the effect of varying the DNA concentration. The ratio of DNA:DOTAP was kept constant at 1:4. Levels of β-galactosidase >0ng represent transfected cells. Levels of β-galactosidase ≥0.15 ng are significant, as they denote an adequate level of transfection for transient transfection assays.

3.8.1. Discussion

Figure 3.8.1. shows the results of a DOTAP transfection optimisation experiment carried out on the A549 cell line. All of the various DNA:DOTAP ratios tested resulted in transfected cells as all levels of β-galactosidase expression were >0 ng. However none of the levels of β-galactosidase expression were significant (as they were all <0.15 ng of β-galactosidase) for carrying out transient transfection assays so further optimisation experiments was carried out. There was a gradual increase in the number of transfected cells as the DNA:DOTAP ratio increased. This number of transfected cells reached a maximum at the 1:3 and 1:4 ratios before starting to decline again. Further optimisation was then carried out with the ratios that had

resulted in maximum levels of transfected protein i.e. the 1:3 and 1:4 ratios. This further optimisation involved keeping the DNA:DOTAP ratio constant and varying the DNA concentration from 2.5 to 20 μg. The results of this optimisation for the 1:3 ratio can be seen in figure 3.8.2. and for the 1:4 ratio in figure 3.8.3. 2.5 μg of DNA did not result in any level of β-galactosidase expression, the protein product of the transfected gene, for either of the DNA:DOTAP ratios being investigated. The next concentration of DNA, 5 μg produced a similar result for both of the ratios being investigated, 0.075 ng for the 1:3 ratio and 0.055 ng of β-galactosidase for the 1:4 ratio. This result is in keeping with those obtained in the original optimisation experiment where 5 μg of DNA resulted in a 0.075 ng level of β-galactosidase expression for the 1:3 ratio and a 0.073 ng level of β-galactosidase expression for the 1:4 ratio. The level of expression of β-galactosidase in the rest of the samples from the 1:3 DNA:DOTAP ratio peaked at 10 μg of DNA, with 0.096 ng of β-galactosidase being expressed. This then dropped to 0.062 ng for 15 μg of DNA and then to practically zero, (0.001 ng) for 20 μg of DNA. None of the levels of transfected β-galactosidase reached significance for transient transfection assays as all the levels of β-galactosidase expression were <0.15 ng.

This was not the case for the rest of the samples from the 1:4 DNA:DOTAP group. The level of β-galactosidase expression rose to much higher levels for 10 and 15 μg of DNA reaching 0.196 and 0.198 ng respectively, which were significant levels of β-galactosidase expression for transient transfection assays as these levels are >0.15 ng. The level of β-galactosidase expression then decreased for the 20 μg of DNA sample, to 0.141 ng level of β-galactosidase.

In conclusion, the optimal conditions for transfection of the A549 cell line using DOTAP were, a concentration of 10 μg of DNA, and a DNA:DOTAP ratio of 1:4 produces a level of expression of β-galactosidase, (0.196 ng, O.D. reading of 0.473 in a 10 μl sample of extract, total volume 60 μl), which would be sufficient for transient transfection assays. A photograph of the A549 cells transfected by DOTAP can be found in figure 3.8.4. (d).

Having successfully demonstrated that the A549 cell line could be transfected using the cationic lipid DOTAP the transcriptional regulation of matrilysin gene expression was investigated.

Method	SW620	WiDr	SW480	A549
DEAE-dextran	-[a]	ND[b]	ND	ND
Glass Beads	-	NS[c]	NS	NS
Polybrene	-	ND	ND	ND
$CaPO_4$	-	-	++++[d]	ND
Electroporation	-	-	+	ND
Lipofectamine	-	-	++	ND
DOTAP	ND	ND	ND	+++

Table 3.8.2. Comparison of transfection methods in human cancer cell lines

(a) -; Negative results

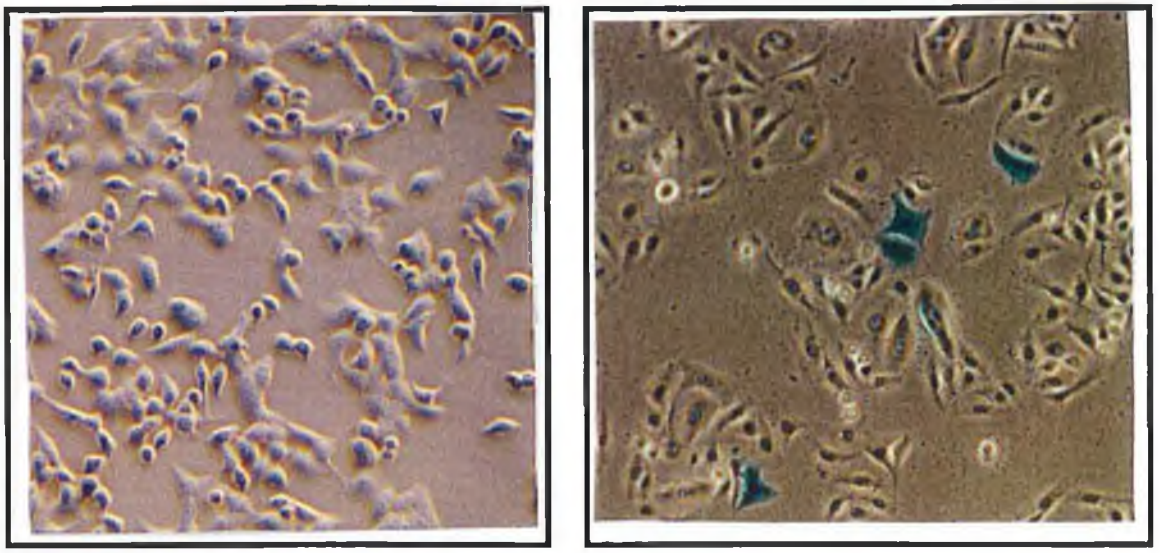
(b) ND; Not determined

(c) NS; Not suitable, as cells detached from surface of plate

(d) + Positive results, +=0.04 ng, ++=0.117 ng, +++=0.196 ng, ++++=0.460 ng of β-galactosidase

This table summarises the results of all the transfection experiments carried out. As can be seen from the table a suitable transfection method for the SW620 and WiDr cell lines was not found. The SW480 cell line could be successfully transfected by a number of methods including the $CaPO_4$, the electroporation, and the lipofectamine methods. Of these the $CaPO_4$ method yielded the highest level of β-galactosidase expression. As can be seen from the table the A549 cell line was successfully transfected by the DOTAP method.

(a) (b)



(c) (d)

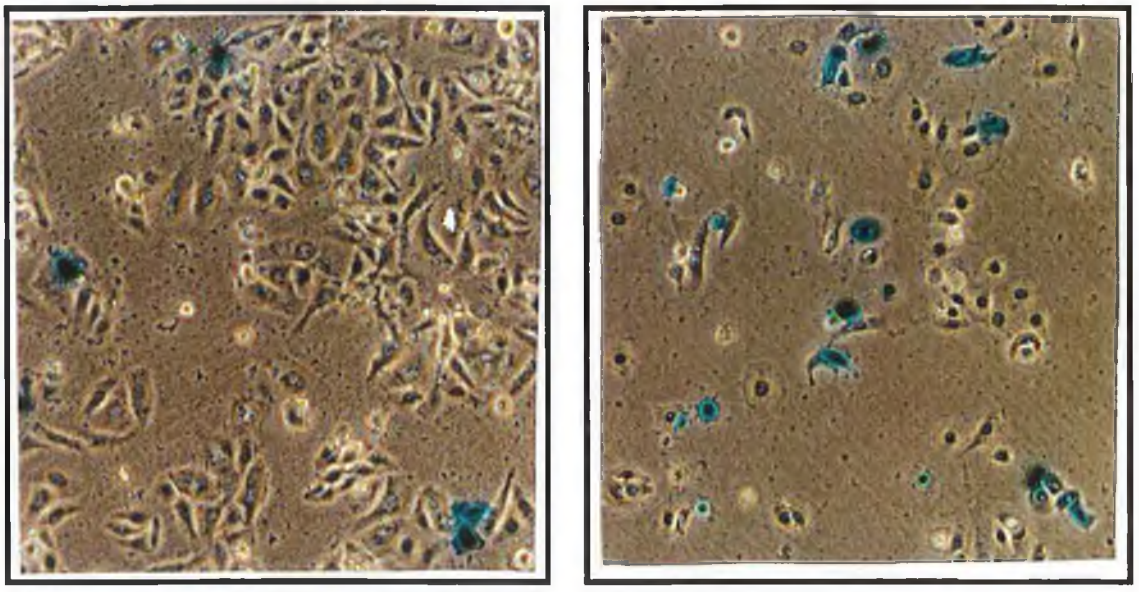


Figure 3.8.4. ***In-situ*** **staining of transfected cells.** (a) SW480 cells which are not transfected, (b) SW480 cells transfected by $CaPO_4$, (c) SW480 cells transfected by lipofectamine and (d) A549 cells transfected by DOTAP. Transfected cells stain blue.

3.9. Investigating the transcriptional regulation of matrilysin gene expression

The transcriptional regulation of matrilysin gene expression was investigated in the A549, a human lung adenocarcinoma cell line, since this cell line endogenously expresses matrilysin (Fingleton 1995). It can also be stimulated with growth factors and cytokines to overexpress matrilysin and can be transfected with the cationic lipid DOTAP.

Sequence analysis has shown that various transcription regulatory elements are present within the matrilysin promoter, including the AP-1 and PEA3 motifs (Gaire *et al.*, 1994). These regulatory sequences, in the collagenase and stromelysin promoters, have been shown to transactivate EGF and TPA regulation of collagenase and stromelysin (for further details consult section 1.3) In order to identify these regulatory sequences as functional regulatory elements in the matrilysin promoter, various segments of the matrilysin promoter were linked to the CAT marker gene, which is easily assayable following transfection. This was carried out by our collaborators in Nashville. Four matrilysin CAT promoter constructs were made (Gaire *et al.,* 1994). The p-95HPCAT construct contains the TATA and AP-1 regulatory sequences. The p-295HPCAT construct encompasses these sequences plus the PEA3 motifs and the p-933HPCAT construct includes those sequences present in the p-295HPCAT construct plus additional TGFβ inhibitory elements (TIE) sequences. The p-4.2HPCAT construct spans 4.2 Kbp of the matrilysin promoter and it contains all the above mentioned sequences. Transfecting these constructs into the A549 cell line and stimulating with EGF and TPA would enable a determination as to whether or not the matrilysin gene is regulated by EGF and TPA to be made and if so through which *cis*-acting elements they are exerting their effect. If EGF or TPA had a stimulatory effect on the matrilysin promoter, it will activate the promoter causing the 3'-flanking CAT gene to be transcribed. The amount of CAT produced is directly proportional to the regulatory effect that the particular factor has on the promoter. Whichever construct showed the greatest stimulation of the promoter, that is the largest amount of CAT production, would tell us that it is in that region of the promoter that EGF or TPA is exerting its effect.

Before proceeding with this experiment it was first necessary to optimise the conditions under which the A549 cells were stimulated. Prior to stimulation, the cells were fed with serum-free medium. This was necessary as growth factors naturally present in the serum may interfere in these experiments. Feeding the cells with serum-free medium before stimulation with the regulatory factors being investigated, ensures that any regulatory effects observed are due to the factors being investigated and not any components in the serum. The length of time the cells were stimulated also had to be investigated. In order to optimise the length of time the cells were incubated in serum-free medium and the duration of the stimulation, transfection experiments varying these parameters using the pCMVCAT plasmid, which codes for the bacterial protein chloramphenicol acetyltransferase, were carried out. The pCMVCAT plasmid contains the cytomegalovirus (CMV) promoter linked to the CAT gene. This plasmid serves as a positive control as the CMV promoter is a constitutive promoter which enables endogenous expression of the gene it is linked to in all cells (Reese et al., 1992). The amount of CAT produced was assayed using a CAT ELISA. The standard curve for the ELISA can be found in figure 3.9.1. The results of the stimulation optimisation are illustrated in figure 3.9.2. Once the stimulation step had been optimised we could then transfect the matrilysin promoter constructs and the pCH110 plasmid, to normalise for variations in transfection efficiency, into the A549 cells, stimulate with EGF (5 ng/ml) and TPA (100 ng/ml) and then assay for CAT production using the CAT ELISA. The results of this experiment can be found in table 3.9.1.

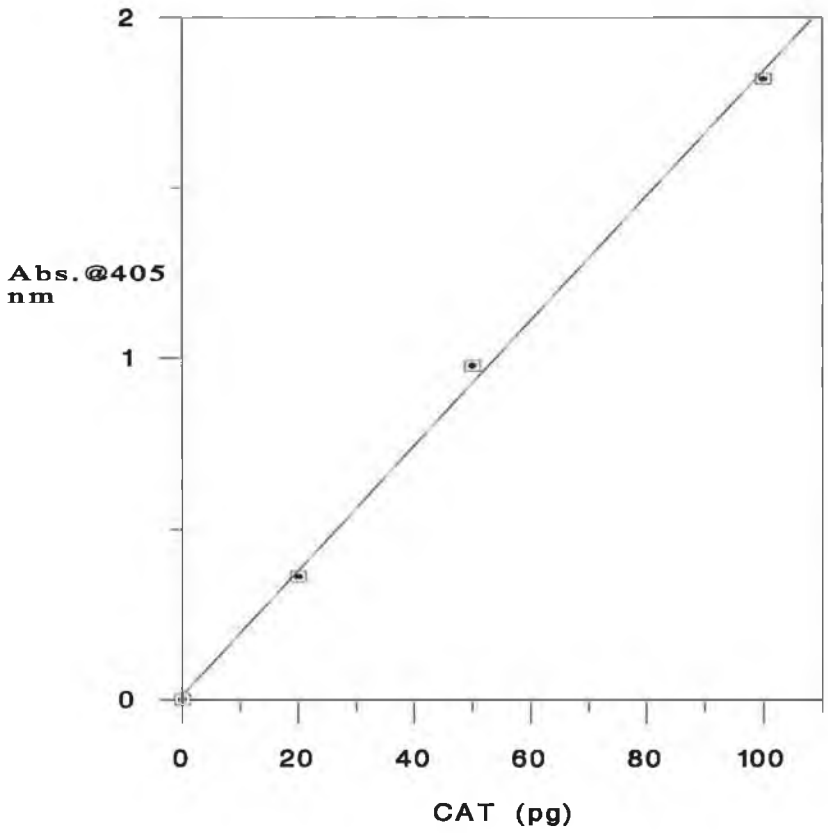
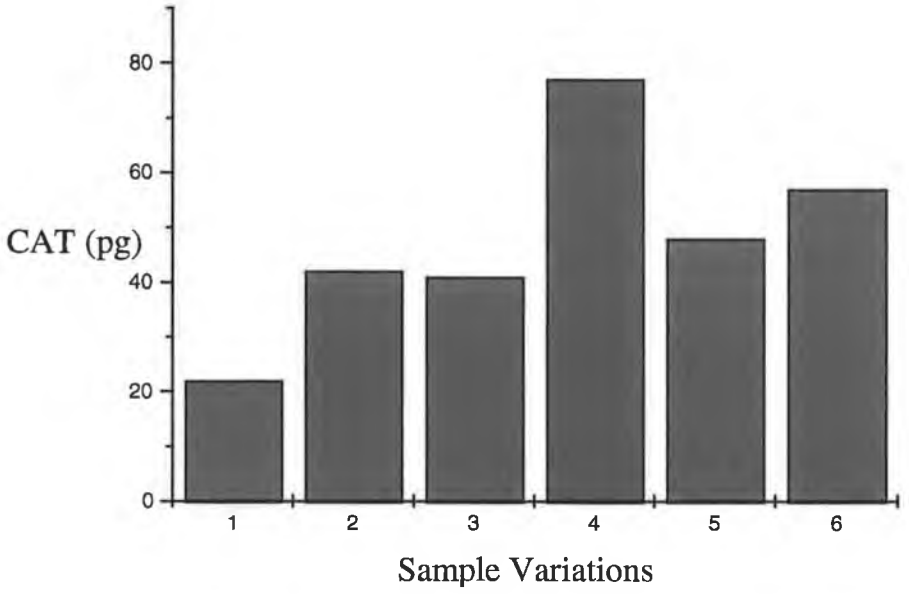


Figure 3.9.1. CAT ELISA standard curve



Sample 1	Total transfection carried out in $DMEMS_0$, no EGF stimulation
Sample 2	Total transfection carried out in $DMEMS_0$, 24 hr EGF stimulation
Sample 3	Transfection carried out in $DMEMS_5$, no EGF stimulation
Sample 4	Transfection carried out in $DMEMS_5$, 8 hrs serum-free, 24 hr EGF stimulation
Sample 5	Transfection carried out in $DMEMS_5$, no EGF stimulation
Sample 6	Transfection carried out in $DMEMS_5$, 24 hr serum-free, 8 hr EGF stimulation

Figure 3.9.2. Optimisation of stimulation conditions. This figure shows the effect of variations in the length of time serum-free media ($DMEMS_0$) is incubated with the A549 cells transfected with the pCMVCAT plasmid and in the length of time EGF (5 ng/ml) is subsequently left to stimulate these cells. $DMEMS_5$ denotes DMEM supplemented with 5% FCS.

Sample	Description	CAT ELISA Abs. at 405 nm	CPRG Assay Abs. at 560 nm
1	p-95HPCAT-basal	-0.038	0.473
2	p-95HPCAT-EGF	-0.031	0.474
3	p-95HPCAT-TPA	-0.010	0.463
4	p-295HPCAT-basal	-0.002	0.395
5	p-295HPCAT-EGF	-0.035	0.354
6	p-295HPCAT-TPA	-0.024	0.481
7	p-933HPCAT-basal	-0.028	0.461
8	p-933HPCAT-EGF	-0.015	0.481
9	p-933HPCAT-TPA	-0.038	0.395
10	p-4.2HPCAT-basal	-0.041	0.387
11	p-4.2HPCAT-EGF	-0.052	0.471
12	p-4.2HPCAT-TPA	-0.033	0.476
13	pFLCAT (neg. con.)	-0.001	0.435
14	pCMVCAT (pos. con.)	0.845	0.389
15	Water Blank	-0.002	0.000
16	Neg. Con. (A549 cells)	0.000	0.000
17	Pos. Con. (20 pg CAT)	0.436 ←	-

Table 3.9.1. Regulation of matrilysin gene expression. This table shows the results of the experiment investigating the regulatory effect of EGF (5 ng/ml) and TPA (100 ng/ml) on the matrilysin promoter. Significant absorbance readings from the CAT ELISA are highlighted with an arrow. All absorbance readings from the CPRG assay were significant as they were all >0.3 (>0.15 ng)

3.9.1. Discussion

As can be seen from figure 3.9.1. the CAT ELISA is very sensitive, detecting levels of CAT as low as 2-5 pg. This standard curve is linear in the range of 0-100 pg. Figure 3.9.2. illustrates the results obtained from the investigations into the stimulation conditions to be used. Sample 1 illustrates the amount of CAT protein expressed when the DOTAP transfection is carried out in serum-free media, which is 22 pg. It was decided to investigate carrying out the transfection in serum-free media as this would automatically eliminate the need to optimise an incubation time for the cells with serum-free media. The level of expression of the transfected protein CAT is approximately doubled (43 pg) when a 24 hr stimulation with EGF (5 ng/ml) is administered (sample 2). When the transfection is carried out in the presence of serum, as were those in the initial DOTAP optimisation experiments on the A549 cells, the level of CAT protein expressed was 41 pg (sample 3), practically the same as that obtained when the transfection is carried out in serum-free conditions and stimulated with EGF (5ng/ml) (sample 2). From this we could conclude that carrying out the transfection in serum-free conditions reduced the level of CAT expression. Sample 5 is a duplicate sample of sample 3, and the result obtained is approximately at a similar level. Sample 4 and 6 investigate the use of an 8 hr serum-free incubation, a 24 hr stimulation and a 24 hr serum-free incubation and a 8 hr stimulation respectively. As can be seen from the graph, sample 4 resulted in the maximum amount of CAT expression after stimulation with EGF (5ng/ml). Thus, the conditions of an 8 hour serum-free incubation and a 24 hr stimulation were used in all of the stimulations. This experiment not only optimised the stimulation conditions it also demonstrated that the transfection conditions used are suitable for transcriptional regulation experiments. The results conclusively prove that firstly, the transfection conditions used are ensuring that a sufficient level of DNA is being transfected into the A549 cells to allow for expression of the CAT protein, and secondly, the induction of the CMV promoter by EGF (~ 2-fold induction) can be detected using the CAT ELISA.

As can be seen from table 3.9.1. the absorbance readings obtained from the matrilysin promoter constructs were not significant. We also co-transfected the plasmid pCH110

which codes for β-galactosidase activity, to normalise for slight variations in transfection efficiency. All the absorbance readings obtained from the CPRG assay were significant. These readings show that the A549 cells were successfully transfected in this experiment. We have shown through these results obtained from the CPRG assay and the results obtained from transfecting in the pCMVCAT plasmid and stimulating with EGF, that the DOTAP transfection method and the CAT ELISA are suitable for use in transcriptional regulation experiments. We concluded that the lack of significant absorbance readings in the CAT ELISA experiment investigating the transcriptional regulation of matrilysin gene expression, was due to a transcriptional inactivity of the matrilysin promoter constructs. Our collaborators in Nashville obtained similar results transfecting these matrilysin CAT promoter constructs into a breast cell line.

The inactivity of the matrilysin promoter constructs was thought to be due to the inclusion of 5'-flanking CAT promoter sequences when the matrilysin promoter CAT reporter constructs were originally constructed. The presence of CAT promoter sequences in this system would interfere with the transcriptional activity of the matrilysin promoter segments. The inactivity of the matrilysin promoter constructs also raises the possibility that the matrilysin promoter is a weak promoter. Linking the matrilysin promoter segments to the more sensitive luciferase marker gene (Luehrsen *et al.*, 1993) could help to alleviate this problem.

4.1. Conclusions

The use of various transfection methods to transfect the SW620, SW480, WiDr and A549 cell lines were investigated. Table 3.8.2. summarises the results obtained. The SW620 and WiDr colon carcinoma cell lines proved to be very resistant to transfection. The DOTAP transfection method was successful in transfecting the A549 cell line and this cell line was used in experiments investigating the transcriptional control of matrilysin gene expression. From these experiments it was concluded that the human matrilysin CAT promoter constructs were not transcriptionally active. Similar results were obtained when the human matrilysin CAT constructs were transfected into a breast cell line (personal communication from Lynn Matrisian).

Human matrilysin reporter promoter constructs linked to the more sensitive luciferase marker gene (Leuhrsen *et al.*, 1993) have been synthesised. Future experiments that can be carried out with these human matrilysin luciferase promoter constructs would include transfecting the promoter constructs into the A549 cell line and stimulating with EGF and TPA. From these experiments it would be possible to show if EGF and TPA regulate human matrilysin gene expression and if so, an approximate estimation could be obtained of where in the promoter these factors affect transcription and through which *cis*-acting elements. These experiments would have narrowed down the area where these factors exert their effect to a few hundred base pairs. The next step would be to confine this area down further to the region of 10-30 bp. This can be achieved by carrying out a time course digest on the matrilysin promoter and subcloning the smaller fragments of interest obtained into the promoter reporter system. These will be transfected into the cell line of interest and stimulated with EGF and TPA to illustrate which of the smaller fragments produced the regulation expected. To conclusively prove that these factors are exhibiting their effect through the sequences found to be responsible, site directed mutagenesis will be carried out on the sequences in question. These mutated sequences will be subcloned into the promoter reporter system and transfected into the cell line of interest. If the mutated promoter constructs did not show any regulation after stimulation with the regulatory

factors than this would confirm that it is through this sequence that the factor of interest was exerting its effect.

There are two additional steps to fully elucidate the transcriptional control of matrilysin gene expression left to complete. The first of these is to determine which transcription factors are binding to this region of the promoter that is the responsive unit for the particular growth factor or other regulatory factor in question. This can be achieved using the DNA electrophoretic mobility shift assay (EMSA) or gel shift assay. The method relies on the ability of a protein to bind to a radiolabelled DNA fragment *in vitro*, followed by electro-phoretic separation. It is based on the fact that during electrophoresis complexes of protein and DNA migrate more slowly through a nondenaturing polyacrlyamide gel than unbound DNA fragments or double-stranded oligonucleotides. One or more proteins binding to the DNA fragment may be identified. In general, the larger the protein, the greater the extent of retardation of mobility within the gel, (Fried *et al.*, 1981). Various transcription factors and antibodies to these transcription factors are commercially available for use in these gel-shift experiments, to enable identification of the transcription factors. These antibodies can be used to determine what transcription factors are naturally present in the nuclear extracts from the cells in which the regulation experiments are being carried out, and aid in the purification of DNA binding proteins from crude cell extracts or from recombinant sources.

Addition of antibodies which recognise protein(s) binding to the DNA to the reaction mixture to be electrophoreised produces an even slower migrating species than the original protein:DNA complex. This phenomenon is known as a "supershift" or shift-shift.

Competitors can also be analysed using this approach. Gutman *et al.*, (1990) applied this technique to establish whether the putative PEA3 motifs in the collagenase and the c-fos promoters bound PEA3 and to what extent. The PEA3 collagenase and fos probes were competed with the PEA3 polyoma virus motif, which is known to bind PEA3. From the results of this assay it was concluded that the PEA3 polyoma virus sequence has a five-fold higher affinity for PEA3 than the PEA3 collagenase and PEA3 fos motifs. This approach which ascertained the binding affinity of PEA3 to

the PEA3 binding sites in the collagenase and fos promoters is an approach which can be applied to any promoter which has a PEA3 binding site.

The mobility shift assay does not allow the identification of the precise sequence to which a protein binds, as it employs relatively large fragments of a gene promoter as a probe for DNA binding activity. So the last step in this process is to carry out DNA footprinting which allows the determination of a short protein binding site within a relatively large DNA fragment, providing an essential step in the characterisation of transcription factors.

The general principle of in vitro DNA footprinting is based on the cleavage of the DNA molecule with either a chemical reagent or an enzyme. The DNA of interest is radioactivity labelled at one end of the molecule and on one strand of the DNA duplex only. Thus limited degradation of DNA such that each molecule of the DNA is cleaved randomly only once or a few times by the chemical or enzyme, resulting in a ladder of DNA fragments of varying size when the DNA is subjected to denaturing polyacrylamide electrophoresis and detected by autoradiography. Thus each band in the DNA ladder is representative of a specific nucleotide where the cleavage agent has cut the labelled strand of the DNA. If Maxam and Gilbert sequencing reactions of the same DNA fragment are run alongside the cleavage products, then the specific nucleotide in the DNA sequence which is represented by each band can be determined.

If however, the DNA is complexed with a protein before being treated with the cleavage agent, the nucleotides to which the protein is bound will be protected against cleavage. Thus, certain bands in the DNA ladder, representative of where the protein binds the DNA duplex, will not be produced due to the protein protecting these nucleotides against cleavage. This results in a gap in the DNA ladder which is representative of a protein binding a specific DNA sequence. By comparing the gap in the ladder to the Maxam and Gilbert sequencing reactions, the precise DNA sequence protected from cleavage, and thus the DNA-binding site of the protein can be determined.

The information obtained at this point would give a clear picture as to what effect a particular growth factor has on the matrilysin promoter - where exactly in the promoter the factor exerts its effect, what transcription factors are binding to this area

and what the precise sequence is that binds the particular transcription factor(s). This information could then be used to provide mechanisms through which the expression of matrilysin in a disease state could be controlled.

List of Publications

Publications

O'Shea, H., and McDonnell, S., (1995) Comparison of transfection methods in human colon cancer cell lines. Ir. Jr. Med. Sci, (*In Press*)

McDonnell, S., **O'Shea,** H., and Fingleton, B., (1996) Regulation of matrilysin gene expression in human tumour cell lines. Proc. Amer. Assoc. for Cancer Res., (*In press*).

Bibliography

Bibliography

An G., Hidaka K., and Siminovitch L. (1982) Expression of bacterial β-galactosidase in animal cells. Mol., Cell., Biol., 2(2), 1628-1632.

Anderson M. L. M., Spandidos D.A., Coggins J.R. (1991) Electroporation of lymphoid cells: factors affecting the efficiency of transfection. J., Biochem., Biophys., Methods, 22, 207-222.

Andreason G. L., and Evans G.A. (1988) Introduction and expression of DNA molecules in eukaryotic cells by electroporation. Biotechniques, 6(7), 650-660.

Andreason G.A., Evans G.A. (1989) Optimisation of electroporation for transfection of mammalian cell lines. Anal., Biochem., 180, 269-275.

Angel P., Baumann I., Stein B., Delius H., Rahmsdorf H.,J., and Herrlich P. (1987) 12-O-tetradecanoyl-phorbol-13-acetate induction of the human collagenase gene is mediated by an inducible enhancer element located in the 5'-flanking region. Mol. Cell. Biol. 7, 2256-2266.

Banda M.J., Howard E.W., Herron G.S. and Apodeca G. (1992) Secreted inhibitor of metalloproteinases (IMP's) that are distinct from TIMP. Matrix (Germany) Suppl. 1, 294-298.

Baragi V.M., Fliszar C.J., Conroy M.C., Ye Q.Z., Shipley J.M. and Welgus H.G. (1994) Contribution of the C-terminal domain of metalloproteinases to binding by tissue inhibitor of metalloproteinases. C-terminal truncated stromelysin and matrilysin exhibit equally compromised binding affinities as compared to full-length stromelysin. J. Biol. Chem., 269, 12692-12697.

Basset P., Bellocq J.P., Wolf C., Stoll I., Hutin P., Limacher J.M., Podhajcer O.L., Chenard M.P., Rio M.C., and Chambon P. (1990) A novel metalloproteinase gene

specifically expressed in stromal cells of breast carcinomas. Nature 348, 20/27 699-704.

Boone T.C., Johnson M.J., DeClerck Y.A. and Langley K.E. (1990) cDNA cloning and expression of a metalloproteinase inhibitor related to tissue inhibitor of metalloproteinases. Proc. Natl. Acad. Sci. USA, 87, 2800-2804.

Bruner K.L., Rodgers W.H., Gold L.I. Korc M. Hargrove J.T. Matrisian L.M. and Osteen K.G. (1995) Transforming growth factor beta mediates the progesterone suppression of an endothelial metalloproteinase by adjacent stroma in the human endometrium. Proc. Natl. Acad. Sci. USA, 92 (16), 7362-7366.

Carmichael D.F., Sommer A, Thompson R.C., Anderson D.C., Smith C.G., Welgus H.G., and Stricklin G.P. (1986) Primary sequence and cDNA cloning of human fibroblast collagenase inhibitor. Proc. Natl. Acad. Sci. USA, 83, 2407-2411.

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

Chu G., Hayakawa H., and Berg P. (1987) Electroporation for the efficient transfection of mammalian cells with DNA. Nucleic Acids Res., 15, 1311-1326.

Cockett M, Birch M, Murphy G, Hart I, and Docherty A. (1993) Metalloproteinase domain structure, cellular invasion and metastasis. Biochem. Soc. Trans., 22, 55-57.

Crabbe T, Smith Bj, O'Connell J and Docherty A. (1994) Human progelatinase A can be activated by matrilysin. FEBS Letters, 345, 14-16.

Curran T. and Franza R. (1989). Fos and Jun; the AP-1 connection. Cell 55, 395-357.

DeClerck Y.A., Yean T.D., Lu H.S., Ting J. and Langley K.E. (1991) Inhibition of autoproteolytic activation of interstitial procollagenase by recombinant metalloproteinase inhibitor MI/TIMP-2. J Biol. Chem. 266, 3893-3899.

DeClerck Y.A., YeanT.D., Ratzkin B.J., Lu H.S. and Langley K.E. (1989) Purification and characterisation of two related but distinct metalloproteinase inhibitors secreted by bovine aortic endothelial cells. J. Biol. Chem., 264, 17445-17453.

Docherty A.J.P., Yean T.D., Chan D, Shimada H and Langley K.E. (1985) Sequence of human tissue inhibitor of metalloproteinases and its identity to erythoid-potentiating activity. Nature, 318, 66-69.

Dollard S.C., Broker T.R. and Chow L.T. (1993) Regulation of the human papillomavirus type-11 E6 promoter by viral and host transcription factors in primary human keratinocytes. J. Virol., 67, 1721-1726.

Felgner J., Martin M., Tsai Y., and Felgner P.L. (1993) Cationic lipid-mediated transfection in mammalian cells: "lipofection". J. Tiss. Cult. Meth., 15,63-68.

Fingleton B. (1995) Detection of matriysin expression in human breast tumours by RT-PCR and ELISA. Poster presented at the prevention and early detection of human tumours conference, Torino Incontra congress center, Turin Italy.

Gaire M, Magbanua Z, McDonnell S, McNeil L, Lovett D and Matrisisan L. (1994) Structure and expression of the human gene for the matrix metalloproteinase matrilysin. J. Biol. Chem., 269, No. 3, 2032-2040.

Glogauer M., and McCulloch C.A.G. (1992) Introduction of large molecules into viable fibroblasts by electroporation: optimisation of loading and identification of labeled cellular compartments. Exp., Cell Res., 200, 227-234.

Goldberg G.I., Marmer B.L., Gregory G.A., Eisen A.Z., Wilhelm S and He C. (1989) Human 72-kilodalton type IV collagenase forms a complex with a tissue inhibitor of metalloproteases designated TIMP-2. Proc. Natl. Acad. Sci. USA, 86, 8207-8211.

Graham F.L. and van der Eb A.J. (1973) A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology, 52, 456.

Grant G.A., Eisen A.Z., MarmerB.L., Roswit W.T. and Goldberg G.I. (1987) The activation of human skin fibroblast procollagenase. Sequence identification of the major conversion products. J.Biol. Chem., 262, 5886-5889.

Gutman A. And Bohdan W. (1990) The collagenase gene promoter contains a TPA and oncogene-responsive unit encompassing the PEA3 and AP-1 binding sites. EMBO J. 9 2241-2246.

He C, Wilhelm S.M., Pentland A.P., Marmer B.L., Grant G.A.,Eisen A.Z. and Goldberg G.I. (1989) Tissue cooperation in a proteolytic cascade activating human interstitial collagenase. Proc. Natl. Acad. Sci. USA, 86, 2632-2636.

Hicks N.J., Ward R.V. and Reynolds J.J. (1984) A fibrosarcoma model derived from mouse embryo cells: growth properties and secretion of collagenase and metalloproteinase inhibitor (TIMP) by tumour cell lines. Int. J Cancer, 33, 835-844.

Holliday R., and Ho T. (1991) Gene silencing in mammalian cells by uptake of 5-methyl deoxycytidine-5'-triphosphate. Somatic Cell Mol. Genet., 17(6), 537-542.

Howard E.W., Bulen E.C. and Banda M.J. (1991) Regulation of the autoactivation of human 72-kDa progelatinase by tissue inhibitor of metalloproteinases-2. J.Biol. Chem., 266, 13064-13069.

Jiang C.,K., Connolly D., and Blumenberg M. (1991) Comparison of methods for transfection of human epidermal keratinocytes. J., Inves., Dermatol., 97(6), 969-973.

Kerr L.D., Miller D.B. and Matrisian M. (1990) TGF-β1 inhibition of transin/stromelysin gene expression is mediated through a Fos binding sequence. Cell, 61, 267-278.

Koudarides T. and Ziff E. (1988). The role of the leucine zipper in the Fos-Jun interaction. Nature 336, 646-651

Kressman A., Clarkson S.G., Pirotta V., and Birnstiel M.L. (1978) Proc. Natl. Acad. Sci. USA, 75, 1176.

Leuhrsen K.R., and Walbot V. (1993) Firefly luciferase as a reporter for plant gene expression studies. Promega notes No.44, 24-29.

Liotta L.A. (1992) Cancer Cell Invasion and Metastasis. Sci. Amer., Feb., 35-41.

Lopata M.A., Cleveland D.W., and Sollner-Webb B. (1984) High-level expression of a chloramphenicol acetyltransferase gene by DEAE-dextran-mediated DNA transfection coupled with a dimethyl sulfoxide or glycerol shock treatment. Nucl. Acids Res., 12, 5707.

Loyter A., Scangos G.A., and Ruddle F.H. (1982) Proc., Natl., Acad., Sci., USA, 79, 422-426.

Mathews K.E., Mills G.B., Horsfall W., Hack N., Skorecki K., and Keating A. (1993) Bead transfection: rapid and efficient gene transfer into marrow stromal and other adherent mammalian cells. Exp. Haem., 21, 697-702.

Matrisian L.M. (1990) Metalloproteinases and their inhibitors in matrix remodeling. Trends Genet., 6, 121-125.

Matrisian L.M., McDonnell S., Miller D.B., Navre M., Seftor E.A. and Hendrix M.J.C. (1991) The role of matrix metalloproteinase stromelysin in the progression of squamous cell carcinomas. Am. J. Med. Sci., 302, 157-162.

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

McDonnell S., Navre M., Coffey R.J. and Matrisian L.M. (1991) Expression and localisation of the matrix metalloproteinase Pump-1 (MMP-7) in human gastric and colon carcinomas. Mol. Carcinogenesis, 4, 527-533.

McNeil P.L., and Warder E. (1987) Glass beads load macromolecules into living cells. J. Cell Sci., 88, 669-678.

Melton D.A. and Cortese R. (1979) Cell, !8, 165.

Mortlock D., Keller E.B., Ziegra C.J. and Suter M.M. (1993) High efficiency transfection of monkey kidney COS-1 cells. J. Tiss. Cult. Meth., 15, 176-180.

Muller D., Breathnach R., Engelmann A., Millon R., Bronner G., Flesch H., Dumont P. and Eber M. (1991) Expression of collagenase-related metalloproteinase genes in human lung or head and neck tumors. Int. J. Cancer, 48, 550-556.

Murphy G., Cawston T.E. and Reynolds J.J. (1981) An inhibitor of collagenase from human amniotic fluid. Biochem. J, 195, 167-170.

Nagase H., Enghild J.J., Suzuki K and Salvesen G. (1990) Stepwise activation mechanisms of the precursor of matrix metalloproteinase (stromelysin) by proteinases and (4-aminophenyl) mercuric acetate. Biochemistry, 29, 5783-5789.

Neumann E., Schaefer-Ridder M., Wang Y., *et al.* (1982) Gene transfer into mouse lyoma cells by electroporation in high electric fields. EMBO J., 1(7), 841-845.

Newell K., McDonnell S., Witty J.P., Gaire M., Rodgers W.H. and Matrisian L.M. (1993) The stromelysin subclass of metalloproteinases in tumour progression and metastasis. In: Barrett A.J. and Bond J, (eds) Proceedings of the 9th International ICOP Conference, Proteolysis and Protein Turnover.

Pajouh M.S., Nagle R.B., Breathnach R, Finch J.S., Brawer M.K. and Bowden G.T. (1991) Expression of metalloproteinase genes in human prostate cancer. J. Cancer Res. Clin. Oncol., 117, 144-150.

Potter H. (1988) Electroportion in biology: methods, applications and instrumentation. Anal., Biochem., 174, 361-373.

Potter H., Weir L., and Leder P. (1984) Enhancer-dependent expression of human immunoglobulin genes introduced into mouse pre-B lymphocytes by electroporation. Proc., Natl., Acad., Sci., USA, 81, 7161-7165.

Powell W.C., Knox J.D., Navre M., Grogan T.M., Kittelson J., Nagle R.B. and Bowden G.T. (1993) Expression of metalloproteinase matrilysin in DU-145 cells increases their invasive potential in severe combined immunodeficient mice. Cancer Res., 53, 417-422.

Pyke C., Ralfkiaer E., Tryggvason K. and Danø K. (1993) Messinger RNA for two type IV collagenases is located in stromal cells in human colon cancer. Amer. J. Pathol., 142, 359-364.

Rauscher F.J., Cohen D.R., Curran T., Bos T.J., Vogt P.K. Bohman D., Tijian R. and Franza B.R. Jr (1988). Fos-associated protein p39 is the product of the *jun*-proto-oncogene. Science 240, 1010-1016.

Reese J.C., and Katzenellenbogen B.S. (1992) Examination of the DNA-binding ability of estrogen receptor in whole cells: implications for hormone-independent transactivation and the actions of antiestrogens. Mol. Cell. Biol., 10, 4531-8.

Reeves R., Gorman C., and Howard B. (1985) Minichromosome assembly of nonintegrated plasmid DNA transfected into mammalian cells. Nucl. Acids Res., 13, 3599.

Rothwell N.V. (1993) Understanding genetics-a molecular approach. Ch. 12.

Sato H., Takino T., Okado Y., Cao J., Shinagawa A., Yamamoto E. and Selkl M. (1994) A matrix metalloproteinase expressed on the surface of invasive tumour cells. Nature, 370, 61-65.

Springman E.B., Angleton E.L., Birkedal-Hanson H and Van Wart H. (1990) Multiple modes of activation of latent human fibroblast collagenase: evidence for the role of a Cys 73 active-site zinc complex in latency and a "cysteine switch" mechanism for activation. Proc. Natl. Acad. Sci. USA, 87, 364-368.

Stetler-Stevenson W, Brown P, Ohisto M, Levy A and Liotta L. (1990) Tissue inhibitor of metalloproteinases-2 (TIMP-2) mRNA expression in tumour cell lines and human tumour tissues. J. Biol. Chem., 265, 13933-13938.

Stetler-Stevenson W.G., Krutzsch H.C. and Liotta L.A. (1989b) Tissue inhibitor of metalloproteinase (TIMP-2): a new member of the metalloproteinase inhibitor family. J. Biol. Chem., 264, 17374-17378.

Stetler-Stevenson W.G., Krutzsch H.C., Wacher M.P., Margulies I.M.K. and Liotta L.A. (1989a) The activation of type IV collagenase proenzyme:sequence identification on the major conversion product following organomercurial activation. J. Biol. Chem., 264, 1353-1356.

Uria J., Ferrando A., Velasco G., Freije J., and Lopez-Otin C. (1994) Structure and expression in breast tumours of human TIMP-3, a new member of the metalloproteinase inhibitor family. Cancer Research 54, 2091-2094.

Walker C. *et al.* (1992) Proc. Natl. Acad. Sci. Cationic lipids direct a viral glycoprotein into the class I major histocompatability complex antigen presenting pathway. USA 89, 7915.

Welgis H.G. and Steicklin G.P. (1983) Human skin fibroblast inhibitor. EMBO J, 10, 1127-1134.

Wickens M.P., Woo S., O'Malley B.W., and Gurdon J.B. (1980) Nature 285, 628.

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

Wilson S.P., Liu F., Wilson R.E., and Housley P.R. (1995) Optimisation of calcium phosphate transfection for bovine chromaffin cells: relationship to calcium phosphate precipitate formation. Anal., Biochem., 226, 212-220.

Yamamoto H., Itoh F., Hinoda Y. and Imai K. (1995) Suppression of matrilysin inhibits colon cancer cell invasion *in vitro.* Int. J. Cancer., 61, 218-222.

Zervos P.H., Morris L.M. and Hellwig R.J. (1988). A novel method for rapid isolation of plasmid DNA. Biotechniques 6, 238-242.

Zhang Z., Winyard P., Chidwick K., Murphy G., Wardell M., Carrell R., and Blake D. (1994) Proteolysis of human native and oxidised α_1-proteinase inhibitor by matrilysin and stromelysin. Biochim. et Biophy., 1199, 224-228.

Zimmermann U., and Vienken J. (1982) Electric field-induced cell to cell fusion. J. Membr. Biol., 67, 165-182.