BIOLOGICAL AND MOLECULAR CHARACTERIZATION OF A MODEL OF CANCER METASTASIS

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ABSTRACT

Metastatic disease represents the greatest cause of morbidity and mortality in most cancer patients. The process of metastasis appears complex and multifaceted and has still to be comprehensively understood. To investigate the metastatic phenotype, a murine model system was employed: the CMT model. The basis of this model is a pair of related epithelial cell lines which were both derived from the same spontaneous tumour in a C57B/T animal, but which exhibit markedly differing metastatic potentials. The CMT 167/C6 subline exhibits a high metastatic potential as measured in the spontaneous metastasis assay in syngeneic, immunocompetent C57B/T animals and the subline CMT 170/E9 exhibits low metastatic potential. Examination and characterization of these two cell lines revealed remarkably few differences between the cell lines. Decreased protein expressions of the cell adhesion molecules E-cadherin and NCAM were detected in CMT 167/C6 cells in comparison with CMT 170/E9 cells. Neither cell line secreted collagenases as assayed by zymography, nor did either cell line exhibit gap-junctional communication. Expression of the putative metastasis-associated gene *mts*-1 was not detected by Northern analysis in either cell line and expression of alternatively-spliced isoforms of CD44, which have been implicated in another metastasis model system, was not found. Examination of gene amplification by in-gel renaturation, which allows for the detection of reiterated DNA sequences without specific analysis of a particular gene, revealed no differences between the two cell lines which may be correlated with their differing metastatic phenotypes. The evidence presented suggests that metastasis as displayed in this model may result from a number of subtle alterations within the cell which may not be an "all-or-nothing" effect. This is supported from preliminary evidence of 2-D gel electrophoresis which detected few differences between the cell lines with apparently no resolved protein identified to be specifically expressed in either cell line; the preliminary identification of differences were quantitative in nature.

Additionally, analysis of transcription factors in the CMT model revealed only subtle quantitative differences in EMSA experiments; qualitative differences were not observed. In this model, the most distinct differences found between the cell lines which may be significant to the differing metastatic phenotypes, were alterations in cell adhesion. The implications of these results and further experiments to examine this are discussed.

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Abbreviations

+'ve	positive
-'ve	negative
2-D	2-dimensional
72T4Cl	72kD type IV collagenase
92T4Cl	92kD type IV collagenase
AMF	autocrine motility factor
amp^r	ampicillin resistance gene
AMV	avian myeloblastosis virus
APC	adenomatous polyposis coli
APMA	4-aminophenylmercuric acetate
A_x	Absorbance (x=wavelength)
bp	base pair(s)
BSA	bovine serum albumin
CAM	chorioallantoic membrane
cDNA	complementary deoxyribonucleic acid
CEA	carcinoembryonic antigen
CO_2	carbon dioxide
cpm	counts per minute
Da	dalton(s)
DCC	deleted in colorectal cancer
DEPC	diethylpyrocarbonate
dH_2O	distilled water
DMBA	9,10-dimethyl-1,2-benzathracene
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	3'deoxyribonucleoside 5'triphosphate
dsDNA	double-stranded deoxyribonucleic acid
DTT	dithiothreitol
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylenediaminetetra-acetic acid, disodium salt
EMSA	electrophoretic mobility shift assay
FAK	focal adhesion kinase
FAP	familial adenomatous polyposis coli
g	gram(s)
GJIC	gap-junctional intercellular communication
HCl	hydrochloric acid
HGF	hepatocyte growth factor
HRP	horse radish peroxidase
i.p.	intraperitoneal
i.v.	intraveneous
kb	kilobase(s)
kD	kilo dalton(s)
LPS	lipopolysaccharide
μ	micro
M	molar
MCMF	mast cell-derived motility factor
min(s)	minute(s)
MMP	matrix metalloprotease
MOPS	4-morpholinepropanesulphonic acid
mRNA	messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide

MW	molecular weight
NCAM	neural cell adhesion molecule
NGF	nerve growth factor
oligo	oligonucleotide
PA	plasminogen activator
PAI	plasminogen activator inhibitor
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pI	isoelectrc point
PMSF	phenylmethylsulphonyl fluoride
PNK	polynucleotide kinase
RNA	ribonucleic acid
rpm	revolutions per minute
RT	reverse transcription
s.c.	subcutaneous
SCLC	small cell lung carcinoma
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SF	scatter factor
ssDNA	single-stranded deoxyribonucleic acid
TCA	trichloroacetic acid
TEMED	tetramethylenediamine
TIMP	tissue inhibitor of matrix metalloprotease
tPA	tissue-type plasminogen activator
uPA	urokinase-type plasminogen activator
UV	ultra violet
v/v	volume for volume
w/v	weight for volume

Amino acid one letter and three letter code used in this thesis is the same as that described by Sambrook *et al.*, 1989.

Throughout this thesis genes/alleles are indicated by italics, whilst proteins are non-italicised.

INTRODUCTION

CHAPTER I METASTASIS

I.1 *Overview*

There is increasing evidence that a malignant cancer predominantly arises through an accumulation of genetic events (Bishop, 1991; Fearon & Vogelstein, 1990). Notably, genes associated with carcinogenesis have been essentially categorized as being either oncogenes or tumour suppressor genes. Oncogenes, whether the cellular proto-oncogenes or the viral counterparts, can generally be defined as genes whose products are tightly associated with cell proliferation. Conversely, tumour suppressor genes encode for proteins that function to confer responsiveness to growth-inhibiting signals.

Inappropriate activation of oncogenes can occur through different genetic mechanisms such as point mutations, as commonly found in the *ras* genes (Bos *et al.*, 1987; Bos, 1989), or chromosomal translocations (Solomon, Borrow & Goddard, 1991) which, for example, occurs in Burkitt's lymphoma whereby a translocation occurs between the c-*myc* gene on chromosome 8 to one of the immunoglobulin loci on chromosomes 2, 14 or 22 (reviewed by Croce, 1991). Classical oncogene activation promotes cell proliferation by abrogating the requirement for exogenous mitogenic stimulation: for example, constitutive expression of an active Ras protein which is conformationally held in the GTP-bound state, displaces the requirement of upstream signalling from the interaction of specific growth factors with the appropriate receptor since the active Ras constantly transduces mitogenic signals downstream. In conjunction, inactivation of the normal function of tumour suppressor proteins is necessary for tumours to escape the regulatory controls inhibiting cell proliferation. This loss of function can occur through different mechanisms including: mutation of both alleles resulting in the expression of a non-functional protein or a protein with aberrant function; loss of one allele with, and possibly in some cases without, mutation of

the remaining allele. Mutation such as insertion and small deletions can inactivate the encoded protein, as has been described of the retinoblastoma (*RB*) gene (Horowitz *et al.*, 1987; Hogg *et al.*, 1993) and the tumour suppressor gene *TP53* (Baker *et al.*, 1989; Chen *et al.*, 1990), or through larger deletions, as discovered of the gene *DCC* (deleted in colorectal carcinoma) (Fearon *et al.*, 1990). In addition, gain-of-function mutations can occur which convert the tumour suppressor gene encoded protein, for example p53, into a dominant negative form which is capable of suppressing the normal activity of the wild type protein (Gannon *et al.*, 1990).

However, the greatest life-threatening aspect to the patient with cancer is the progression of a hyperproliferative lesion to a malignant tumour. It is this which remains the least understood process of carcinogenesis, even though invasive tumours and metastatic disease represent the major causes of morbidity and mortality in most cancer patients. Even though primary tumours of different origins can be successfully resected by surgeons, patient survival is still determined largely on malignancy of the cancer and presence of metastatic disease. It is therefore critically important and clinically relevant to understand how a tumour disseminates resulting in secondary tumours distant to the primary site, in order to determine therapeutic strategies aimed at relief, regression and prevention of metastatic disease for the patient.

While there is relatively little evidence of the underlying genetic mechanisms involved in metastasis, it is becoming more apparent that it will be the result of several events integrated together (Fidler & Randinsky, 1990). Alterations in the cell which may contribute to the metastatic phenotype, will be addressed later in this text (see chapter II).

I.2 *THE METASTATIC PROCESS*

The initial neoplasm is a number of cells, probably arising from a single cell, which has escaped the normal controls of cell growth, division and death, resulting in a growth which is outwith normal regulation and presenting as a cancer. However, unrestrained growth alone does not impart the malignant phenotype; further aberrations within the cell must occur in order for the hyperproliferative cell to progress further towards malignancy.

A brief and reductionist view of the metastatic process involves the metastatic cell invading into surrounding normal stroma, degrading the extracellular matrix (ECM) in order to facilitate migration of the tumour cell through normal tissue, the gain of access to the circulation, whether haematogenous or lymphatic, survival during circulation until another suitable organ environment is encountered where extravasation occurs, continued survival and proliferation of the tumour cell in the foreign microenvironment with stimulation of angiogenesis (see Figure 1). From this point, the metastatic cascade may repeat, forming metastases of metastases. Throughout this whole process, the malignant cell requires to survive or escape immunological attack.

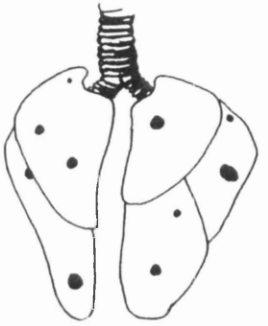
With such a particularly complex process as metastasis, which most tumorigenic cells shed from the primary tumour fail to complete (Fidler, 1970; Glaves, 1983; Weiss, 1986), research has tended to be focused on particular aspects of the metastatic process. This in turn, has led to the combining of research from different disciplines, such as molecular biology, protein chemistry, biochemistry, immunology and cell biology. Each scientific approach provides further insights into metastasis and is essential for them to be viewed together in order to gain a stronger and more coherent understanding of metastasis.

Figure 1

Figure 1. *Diagrammatic Representation Of The Metastatic Process*

For a tumour cell to successfully produce a metastatic deposit at a site distant to the primary tumour, it has been proposed that the cell requires to complete a series of sequential steps as illustrated. Alterations in a variety of cellular properties including:- decreased cell-cell adhesion, increased cell-matrix adhesion, increased matrix metalloprotease (MMP) expression and / or decreased tissue inhibitors of matrix metalloprotease (TIMP) expression, are proposed to enhance the metastatic potential of a tumorigenic cell.

The Metastatic Process



Primary Tumour → ***Invasion*** → ***Circulation*** → ***Extravasation*** → ***Secondary Tumours***

Invasion

- *Increased cell-matrix adhesion*
- *Increased MMP expression*
- *Decreased cell-cell adhesion*

Circulation

- *Survival*

Extravasation

- *Adherence*
- *MMP expression*

Secondary Tumours

- *Proliferation*
- *Angiogenesis*

I.2.1 *The Extracellular Matrix*

Invasive cells are characterized by their ability to penetrate surrounding normal tissue stroma through the extracellular matrix (ECM) and traverse the basement membrane. The ECM is recognised to be an important component in normal cellular processes such as the regulation of development and control of differentiation (reviewed by Adams & Watt, 1993; Mooney *et al.*, 1992). In addition to the complex array of glycoproteins, proteoglycans, collagens and glycosaminoglycans which constitute the major components of the ECM, many growth factors are also found to be integrated within the ECM structure (Ruoslahti & Yamaguchi, 1991; Benezra *et al.*, 1993; Masumoto & Yamamoto, 1993). In order to facilitate the active migration of invasive cells within this "exoskeleton" of the ECM, three sequential and integrated events have been hypothesized (Liotta, 1986). Initially, cell adhesion is required to the underlying substratum, perhaps through the integrins, to facilitate the necessary tractional forces generated through locomotion with the continuous formation of new adhesions at the invading face of the cell and loss of adhesion at the trailing edge of the cell; secretion of enzymes capable of degrading the ECM, for example the collagenases, in the immediate vicinity of the invading cell front, one possible consequence of which may be the release and activation of factors present in the ECM (Masumoto & Yamamoto, 1993); finally, the response of the cell to motility factors such as autocrine motility factor or scatter factor, which promote cell locomotion, their actions being mediated either in an autocrine or paracrine fashion. The processes here are not unique to invading tumour cells, but instead are associated with normal cellular processes such as development, inflammation, wound healing and angiogenesis, however in normal physiological situations these processes are under highly stringent controlling mechanisms. It is these controls that are circumvented or

aberrantly manipulated in malignancy leading to the devastating effects of metastatic disease.

I.2.2 *Cell Adhesion*

Cell adhesion, both cell-cell and cell-matrix interactions, are mediated by an amalgamation of cell adhesion molecules, many of which are specific for cell types and their interactive ligands. As a consequence of the large numbers of cell adhesion molecules, classifications have been derived, primarily based upon structural similarities.

I.2.2.1 *Cadherins*

This superfamily of transmembrane cell surface molecules mediate Ca^{2+}-dependent homophilic cell-cell interactions which are normally developmentally regulated controlling cell polarity and tissue morphology (Takeichi, 1991). Of the cadherins, E-cadherin (uvomorulin) has probably been the most extensively studied and is found commonly on epithelial cells. Through structural similarities of the different cadherins, each could be predicted to be functioning in a similar manner for each particular cell type on which they are expressed, for example, N-cadherin on neural cells (Ozawa & Kemler, 1992), P-cadherin of placental cells (Nose *et al.*, 1987) and M-cadherin of muscle cells (Donalies *et al.*, 1991), although they may not necessarily be functionally interchangeable between cell types (Levine *et al.*, 1994).

The cadherins are synthesized as precursor polypeptides which undergo post-translational modifications. Without the correct proteolytic cleavage of a 12 amino acid precursor segment from E-cadherin, the resulting expressed molecule is rendered non-functional in its adhesive properties (Ozawa & Kemler, 1990). Additionally, three cytosolic proteins termed catenins α, β and γ of molecular

weight sizes 102, 88 and 80kD respectively, are implicated to covalently associate with a specific 72 amino acid region in the cytoplasmic domain of cadherins, stabilizing their association to the cytoskeleton and mediating the cadherins' adhesive properties (Ozawa, Baribault & Kemler, 1989; Ozawa, Ringwald & Kemler, 1990; Gumbiner & McCrea, 1993). β-catenin has been demonstrated to associate with the E-cadherin precursor immediately after synthesis of this precursor molecule (Ozawa & Kemler, 1992). Following a lag period of ~10 minutes, α-catenin appears to associate with this complex (Ozawa & Kemler, 1992), which may correlate to the post-translational modifications of precursor E-cadherin, including endoproteolytic cleavage (Ozawa & Kemler, 1990). At present, it is not known whether α-catenin interacts directly or indirectly through β-catenin with E-cadherin. γ-catenin appears to weakly associate on the periphery of this complex. Deletion of the COOH-terminal portion of the intracellular domain of E-cadherin, or single amino acid substitutions in one of the Ca^{2+}-binding domains, abolishes the receptor's function in cell-cell adhesion even though the extracellular components remain intact and can be detected on the cell surface (Nagafuchi & Takeichi, 1988; Ozawa, Engel & Kemler, 1990). This emphasizes the importance of intracellular interactions with the cytoplasmic domain of E-cadherin in addition to specific receptor recognition on the cell surface for cell-cell adhesive function. However, although the catenins obviously play an important role in cadherin function (Hirano *et al.*, 1992), other as yet unkmown mechanisms are implicated which anchor cadherin-catenin complexes to cytoskeletal structures since it is known that detergent-insoluble cadherin complexes are found at stable cell-cell contacts (McNeill *et al.*, 1993) and yet cadherin-catenin complexes can be found in both detergent-soluble and -insoluble extractions (Ozawa *et al.*, 1989). Detergent insolubility is regarded as being indicative of proteins complexed to cytoskeletal components. The phosphorylation status of cadherins and catenins may influence adhesive function since v-Src

v-Src tyrosine phosphorylation of these proteins have been demonstrated to disrupt and inactivate E- and N-cadherin mediated adhesion (Hamaguchi *et al.*, 1993; Matsuyoshi *et al* 1992; Brehens *et al.*, 1993).

Evidence to support the involvement of cadherin mediated cell adhesion in invasion and therefore the overall process of metastasis has arisen from *in vitro* studies using cell lines to transfect with either E-cadherin, rendering the cells non-invasive in collagen matrix gel assays and in chick embryo heart fragment invasion assays (Vleminckx *et al.*, 1991); or transfection with anti-sense E-cadherin into E-cadherin expressing cells rendering them invasive as assayed by the same methods (Vleminckx *et al.*, 1991). Indeed, treatment of cells with neutralizing antibodies to E-cadherin also allows the invasive phenotype to arise (Vleminckx *et al.*, 1991). Studies of tumours from patients presenting with breast, gastric or head and neck squamous cell cancers indicate that loss or reduction of E-cadherin expression correlates with cellular dedifferentiation of the tumour (Oka *et al.*, 1993; Meyer *et al.*, 1993; Schipper *et al.*, 1991). Cellular dedifferentiation of tumours is often associated with increased malignancy.

I.2.2.2 *Integrins*

The integrin receptors are a family of transmembrane glycoproteins which are non-covalently associated $\alpha\beta$ heterodimers. Currently there are at least 14 known α subunits and 8 known β subunits (Hynes, 1992), of size ranges 120-180kD and 90-110kD respectively. Several of the α subunits can associate with different β subunits, furthermore, in mammals, several subunits have alternatively spliced cytoplasmic domains (Hynes, 1992). It appears that individual integrins are capable of binding more than one ligand and equally, particular ligands can be recognized by different integrins. Significantly, the majority of interactions mediated by integrins are cell-matrix.

The first integrin recognition site to be defined was the Arg-Gly-Asp (RGD) sequence (Pierschbacher & Ruoslahti, 1984a; Pierschbacher & Ruoslahti, 1984b) which is present in fibronectin, vitronectin and thrombospondin, for example. It has been demonstrated that experimental metastasis can be inhibited using synthetic peptides encompassing this RGD sequence (Humphries *et al.*, 1986). Poly(RGD) peptides have also been shown to reduce the number of lung metastatic colonies formed in a spontaneous assay (Dsaiki *et al.*, 1989). Both assay systems employed murine B16 melanoma cell lines. The decrease in number of lung colonies formed was observed when poly(RGD) peptides were co-injected with the B16 melanoma cells, as well as when the tumour cells were preincubated with RGD before injection. The inference from these results was that peptides which can mimic the natural ligands for integrins, can inhibit the formation of lung metastases by interfering with normal cell adhesion processes. By occupying the integrin receptor sites, the cells are prevented from further cell attachment to other components of the ECM or basement membranes and thus cannot invade.

Both α and β subunits of integrins are transmembrane glycoproteins. The active integrin receptor is of high affinity, whereas the inactive state has low affinity. The cytoplasmic domains of each are believed to interact with cytoskeletal proteins such as talin (Horwitz *et al.*, 1986) and α-actinin (Otey *et al.*, 1990) and potentially mediate specific signalling pathways within the cell. The affinity and specificity of the integrin receptor can be modulated by the cytoplasmic domains as demonstrated by O'Toole and colleagues (O'Toole *et al.*, 1991). Truncation of the cytoplasmic domain of α_{IIb} of the integrin $\alpha_{IIb}\beta_3$ resulted in a receptor which was constitutively active. Moreover, substitution of the deleted α_{IIb} cytoplasmic domain with α_5 cytoplasmic domain did not reverse the activated state of the receptor. These results and results from other similar experiments (O'Toole *et al.*, 1994) substantiate the belief that the specificity of the cytoplasmic domain can

influence the ligand affinity of the extracellular domain, thus maintaining control over the receptor status, either active or inactive. This type of signalling is recognised as the inside-out signalling of integrins.

Outside-in signalling by integrins is also thought to occur. Ligand occupancy of integrin receptors leading to clustering of these receptors at focal adhesions has been proposed to activate signal transduction into the cell, causing an increase in protein tyrosine phosphorylation of a number of proteins ranging between 115-130kD in size (Kornberg *et al.*, 1991). Precisely how this is effected is not yet understood since the integrins themselves, with short cytoplasmic domains, are not tyrosine kinases, however some preliminary evidence is now emerging. Similar sized proteins have also been identified to be tyrosine phosphorylated in NIH 3T3 cells after adhesion and spreading on fibronectin or via anti-integrin antibodies (Guan *et al.*, 1991). One of these proteins has now been identified and is itself a tyrosine kinase and is therefore implicated in being directly involved in the tyrosine phosphorylation of the other proteins upon signalling through the integrins. Due to its size and cellular localization, it has been termed pp125^{FAK} (focal adhesion kinase) (Schaller *et al*, 1992).

pp125^{FAK} was first identified as a tyrosine phosphorylated protein substrate of chick embryo fibroblasts transformed with oncogene-encoded tyrosine kinases *v-src*, *v-yes* and *v-crk* (Kanner *et al.*, 1990). It has been since characterized to be a tyrosine kinase localized at focal adhesions (Schaller *et al.*, 1992). The phosphorylation of pp125^{FAK} appears to correlate with its tyrosine kinase activity (Guan & Shalloway, 1992) and it has been shown that *v-src* transformed chick embryo cells exhibit enhanced tyrosine phosphorylation content of pp125^{FAK} over untransformed cells (Schaller *et al.*, 1992) and in addition, NIH 3T3 cells plated on fibronectin exhibit enhanced phosphotyrosine content over cells plated on poly-L-lysine (*i.e.*, no integrin-mediated adhesion) (Guan & Shalloway, 1992).

Indeed, Guan and Shalloway propose that signalling via the integrins to regulate attachment to the ECM with possible consequent involvement in the control of cellular proliferation is in part regulated by pp125^{FAK} and its substrates. Transformation by oncogenes such as *v-src* overrides the requirement of attachment to the ECM leading to anchorage-independent growth by constitutively activating pp125^{FAK} or by phosphorylating its substrates (Guan & Shalloway, 1992). As yet, these substrates have still to be conclusively identified. Platelets also exhibit pp125^{FAK} activation upon $\alpha_{IIb}\beta_3$ integrin stimulation, although only if platelet aggregation is allowed to occur (Lipfert *et al.*, 1992). If aggregation is inhibited or actin polymerization is prevented using cytochalasin D, then tyrosine phosphorylation of pp125^{FAK} is abrogated (Lipfert *et al.*, 1992). Also, platelets from patients with Glanzmann's thrombasthenia, which are deficient in the integrin $\alpha_{IIb}\beta_3$, do not display the tyrosine phosphorylation of pp125^{FAK} upon stimulation with fibrinogen, thus demonstrating the integrin dependence of activation in platelets (Lipfert *et al.*, 1992). However recent evidence indicates that prior to platelet aggregation and pp125^{FAK} tyrosine phosphorylation, fibrinogen binding to $\alpha_{IIb}\beta_3$ initiates the tyrosine phosphorylation of other proteins of sizes 50-68kD and 140kD (Huang *et al.*, 1993), therefore pp125^{FAK} activation is not an immediate event upon integrin binding and may depend on integrin oligomerization and actin reorganization and polymerization. Interestingly, the neuropeptides bombesin, vasopressin and endothelin also stimulate the rapid tyrosine phosphorylation of pp125^{FAK} in Swiss 3T3 cells at concentrations which closely parallel those required for mitogenic stimulation (Zachary, 1992). It appears that pp125^{FAK} activity is relevant to many cell types and may represent a point of convergence in many different signalling pathways.

Another intracellular event triggered via integrins is cytoplasmic alkalinization. Intracellular pH of bovine capillary endothelial cells can be elevated *in vitro*,

through the binding of different ligands (for example, fibronectin) with the endothelial cell surface expressed integrins or through specific anti-integrin antibodies resulting in the activation of the Na-H antiporter (Schwartz *et al.*, 1991). This cytoplasmic alkalinization can be inhibited with hexamethylene amiloride (HMA), a specific inhibitor of the Na-H antiporter (Schwartz *et al.*, 1991). Furthermore, fibronectin binding to $\alpha_5\beta_1$ on T cells induces the AP-1 transcription factor necessary for IL-2 transcription (Jamaica *et al.*, 1991); crosslinking of integrins on monocytes induces immediate-early gene expression which correlates with initiation of an inflammatory response (Yurochko *et al.*, 1992); and fibronectin stimulation of rabbit synovial fibroblasts induces the expression of two matrix metalloproteinase enzymes, collagenase and stromelysin (Werb *et al.*, 1989). Clearly, ligand binding to integrins can result in further downstream events within the cell, therefore outside-in signalling can also function via integrins.

I.2.2.3 *Selectins*

There are three known selectins; P, L and E. These adhesion molecules recognise specific carbohydrate structures on glycoproteins and have been implicated in a direct involvement in the process of leukocyte trafficking (reviewed in Lasky, 1992). The initial step required for the arrest and extravasation of circulating leukocytes at sites of inflammation is their transient interaction with the activated endothelium of blood vessels in the vicinity of the inflammatory response. This transient interaction, recognised as leukocyte rolling, accommodates the slowing of the leukocytes in circulation thus providing the opportunity for the β2 integrins expressed on the leukocytes to become activated and mediate their subsequent arrest and extravasation. It has only recently become evident that the transient interactions of the leukocytes with activated endothelial cells is determined significantly through endothelial expression of P-selectin (Mayadas *et al.*, 1993). Mice deficient in P-selectin, engineered through genetic manipulation, have

provided conclusive evidence that P-selectin has a critical role in leukocyte recruitment since, in these animals, leukocyte rolling is not evident as measured by intravital microscopy. Additionally, during an experimentally induced inflammatory reaction in mice, neutrophil recruitment to the inflamed peritoneal cavity has a lag phase of four hours before neutrophil numbers are attained to less than a two-fold difference of normal controls (Mayadas *et al.*, 1993). It may be postulated that during early responses to inflammation, recruitment is dependent on P-selectin, possibly in conjunction with L-selectin (Watson *et al.*, 1991) since these adhesion proteins are either expressed within minutes of stimulation or are constitutively expressed. Later leukocyte recruitment may be regulated through E-selectin which is maximally expressed on the cell surface 4-6 hours after stimulation with inflammatory cytokines (Beviacqua, 1987).

An important criterion for dissemination of the primary tumour is extravasation into secondary organs. It may be therefore anticipated that haematogenous spread of tumour cells could similarly undergo a process resembling that of leukocyte recruitment. This may be an area deserving of further investigation in relation to metastasis since the prevention of tumour cells gaining access to secondary sites could have profound implications in the treatment of cancer patients.

I.2.2.4 *Immunoglobulin Superfamily*

This superfamily consists of genes whose encoded proteins share structural similarities with the immunoglobulins. The polypeptide backbone of these proteins contain one or more domains which are formed from two anti-parallel β-pleated sheets and are often stabilized through interconnecting disulphide bridges. Many proteins identified as members of this superfamily are involved in the regulation of the immune system, for example, the major histocompatability complex antigens (MHC class I and class II), Thy1 and the T cell receptor. Expression of other

members of this family is not restricted to the immune system, for example, the neural cell adhesion molecules (NCAMs) are expressed in the nervous system (Walsh & Docherty, 1992), and carcinoembryonic antigen (CEA), MUC 18 and DCC (deleted in colorectal cancer) have all been proposed to be cell adhesion molecules, although their roles are yet to be clearly defined.

I.2.2.4.1 *NCAM*

Neural cell adhesion molecule (NCAM) possesses five immunoglobulin (Ig) domains and two type III fibronectin repeats. Through alternative splicing of the single gene encoding 26 exons (Colwell *et al.*, 1992), NCAM can exist in several isoforms (Reyes, Small & Akeson, 1991). The major classes are:- (i) transmembrane, of M.W.s 140kD and 180kD, the larger containing an extended cytoplasmic tail which may be constitutively anchored to the cytoskeleton and consequently be restricted in membrane mobility; (ii) glycophosphatidylinositol (GPI)-linked isoforms, M.W. 120-125kD and (iii) soluble isoforms of ~110kD.

In addition to alternative splicing, NCAM varies in polysialic acid content, an important carbohydrate moiety which can influence NCAM function (Docherty, Cohen & Walsh, 1990).

NCAM mediates Ca^{2+}-independent homophilic cell-cell interactions and can also promote neurite outgrowth. This latter property is restricted to particular NCAM isoforms with only the smaller transmembrane forms containing polysialic acid being capable of fully inducing neurite outgrowth (Docherty *et al.*, 1992a). In early development, these isoforms predominate as would be expected for the maturation of the central nervous system (CNS). In the adult, polysialic acid content in NCAM decreases and a greater proportion of NCAM isoforms contain alternatively spliced VASE (variable alternative splice exon) in the fourth Ig repeat, which is known to downregulate neurite growth-promoting activity, but

increase adhesiveness (Docherty et al., 1992b). This would provide a mechanism by which the adult CNS maintains stable cell-cell interactions but preventing continued neurite development.

To further support NCAMs association with development is the transcriptional regulation of NCAM which can be potentially regulated, at least in part, by the developmental and morphoregulatory associated mouse homeobox gene, *hox-2.5* (Jones *et al.*, 1992). *Hox-2.5* is the homologue to the human *hox-2E*, which are recently revised under the new nomenclature as *hox-B9* (Scott, 1992).

NCAM-stimulated neurite outgrowth appears not to be mediated by adhesion *per se*, since the effect of NCAMs can be mimicked by the activation of L- and N-type neuronal Ca^{2+} channels resulting in Ca^{2+} influx (Docherty *et al.*, 1991). This effect can be abrogated using Ca^{2+} antagonists and is reported to be pertussis toxin sensitive.

In malignancy, the majority of human small cell lung carcinomas (SCLC) and around one fifth of non-SCLCs express NCAM. These NCAM-positive patients have a poorer prognosis than those who are NCAM negative (Moolenaar *et al.*, 1992; Kibbelaar *et al*, 1991). However, the biological consequences of this expression in cancers are not understood, but nevertheless, is a useful diagnostic marker (Aletsee-Ufrecht *et al.*, 1990).

I.2.2.4.2 *CEA*

Carcinoembryonic antigen, identified as a member of the immunoglobulin superfamily (Paxton *et al.*, 1987), is a highly glycosylated cell surface protein of M.W. 180kD, implicated in Ca^{2+}-independent intercellular adhesion (Benchimol *et al.*, 1989). Expression is normally detected during embryogenesis and only low levels detected in normal adult tissues. However, overexpression of CEA is

frequently observed in tumours of epithelial origin, including breast, lung and colorectal cancers (reviewed by Shuster *et al.*, 1980) and correlation has been found between high serum levels of CEA and metastatic disease of SCLC (Chevinsky, 1991). As such, CEA is widely used as a clinical marker, although due to the high glycosylation status of CEA, antibodies used in these assays tend to have high cross-reactivity, therefore lowering the specificity and accuracy of these assays. Preliminary experiments present evidence to suggest that expression of functional CEA can prevent terminal differentiation and allow for continued cell proliferation (Eidelman *et al.*, 1993). This study incorporated the use of a rat L6 myoblast cell line which can be induced to differentiate under the appropriate conditions. Stable transfection of this cell line with CEA prevented terminal differentiation and abrogated myogenin up-regulation, creatine phosphokinase up-regulation and β-actin down-regulation which are all associated with the normal program of myoblast differentiation (Eidelman *et al.*, 1993). In addition, transfected cells retained their capacity to proliferate, therefore expression of CEA in tumour cells could be postulated to disrupt normal cell regulation allowing for inappropriate cell growth and obstruct normal differentiation processes.

I.2.2.4.3 *MUC18*

MUC18 antigen is a membrane glycoprotein of 113kDa size with sequence similarity to NCAM (Lehmann *et al.*, 1989). Direct correlation has been demonstrated with tumour progression of human melanoma; increased expression is associated with advanced primary tumours which have high metastatic probabilities and metastases (Lehmann *et al.*, 1989). Studies of human melanoma cell lines with known metastatic abilities in nude mice (using both spontaneous and experimental assays) also correlate MUC18 expression of both mRNA and the glycoprotein with metastatic potential, to statistical significance (Luca *et al.*, 1993).

I.2.2.4.4 *DCC*

Allelic deletions involving chromosome 18q have been shown to occur in more than 70% of colorectal cancers (Vogelstein *et al.*, 1988). Within this region, the candidate tumour suppressor gene DCC (deleted in colorectal carcinoma) was identified (Fearon *et al.*, 1990). Contrasting with the above described members of the immunoglobulin superfamily, DCC is frequently lost or reduced in its expression in the latter stages of colorectal carcinogenesis (Fearon *et al.*, 1990). These alterations in expression of DCC have also been described in breast cancer (Thompson *et al.*, 1993), prostatic carcinomas (Gao *et al.*, 1993), and in a small study of leukaemias (Porfiri *et al.*, 1993).

Insight into DCC function is presently tentative, however through its sequence similarity to NCAM, it was first designated as a putative cell adhesion molecule. Transfection of anti-sense DCC constructs into Rat-1 cells, which express low levels of DCC, resulted in a stable cell line which exhibited increased growth rates, anchorage-independence and tumorigenicity in nude mice (Narayanan *et al.*, 1992). Exposure of the parental Rat-1 fibroblasts to DCC anti-sense oligonucleotides caused their detachment from the substratum, an effect which can be overcome through plating the cells onto laminin, fibronectin, collagen type IV and poly-D-lysine (Narayanan *et al.*, 1992).

In a further study, rat pheochromocytoma (PC12) cells, when stimulated to differentiate with nerve growth factor (NGF), exhibit increased DCC expression which is maximal at 72-96 hours. In DCC anti-sense transfected PC12 cells, morphological differentiation is prevented when this construct is induced and the cells continue to proliferate in the presence of NGF (Lawlor & Narayanan, 1992). Also reported by these authors is the inhibition of interleukin-6 (IL-6) and basic growth factor (bFGF) induced neuronal differentiation in these anti-sense

transfected cells, thus it would seem that the expression of DCC is of fundamental importance in the establishment of neurite extensions. Indeed, differentiated PC12 cells will retract neurite outgrowths upon exposure to anti-sense oligonucleotides (Lawlor & Narayanan, 1992). In a further study from Pierceall *et al.* (1994), neurite outgrowth could be stimulated in PC12 cells when co-cultured with NIH 3T3 cells transfected with DCC expressing constructs. This neurite stimulation appeared to be dependent on cell-to-cell contact since conditioned media from the transfected NIH 3T3 cells did not induce neurite outgrowth. In addition, transfection with a truncated DCC construct which lacked the majority of the cytoplasmic domain also failed to stimulate neurite outgrowth (Pierceall *et al.*, 1994), which suggests that the cytoplasmic domain is of critical importance, perhaps through the stabilization of cell-to-cell contacts. As already noted, cell interactions with the extracellular matrix have critical roles in maintenance of cell behaviour and this appears to be the situation with DCC as a cell adhesion molecule involved in the maintenance of morphological differentiation of PC12 cells.

I.2.2.5 *Other Cell Adhesion-Related Molecules: CD44*

CD44 comprises a large family of cell surface glycoproteins implicated in cell adhesion and known to function in a variety of processes including lymphocyte homing and trafficking, T cell activation and metastatic spread (Arch *et al.*, 1992; Haegel *et al.*, 1993; Gunthert *et al.*, 1991).

Multiplicity of CD44 variants arise predominantly through alternative splicing in addition to differential glycosylation and alternative poly A^+ sites (Screaton *et al.*, 1992; Camp, Kraus & Pure 1991; Gunthert *et al.*, 1991). Alternative splicing appears to occur in at least two main regions of the CD44 molecule; either proximal to the membrane in the extracellular domains or within the cytoplasmic

tail (Screaton *et al.*, 1992). In studies to date, it would appear that there are at least ten alternatively spliced exons of the single CD44 gene in the extracellular domain and these exons are conserved between mouse, rat and human (Screaton *et al.*, 1993). The apparent ability for alternative splicing to occur in numerous permutations of these exons gives rise to enormous possibilities of different isoforms to exist and for these to contribute to numerous functions. The biological consequences of variant CD44 expressions remain to be elucidated, however tissue-specific expression patterns are becoming apparent (Kennel *et al.*, 1993; Wirth *et al.*, 1993; MacKay *et al.*, 1994) and possible alterations in associated function are presently the focus of much attention. It is already known that the standard form of CD44 (devoid of variant exons) which has widespread expression, and particularly associated with lymphocytes, possesses the ability to bind hyaluronic acid (Aruffo *et al.*, 1990; Stamenokovic *et al.*, 1991). When exons v8-v10 are alternatively spliced into CD44, this molecule, which is predominantly expressed on normal epithelial cells, cannot bind hyaluronic acid (Stamenokovic *et al.*, 1991).

Recent interest in CD44 has intensified after the identification of a CD44 isoform containing variant exons v4-v7 within the extracellular domain which was discovered to be expressed on a metastatic rat pancreatic carcinoma cell line and not the corresponding tumorigenic, but non-metastatic cell line (Gunthert *et al.*, 1991). Furthermore, overexpression of this variant CD44 in the non-metastatic cell line conferred the metastatic phenotype, as measured by a spontaneous assay (Gunthert *et al.*, 1991; Rudy *et al.*, 1993). The question of how this variant molecule can confer metastatic properties is still unanswered, and may only give this significant effect when in conjunction with other properties already present in these particular cell lines. However, the finding that alterations of variant CD44 expression may be associated with the progression of certain tumours, and the

evidence of a "metastatic-associated / enhancing" CD44 variants could have profound implications in human cancers for clinical diagnosis and treatment (Matsumura & Tarin, 1992; Wielenga *et al.*, 1993; Matsumura *et al.*, 1994).

I.2.2.6 *Connexins*

These are a class of molecules implicated in cell-cell communication through the formation of intercellular gap junctions, with integrated involvement in cell adhesion. Gap junctions facilitate the transfer of small molecules and ions thus mediating signalling between adjacent cells. Such physical transfer of molecules directly between cells can be elegantly demonstrated *in vitro* by microinjection of fluorescent dyes into a single cell. The ability to communicate through these gap junctions is reflected in the number of cells which fluoresce as a result of dye transfer through adjacent cells.

The identity of the subunit components of connexons remains the target of controversy, however evidence lends to the argument that gap junctions consist of ductins rather than the more popularly believed connexins (Finbow & Pitts, 1993). Either way, gap junctions are of critical importance in intercellular communication and contribute to cellular adhesion.

It has been considered that highly metastatic cells could avoid inhibitory signals from adjacent cells, thus aiding their malignant phenotype, by reducing or completely abolishing their ability for gap-junctional communication. Preliminary data has indicated that reduction and / or loss of junctional communication may, in some instances, be correlated with the metastatic phenotype (Hamada *et al.*, 1991)

I.2.3 *Extracellular Matrix Degradative Enzymes*

The presence of proteinases capable of degrading components of the extracellular matrix is a characteristic feature of invasive carcinomas. However, this is not a

unique phenomenon of malignant cells: proteolysis of tissue barriers is also seen in the normal situations of trophoblast implantation, embryo morphogenesis, angiogenesis and bacterial and parasitic invasion, for example.

Various matrix degrading enzymes have been implicated in tumour invasion and metastasis. These include the matrix metalloproteinases (MMPs), members of which include the collagenases and stromelysin enzymes; serine proteases which include the plasmin/plasminogen activator system; cysteine and aspartic proteases termed cathepsins; and the glycosidases which include heparanase and hyaluronidase (Nakajima & Chop, 1991). Of these, the most thoroughly studied are the matrix metalloproteinases and the plasminogen activator/plasmin system, which are given further discussion below.

I.2.3.1 *Matrix Metalloproteinases*

Particular attention has focused on the matrix metalloproteinase (MMP) family. Currently, there are eight known members:- interstitial collagenase (MMP-1), neutrophil collagenase, stromelysins-1,-2 and -3 (MMP-3, MMP-10 respectively, with no MMP nomenclature for st-3 since it has only been characterized at the mRNA level with no native protein isolated to date), matrilysin, and 72kDa and 92kDa type IV collagenases (72T4Cl and 92T4Cl respectively, also termed MMP-2 or gelatinase A and MMP-9 or gelatinase B). Each member of this family have in common a signal peptide segment, propeptide domain and a catalytic domain containing the zinc-binding region. In all except the smallest member, matrilysin, each also possesses a carboxyl-terminal domain which shares sequence homology with haemopexin and vitronectin. This carboxyl-terminal domain appears to determine the binding specificity of tissue inhibitor of metalloproteinases (TIMPs), with each TIMP carboxyl-terminal binding specifically to only one of the gelatinases at its carboxyl-terminal. The

amino-terminal of the TIMPs appear to be capable of interacting with the open and active sites of all the metalloproteinases and cause inhibition of enzymatic activity.

Regulation of these enzymes occurs both at the level of gene expression, with induction through growth factors and cytokines, and post-translational control exerted at three levels: (1) each enzyme is synthesized and secreted as a zymogen which requires to undergo proteolytic cleavage of an 80-84 residue amino-terminal domain to generate a conformationally altered and active enzyme (*in vitro* activation can be attained with organomercurial treatment of the latent enzymes); (2) once activated, the metalloproteinases can be subjected to inactivation by specific inhibitors, e.g., TIMP-1 or TIMP-2, and possibly the recently identified TIMP-3 (Leco *et al.*, 1994), at a stoichiometry of 1:1; (3) autodegradation. However, to further complicate this, both latent gelatinases can be secreted already in complex with their respective TIMP (Wilhelm *et al.*, 1989; Stetler-Stevenson Krutzsch & Liotta, 1989). This complex is thought to function by stabilizing the enzyme through inhibitory binding of the amino-terminal domains and prevent its autodegradation Kleiner *et al.*, 1993). It could be postulated that stabilization is indeed the primary function of TIMPs and activation of the enzyme results from the (transient?) interaction of a third factor which partially displaces TIMP thus providing opportunity for enzyme activation and also maintaining localized enzymatic activity. Interestingly, 72T4Cl has been discovered to be activated in a plasmin-independent manner (unlike the other interstitial collagenases and prostromelysins) (Brown *et al.*, 1993; Strongin *et al.*, 1993). Initiation of enzyme activation can be mediated by a cell membrane-dependent mechanism as demonstrated with the co-incubation of purified 72T4Cl in complex with TIMP-2 together with residual cell fractions of phorbol ester treated HT-1080 cells (Brown *et al.*, 1993). Conversion of the proenzyme 72T4Cl to the active enzyme was monitored with a gelatin degradation assay. Similar co-incubation experiments

with HT-1080 cell lysates or conditioned medium instead of membrane fractions did not display gelatinase activities. It may be postulated that interaction of 72T4Cl / TIMP-2 with the plasma membrane of stimulated cells is mediated through a specific receptor on the cell surface which binds the complex causing a conformational alteration which induces autoproteolytic activity thus leading to cleavage of the propeptide and subsequent enzymatic activation. This sequence of events would allow for the discrete degradation of ECM components in the immediate vicinity of cells which have been suitably stimulated to initiate these events. However, there is conflicting evidence suggesting that plasma membrane activation of 72T4Cl is inhibited by TIMP-2 complex formation (Strongin *et al.*, 1993). It is difficult to reconcile these opposing findings and it needs further investigation in order to clarify the situation. To speculate, it may be possible that during the purification procedures of 72T4Cl / TIMP-2 complexes by Brown and colleagues, a proportion of the complex was dissociated producing free enzyme and the observed plasma-dependent activation was of the non-complexed enzyme.

Since controlled degradation of the ECM is important in normal cellular processes, including angiogenesis and trophoblast implantation, it is not surprising that the inhibitors of the metalloproteinases have been associated with controlled inhibition of these processes (Schnaper *et al.*, 1993; Murphy, Unsworth & Stetler-Stevenson, 1993). Further still, transfection of *ras*-transformed rat embryo cells (*ras*-4R) with an expression vector encoding TIMP-2 caused a suppression in these cells ability to form experimental metastatic lung deposits (DeClerck *et al.*, 1992). Upon sub-cutaneous injection of the transfected cells, the invasive characteristics of the tumour were completely suppressed whereas primary tumours formed from non-transfected *ras*-4R cells were highly invasive into surrounding muscle tissue. Similarly, intraperitoneal injection of recombinant TIMP-1 two hours prior, upon

and following intravenous injection of *ras*-4R cells inhibited experimental metastatic deposit formation by 83% (Alvarez, Carmichael & DeClerck, 1990).

In situ hybridization studies for 74T4Cl and 92T4Cl mRNAs in human skin carcinomas detected signals at tumour / stromal interfaces (Pyke *et al.*, 1992). The mRNAs were particularly abundant in normal stroma adjacent to infiltrating squamous and basal cell carcinomas, whereas normal skin from control patients did not exhibit expression. This clearly demonstrates that modulation of MMP expression may not only occur in the tumour cells themselves, but may involve surrounding normal tissue which may be recruited by the tumour cells to express products enabling ECM degradation thereby enhancing migratory potential of invasive cells. The mechanism by which this "recruitment" of normal cells occurs remains to be elucidated, but it is conceivable that the tumour cells may secrete a diffusible factor which stimulates the surrounding cells, inducing MMP expression. This induction appears to also occur in breast cancers with stromelysin-3 gene expression reported as being restricted to normal surrounding stromal tissues as detected by the methods of *in situ*-hybridization and immunohistochemical analyses (Basset *et al.*, 1990; Wolf *et al.*, 1993). However, analysis of metastatic deposits and comparison with the primary tumour from the same patient did not consistently correlate with their stromelysin-3 mRNA abundance (Wolf *et al.*, 1993). But, concerning the primary tumour and its classification type, correlation was found between invasive and metastatic potential with positive expression of stromelysin-3: those tumours which rarely progress or progress to malignant carcinoma only over prolonged periods of time, such as the *in situ* lobular carcinomas, scoring as negative (Wolf *et al.*, 1993). In this latter situation, it may be that during these later stages of progression, stromelysin-3 expression will also be found, although this is still to be examined.

I.2.3.2 *Serine Proteases*

Most widely studied of the serine proteases is the plasminogen activator / plasmin system. Both urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) are capable of activating the zymogen plasminogen through proteolytic cleavage at arginine 560 giving rise to active plasmin which characteristically has broad substrate specificity (Liotta *et al.*, 1981). In particular, tPA exhibits enhanced activity in the presence of fibrin; it may therefore serve a critical function as a fibrinolytic agent. In contrast, uPA is not fibrin modulated, and this would suggest that its major roles may be in activities such as tissue remodelling and cell migration. Notably, when uPA / pro-uPA and plasminogen are cell surface bound by specific receptors concentrated at focal adhesions, uPA plasminogen activation is enhanced (Ossowski *et al.*, 1991).

Post-transcriptional regulation of plasminogen activators includes the specific inactivation by PA inhibitors (PAIs) which mediate rapid PA internalization and degradation (Cubellis, Wun & Blasi, 1990). Enhanced uPA transcription stimulated by phorbol ester treatment is mediated in part through the cooperation of a PEA3/AP-1 element and an AP-1 site (Nerlov *et al.*, 1992). Similar PEA3/AP-1 elements are present in the promoters of stromelysin and collagenase genes (Gutman & Wasylyk, 1990; Sirum-Conolly & Brinckerhoff, 1991). In addition to these elements, NFκB has also been demonstrated to be involved in uPA transcription (Hansen *et al.*, 1992). Interestingly, κB elements have also been identified in the promoter regions of some cell adhesion molecule encoding genes (Shu *et al.*, 1993, Smith *et al.*, 1993; Whelan *et al.*, 1991). Inhibition experiments of NFκB mediated transcription either through the use of antisense NFκB constructs or phosphorothioate oligonucleotides composed of either single-stranded oligonucleotides specific to different NFκB subunits at their 5' mRNA termini and inclusive of ATG initiation sites, or as double-stranded

oligonucleotides capable of competitively binding endogenous NFκB, have clearly demonstrated inhibition of cellular adhesion (Eck *et al.*, 1993; Higgins *et al.*, 1993). The reduction of adhesion seen in phorbol ester-stimulated HL60 cells treated with competitive NFκB double stranded oligonucleotides may be due to reduced CD11-mediated cell adhesion in these cells (Eck *et al.*, 1993). Reduced cell-matrix adhesion, soft agar colonization ability and tumorigenicity of a fibrosarcoma cell line (K-BALB, a *ras*-transfected NIH 3T3 cell line) and of a melanoma cell line (B16) in nude mice was observed by Higgins *et al.* (1993) and indeed these authors demonstrate with inducible antisense constructs to a subunit of NFκB, p65, the regression of established primary tumours in nude mice and extended survival times. Although not addressed in these studies, inhibition of NFκB would inevitably also affect the transcription of other genes including uPA. If synthesis of uPA was reduced, it could be proposed that the effects on cellular behaviour may include reduced tumorigenicity *in vivo* and reduced colonization ability in soft agar *in vitro* assays due to a decrease in enzymatic ECM degradation which aids the expansion of tumour cell growth. Thus the reported alterations using antisense approaches could be a complementation of effects on expression of cell adhesion molecules and enzymes involved in ECM degradation.

I.2.4 *Tumour Cell Motility*

Active motility coupled with proteolysis is required for the tumour cell to invade normal stroma. These processes are also necessary in later events of metastasis during the extravasation of the malignant cell into a secondary site. Again, cell locomotion is not a unique characteristic of metastatic cells, normal cellular processes also involve cellular movement, such as in wound healing, morphogenesis, embryo implantation and the migration of immunological cells such as neutrophils and macrophages. However, despite many studies on such processes and control of these mechanisms, very little is known.

I.2.4.1 *Autocrine Motility Factor*

In the past few years, a group of cytokines have been described which can stimulate motility in various cells. One such cytokine is autocrine motility factor (AMF), isolated by Liotta and co-workers from a melanoma cell line derived from a brain metastasis (Liotta *et al.*, 1986). This protein is not unique to melanoma cells and has also been purified from human HT-1080 fibrosarcoma cells along with its receptor, gp78 (Watanabe *et al.*, 1991). AMF migrates as a single band of 55kD in SDS-PAGE, under non-reducing conditions. In reducing conditions, it migrates as a single polypeptide of 64kD, suggesting that AMF has intrapeptide disulphide bonds (Liotta *et al.*, 1986; Watanabe *et al.*, 1991).

Zetter and Yano (1992) have reported the purification of a molecule termed mast cell motility factor (MCMF) which exhibits high amino acid similarity with AMF and presents identical molecular weights in SDS-PAGE as AMF. It seems highly probable that MCMF is the same as AMF, or indeed is closely related. Since mast cells normally produce many factors upon degranulation, some of which are considered to be important for the induction of blood vessel formation (see section I.2.6 on angiogenesis), it may be that tumour cells can recruit such normal cells during the process of carcinogenesis and metastasis.

Precisely how motility is induced by AMF is not yet understood. However, studies have revealed that AMF stimulation of the human breast carcinoma cell line, MDA 435, with AMF purified from MDA 435 conditioned media, resulted in pseudopodial protrusion of the tumour cells, leading to cell motility (Guirguis *et al.*, 1987). These AMF-induced pseudopodia contain a twenty-fold increase in laminin and fibronectin RGD recognition receptors over the levels found in the plasma membrane of unstimulated cells. The concentrated presence of these

receptors could facilitate cell-matrix adhesion necessary for traction in cell motility, allowing for directional movement leading to invasion by tumour cells.

I.2.4.2 *Scatter Factor / Hepatocyte Growth Factor*

Scatter factor (SF) is a secretory product of fibroblasts which induces epithelial cells to dissociate, increasing their motility and invasiveness (Stoker et al., 1987; Weidner et al., 1990) and as such has been proposed to be an important factor in invasion and metastasis. In addition, another factor reported to be a powerful mitogen for hepatocytes in primary cultures, known as hepatocyte growth factor (HGF), has recently been found to be indistinguishable from SF (Weidner *et al.*, 1991). This factor is synthesized as a precursor of 92kD size which undergoes proteolytic cleavage (Naka *et al.*, 1992) to yield a disulphide linked $\alpha\beta$ heterodimer. This proteolytic cleavage has been demonstrated to be inhibited by serine proteases (Naka *et al.*, 1992) and can be catalysed by both urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) (Naldini *et al.*, 1992; Mars, Zarnegar & Michalopoulos, 1993). The α chain contains four kringle domains and functional studies indicate that the first two domains are required for the scatter factor activity, whereas it appears that the complete mature form is necessary for mitogenic activity (Hartmann *et al.*, 1992; Lokker *et al.*, 1992). Both forms appear to compete with identical affinity for the $p190^{Met}$ receptor and consequently, both equally trigger the tyrosine phosphorylation of the Met receptor β subunit (Naldini *et al.*, 1991).

The different biological properties ascribed to SF/HGF, although both are almost certainly the same molecule (both factors have been identified as products of a single gene located on chromosome bands 7q11.2-21 (Weidner *et al.*, 1991)), may be the consequence of the particular cell type stimulated and the presence of other

cell type-specific cooperating proteins acting to modify the ligand-receptor interaction leading to divergent signalling pathways. However, the mechanism for this, at present, remains open to speculation.

It is worth noting at this point that the *met* gene has been reported to be amplified and overexpressed in some cell lines derived from human tumours (Giordano *et al.*, 1989) and in some spontaneously transformed NIH 3T3 cells after *in vitro* culture (Cooper *et al.*, 1986). Cooper and colleagues report the amplification of the *met* gene between 4- to 8-fold, with at least a 20-fold overexpression of the *met* mRNA compared to untransformed NIH 3T3 cells. In another study of human thyroid carcinomas, overexpression of the Met receptor correlated with advanced stage tumours which had locally invaded and/or had distant metastases (Di Renzo *et al.*, 1992).

Another molecule has been isolated which appears to be similar in sequence to SF/HGF (Rubin *et al.*, 1991) and is lung fibroblast derived. However, there are some distinguishing features for both: SF/HGF is synthesized as a precursor molecule which requires to undergo proteolytic cleavage but the lung fibroblast-derived factor appears to be synthesized and secreted as a single polypeptide chain of 87kD size (Rubin *et al.*, 1991). Additionally, p87 appears to have some amino acid substitutions in comparison to SF/HGF, one of which interrupts a potential integrin-binding RGD sequence and another that adds a cysteine residue which could potentially alter disulphide bridge formations (Rubin *et al.*, 1991).

I.2.4.3 *Migration Stimulating Factor*

Migration stimulating factor (MSF) is another cytokine identified for its ability to stimulate cell migration (Grey *et al.*, 1989). It has been purified from foetal and breast cancer patient fibroblasts and appears to operate in an autocrine fashion as

observed for AMF (Liotta *et al.*, 1986). It does not exhibit sequence similarity to SF/HGF or AMF (Grey *et al.*, 1989).

I.2.5 *Organ-Specific Metastasis*

Many tumours characteristically metastasize in an apparently non-random manner to particular organs. The specific colonization of secondary sites cannot be consistently explained by the simple mechanical entrapment of circulating tumour cells in the first microcirculatory network encountered. For example, cutaneous malignant melanoma can spread to many sites including the brain, ovaries, spleen and thyroid; bone metastases can specifically result from breast adenocarcinomas and kidney clear cell carcinomas (reviewed by Rusciano & Burger, 1992).

Although organ-specificity is a recognised phenomenon of tumour metastasis, and has been for many years whereupon the first hypothesis of the "seed and soil" nature of metastasis was proposed (Paget, 1889), the questions of how and why this occurs remain unanswered. Indications from experiments examining the influence of conditioned medium from cultured organs (Nicolson & Dulski, 1986) and the ability of various organ-derived biomatrices (Doerr *et al.*, 1989) to support tumour cell growth suggest that particular properties exist for organs which are preferential sites of metastasis. But evidence of what these properties are, remains to date inconclusive. Additionally it has been proposed by some investigators that progression of tumours to the metastatic state involve the transition of cells from a paracrine responsive state to an ultimately "acrine" state (Nicolson, 1993). This would suggest that tumour cells could therefore be unresponsive to inhibitory signals in different organ environments, however, it does not provide the mechanism underlying organ specificity.

I.2.6 *Angiogenesis*

For a tumour cell population to proliferate *in vivo* beyond a volume restricted by oxygen and nutrient diffusion, there must be new blood vessel formation (Bouck, 1990). This applies both to the primary tumour and disseminated tumours. As noted before for other normal processes involved in metastasis, angiogenesis is a normal cellular process involved in reproduction, development and wound repair.

Angiogenesis is a cascade of events by which pre-existing, resting endothelial cells respond to angiogenic factors causing degradation of the endothelial basement membrane, migration of the cells into the stroma in the direction of the angiogenic stimulus and formation of a capillary sprout. This new capillary sprout expands and forms into a tubular structure. The continued endothelial proliferation allows the vascular tubules to extend and form loops which can then function as a capillary network (Liotta, Steeg & Stetler-Stevenson, 1991). Again, degradation of the extracellular matrix and cell migration are not dissimilar to the processes involved in invasion.

Some cells, notably malignant cells, are capable of producing several angiogenic factors (Folkman & Klagsbrun, 1987). These angiogenic factors in addition to stimulating angiogenesis, also promote the recruitment of other cell types (e.g. macrophages and mast cells) which can in turn produce their own angiogenic factors (Meininger & Zetter, 1992). Many different factors have now been ascribed to possess angiogenic properties, including macrophage-derived interleukin-8, IL-8 (Koch *et al.*, 1992) and vascular endothelial growth factor, VEGF (Plate *et al.*, 1992; Shweiki *et al.*, 1992). Interestingly, one factor recently identified is scatter factor, SF/HGF (Grant *et al.*, 1993), previously shown to have the ability to dissociate epithelial cells and to be mitogenic for hepatocytes (see section I.2.4.2). The normal function of SF/HGF may therefore be to promote angiogenesis under

normal physiological circumstances such as wound healing (Miyazawa *et al.*, 1994) through the simultaneous recruitment of cells into the wound area (induction of scattering) and the stimulation of cell proliferation (as required for blood vessel expansion and the formation of scar tissue). Furthermore, other factors required in angiogenesis include the matrix degradative proteases which have been demonstrated to be produced by stimulated endothelial cells allowing for the expansion of the capillary tubule into the ECM (Kalebic, 1983).

Correlation between angiogenesis and tumour invasion can be readily visualized, since both processes require cell motility, degradation of the ECM and cell proliferation. Therefore, inhibitors of angiogenesis may also be potent inhibitors of tumour invasion and metastasis.

I.2.7 *Metastases of Metastases*

Once metastases have established at distant sites to the primary tumour, it could be postulated that these tumours can in turn metastasize, thus taking the metastatic process around in full circle. However, at this stage of the metastatic disease, long term patient survival must be improbable.

I.2.8 *Conclusion*

It seems unequivocal that the cascade of events which ultimately leads to the successful dissemination of a primary tumour to form metastatic deposits must involve a plethora of signalling events and responses, and indeed the inhibition of normal regulatory signals and responses. Many host-tumour cell interactions are required to occur, some of which have been outlined here, but the progression of the tumour depends on the outcome of an imbalance of normal control mechanisms, giving rise to inappropriate responses and the misdirecting of normal cellular functions. It is this imbalance of homeostasis which may fundamentally lie

in genetic alterations. Some of these possible genetic alterations will be addressed in the following chapter.

CHAPTER II GENETIC ALTERATIONS ASSOCIATED WITH TUMOUR PROGRESSION

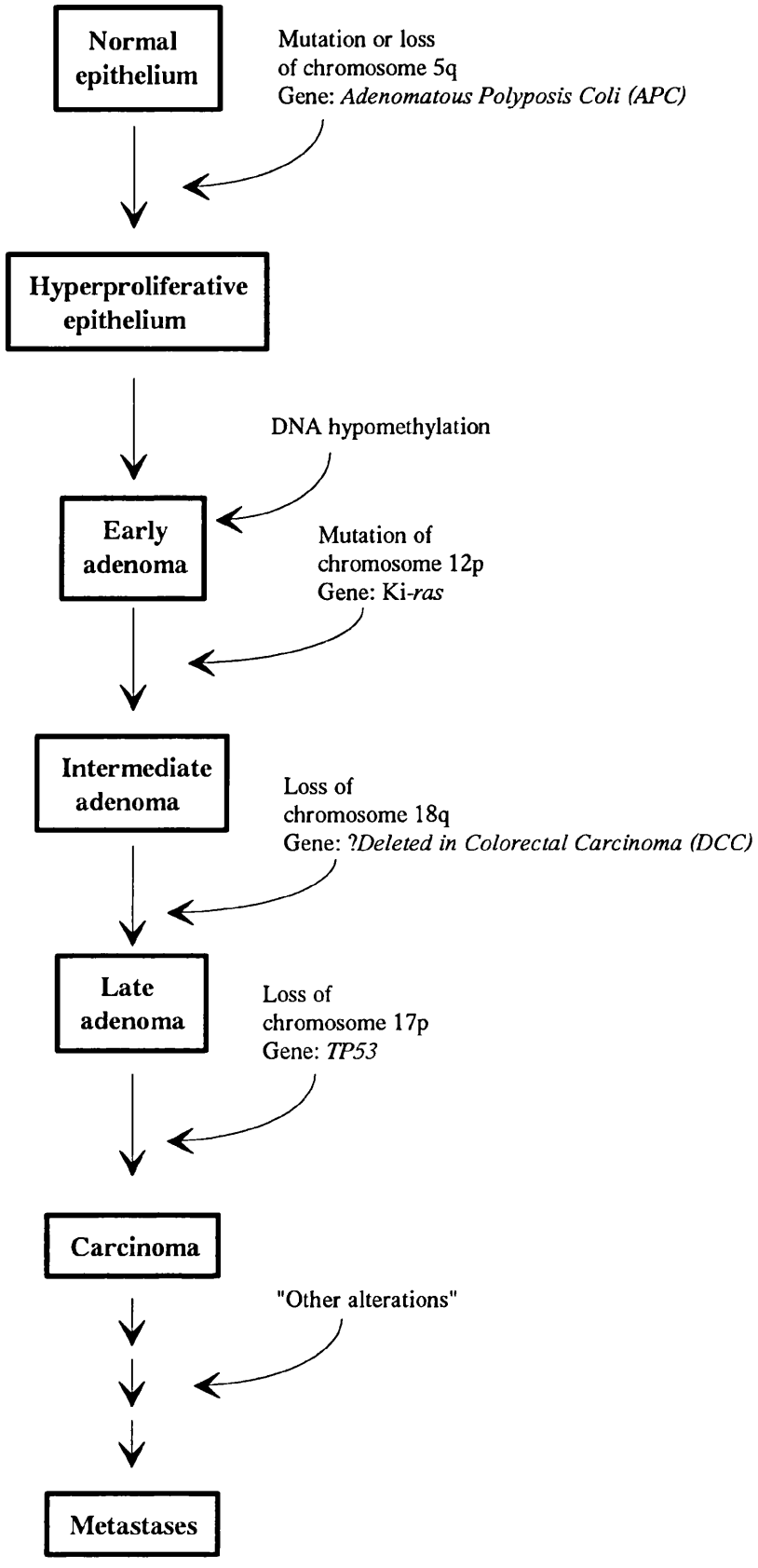
II.1 *Multistep Model Of Colon Carcinogenesis*

Progression of a primary tumour to malignancy is frequently associated with alterations within the cancerous cell at the genetic level. One such alteration is gene amplification and this will be discussed in more detail in section II.2. An intensively studied human cancer, with respect to the characterization of genetic alterations associated with tumour progression, is colon cancer (Fearon & Vogelstein, 1990). The model, now widely adopted as Vogelstein's model of colon carcinogenesis, was initially derived from studies of numerous colorectal tumour samples representative of different stages of tumour progression, including benign polyps, adenomas and carcinomas (Fearon, Hamilton & Vogelstein, 1987; Vogelstein *et al.*, 1988; Vogelstein *et al.*, 1989; Kern *et al.*, 1989). A number of genetic alterations have been associated with the progression of an adenoma to an invasive and metastatic carcinoma, including the mutation of *TP53* (Baker *et al.*, 1989; Rodrigues *et al.*, 1990; Campo *et al.*, 1991), Ki-*ras* (Forrester *et al.*, 1987; Bos *et al.*, 1987), *DCC* (Fearon *et al.*, 1990) and *MCC* (Kinzler *et al.*, 1991; Ashton-Rickardt *et al.*, 1991). Evidently, it is not the sequential mutation of particular genes in any given order which is of prime importance, since early lesions can have loss of p53 without mutation of Ki-*ras* and late stage carcinomas may not have all of the mutations implicated in colon tumour progression, rather it is the overall accumulation of genetic aberrations (Fearon & Vogelstein, 1990). The classically represented model of colorectal tumour progression (see Figure 2) is therefore misleading such that there is no rigid requirement for each genetic alteration to occur at a precisely pre-determined step in tumour progression. However, the model does provide a concept upon which to found further studies on the process of carcinogenesis. But, the progression of a non-invasive tumour to the

Figure 2

Figure 2. *Multistep Model Of Colon Carcinogenesis*

Progression of colon cancer has been viewed as a multistep event with several genetic alterations involved at different stages, as shown in this figure. See section II.1 for details.


Normal epithelium
Mutation or loss of chromosome 5q
Gene: *Adenomatous Polyposis Coli (APC)*
Hyperproliferative epithelium
DNA hypomethylation
Early adenoma
Mutation of chromosome 12p
Gene: Ki-*ras*
Intermediate adenoma
Loss of chromosome 18q
Gene: ?*Deleted in Colorectal Carcinoma (DCC)*
Late adenoma
Loss of chromosome 17p
Gene: *TP53*
Carcinoma
"Other alterations"
Metastases

metastatic state is not addressed in this model and is broadly labelled as "other mutations". Many studies have been attempted to elucidate the metastatic phenotype and some genes have been identified as being putatively metastasis-associated. Some of these will be discussed (see section II.3).

II.2 *Gene Amplification*

A recurrent feature of malignant tumours is gene amplification (reviewed by Brison, 1993). Cytogenetically, two different structures have been identified which are believed to harbour amplified DNA; homogeneously staining regions (HSRs) integrated with the chromosomal structure (Alitalo *et al.*, 1983) and extrachromosomal elements termed double minutes (DMs) (Hahn, 1993; Barker, 1982). Most frequently, amplification has been described of the *ras*, *myc* and *erbB* proto-oncogene families (Yokota *et al.*, 1986; Brodeur *et al.*, 1984; Nau *et al.*, 1986; Mizutani *et al.*, 1993), and of the human chromosomal locus 11q13 (Lammie & Peters, 1991). The 11q13 region spans ~15 million base pairs (mb) of DNA and the genes found within this region are still being characterized. However, a strong candidate gene which is thought to contribute significantly to the development of some tumours is cyclin D1 (Motokura *et al.*, 1991; Lovec *et al.*, 1994; Zhang *et al.*, 1993; Jiang *et al.*, 1993). In addition to being amplified in tumours such as breast cancer (Buckley *et al.*, 1993; Lammie *et al.*, 1991), the 11q13 locus is also translocated in some B cell lymphomas (Koduru, Offit & Filippa, 1989; Rabbitts *et al.*, 1988), further substantiating the prediction of a proto-oncogene within this locus (Lammie & Peters, 1991).

The mechanism of gene amplification in mammalian cells remains elusive since there is no known example of normal mammalian cells which undergo programmed gene amplification (Stark *et al.*, 1989). Several models have been proposed to explain the formation of both DMs and HSRs (reviewed by Stark

et al., 1989; Hahn, 1993) but none have been proven. Events leading to gene amplification in the proposed models include both DNA damage and recombination (Hahn, 1993; Stark *et al.*, 1989). Amplicons which contain amplified genes can often have duplicate DNA sequences in an inverted / palindromic "head-to-head" orientation (Ford & Fried, 1986; Ford *et al.*, 1985).

Regardless of the mechanism of gene amplification, increased copy number of particular genes has been identified to be clinically correlated with particular tumours and their disease stage. A notable example is neuroblastomas, whereby N-*myc* amplification was found to be increased 3-100 fold, as detected by Southern analysis, in patients with advanced-stage disease, *i.e.*, invasion and metastasis had occurred (Brodeur *et al.*, 1984). More recently, a gene named *MDM2* by virtue of its identification in DMs contained in a murine cell line 3T3DM, which was derived from spontaneously transformed Balb/c cells (Fakharzadeh *et al.*, 1993), has been discovered to be a regulator of p53 (Oliner *et al.*, 1993). The protein encoded by *MDM2* inhibits p53 activation of transcription by binding and concealing the p53 activation domain (Oliner *et al.*, 1993), therefore it can be surmised that gene amplification of MDM2 will increase MDM2 protein levels within the cell and result in the sequestration of p53, thus potentiating deregulated cell growth. Amplification of *MDM2* has been associated with human sarcomas (Oliner *et al.*, 1992; Cordon-Cardo *et al.*, 1994). However, with the observation that amplification of *MDM2* may lead to deregulated cell growth, it is not surprising that direct correlation of this amplification has not been reported with tumour progression to invasiveness and metastatic potential, but it may indeed be an earlier event leading to this phenotype.

II.3 *Putative Metastasis Genes*

Already apparent is that the metastatic phenotype arises through a cascade of events, affecting numerous aspects of normal cellular behaviour including cell adhesion, production of matrix degrading enzymes and cellular motility, as detailed in Chapter I. As a consequence of the multifaceted nature of metastasis, putative metastasis-suppressor genes may affect different individual aspects of metastasis, but the overall result is the suppression of the metastatic phenotype. With this regard, metastasis-suppressor genes may be multiple in number and divergent in function, but with only a single functional metastasis-suppressor gene required for metastasis to be overcome in any one tumour cell. Conversely, alterations of numerous metastasis-associated genes may be necessary for the metastatic phenotype to be manifested, since it has yet to be proven that any single gene is the universal metastasis-gene. However, in some systems studied, there may be the apparent induction of metastatic ability by mutation of a single gene or the modulation of gene expression, this can generally be restricted to particular cell types of particular origins. Thus, these cells may already possess many of the other requirements for metastasis and need only one further factor, or up-regulation / down-regulation of factors already present within the cell for full conversion to the metastatic phenotype. This therefore presents distinct difficulties in determining which, if any genes, are responsible for the metastatic phenotype.

II.3.1 *Nm23*

Nm23 was identified by differential colony hybridization of related murine melanoma K-1735 cell lines of high and low metastatic potentials (Steeg *et al.*, 1988a). Reduced expression of *nm23* was correlated with high metastatic potential and *nm23* was therefore proposed to be a candidate metastasis suppressor gene. Early reports indicated that in some rodent systems and in human breast cancer, inverse correlation could be found between Nm23 expression and metastatic potential (Steeg *et al.*, 1988b; Su *et al.*, 1993; Honoki *et al.*, 1993; Bevilacqua *et al.*, 1989). In colorectal cancer, correlation has been noted between *nm23* mutations and metastasis (Cohn *et al.*, 1991; Wang *et al.*, 1993) in addition to inverse association of *nm23* mRNA expression and liver metastasis (Yamaguchi *et al.*, 1993). However, further studies in other models and tumour types have not found the same correlation (Sastre-Garau *et al.*, 1992; Haut *et al.*, 1991; Myeroff & Markowitz, 1993). Controversy therefore exists about the role of the *nm23* gene in tumour metastasis.

The *Drosophila* gene abnormal wing discs (*awd*), encodes for a developmentally regulated protein which shares 78% identity of the predicted amino acid sequence with Nm23 (Rosengard *et al.*, 1989; Biggs *et al.*, 1990). Mutations or decreased expression of *awd* causes abnormal morphology, aberrant differentiation and cell necrosis during metamorphic stages of *Drosophila* development (Dearolf *et al.*, 1988a, 1988b). It has since been realised that Gip 17, a protein purified from *Dictyostelium discoidium*, identified as a nucleoside diphosphate (NDP) kinase, has 77% similarity to the human Nm23 protein and 75% similarity with Awd from *Drosophila* (Wallet *et al.*, 1990). Furthermore, two *nm23* genes have been identified, *nm23-H1* and *nm23-H2*, which share 88% amino acid identity (Stahl *et al.*, 1991) and these have been identified to be the A and B polypeptide chains respectively, of the human erythrocyte NDP kinase (Gilles *et al.*, 1991). The

human erythrocyte NDP kinase is a hexameric enzyme which can form several isoforms through permutations of the A and B polypeptide chains (Gilles *et al.*, 1991).

Phosphorylation studies of NDP kinase have identified the catalytic intermediate which is phosphorylated on histidine residue 118 during the "ping-pong" mechanism of nucleoside diphosphate phosphorylation (Gilles *et al.*, 1991). Also, an acid-stable auto-phosphorylation product of NDP kinase at residue serine 44 has been identified (MacDonald *et al.*, 1993). This serine 44 residue is conserved in mouse, human, rat and *Drosophila* Nm23 / NDP kinase sequences and conservatively substituted for threonine in *Dictyostelium* and *Escherichia coli* (MacDonald *et al.*, 1993). Nm23 / NDP kinase activity has not been consistently correlated with metastatic potentials (Sastre-Garau *et al.*, 1992) and one recent publication suggests that the acid-resistant auto-phosphorylated Nm23 at serine 44 exhibits correlation with the suppression of lung colonization potential as assayed by tail vein injection (MacDonald *et al.*, 1993). Transfection experiments with a mutated histidine 118 residue which abrogates the NDP kinase activity, did not produce viable colonies indicating that this kinase activity is absolutely required by the cell (MacDonald *et al.*, 1993). Nucleotide binding to NDP kinase is within a cleft of each subunit (Dumas *et al.*, 1992) and it is within this cleft that phosphorylated serine 44 also resides (Dumas *et al.*, 1992, MacDonald *et al.*, 1993). *In vitro* evidence suggests that binding of nucleotides to the NDP kinase releases the serine phosphorylation (MacDonald *et al.*, 1993), implying that serine 44 phosphorylation is separate from the kinase activity. This supports the observation that NDP kinase activity may not be the important parameter in metastasis, however this leaves open the question of what is the role of phosphorylated Nm23 in the cell and in metastasis.

Microinjection of antibodies specific to Nm23 into rat embryo fibroblasts inhibited cell division (Sorscher *et al.*, 1993). Expression of Nm23 has been detected on the cell surface as measured by flow cytometry, using specific antibodies to each of Nm23-H1 and Nm23-H2 (Urano, Furukawa & Shiku, 1993). Cell surface expression was of combinations Nm23-H1+H2+, Nm23-H1+H2- or Nm23-H1-H2- but never Nm23-H1-H2+ (Urano *et al.*, 1993). Interestingly, Nm23-H2 has been discovered to be identical to the human *c-myc* purine-binding transcription factor PuF (Postel *et al.*, 1993) and therefore it may not be surprising that Nm23-H2 is not cell surface expressed unless complexed with Nm23-H1, since its normal localization may be nuclear in order to function as a transcription factor. Unfortunately, detailed cellular localization studies of each Nm23 protein have not been reported to date, which may resolve some of these observations.

II.3.2. *Mts-1*

This gene was reported by Lukanidin and colleagues to be expressed specifically in metastatic cells (Ebralidze *et al.*, 1989). *Mts-1* encodes for a protein with 55% amino acid identity within the calcium-binding domains of the S100 family of calcium-binding proteins (Ebralidze *et al.*, 1989) and is very similar to the rat proteins p9Ka (Barraclough *et al.*, 1987) and 42A (Masiakowski & Shooter, 1988) which have also been identified to be related to the S100 family. Mts-1 is also apparently identical to a previously identified mouse calcium-binding protein, 18A2 (Jackson-Grusby, Swiergiel & Linzer, 1987). However the reported expression patterns in normal tissues of Mts-1 and 18A2 appear to differ. Transfection studies with the rat homologue, p9Ka, has been reported to induce the metastatic phenotype in a benign rat mammary epithelial cell line, RAMA 37 (Davies *et al.*, 1993). However, transfection of p9Ka also dramatically reduces the tumour latency period suggesting that the primary effect may be tumorigenicity-related or a proliferative effect. Indeed, two of the transfected cell lines, KP1-LT2

and KP1-LNT1, derived from metastases of primary tumours induced by pooled transfected cells, demonstrate no increased p9Ka mRNA levels or protein levels over the benign parental cell line but still exhibit similar (if not enhanced) metastatic abilities in comparison to another similarly metastasis-derived cell line containing elevated levels of p9Ka (Davies *et al.*, 1993). Further evidence to suggest that *mts-1* is not a directly related metastasis gene includes the rat equivalent, 42A, which differs from p9Ka by a single amino acid (Masiakowski & Shooter, 1988), and which was found to be induced in PC12 cells induced to differentiate with nerve growth factor (NGF). It would seem more plausible that these calcium-binding proteins are important structurally as they are found in association with F-actin (Davies *et al.*, 1993) and are induced under conditions which may involve cell shape change such as induced by growth factors and serum (Masiakowski & Shooter, 1988; Barraclough *et al.*, 1987; Jackson-Grusby, Swiergiel & Linzer, 1987). Although this is important in invasion and metastasis, it appears that these proteins are not necessarily causative of the metastatic phenotype, rather they may be consequentially affected as a result of this phenotype.

II.3.3 *CD44*

As earlier described (section I.2.2.5), CD44 is a transmembrane, highly glycosylated protein which can be alternatively spliced (Screaton *et al.*, 1992, 1993; Tolg *et al.*, 1993). Particular variant exons have been associated with the metastatic phenotype in a rat model system (Gunthert *et al.*, 1991; Rudy *et al.*, 1993) and with some human cancers (Heider *et al.*, 1993; Matsumura & Tarin, 1992; Li *et al.*, 1993), including colorectal cancer (Heider *et al.*, 1993; Wielenga *et al.*, 1993), although not in all human cancers (Chaudhry *et al.*, 1994; Salmi *et al.*, 1993). Notably, in early colorectal adenomas, CD44 is found to contain alternatively spliced exons including variant exon V5 which is not present in

normal mucosa, but not the metastatically-associated variant exons V6 or V7 (Rudy *et al.*, 1993; Wielenga *et al.*, 1993). However, analysis of later stages of colorectal tumour progression correlate with the expression of variant exon V6 (Weilenga *et al.*, 1993). Clues to the function of CD44 alternative splicing come from the observation that these different CD44 isoforms are found transiently expressed on activated T cells (Arch *et al.*, 1992; Haegel *et al.*, 1993). Indeed it has been suggested that one role for these isoforms may be in homing of the T cells to the lymph nodes (Wu, Kincade & Shortman 1993; Arch *et al.*, 1992). It can therefore be envisualized that a similar role may be employed by the metastatic cell with expression of these variant exons. Also proposed of some glycosaminoglycans such as CD44 is the ability to bind cytokines (Tanaka *et al.*, 1993). It may be that different variant isoforms can bind different cytokines, thereby influencing the interactions of these expressing cells with other cells responding to the cytokine. Alternatively, cytokines bound to CD44 may be internalized and mediate an intracellular effect. These possibilities have yet to be resolved or disregarded.

However, it is apparent that expression of particular CD44 isoforms are, in some instances, specifically associated with the metastatic phenotype, although in some systems, it may be the level of expression of the standard form of CD44 which is correlated with the metastatic process. As a prognostic marker, CD44 appears to be useful in certain human tumours to correlate the disease stage with expression of variant CD44, which can be detected in the blood of patients by the polymerase chain reaction (PCR) technique.

II.3.4 *Cell Adhesion Molecules*

E-cadherin, together with other cell adhesion molecules including the integrin $\alpha_4\beta_1$ (Qian, Vaux & Weissman, 1994), have been demonstrated to suppress the

invasive capacities of tumour cell lines (Vleminckx *et al.*, 1991; Frixen *et al.*, 1991). These genes are sometimes proposed as metastasis suppressor genes, but should be more accurately termed invasion suppressor genes. As already mentioned in section I.2.2.1, transfection of a cDNA encoding for E-cadherin both into v-*ras* transformed derivatives of Madin-Darby canine kidney (MDCK) cells which normally express very low levels of E-cadherin and into a subclone of the cell line NMuMG (normal murine mammary gland) which also lacked E-cadherin expression, resulted in the abrogation of their invasive phenotype as estimated in the invasion assays of chick embryo heart fragments and collagen matrices (Vleminckx *et al.*, 1991). Studies on human cancers and cell lines derived from human cancers including breast, gastric, gynaecologic and squamous cell carcinoma of the head and neck (Mayer *et al.*, 1993; Oka *et al.*, 1993; Kinsella *et al.*, 1993; Pignatelli *et al.*, 1992; Schipper *et al.*, 1991), often display altered expression of E-cadherin. Correlation is inverse with expression of E-cadherin protein in comparison with the status of tumour differentiation or lymph node involvement. One recent study of the E-cadherin gene on chromosome 16q22 revealed in 3% of gynaecological carcinomas examined (n=135) somatic mutations which were either missense or nonsense (Risinger *et al.*, 1994). In another study of cell lines derived from human gastric carcinomas, mRNA processing appeared to be disrupted in two cell lines; MKN45, a poorly differentiated adenocarcinoma and KATO-III, a signet ring cell carcinoma (Oda *et al.*, 1994). Together, these results support the invasion-suppressor role of E-cadherin and it can be readily envisaged that breakdown of cell-cell contacts could increase the propensity of a tumour cell to detach from the primary mass and commence invasion into normal local stroma. Aberration of these cell-cell contacts can also arise from disruption of intracellular interactions with the cytoskeleton, such as cadherins with the catenins (described in section I.2.2.1). The human lung cancer cell line PC9, although E-cadherin positive, was not cell-cell adhesive and was found to have homologous deletion of

part of the α-catenin gene (Shimoyama *et al.*, 1992). Cell-cell adhesion was restored upon transfection of a functional α-catenin gene (Hirano *et al.*, 1992). This would mean that immunohistochemical analysis of tumours for E-cadherin expression could be positive, but E-cadherin may still not be functional. Therefore the invasion suppressor role of E-cadherin can be extended to other components necessary for functional E-cadherin cell-cell adhesion.

A similar situation appears to exist for the $\alpha_4\beta_1$ integrin, recently described to be suppressive of invasion (Qian *et al.*, 1994). Although many of the integrins are involved in cell-matrix interactions (see section I.2.2.2), some have also been implicated in cell-cell adhesion, including $\alpha_4\beta_1$ (Pulido *et al.*, 1991; Campanero *et al.*, 1990; Takada *et al.*, 1989). A B16 melanoma variant cell line, B16a, was highly invasive and spontaneously metastasized from subcutaneous injection and did not express α_4 (Qian *et al.*, 1994). Upon retroviral infection of this cell line with an α_4 cDNA, heterodimer $\alpha_4\beta_1$ integrin expression was restored and these transfected cells were non-invasive when subcutaneously injected (Qian *et al.*, 1994). Furthermore, intravenous injection of transfected cells displayed similar colonization ability of the lung as mock-transfected cells, indicating that the functional integrin heterodimer was influencing the early invasive steps in metastasis and not the later events (Qian *et al.*, 1994).

II.3.5 *Conclusion*

Evidently some genes are directly implicated in tumour invasion and metastasis, including some of those described above, and genes encoding for proteins such as the extracellular matrix degradation enzymes (section I.2.3) and motility factors (section I.2.4), but no single gene has truly been classified as a "metastasis gene". It may be that metastasis is indeed a collaboration of events within the cell, and certain genetic alterations can potentiate the ability for a cell to metastasize, but

overall it may be the balance of the various factors which determines metastasis. This obviously presents significant difficulties in determining exactly what it is that makes a tumour cell metastatic and perhaps greater insight will result from research into cells such as lymphocytes which normally migrate throughout the body, but are obviously not tumorigenic and therefore do not form metastases.

CHAPTER III MODEL SYSTEMS FOR THE ANALYSIS OF INVASION & METASTASIS

III.1 *Introduction*

To allow for the study of the complex processes of invasion and metastasis, many model systems have been developed which involve both *in vivo* and *in vitro* approaches (Evans, 1991). Because of the nature of metastasis, it is difficult to comprehensively represent the whole process in a single model, although there are advances in this field with *in vivo* model systems and orthotopic implantation techniques, as will be discussed later in this chapter (III.2). However, it is also advantageous to attempt to dissect the invasion and metastatic processes with *in vitro* analyses, which provides a favourable position for experimental manipulation. The major disadvantage of the "simplified" *in vitro* model systems is the physiological relevance, since these systems circumvent many of the complex parameters found *in vivo*, such as the extracellular matrix, appropriate tissue / cell types, host response, *etc.* However, without an ideal system which addresses all aspects of invasion and metastasis, many of the model systems viewed together should aid research into these multifaceted processes, bearing in mind the limitations of each model and therefore the interpretations of the results. The following sections give a brief overview of different model systems used to study invasion and metastasis.

III.2 *In Vivo Model Systems*

These model systems are largely dependent upon laboratory mice which are highly inbred strains, with largely known genetic backgrounds (Lyon & Searle, 1989). The two major systems for the initiation of primary tumours are chemically-induced, for example with the use of DMBA in multistage mouse skin carcinogenesis (reviewed by Bowden *et al.*, 1994), or by direct injection of tumour

cells, either of cell lines, for example in the CMT model (see section III.3.5) or cells from a tumour. Initiation of a primary tumour and the formation of metastases at secondary distant sites is known as the spontaneous metastasis assay. The experimental assay, or the colonization assay, does not involve the initiation and formation of a primary tumour, but instead the tumour cells are injected directly into the blood circulation, for example *via* the tail vein. This latter metastasis assay does not represent the initial tumour formation and invasion stages of metastasis, but does reflect the later stages of metastasis, which is more accurately described as the colonization potential of circulating tumour cells. It should be noted here that some of the cell lines used in metastasis research display colonization potential as assayed by the experimental metastasis assay, but cannot form distant metastases spontaneously from subcutaneous injection, for example many of the B16 melanoma sublines (Evans, 1991), and therefore these cells lack a possibly fundamental property of metastasis and this should be taken into consideration when interpreting results.

Recently, it is becoming more widely recognized that the site of injection in the spontaneous assay can affect the subsequent behaviour of the tumour cells (reviewed by Manzotti, Andisio & Pratesi, 1993). Traditionally, the site of injection has been subcutaneous, however recent model systems are employing orthotopic implantation as opposed to ectopic injection (Furukawa *et al.*, 1993a & b; Stephenson *et al.*, 1992). The orthotopic implantation models frequently utilize nude mice which are immunocompromised since they are athymic. When tumour cells are injected subcutaneously into nude mice, metastasis rarely occurs, but orthotopic implantation has been reported to mimic successfully the clinical disease with tumour spread to lymph nodes and organ-specific metastasis typical of the tumour cell origin (Furukawa *et al.*, 1993a & b; Stephenson *et al.*, 1992; Fidler, 1991). This therefore represents a more clinically

relevant model system with which to analyse the metastatic phenotype and examine the potential of therapeutic agents.

A disadvantage of the *in vivo* model systems using immortalized tumour cell lines with known metastatic potentials is phenotypic drift consequent to long-term cell culture. The original parental tumours, from which many of the present cell lines were derived, are no longer available which could be used to determine the degree of phenotypic drift in the cell lines. However, this is a necessary sacrifice for having a stable model system with which repeated experimentation can be tested and allowing for the same model to be examined in different laboratories. Additionally, cell lines allow for substantial molecular and biochemical analyses since the cells can be grown to large numbers. The use of primary tumour material for orthotopic implantation overcomes the problems associated with *in vitro* tissue culture, however it too has a number of associated disadvantages. The primary disadvantage is the limited quantity of tumour sample which cannot be so readily analysed in great detail at the molecular and biochemical levels. In conjunction, due to tumour heterogenity, different sections of a tumour sample may exhibit different phenotypical charateristics (Heppner, 1984) and thus produce variablity into the assay. The obvious advantage to orthotopic implantation of tumour material is the mimicry of clinical metastasis.

The chick embryo is another *in vivo* model system for the study of colonization potential / experimental metastasis and spontaneous metastasis (Evans, 1991). However, although tumour cells seeded onto the chorioallantoic membrane (CAM) can invade and spread to organs of the developing chick, this event tends to take a considerable time. Since this is a xenogeneic system and the study of these processes are often preferred to be without immunological intervention, the tumour cells need to establish and exhibit their invasive and / or metastatic phenotype before the chick becomes immunocompetent at 18 days (Evans, 1991). A further

constraint of this system is the CAM: the CAM is not suitable for seeding of primary tumour cells until it is sufficiently developed at 8 days. In addition, the status of the CAM can affect the experiment; slight damage to the CAM appears to enhance tumour growth (Armstrong, Quigley & Sidebottom, 1982). The mechanism by which this operates is unclear. It may be postulated that the damage induces the normal repair processes which results in the stimulation of growth factor secretion and possibly of angiogenic factors which can in turn be utilized by the tumour cells.

As a result of the disadvantages of this model system (the short time window of 8-18 days within which the chick embryo and the CAM are compatible for experimentation, and the poor correlation with other model systems, e.g. syngeneic animals, of the invasive phenotypes) the chick embryo is not a preferentially used model system.

III.3 *In Vitro Model Systems*

A number of *in vitro* model systems have been developed and all study aspects of invasion (Evans, 1991). Due to the complex process of metastasis, it has not been possible to design a system which could operate *in vitro* and represent the whole process of metastasis. However, although the *in vitro* model systems are limited in their applicability to later events of the metastatic process, they may provide some valuable insight into the initial stages of invasion. Unfortunately, due to the simplified nature of these models - an advantage in itself for the greater control of experimental parameters than *in vivo* - it is poorly representative of the physiological situation and therefore caution needs to be taken in the interpretation of results.

III.3.1 *Confrontation Assays*

Explants are taken from tissues such as the chick embryo heart in this assay, and placed in culture ~1mm distant to a tumour explant. From these explants, cells can migrate and grow out to "confront" cells from the opposing explant. This assay is meant to be representative of the invasion characteristics of tumour cells, however "invasion" can often be seen equally from both sources (Stephenson & Stephenson, 1978), thus questioning the validity of this experimental approach.

III.3.2 *Collagen Gels / Matrigels*

These assays provide a 3-dimensional invasion assay as opposed to the essentially 2-dimensional confrontation assay (III.3.1). The use of native collagen to form a gel or reconstituted basement membrane gel prepared from EHS tumours (matrigel) (Kleinman *et al.*, 1986), allows for the examination of tumour cells penetrating the gel and tumour cell migration through the gel. With the use of fluorescent stains or tagged antibodies, invading cells can be visualized with a confocal microscope. This assay system allows for the addition of chemoattractant factors and specific inhibitors to experimentally manipulate the invasion process and therefore attempt to dissect the process. However, as with all *in vitro* invasion assays, the system is highly simplified in comparison to the *in vivo* situation and caution must be maintained in extrapolating results.

III.3.3 *Invasion Of Tissue Fragments*

Different types of normal tissue fragments have been employed to assess invasive potentials of tumour cells. These include chick embryo heart, chick embryo CAM, amnion and blood vessels (Evans, 1991). Invasion of the chick embryo heart has been tested in both stationary and gyratory coculture with tumour cells (Evans, 1991). Generally, tumour cells are incubated with whole or fragments of chick

embryo heart and invasion is assessed by sectioning of the tissue and examination for invading cells. This is a rather laborious method for analysis and the implications from the results of these experiments are questionable for their validity since the heart is rarely a site for metastasis.

Invasion of the amnion is essentially performed in a chemotactic chamber with the amnion clamped over a millipore filter and the tumour cells are seeded upon the upper surface (Thorgeirsson *et al.*, 1982). At various time intervals, the amnion can be removed and analysed for cells which have successfully invaded. Cells which have succeeded in invading the amnion completely can be collected and identified from the filter. Since the upper amnion surface is epithelium and is generally a poor substrate for adhesion, it is usually removed by treatment with 0.1M ammonium hydroxide. It would appear that in this assay system the vital criterion for invasion is the expression of matrix metalloproteases, since uninhibited expression of these enzymes correlate with invasion of the amnion (Thorgeirsson *et al.*, 1982). However, as already noted with other *in vitro* model systems, some tumours do not exhibit the same invasive phenotype in this *in vitro* assay as characteristic of their tumours in *in vivo* models, for example MCF-7 cells form highly invasive tumours, but only display a limited basement membrane degradation ability in this assay.

Poste *et al.* (1980) developed an *in vitro* assay system examining the invasion of blood vessels by tumour cells. Excised blood vessels were inserted with a porous cylinder capable of allowing the passage of single cells. The blood vessel itself could be orientated with the endothelium facing either inwards or outwards after eversion. This was then linked with a flow through system which could maintain an internal circulation and the external surface of the blood vessel was bathed in non-circulating medium. Tumour cells were inoculated into the surrounding medium and the cells which successfully traversed the blood vessel were recovered

from the internal circulating system. Although this is an ingenious system for the study of blood vessel traversion by tumour cells, there are certain physiological deviations: the blood vessel which is used is usually a vein which is different from the capillary bed normally associated with the extravasation of metastasizing tumour cells. Also, a modification of the system would have allowed for the incorporation of blood flow and shear stress to be examined by the inoculation of the tumour cells into the circulating system and the recovery of cells from the surrounding medium.

III.3.4 *Summary*

To reiterate, *in vitro* model systems focus on particular aspects of the metastatic process, most notably invasion. Consequently, the effects of many of the normal physiological conditions are omitted from these studies, such as interactions with host cells, immune reactions, circulation and the extracellular matrix. However, due to the vastly complex nature of metastasis, dissection of this process may be advantageous and *in vitro* model systems will prove advantageous if viewed in perspective.

The *in vivo* model systems are presently in two main categories for the study of metastasis: "spontaneous" and "experimental" assays. The experimental assay system essentially reflects colonization potential, but not metastatic ability. The spontaneous metastasis assay, although more analogous than other systems to the complete metastatic process, generally does not allow for detailed investigations of the individual steps necessary for metastasis.

Overall, until superior assay systems for the analysis of the metastatic process are developed, if possible, then metastasis research is limited to the aforementioned assay systems. However, these should prove worthwhile and provide valuable insight when the limitations of the systems are taken into consideration. The model

which does provide the greatest representation of metastasis is the spontaneous metastasis assay. These systems are also valuable for the evaluation of possible therapeutic trials since complete understanding of the biology of metastasis need not be fully comprehended before such experimentation can be attempted. For this thesis, a murine model representative of the spontaneous metastasis phenotype was employed and further details of this model are described in the following section (III.3.5).

III.3.5 *CMT Model*

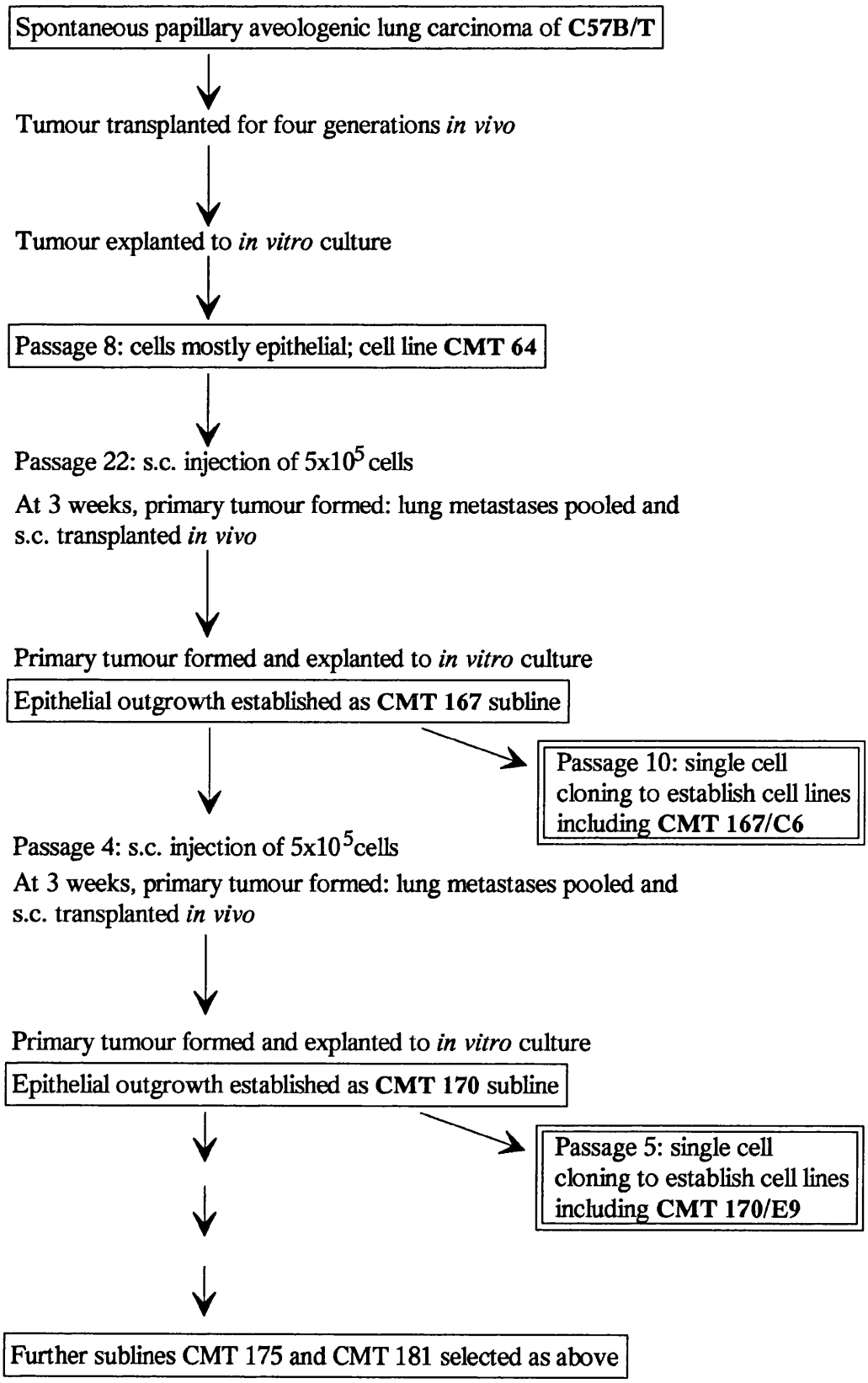
This mouse model system for the investigation of the metastatic phenotype was first described in 1976 (Franks *et al.*) and characterized for stable metastatic variants in 1984 (Layton & Franks). The basis of this model is a series of epithelial cell lines which were originally derived from a spontaneous alveologenic carcinoma of a female C57B/T mouse (Franks *et al.*, 1976). The derivation of the cell lines is illustrated in Figure 3. Briefly, a heterogeneous cell line (CMT 64) was first established from explant cultures of fourth transplant generation tumour tissue (Franks *et al.*, 1976). Subsequent heterogeneous sub-lines of this parent cell line were then derived by means of subcutaneous injection of CMT 64, the resultant lung metastases were pooled and re-injected subcutaneously to form another primary tumour. From this tumour, explants were taken and cultured *in vitro* to generate the first sub-line CMT 167. This process was repeated with CMT 167 cells being injected subcutaneously, resulting in the derivation of the sub-line CMT 170, which gave rise to other sub-lines following the same procedure. These sub-lines were cloned to produce a variety of cell lines which were then characterized for their metastatic abilities (Layton & Franks, 1984).

In the work described in this thesis, two sub-line clones were used for analysis: CMT 167/C6 (high metastatic capacity, as assayed by the spontaneous metastasis

Figure 3

Figure 3. *Schematic Illustration Of The Derivation Of The CMT Cell Lines*

The CMT cell lines were derived from the same spontaneous lung carcinoma of a female C57B/T animal through the sequential selection of cell lines established in cell culture (Layton & Franks, 1984). The cell lines CMT 167/C6 and CMT 170/E9 which, among many other cell lines, were characterized for their metastatic potential (Layton & Franks, 1984). These CMT cell lines were subsequently chosen as the basis of the CMT model of metastasis through their differing metastatic phenotypes (CMT 167/C6: high metastatic potential; CMT 170/E9: low metastatic potential).



Derivation of Cell Lines from a Spontaneous Lung Tumour

assay) and CMT 170/E9 (low metastatic capacity). It was reasoned that these two cell lines with their markedly differing metastatic potentials would be the most informative for analysing the metastatic phenotype.

To date, this model system has not been extensively characterized. Initial studies have determined the effects of cell fusion upon the metastatic phenotype with the general conclusion that fusion of metastatic cells (CMT 167) with non-metastatic L cells or EJ bladder carcinoma cells results in a suppression of the metastatic phenotype (Layton & Franks, 1986). However, other studies have indicated that fusion studies and chromosome transfer mediated by microcells, resulted in an instability of the metastatic phenotype (Pickford, 1987). Indeed, the procedure of single cell cloning could in some instances result in an apparent destabilization of the metastatic potential of CMT 167/C6 cells. There have been no reported studies on the biological and molecular characteristics of both CMT 167/C6 and CMT 170/E9 cell lines which could identify the cause of their differing metastatic abilities. Therefore, the major aim of this research was to attempt to define the determining factors involved in the metastatic phenotype of this system. An advantage of this system is the ability of the cell lines to metastasize spontaneously and therefore these cells are representative of the complete metastatic process. Also, since both cell lines are derived from the same spontaneous primary tumour, the basic genetic background of these cells are essentially identical with hopefully only the key differences which are pertinent to the metastatic phenotype present. In addition, unlike many other models of metastasis based upon cell lines, these cells have not been in *in vitro* cell culture for many decades, thereby minimizing phenotypic drift which may be a significant factor in other systems.

MATERIALS AND METHODS

CHAPTER IV *MATERIALS*

IV.1 *ANTISERUM*

The rat monoclonal against E-cadherin (ECCD-2) (Shirayoshi *et al.*, 1986) and rat monoclonal against P-cadherin (PCD-1) (Nose & Takeichi, 1986) were gifts from Prof. M. Takeichi and Dr. D. Prowse.

Supplier: *ICN Biomedicals Inc., California, USA*

-anti-β1 integrin, mouse monoclonal; clone DF5

-anti-β3 integrin, mouse monoclonal; clone BB10

-anti-αIIb integrin, mouse monoclonal; clone CA3

Supplier: *Novocastra Laboratories Ltd., Newcastle upon Tyne, England*

-CM1; anti-p53, rabbit polyclonal

Supplier: *Sigma Immunochemicals, Poole, Dorset, England*

-anti-NCAM, mouse monoclonal; clone NCAM-OB11

-anti-N-cadherin, mouse monoclonal: clone GC-4

-goat anti-mouse IgG (Fab specific) - peroxidase conjugate; product No. A-9917

-goat anti-mouse IgG (whole molecule) - alkaline phosphatase conjugate; product No. A-3688

-goat anti-mouse IgG (whole molecule) - FITC conjugate; product No. F-2012

-goat anti-rat IgG (whole molecule) - FITC conjugate; product No. F6258

IV.2 *CELL LINES, MEDIA & TISSUE CULTURE SUPPLIES*

Murine cell lines CMT 167/C6 and CMT 170/E9 were obtained either from laboratory stocks or from stocks held at Glasgow Royal Infirmary Department of Surgery. All other cell lines used in this thesis were from laboratory stocks.

Supplier: *Beatson Institute Central Services*

-sterile dH_2O

-sterile PBS

-sterile CT buffer

-sterile glassware and glass pipettes

Supplier: *Becton Dickinson Labware, Plymouth, England*

-60mm and 100mm diameter tissue culture dishes

Supplier: *Costar, 1 Alewife Ctr, Cambridge, MA 02140, USA*

-6 well cluster and 24 well cluster tissue culture dishes

-sterile disposable cell scrapers

Supplier: *Fisons Scientific Equipment, Loughborough, Leics., England*

-dimethylsulphoxide (DMSO)

Supplier: *Gibco Europe Life Technologies Ltd., Paisley, Scotland*

-10x DMEM concentrate

-methionine-free DMEM

-Newborn calf serum

-200mM glutamine

-100mM sodium pyruvate

-7.5% (w/v) sodium bicarbonate

-1M HEPES

-2.5% (w/v) trypsin

-100x non-essential amino acids

Supplier: *A/S Nunc, Roskilde, Denmark*

-Tissue culture flasks

-Nunc tubes

IV.3 *CHEMICALS*

Supplier: *Amersham International plc, Amersham, Bucks, England*

-[α-^{32}P] dCTP ~3000Ci/mmol

-[γ-^{32}P] ATP ~5000Ci/mmol

-[α-^{35}S] dATP ~600Ci/mmol

-^{32}P-*ortho*phosphate, carrier-free

Supplier: *BDH Chemicals Ltd., Poole, Dorset, England*

Unless otherwise stated, all chemicals were obtained from BDH and were of AnalaR grade or better.

Supplier: *Boehringer Mannheim UK Ltd., Lewes, East Sussex, England*

-cesium chloride

Supplier: *Bethesda Research Laboratories, Life Technologies, Inc.,USA*

-agarose, ultrapure electrophoresis grade

-LMP agarose, ultrapure electrophoresis grade

Supplier: *James Burrough Ltd., Witham, Essex, England*

-ethanol

Supplier: *Cinna/Biotecx Laboratories Inc., Houston, Texas, USA*

-RNAzolB

Supplier: *Difco Laboratories, Detroit, Michigan, USA*

-Bactoagar

Supplier: *Fisons Scientific Equipment, Loughborough, England*

-38% (w/v) formaldehyde

Supplier: *Fluka Chemika-Biochemika AG, Buchs, Switzerland*

-formamide

Supplier: *Koch-Light Ltd., Haverhill, Suffolk, England*

- isoamyl alcohol

Supplier: *Merck, Darmstadt, Germany*

-ninhydrin

Supplier: *Millipore UK Ltd., Watford, Herts., England*

-all reagents for 2D-gel electrophoresis

Supplier: *National Diagnostics, Manville, New Jersey, USA*

-Ecoscint A

Supplier: *New England Nuclear, DuPont UK Ltd., Stevenage, Herts, England*

-^{35}S-methionine

Supplier: *Pharmacia Ltd., Milton Keynes, Bucks., England*

-dextran sulphate

-50mM dNTPs

-poly (dI-dC)

Supplier: *Rathburn Chemicals Ltd., Walkerburn, Scotland*

-phenol (water-saturated)

Supplier: *Sigma Chemical Co. Ltd., Poole, Dorset, England*

-ampicillin

-bromophenol blue

-BSA (fraction V)

-coomassie brilliant blue R

-dithiothreitol

-ethidium bromide

-nitroblue tetrazolium

-NP40

-methylene blue

-MOPS

-phosphoserine

-phosphothreonine

-phosphotyrosine

-polyvinylpyrrolidone

-salmon sperm DNA

-spermidine

-TEMED

-triton X-100

Supplier: *Unipath Ltd., Basingstoke, Hants., England*

-PBS (Dulbecco A, tablet form)

IV.4 *COLUMNS*

Supplier: *Pharmacia Ltd., Milton Keynes, Bucks., England*

-Nick columns

IV.5 *ENZYMES & INHIBITORS*

Supplier: *Bethesda Research Laboratories, Gibco Ltd., Paisley, Scotland*

All DNA modifying enzymes were obtained from BRL and supplied with the appropiate buffer concentrates, with the following exceptions:-

Supplier: *Boehringer Corporation (London) Lyd., Lewes, East Sussex, England*

-Klenow

-Proteinase K

-RNase T1

Supplier: *Nothumbria Biologicals Ltd., Cramlington, Northumberland, England*

-AMV reverse transcriptase

-RNase A

-T4 polynucleotide kinase

Supplier: *Pharmacia Ltd., Milton Keynes, Bucks., England*

-S1 nuclease

-RNA Guard

Supplier: *Sigma Chemical Co., Ltd., Poole, Dorset, England*

-aprotinin

-diethylpyrocarbonate (DEPC)

-leupeptin

-lysozyme

-pepstatin A

IV.6 *KITS*

Supplier: *Amersham International plc, Amersham, Bucks., England*

-ECL kit

Supplier: *Bio 101 Inc., Stratatech Scientific, Luton, England*

-Geneclean kit

Supplier: *Boehringer Mannheim UK, Lewes, East Sussex, England*

-DNA random priming labeling kit

Supplier: *Perkin Elmer Cetus, Norwalk, CT 06859, USA*

-GeneAmp PCR reagent kit

Supplier: *Pierce, Life Science Laboratories Ltd., Luton, England*

-MicroBCA protein assay kit

Supplier: *United States Biochemical, Cleveland, Ohio, USA*

-Sequenase version 2.0 kit

Supplier: *Vector Laboratories, Burlingame, California, USA*

-Vectastain ABC-AP kit

IV.7 *MARKERS*

Supplier: *Amersham International plc., Amersham, Bucks., England*

-Prestained protein standards: Rainbow markers,

range 14 300 - 200 000Da

Supplier: *Bethesda Laboratories Research, Gibco Ltd., Paisley, Scotland*

-ϕX174 RF DNA / Hae III fragments

-λ DNA / Hind III Fragments

-RNA ladder

Supplier: *Sigma Chemical Co. Ltd., Poole, Dorset, England*

-High molecular weight standard mixture (non pre-stained)

-Standards for non-denaturing electrophoresis: BSA dimer (132kD), BSA monomer (66kD), chick egg albumin (45kD), carbonic anhydrase (29kD)

IV.8 *MEMBRANES, PAPER, TLC PLATES & X-RAY FILM*

Supplier: *Amersham International plc, Amersham, Bucks., England*

-Hybond N+

Supplier: *Eastman Kodak Co., Rochester, New York, USA*

-Duplicating film (DUP-1)

-X-ray film (XAR)

Supplier: *Fuji Photo Film Co., Ltd., Japan*

-X-ray film (RX)

Supplier: *Presentation Technology Ltd., Clydebank, Scotland*

-AGFA Rapitone photographic paper (black & white)

Supplier: *Phase Separations Ltd., Deeside, Clywd, Wales*

-20x20cm cellulose TLC plates (w/o fluorescent indicator)

Supplier: *Sartorius Ltd., Epsom, Surrey, England*

-dialysis bags

-nitrocellulose membranes

Supplier: *Whatman International Ltd., Maidstone, Kent, England*

-GF-C filters

-3MM chromatography paper

IV.9 *WATER*

Distilled water for buffers and all general solutions was purified through a Millipore MilliRO 15 system. Water for protein or recombinant DNA procedures which required further purifcation was obtained from a Millipore MilliQ system at 18MΩ cm.

CHAPTER V *METHODS*

V.1 *CELL CULTURE*

V.1.1 *Cell Maintenance*

CMT cell lines were maintained in Dulbecco's modified Eagles medium (DMEM) (see below), supplemented with 10% new born calf serum, in conditions of a humidified atmosphere with 10% (v/v) CO_2 at 37°C. Cells were subcultured every 4-5 days as follows:- growth medium was aspirated from the monolayer of cells, the cells were briefly rinsed with sterile phosphate buffered saline (PBS) then incubated with a minimum volume of 0.25% (v/v) trypsin in CT buffer (146mM sodium chloride, 14mM tri-sodium citrate, 14mM tricine, 0.5% (w/v) phenol red, pH 7.8) at 37°C for 3-5mins. Cells were detached with gentle agitation, resuspended in fresh growth medium and replated at the appropriate density.

10x DMEM	50mls
200mM glutamine	5mls
100mM sodium pyruvate	5ms
7.5% sodium bicarbonate	22mls
sterile dH_2O	418mls

pH 6.8 - 7.2 with sterile 1N NaOH

V.1.2 *Mycoplasma Testing Of Cell Lines*

All cell lines used throughout this thesis were routinely checked for mycoplasma contamination. Conditioned medium was collected from cells which had been cultured for at least 2-3 days by centrifugation at 1300rpm for 8-10 mins. ~2mls of this conditioned medium was co-incubated with 2 x 10^5 NRK49 fibroblasts in 2mls fresh medium. NRK49 fibroblasts are known to be mycoplasma free, although a negative control was always set up in parallel: NRK49 cells without

conditioned medium. After incubation at 37°C in a humidified atmosphere with 5% (v/v) CO_2 for 3 days, cells were fixed by the addition of fixative (1 volume glacial acetic acid : 3 volumes methanol) for 5mins. This was followed with a further two incubations with fresh fixative for 5mins and the cells allowed to air - dry. Hoechst 33258 is a fluorescent stain which detects DNA and was prepared from a 1mg/ml stock by diluting 1:20 000 in PBS. This solution was added to the fixed cells and incubated at room temperature for 10mins. After staining, excess stain was removed and cells washed 2x with dH_2O, then viewed by fluorescent microscopy using a water immersion lens. Mycoplasma contamination was detected as fluorescence in the cytoplasm of NRK49 cells.

V.1.3 *Treatment of Culture Surfaces*

In some experiments, tissue culture plastics were coated with either fibronectin or collagen. Lyophilized fibronectin was resuspended at 30μg/ml with sterile distilled water (dH_2O). Under sterile conditions, fibronectin solution was added to the flasks / dishes in a volume sufficient to cover the surface area and incubated at room temperature for 2 hours. Excess solution was then aspirated off and the flasks / dishes rinsed with sterile PBS.

Concentrated rat tail type 1 collagen was diluted with 0.02M NaOH to 100μg/ml. As above, the collagen solution was incubated on the required plastics, except incubation was for 20mins at room temperature before excess solution was removed. The plastics were then rinsed with sterile PBS.

Stock solutions were stored at 4°C ; flasks / dishes were either air - dried and stored at 4°C until required, or used immediately for the culture of cells.

V.1.4 *Cell Storage*

Cells were frequently stored in order to maintain stocks of cells at early passage. Trypsinised cells were resuspended as a single cell suspension in storage medium (DMEM supplemented with 10% (v/v) dimethylsulphoxide [DMSO]) at ~$5x10^6$ - 10^7 cells / ml. Cell suspensions were aliquoted into 1ml Nunc cryotubes and allowed to slowly cool to -70°C overnight in a well-insulated container before transferring to storage in liquid nitrogen.

V.1.5 *Soft Agar Assays*

Soft agar assays were performed to assess anchorage-independent cell growth. In a 200ml Duran bottle, 1g of Difco bacto-agar in 40mls sterile dH_2O was melted using a microwave oven and cooled to 44°C. Added to this was 40mls of 2x DMEM (see below), 20mls newborn calf serum and 100mls 1xDMEM. This 0.5% (w/v) agar solution was maintained at 44°C for pouring, ~5mls / 60mm dish, and these base plates allowed to set at room temperature or 4°C. 0.5mls of CMT cells or control cells, at a concentration of $2x10^5$ cells / ml in normal growth medium, were mixed with 1ml of cooled (~37°C) base agar in a sterile plastic universal, resulting in a 0.3% soft agar / cell mix. Each base plate was carefully overlaid with this mix and allowed to set at room temperature. Plates were subsequently incubated at 37°C, in a 10% (v/v) CO_2 humidified incubator. Each assay was typically performed in at least triplicate and colony formation was assessed following 2 weeks incubation.

2x DMEM:	10x DMEM	20mls
	200mM glutamine	2mls
	100mM Na pyruvate	2mls
	7.5% sodium bicarbonate	10mls
	1M HEPES	4mls
	sterile dH_2O	58mls

V.1.6 *MTT Assay*

The MTT assay is a rapid colorimetric assay for cell growth and the estimation of viable cell numbers. Its advantages over other biological assays include a time saving factor, allowing for numerous samples to be assayed simultaneously and the eliminated use of radioactive material. Enzymatic cleavage of the tetrazolium ring present in the compound MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; also named thiazol blue), by mitochondrial dehydrogenases produces a formazan precipitate around the mitochondria of respiring cells. This blue crystallized salt can be dissolved with DMSO and the resulting colour measured; the degree of absorbance at wavelength 595nm relates to viable cell number.

Cells were seeded in flat-bottomed 96-well tissue culture plates at the appropriate concentration in a volume of 100µl per well. Replica plates were set up for each time point. Plates were incubated in a humidified atmosphere with 10% (v/v) CO_2 at 37^{o}C. At each time point, 10µl of MTT (5mg/ml in PBS) was added to each well and incubation of the cells continued for a further 4 hours. The excess medium was carefully pipetted out, leaving the adherent cells and precipitate in the wells. 100µl of DMSO was then added per well, dissolving the precipitate and the plates read in a 96-well plate scanner at 595nm.

Absorbance values were converted to equivalent viable cell numbers using a standard curve of known cell numbers plotted against their absorbance values as measured by the MTT assay.

V.2 *SPONTANEOUS METASTASIS ASSAY*

A single cell suspension of $5x10^5$ cells in 0.1mls DMEM was injected subcutaneously (s.c.) into the right flank of mice, without anaesthesia. Tumour

growth was monitored regularly and once growth was of palpable size, measurements were taken of both the largest diameter and smallest diameter of the primary tumour. These measurements were computed in the equation:-

$\mathbf{V = \pi.D.d^2/6}$, where **V** = volume (mm^3); $\pi = 3.14$; **D** = largest diameter; **d** = smallest diameter (Ferguson, Carmichael & Smith, 1986).

Animals were sacrificed at 3-5 weeks, or when tumour diameter reached 2cm or if animals became moribund.

In order to examine for metastatic spread, animals were killed in a euthanasia chamber. Using standard dissection techniques, internal organs were examined for lesions, these organs were dissected out, preserved in Fekete's solution (100mls 70% ethanol, 10mls 38% formaldehyde solution, 5mls glacial acetic acid) for later histological examination. To quantify metastatic deposits in the lung, the thoracic cavity was opened and the trachea exposed. Ligatures were placed around the trachea both proximal and distal, an incision made between ligatures and a blunt ended hypodermic needle inserted which was then secured by the proximal ligature. The lungs were subsequently inflated with ~1.5mls Indian ink solution (15% (v/v) Indian ink, 0.5% (v/v) ammonia solution), the distal ligature tied, needle withdrawn and proximal ligature tied. The lungs were then dissected out, rinsed briefly in dH_2O, Fekete's solution and then stored in Fekete's solution. Lungs were stored for a minimum of 24 hours for bleaching and fixation before the lobes of the lungs were dissected under 70% ethanol, and metastatic deposits counted using a microscope. The metastatic deposits on the surface of the lung are visualised as white lesions against a black background of normal lung tissue.

V.3 *DNA METHODOLOGIES*

V.3.1 *Genomic DNA Extraction & Purification*

Cells were rinsed with sterile PBS before lysing in 10mls lysis buffer / 10^8 cells (lysis buffer:- 10mM NaCl, 10mM EDTA, 10mM tris-HCl, pH8.0) containing 4% (v/v) sarcosine and 100μg/ml proteinase K. This viscous solution was incubated overnight at 37°C, tumbling gently in 50ml Falcon tubes. One volume of TE- (10mM tris, 1mM EDTA, pH8.0) equilibrated phenol / chloroform was added to extract proteins and tumbled gently for 10-30mins. Centrifugation was carried out at 1500rpm for 30mins at room temperature. The aqueous phase was transferred to a fresh tube using wide bore pipettes and precipitated with one tenth volume 3M sodium acetate and 2.5 volumes ethanol. High molecular weight nucleic acids were spooled out using a hooked pasteur pipette (fashioned using a Bunsen flame), sequentially dehydrated in 70% ethanol, 95% ethanol and chloroform, then resuspended in 0.1x SSC (20x SSC:- 0.3M sodium chloride, 0.03M tri-sodium citrate, pH 7.0) at twice the original volume of lysis. The suspension was further incubated with 50μg/ml heat-treated RNase A, 1μg/ml RNase T1 for 3 hours at 37°C, tumbling gently, followed by another 3 hour incubation at 37°C with one tenth volume 0.1M EDTA, 4% (v/v) sarcosine and 50μg/ml proteinase K. Phenol / chloroform extraction was repeated twice as before, followed with precipitation and spooling out of high molecular weight DNA. The dehydrated DNA was resuspended in TE buffer to a concentration of 0.1 - 0.5mg/ml.

V.3.2 *Spectrophotometric Quantitation Of DNA*

The concentration of aqueous solutions of DNA was measured spectrophotometrically. Absorbance readings at 260nm and 280nm were taken of samples diluted in TE buffer using a quartz curvette with a 1cm pathlength. The spectrophotometer was calibrated with a TE blank. An absorbance value of 1 at

260nm was taken to be equivalent to 50μg/ml and an A_{260} / A_{280} ratio of 1.8 indicative of an essentially pure preparation of DNA.

V.3.3 *Restriction Enzyme Digestion Of DNA*

Restriction enzyme digestion of ~1μg plasmid DNA was typically carried out in a total volume of 10μl using 1-10 units of the appropriate enzyme with the manufacturer's supplied reaction buffer at 37°C for 1-2 hours. Digestion of genomic DNA (usually 50μg in reaction volume of 500μl), was essentially the same, except 3mM spermidine was added to the reaction and incubation was overnight. For digestion with more than one enzyme, manufacturer's information was consulted for choice of optimal reaction buffer. Reactions were ceased with one tenth volume 0.1M EDTA (pH 7.0). Genomic DNA digests were purified by phenol / chloroform extraction, followed with chloroform washes and concentrated by ethanol precipitation. Plasmid digests did not undergo purification and precipitation but were instead directly electrophoresed with the addition of 1x loading buffer (10x loading buffer: 50% (v/v) glycerol, 0.4% (w/v) bromophenol blue in TE buffer).

V.3.4 *Agarose Gel Electrophoresis*

DNA fragments were resolved according to apparent molecular weights on agarose gels. The percentage of agarose varied according to the size(s) of DNA of interest; generally, 1% (w/v) agarose dissolved in 1x TAE buffer (40mM tris acetate, 20mM sodium acetate, 2mM EDTA, pH 7.4) or 1x TBE buffer (90mM tris-base, 90mM boric acid, 2mM EDTA, pH 8.0) was used in preparation of Southern blotting procedures. For purification of DNA fragments, 1.5 - 2% (w/v) agarose gels were generally utilized. Once the agarose was dissolved in buffer by microwaving, the solution was allowed to cool to ~45 - 50°C before the addition of ethidium bromide at final concentration of 0.5 μg/ml. Gels were cast in the

appropriate gel former with appropriate well former and allowed to set at room temperature. Samples for electrophoresis were mixed with sample loading buffer, to a final concentration of 1x (10x loading buffer: 50% (v/v) glycerol, 0.4% (w/v) bromophenol blue in TE buffer), loaded into sample wells and electrophoresed in either 1x TAE or 1x TBE buffer as appropriate. Molecular weight markers were run in parallel, usually Hind III digested bacteriophage λ DNA and Hae III digested ϕX174. After electrophoresis, resolved DNA was visualised by UV transillumination and a permanent record kept as either a Polaroid photograph or photoimage.

V.3.5 *Southern Transfer & Hybridization*

Following electrophoresis of 15-20μg digested genomic DNA, as outlined above (II.3.4), the gel was washed for 2x 20mins in 1.5M sodium chloride, 0.5M sodium hydroxide at room temperature with slow shaking in order to denature the DNA. The gel then neutralized with washes in 1M ammonium acetate, 0.02M sodium hydroxide for 2x 30mins, at room temperature with slow shaking. Transfer to Hybond N+ was by capillary action and proceeded for a minimum of 16 hours using 1M ammonium acetate, 0.02M sodium hydroxide as transfer buffer. After transfer, the Hybond N+ membrane was briefly rinsed with 2x SSC (20x SSC: 3M sodium chloride, 0.3M sodium tri-citrate, pH 7.0), air dried, then UV crosslinked to fix the DNA to the membrane.

For hybridization, the membrane was pre-hybridized for a minimum of 4 hours with continuous shaking, at 42^{o}C in formamide hybridization buffer (50% (v/v) formamide, 4x SSPE [20x SSPE: 3.6M sodium chloride, 0.2M sodium phosphate, 0.02M EDTA, pH 8.3], 0.5% (w/v) Marvel, 10% (w/v) dextran sulphate, 1% (w/v) SDS) supplemented with 500μg/ml sonicated salmon sperm ssDNA, boiled just prior to use. After pre-hybridization, radioisotopically labelled probe was heated to

100°C and chilled on ice for 5mins before addition to the hybridization buffer. The membrane was subsequently hybridized for a minimum of 16 hours at 42°C with continuous shaking. Following hybridization, the membrane was washed under increasingly high stringency conditions with the following washes:-

2x 15mins at room temperature with 2x SSC, 0.1% (w/v) SDS

2x 30mins at 65°C with 0.5x SSC, 0.1% (w/v) SDS

1x 30-45mins at 65°C with 0.1x SSC, 0.1% (w/v) SDS.

The membrane was then exposed to X-ray film at -70°C for the appropriate times.

V.3.6 *Plasmid DNA Minipreparations*

Single bacterial colonies were picked from agar plates and grown overnight in 5mls L-broth supplemented with the appropriate antibiotic. 1.5mls of the overnight culture was pelleted in a benchtop microfuge at 14K for 10secs. The supernatant was discarded and the pellet resuspended in 100µl GTE (50mM glucose, 25mM tris-HCL, 10mM EDTA, pH 8.0) containing 4mg/ml lysozyme with vortexing and incubating at room temperature for 5mins. For alkaline lysis, 200µl of 0.2M sodium hydroxide, 1% (w/v) SDS was added, mixed gently and chilled on ice for 5mins. 150µl of 5M potassium acetate was added to precipitate proteins and detergent, mixed by inversion and chilled on ice for a further 5mins. Centrifugation was carried out for 5mins at 14K and 380µl of supernatant removed and further purified by phenol / chloroform extraction using equal volumes. The aqueous phase containing nucleic acids was then precipitated with 0.6 volumes *iso*-propanol, incubated at room temperature for 2mins, pelleted by centrifugation for 15mins at room temperature and the pellet allowed to air-dry. This pellet was finally resuspended in 50µl TE buffer and treated with 1µg heat-treated RNase A.

V.3.7 *Large Scale Plasmid DNA Preparations*

Large overnight cultures (500-1000mls) were extracted on the same principle as the method for small scale preparations (see II.3.6), but with some modifications. Cultures were pelleted in 500ml polypropylene bottles by centrifugation in a Sorvall GS3 rotor at 5K for 10mins at 4°C. The pellet was resuspended in 20mls GTE plus 5mg/ml lysozyme by pipetting and vortexing, then allowed to stand on ice for 30mins. 40mls of pre-chilled 0.2M sodium hydroxide, 1% (w/v) SDS was added, mixed gently by inversion and incubated on ice for 5mins. Precipitation of proteins and detergent was facilitated by the addition of 30mls 5M potassium acetate and incubation on ice for a further 15mins. Cellular debris and precipitate were pelleted by centrifugation at 8K for 10mins at 4°C. The supernatant was then filtered through a double layer of gauze and precipitated with 0.6 volumes of *iso*-propanol at room temperature for 15mins. Precipitate was collected by centrifugation for 10mins at 8K, 4°C, the pellet drained thoroughly before resuspension in 8.5mls TE buffer. Precisely 8mls of this crude plasmid DNA preparation was then added to 8g of caesium chloride in a plastic universal, transferred to a screw cap polycarbonate ultracentrifuge tube and balanced to within 0.05g of other tubes with paraffin oil. 0.5mls of 10mg/ml ethidium bromide was finally added and centrifugation carried out in a Sorvall T1270 rotor at 40K for 40-48 hours at 20°C. Plasmid DNA was carefully removed from the caesium chloride gradient and repeatedly extracted with caesium chloride-saturated *iso*-propanol to remove ethidium bromide before dialysis against TE buffer which was continued for 24 hours at 4°C, with 3-4 changes of buffer. Dialysed plasmid DNA was subsequently ethanol precipitated and resuspended in 1ml TE buffer. DNA concentration was estimated spectrophotometrically as described in V.3.2.

V.3.8 *Purification Of DNA Fragments From Agarose Gels*

DNA fragments were recovered from agarose by the methods of "Genecleaning" or centrifugation. The procedure of "Genecleaning" was according to the manufacturer's instructions. The method of centrifugation involved the excision of the appropriate band from the gel with the aid of UV illumination. This gel slice was then cut into smaller fragments and placed on top of a glass wool plug contained in a 0.5ml eppendorf tube. This 0.5ml tube was previously pierced at the base using a syringe. The plugged tube with the gel slice was then placed in a 1.5ml eppendorf tube and centrifuged for 5-10mins at 5K, room temperature in a benchtop microfuge. This resulted in the DNA being eluted from the agarose in the gel buffer and collected in the 1.5ml eppendorf. The agarose remained in the glass wool plug. The DNA was further purified by phenol / chloroform extraction and ethanol precipitated when necessary.

V.3.9 *Preparation & Quantitation Of Oligonucleotides*

Oligonucleotides were synthesized on an Applied Biosystems model 381A DNA synthesizer. 5' trityl groups were removed on the synthesizer before the oligonucleotide was eluted from the column in concentrated (29%) ammonia solution for 1.5 hours at room temperature. Oligonucleotides were deprotected by incubation of the oligonucleotides in ammonia contained in a sealed glass vial overnight at 55^{o}C. This was followed by ethanol precipitation with extensive washing of the pellet with 70% ethanol to remove salt. Oligonucleotides were resuspended in TE buffer and quantified spectrophotometrically. An A_{260} value of 1 was taken to equal 33μg/ml of oligonucleotide. A ratio of A_{260} / A_{280} was ~1.8 when sufficiently purified.

V.3.10 *Chain-Termination Sequencing*

Single strand sequencing was carried out using a commercially available kit "Sequenase version 2.0" marketed by United States Biochemical Corporation. This kit contains a genetic variant of bacteriophage T7 DNA polymerase which has had its 3'-5' exonuclease activity removed by genetic manipulation. The protocol followed was as manufacturer's instructions using the universal primer supplied (-40) which was suitable for sequencing the pUC19-based vector containing the insert of interest.

V.4 *RNA METHODOLOGIES*

V.4.1 *RNA Extraction, Purification & Quantitation*

In preparations of RNA and handling of RNA, some precautionary steps were taken in order to avoid degradation by contaminating RNases. All manipulations were carried out wearing gloves, tubes and solutions were pre - cooled on ice and centrifugations carried out at $4^{o}C$. All tubes were also pre - treated with diethylpyrocarbonate (DEPC), an irreversible inhibitor of RNases. The tubes were immersed in a solution of 0.1% (v/v) DEPC for a minimum of 2 hours in a fume hood, the solution decanted off and the tubes autoclaved followed by drying in an oven at $80^{o}C$. These tubes were subsequently stored in sealed DEPC - treated containers. Distilled water was also DEPC - treated: a 0.1% (v/v) solution was prepared, incubated overnight and autoclaved.

RNA extraction using RNAzol™B and purification was carried out according to the manufacturer's instructions. Quantitation was determined spectrophotometrically essentially as for DNA (see V.3.2), except calibration blank was DEPC treated dH_2O, A_{260} value of 1 was taken to be equivalent to 40μg/ml RNA and A_{260} / $_{280}$ ratio of 2.0 represented essentially pure RNA.

V.4.2 *RNA Electrophoresis*

RNA samples, typically 15-20μg for Northern analysis, were freeze-dried and resuspended in 3μl DEPC-treated dH_2O. Freshly prepared sample buffer (0.70mls formamide, 0.15mls 10x MOPS buffer [10x MOPS buffer: 200mM MOPS, 50mM sodium acetate, 10mM EDTA, pH7.0], 0.24mls formaldehyde, 0.1mls DEPC-treated dH_2O, 0.1mls glycerol, 0.05% (w/v) bromophenol blue), was added to the resuspended RNAs at 5x sample volume. RNA samples were denatured at 65°C for 15mins, chilled on ice for 10mins, followed with a brief microfuge and the addition of 1μl of 1mg/ml ethidium bromide. Electrophoresis was performed in a formaldehyde gel, typically of 1 - 1.5% (w/v) agarose concentration (1 - 1.5g agarose, 10mls 10x MOPS buffer, 87mls DEPC-treated dH_2O, heated in a microwave oven, cooled to ~50-55°C before the addition of 5.1mls formaldehyde). Electrophoresis running buffer was 1x MOPS buffer with recirculation using a peristaltic pump.

V.4.3 *Northern Transfer & Hybridization*

Following electrophoresis, Northern gels were washed 2x 30mins with dH_2O before capillary action transfer to Hybond N^+. Transfer buffer was 20x SSC, and transfer was allowed to proceed for a minimum of 16 hours. After transfer, the membrane was UV crosslinked then briefly rinsed with 2x SSC before pre-hybridization. Molecular weight markers were run in parallel, usually commercially available RNA ladders.

Hybridization buffer (5x SSPE [20x SSPE: 3.6M sodium chloride, 0.2M sodium phosphate, 0.02M EDTA, pH8.3], 5x Denhardts [50x Denhardts: 1% (w/v) ficoll, 1% (w/v) polyvinylpyrrolidone, 1% (w/v) bovine serum albumin (BSA)], 50% (v/v) formamide, 0.5% (w/v) SDS), was supplemented with 250μg/ml sonicated salmon sperm ssDNA, boiled just prior to addition, and incubated with the

membrane at 42°C for a minimum of 4 hours. Following pre-hybridization, radioisotopically labelled probe was heated to 100°C for 10mins then chilled on ice for 5 mins before addition to the hybridization buffer. The membrane was subsequently hybridized for a minimum of 16 hours at 42°C with continuous shaking. After hybridization, the membrane was washed under increasingly stringent conditions with the following washes:-

2x 20mins at room temperature with 2x SSC, 0.1% (w/v) SDS

1x 30-45mins at 65°C with 0.1x SSC, 0.1% (w/v) SDS

The membrane was then exposed to X-ray film at -70°C for the required times.

V.4.4 *cDNA Synthesis & Polymerase Chain Reaction (RT-PCR)*

cDNA synthesis was carried out as follows: 10μg of RNA was freeze-dried and resuspended in 20μl of annealing buffer (400mM potassium chloride, 10mM PIPES, 0.25 units/μl RNA Guard^R [an RNase inhibitor], pH 6.4). 100ng of the appropriate 3' primer was added, then incubated at 85°C for 5mins, followed with immediate incubation at 65°C for a further 15mins. The annealed product was then diluted in 200μl of extension buffer (50mM tris-HCL, 10mM dithiothreitol [DTT], 6mM magnesium chloride, 0.5mM dNTPs, 0.25 units/μl RNA Guard^R, pH 8.2) and divided into two aliquots: one aliquot for reverse transcription, the other a control without reverse transcription. For reverse transcription, 10 units of avian myeloblastosis virus (AMV) reverse transcriptase was added, both aliquots were then incubated at 42°C for 1 hour. Products and control were ethanol precipitated and resuspended in 10μl TE buffer. These were then ready for PCR.

Polymerase chain reaction (PCR) utilized the kit, GeneAmp PCR reagent kit, supplied by Perkin Elmer Cetus. The protocol followed the manufacturer's instructions using the appropriate 5' and 3' primers. For most reactions, PCR cycle

conditions were 25 cycles of:- denaturation at 94°C for 1min, annealing at 55°C for 1min, extension at 72°C for 3mins, followed by a final extension incubation at 72°C for 10mins then maintained at 4°C until analysis. When necessary, annealing temperatures were modified to optimise the PCR reaction, taking into account the Tm of oligonucleotide primers. Analysis of PCR products by agarose gel electrophoresis was as previously described (II.3.4). Confirmation of the products to be those which the primers were designed to amplify was established by Southern blotting techniques (II.3.5) and probing with another specific internal oligonucleotide (different to the oligonucleotides previously used as 3' or 5' primers).

V.5 *RADIOISOTOPIC LABELLING METHODS*

V.5.1 *Random Priming*

Random priming of probes for Northern and Southern analysis used the commercially available kit marketed by Boehringer Mannheim. The protocol followed was according to the manufacturer's instructions, incorporating the radioisotope [α-^{32}P] dCTP. Probes were separated from unincorporated radioisotope using Pharmacia Nick columns. An aliquot of probe was counted on a liquid scintillation counter to estimate specific activity and the probe heated to 100°C for 10mins and chilled on ice for 5mins before incubation with pre-hybridized membranes.

V.5.2 *Replacement Synthesis*

This method of labelling was employed for the technique of in-gel renaturation (see V.7.1). The properties of bacteriophage T4 DNA polymerase allowed for the retention of the original size of the DNA fragment, which is one criteria of absolute requirement for the success of in-gel renaturation. Limited 3' exonuclease

digestion of ~100 nucleotides of DNA fragments was achieved by the addition of 0.5μg of restriction digested DNA to 2μl of 5x react buffer (165mM tris-acetate [pH 7.9], 330mM sodium acetate, 50mM magnesium acetate, 2.5mM DTT, 0.5mM BSA), in a total volume of 9μl. 0.625 units of T4 DNA polymerase was added to the reaction, mixed and briefly microfuged, then incubated at 37°C for exactly 5mins. Immediately after incubation, reaction tubes were chilled on ice and 1μl of dNTPs (dATP, dTTP, dGTP, each at 2mM) and 30μCi [α-^{32}P] dCTP were added, mixed and briefly microfuged, then incubated at 37°C for 60mins. Following this resynthesis reaction, 1μl of cold 0.2mM dCTP was added in order to ensure complete resynthesis, incubation continued at 37°C for 20mins. Reactions were terminated by incubation at 70°C for 5mins. Separation of unincorporated radioisotope from labelled DNA was achieved with Nick columns and specific activity was estimated by counting an aliquot in a liquid scintillation counter.

V.5.3 *Klenow Labelling Of Double - Stranded Blunt Ends*

This method of labelling replaced the 3' hydroxyl group with [α-^{32}P] dNTP. The DNA to be labelled was diluted to 17.5μl in TE buffer; 2.5μl of 10x reaction buffer (any common restriction enzyme buffer sufficed), 50μCi [α-^{32}P] dCTP and 1 unit of Klenow enzyme were added to the reaction mixture and incubated at room temperature for 15mins. This was followed with 1μl of dNTP mix (dGTP, dCTP, dATP, dTTP; each of 2mM concentration) to fill in nicked ends; incubation at room temperature for 5mins. The reaction was terminated by heating to 70°C for 5mins. DNA was separated form unincorporated nucleotides by ethanol precipitation with one tenth volume 3M sodium acetate and 2.5 volumes ethanol. Centrifugation was carried out in a bench top microfuge for 45mins at 14K, the pellet washed with 70% ethanol, and air-dried before resuspension in TE buffer and an aliquot read in a scintillation counter.

V.5.4 *End Labelling Of Oligonucleotides*

In a reaction volume of 50µl, 300ng of oligonucleotide, 1x T4 polynucleotide kinase buffer, (final concentration) (10x T4 PNK buffer: 0.5M tris-HCL [pH 7.6], 0.1M magnesium chloride, 50mM dithiothreitol [DTT], 1mM spermidine, 1mM EDTA), 50µCi [γ-^{32}P] ATP and 20 units of T4 polynucleotide kinase enzyme were mixed. Incubation was carried out at 37^{o}C for 1 hour. The reaction was terminated with 2.5µl of 0.25M EDTA. Unincorporated nucleotides were removed from the oligonucleotide by ethanol precipitation as detailed above (II.5.3), and ^{32}P incorporation estimated using the scintillation counter.

V.6 *PROTEIN METHODOLOGIES*

V.6.1 *Whole Cell Protein Extraction*

Unless otherwise stated, whole cell protein extraction was carried out as follows:- Cells were grown to sub-confluence or to just confluence, in 10cm tissue culture dishes, using pre-chilled sterile PBS, cells were rinsed twice and the excess PBS aspirated off. Plates were immediately transferred onto ice and 0.5mls of SDS-PAGE sample buffer pipetted onto each plate (1x sample buffer: 20% (v/v) glycerol, 2% (w/v) SDS, 100mM tris pH6.8); and added fresh, 5% (v/v) β-mercaptoethanol). Cells were scraped using a disposable cell scraper and lysed cells transferred to pre-chilled screw cap eppendorf tubes. Samples were boiled for 5mins, chilled on ice and followed with sonication in order to shear high molecular weight DNA. After centrifugation at 14K for 2mins at 4^{o}C, supernatants were transferred in aliquots to fresh eppendorf tubes and stored at -70^{o}C until required.

V.6.2 *Nuclear Protein Extraction*

This procedure was essentially carried out as described by Andrews & Faller, 1991, utilizing hypotonic lysis of cells followed by a high salt extraction of the

nuclei. Adherent cells (~10^6 - 10^7) were washed once with cold PBS before the cells were scraped off with 1.5mls cold PBS and transferred to a pre-chilled screw-cap eppendorf tube. Cells were pelleted in a benchtop microfuge for 10s at 14K and resuspended in 400µl buffer A (10mM HEPES-KOH, pH7.9 at 4°C, 1.5mM magnesium chloride, 10mM potassium chloride and added fresh: 0.5mM dithiothreitol [DTT], 0.2mM phenylmethylsulphonyl fluoride [PMSF], 1µM leupeptin, 0.3µM aprotinin, 1µM pepstatin A). Cells were allowed to swell on ice for 10mins then vortexed for 10s. Samples were then centrifuged for 10s at 14K and the supernatant discarded. The residual pellet was resuspended in 100µl pre-chilled buffer B (20mM HEPES-KOH, pH7.9, 25% glycerol, 420mM sodium chloride, 1.5mM magnesium chloride, 0.2mM EDTA and added fresh: 0.5mM DTT, 0.2mM PMSF, 1µM leupeptin, 0.3µM aprotinin, 1µM pepstatin A) with gentle flicking of the tube and incubated in ice for 20mins for high salt extraction. Cellular debris was removed by centrifugation for 2mins at 14K at 4°C. The supernatant containing crude nuclear protein extract was aliquoted and stored at -70°C until required.

V.6.3 *Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)*

Proteins were resolved on SDS-polyacrylamide gels by electrophoresis according to their apparent molecular weights. Resolving gels were typically between 7 - 10% with respect to the acrylamide concentration (30% stock acrylamide solution : 29.6% [w/v] acrylamide, 0.4% [w/v] *bis*-acrylamide) and stacking gels ranged between 4 - 5% acrylamide. Acrylamide solutions of the required concentration were mixed with the appropriate gel buffer to a final concentration of 1x with dH_2O.

4x resolving gel buffer: 1.5M tris (pH 8.9), 0.4% (w/v) SDS

4x stacking gel buffer: 0.5M tris (pH 6.7), 0.4% (w/v) SDS

Prior to pouring of either resolving or stacking gels, 100µl of a 10% (w/v) ammonium persulphate solution and 10µl of TEMED were added per 10mls of resolving or stacking gel solution, briefly mixed by gentle swirling then poured between clamped vertical plates. For most SDS-PAGE procedures in this thesis, ATTO mini-gel apparatus was used which utilized plates giving gels of ~0.5mm thickness.

Resolving gels were overlaid with 1-2mls of butan-2-ol and allowed to polymerize at room temperature for 20 - 45 mins. Once polymerized, excess butan-2-ol was removed with 3MM Whatman filter paper and the appropriate comb inserted. The stacking gel was subsequently poured and allowed to polymerize for 15 - 20 mins. After polymerization, clamps and the sealing gasket were removed and the plates assembled in the gel tank. Both reservoirs were filled with 1x SDS-PAGE running buffer (10x SDS-PAGE running buffer: 522mM tris, 4% (w/v) glycine, 1% (w/v) SDS), the bubbles removed from the base of gels and the comb carefully removed. Using a needle and syringe, unpolymerized acrylamide was rinsed from the wells with running buffer before the samples were loaded.

Samples were denatured prior to loading by heating to ~100^{o}C for 3-5mins in sample buffer with β-mercaptoethanol and ~0.1% (w/v) bromophenol blue. Equal amounts of sample protein were added to each lane as calculated using a protein determination kit marketed by Pierce and as confirmed by visualization of a Coomassie brilliant blue stained back gel when gels were electrophoresed in duplicate.

Following electrophoresis, the front gel was used for Western transfer (see below, V.6.4) and the duplicate back gel was stained with Coomassie brilliant blue stain as follows: the gel was carefully removed from the plates and incubated at room temperature in staining solution (0.1% (w/v) coomassie brilliant blue in 45: 45: 10

(v/v) methanol: dH_2O: acetic acid) for 45-60mins with gentle shaking. Subsequently, the gel was destained in 45: 45: 10 (v/v) methanol: dH_2O: acetic acid with several changes of destain. Alternatively, gels were stained overnight in a weaker solution of 0.5% (v/v) coomassie brilliant blue in 45: 45: 10 (v/v) methanol: dH_2O: acetic acid, and destained the following day as above.

V.6.4 *Western Transfer & Blotting*

Protein extracts were resolved together with the appropriate molecular weight standards on SDS polyacrylamide gels. Western transfer to nitrocellulose membranes was carried out using one of two methods: semi-dry transfer (~1 hour) or wet transfer (overnight).

Semi-dry transfer was carried using a Sartorius semi-dry blotting apparatus according to the manufacturer's instructions. Twelve pieces of Whatman 3MM paper and a single piece of nitrocellulose were cut to the size of the gel and equilibrated in 1x transfer buffer (10x transfer buffer: 600mM tris, 500mM glycine, 16mM SDS) with 20% (v/v) methanol. The gel was rinsed in 1x transfer buffer and placed upon six pieces of pre-soaked Whatman 3MM, laid on top was the nitrocellulose and finally the remaining six pieces of 3MM. Air bubbles were carefully removed and transfer effected towards the anode at full power for 30-40mins. Transfer could be monitored if pre-stained markers were used; once the markers were transferred completely, the nitrocellulose could be either used immediately for blotting or alternatively, air-dried and stored at $4^{o}C$ until required.

Wet transfer was usually undertaken as transfer quality was consistently higher than semi-dry transfer. Bio-Rad transfer apparatus was used in this procedure. Four pieces of Whatman 3MM paper and one piece of nitrocellulose were cut to the size of the gel and equilibrated in Towbin (12.11g tris, 57.6g glycine, 800mls methanol, 1.2mls conc. HCl; volume made to 4l). The gel was rinsed in Towbin briefly and

placed upon two pieces of pre-soaked 3MM. Pre-soaked nitrocellulose was placed on top of the gel and then the remaining two pieces of pre-soaked 3MM. Air bubbles were removed carefully and this whole "sandwich" placed between the supplied sponges and sandwich plate of the wet transfer apparatus. This was then inserted into the tank filled with Towbin and transfer effected towards the anode overnight at 30 volts at $4^{o}C$. Following transfer, the nitrocellulose was either blotted immediately or air-dried and stored at $4^{o}C$ until required.

Efficiency of transfer was checked and location of non - pre-stained markers was determined by staining the nitrocellulose with 1x Ponceau S stain (10x: 2g Ponceau S, 30g trichloroacetic acid, 30g sulphosalicyclic acid, made to 100mls with dH_2O). 5 - 10mls of 1x stain was added to the nitrocellulose for ~3 - 5mins before rinsing with dH_2O until the protein bands became visible. Marker sizes were marked with a ballpoint pen and blotting continued as protocol. Ponceau S staining is temporary and is washed off during blotting washes.

Blotting of nitrocellulose membranes followed a standard protocol using the appropriate antibodies. First stage was blocking of the membrane for 1 hour in blotto (5% (w/v) non-fat milk powder [Marvel], 0.05% (v/v) Tween 20 in PBS) at $4^{o}C$ with continuous shaking. Primary antibodies were diluted in blotto at optimal concentrations as recommended by the suppliers (ECCD-2 -- 1:100, PCD-1 -- 1:50, D. Prowse, personal communication) and incubated with the membrane for 4 hours at $4^{o}C$ with continuous shaking. The membrane was subsequently washed 1x 15mins and 2x 5mins with blotto at room temperature, before incubation with the appropriate secondary horseradish peroxidase (HRP) conjugated antibody diluted in blotto at 1:2000. Incubation continued at $4^{o}C$ for 1 hour, again with continuous shaking. The membrane was finally washed extensively with at least two rinses and washes of 1x 15mins and 4x 5mins in PBS / 0.05% Tween 20. Detection used the enhanced chemiluminescence (ECL) kit as marketed by

Amersham International plc. Equal volumes of solution A and solution B were mixed and incubated on the membrane for ~1 min before briefly drying the membrane, covering with SaranWrap™ and exposing to X-ray film for 30s - 15mins.

V.6.5 *SDS-Substrate Gel Analysis Of Proteinases - Zymography*

The production of secreted proteinases by cells can be analysed by the method of zymography. Non-denaturing SDS-polyacrylamide gels are impregnated with substrate (such as gelatin or casein) and samples which may contain active enzymes are subjected to electrophoresis. After incubation to allow for enzymatic activity, the gel is stained and enzymatic activity is visualized as zones of clear areas against a stained background where the impregnated substrate has undergone degradation. The active enzyme can be identified according to the substrate used and its apparent molecular weight following electrophoresis.

Cells were plated in 6 well tissue culture plastic plates for 24 - 48 hours in normal medium (see V.1.1), and incubated at 37°C in 10% (v/v) CO_2. Medium was then replaced with serum-free DMEM supplemented with 0.5% BSA after 2 washes of cultured cells with sterile PBS; serum was removed from cultures since it was found to contain high amounts of the proteinases assayed for in this technique and produced too high a background level. Incubation continued for 24 hours, conditioned medium was then harvested and cleared by centrifugation at 1000rpm for 10mins at room temperature. Aliquots of conditioned medium could be stored at -70°C.

Metalloproteinases can be activated from their pro-enzyme form by treatment with 4-aminophenylmercuric acetate (APMA). APMA was kept as a stock solution of 0.02M in 0.5% sodium hydroxide. At a final concentration of 1mM, APMA was incubated with conditioned medium at 37°C for 1 hour. Non-denaturing sample

buffer was added to a final concentration of 1x (5x sample buffer: 10% (w/v) SDS, 50% (v/v) glycerol, 0.312M tris-HCL (pH 6.8), 0.05% (w/v) bromophenol blue), and a further incubation at 37°C for 10mins before electrophoresis. Molecular weight markers for non-denaturing SDS-PAGE were electrophoresed in parallel.

Substrate SDS-polyacrylamide gels were as described in II.7.4, except substrate was added to gels prior to pouring and polymerization at a final concentration of 1mg/ml. Stacking gels were 5% and resolving gels were 10%.

Following electrophoresis, the gel was incubated in 2% (v/v) triton X-100 for 30 mins at 37°C with gentle shaking to remove SDS. Incubation continued at 37°C for 18-24 hours with gentle shaking in substrate buffer (50mM tris-HCL [pH 8.0], 5mM calcium chloride) allowing for enzyme activity. The gel was finally stained at room temperature with gentle shaking for 45-60mins in coomassie blue stain (0.1% (w/v) coomassie blue R250 in 45: 45: 10 (v/v) methanol: dH_2O: acetic acid) and destained in 45: 45: 10 (v/v) methanol: dH_2O: acetic acid. Proteolytic activity indicated as clear zones.

V.6.6 *Immunocytochemistry*

Vectastain kits were used for the immunocytochemistry procedures in this thesis. Cells were grown to near confluence in plastic, 8 well chamber slides. Cells were washed in pre-chilled PBS, dipped once into ice-cold methanol (chilled in -20°C freezer overnight) then immediately tranferred to fresh, ice-cold methanol. Cells were allowed to fix for 20mins. After fixing, the cells were allowed to air-dry for ~20mins before blocking with the vectastain supplied blocking serum (1 drop serum per ml of 0.1% (w/v) BSA in PBS). Incubation was carried out at room temperature for 30mins in a moist chamber. Blocking solution was then drained off and the primary antibody added at the appropriate concentration in 0.1% (w/v) BSA in PBS to the cells. Incubation continued for 1 hour in a humidified

atmosphere at room temperature. Cells were subsequently washed 3x 10mins in wash buffer (0.05% (v/v) Tween 20, 0.15M sodium chloride in PBS, pH 7.6), and the cells incubated with the secondary peroxidase-conjugated antibody (1 drop of antibody supplied in the kit per 10mls blocking solution) for 1 hour in a moist chamber at room temperature. The washes were repeated as before, followed with incubation with the ABC reagent: 1 drop of solution A and 1 drop of solution B in 5mls blocking solution. Incubation continued for 1 hour in a moist chamber at room temperature. Washes were again repeated as before and stain developed with peroxidase substrate (1 tablet (10mg) of 3,3'-diaminobenzidine hydrochloride (DAB), dissolved in 16.7 mls of distilled water with 10µl hydrogen peroxide added just prior to use). Incubation continued in complete darkness for 7.5mins. The reaction was terminated by washing the slides in distilled water and the slides mounted using an aqueous mountant. Negative controls were as above with the omission of the primary antibody: incubation was with blocking solution only.

V.6.7 *Immunofluorescence*

Cells were grown on plastic, 8 well chamber slides to near confluence. The medium was removed and the cells were washed twice with PBS. The cells were then fixed in ice-cold acetone for 30mins and allowed to briefly air-dry before incubation with blocking solution (1.5% (w/v) BSA in PBS) for 5-10mins at room temperature. Using the appropriate concentrations, the primary antibody was incubated on the cells for 1 hour in a moist chamber at room temperature (ECCD-2 used at 1:500, NCAM-OB11 used at 1:100). Following incubation with the primary antibody, cells were washed three times in blocking solution before incubation with the secondary FITC-conjugated antibody diluted in blocking solution at the manufacturer's recommended dilution, for 1 hour at room temperature in a moist chamber. Finally cells were washed with PBS and a coverslip mounted with aqueous mountant. Staining was visualized with a UV

microscope. Negative controls followed the same procedure in parallel, with the omission of the primary antibody; incubation was with blocking solution only.

V.6.8 *Phosphoamino Acid Analysis Of Whole Cell Lysates*

Phosphoamino acid analysis was carried out according to the method described by Cooper, Sefton & Hunter, 1983, as follows:- cultures of exponentially growing, sub-confluent CMT 167/C6 and CMT 170/E9 cell lines were rinsed twice with pre-warmed phosphate-free DMEM supplemented with 10% (v/v) newborn calf serum and 2mM glutamine. To each culture in T25 flasks, 2mls of fresh phosphate-free medium was added and incubation continued at 37°C for 1 hour. After phosphate starving of the cells, 1mCi/ml of carrier-free ^{32}P-*ortho*phosphate was added to cultures and labelling of the cells continued for 18-20 hours at 37°C. Cultures were then transferred to ice and washed twice with pre-chilled PBS before lysis in 1ml of modified lysis buffer (10mM tris-HCl (pH 7.0), 150mM sodium chloride, 1% (v/v) NP40, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 2mM EDTA, 10mM sodium pyrophosphate, 10mM β-glcerophosphate, 10μM sodium *ortho*vanadate, 2mM PMSF, ~1μg/ml aprotinin). Cells were incubated on ice for 10mins then scraped using disposable cell scrapers and the cells incubated for a further 10mins on ice to allow for complete solubilization of adherent structures. Lysed cells were subsequently transferred to pre-chilled screw-cap eppendorfs and lysates centrifuged at 4°C at 14K for 20mins in a Sorvall SS34 rotor. From the supernatant, 300μl was carefully pipetted off and transferred to a fresh eppendorf containing 400μl NTE (10mM tris-HCl (pH 7.5), 100mM sodium chloride, 1mM EDTA) and 400μl NTE-saturated phenol at room temperature. Tubes were capped tightly and vortexed for 30 secs before centrifugation for 1min in a bench top microfuge at 14K at room temperature. The aqueous phase was discarded and the phenol phase extracted with a further 800μl NTE. The phenol phase and interface material was then transferred to a 30ml Corex tube, to which 13mls dH_2O and

2mls 100% TCA were carefully added. After careful thorough mixing, the tubes were placed on ice for 1 hour to allow for precipitation. The precipitated material was collected by centrifugation at 8K for 10mins at 4°C in a Sorvall SS34 rotor. The supernatant was decanted off and the pellet extracted with 5mls chloroform / methanol (2:1) at room temperature with vortexing. Centrifugation was repeated as above but at room temperature. Supernatant was decanted off and the pellets allowed to air-dry before being dissolved in 200µl 6M HCl, then transferred to Wheaton vials with 2x 100µl 6M HCl washes. Vials were sealed and hydrolysis carried out for 2 hours at 110°C. Samples were allowed to cool to room temperature before all the liquid was collected at the bottom of the vials. The vials were then broken open and the hydrolysate diluted with an equal volume of distilled water and transferred to screw-cap eppendorfs before drying overnight under vacuum in a desiccator over solid sodium hydroxide and phosphorus pentoxide. Dried residues were washed twice by redissolving in 200µl dH_2O and drying under vacuum. Dried residues were finally redissolved in 30µl pH 1.9 buffer (98-100% formic acid: glacial acetic acid: dH_2O, at ratios 45: 156: 1799) and stored at -20°C.

Thin layer separation of phosphoamino acids was carried out as follows:- approximately 30-50% of hydrolysates were spotted onto the origin of single 20 x 20cm cellulose thin layer plates (Kodak, 100µm) together with 0.5 µg of each unlabelled standards (phosphoserine, phosphothreonine, phosphotyrosine) in 0.3 - 0.5µl aliquots. Hydrolysates were resolved by electrophoresis at pH 1.9 towards the anode at a constant 700volts for 2 hours. After thorough drying, the plates were rotated 90° and electrophoresis carried out at pH 3.5 (pH 3.5 buffer:- pyridine: glacial acetic acid: dH_2O, at ratios 10: 100: 1890) towards the anode at a constant 600volts for 1 hour 40mins. The thin layer plate was maintained below 10°C during electrophoresis using a block cooled with tap water circulating at

approximately 1 litre / min. Following electrophoresis, the plate was dried thoroughly before the standards were visualized using ninhydrin (0.2% in ethanol, developed at 80^{o}C). Dried plates were subsequently exposed to X-ray film.

A quantitative estimate of the relative abundance of the individual phosphoamino acids was taken after separation of total cell hydrolysates. Following radiography, the areas of cellulose containing the ninhydrin stained standards, together with the appropriate background areas were scraped and collected using a glass wool-plugged 1ml pipette tip attached to a vacuum line and the collected material transferred to a scintillation vial containing 10mls Ecoscint and vortexed vigorously before counting for 10mins. Abundance was expressed as a percentage of total radioactivity incorporated into the three phosphohydroxyamino acids.

V.6.9 *2-D Gel Analysis*

Cells were grown to sub-confluence in 6 well tissue culture plastic plates. The medium was aspirated off and the cells rinsed twice with sterile PBS before incubation of the cells with methionine-free DMEM containing 200μCi / ml [^{35}S] methionine for four hours at 37^{o}C in a humidified atmosphere. Following methionine labelling, cells were lysed in 150μl sample buffer (9.9M urea, 4% (v/v) NP-40, 2.2% (v/v) ampholytes pH 3-10 / 2D, 100mM DTT) and the acid insoluble counts determined. The 2-D gel analysis was expertly performed by L. McGarry as follows:- first dimension gels contained: 9.5M urea, 2% (v/v) NP-40, 4.1% (v/v) acrylamide (from stock acrylamide solution containing 30.8% (w/v) acrylamide, 2.6% (w/v) bis-acrylamide), 2% (v/v) ampholytes pH 3-10 / 2D and polymerized with 0.67% (w/v) ammonium persulphate. These gels were overlayed with: 0.5M urea, 0.2% (v/v) NP-40, 0.1% (v/v) ampholytes pH 3-10 / 2D, 5mM DTT, 0.7M β-mercaptoethanol, then prefocused to 2000V using a constant current of 110μA per gel. Samples were then applied beneath the

overlay buffer; each sample contained 380 000cpm in 50µl sample buffer. Focusing was carried out for 18000volts-hours (17.5 hours at 1000volts then 30mins at 2000volts), after which the gels were extruded into gel equilibriation buffer (0.3M tris-base, 75mM tris-HCl, 3% (w/v) SDS, 50mM DTT, 0.001% (w/v) bromophenol blue) and allowed to equilibriate for 2mins before being placed horizontally on the upper surface of a vertical SDS polyacrylamide gel (10% (v/v) acrylamide/bis-acrylamide (stock: 30.8% / 2.6%), 0.37M tris pH 8.8, 0.1% (w/v) SDS and polymerized with 0.05% (v/v) TEMED and 0.025% (w/v) ammonium persulphate). Electrophoresis continued for ~4 hours at a constant power of 16W per gel. All 2-D gel chemicals were supplied by Millipore (UK) Ltd.

V.7 *OTHER TECHNIQUES*

V.7.1 *In-Gel Renaturation Analysis Of Gene Amplification*

Analysis of gene amplification was carried out without the prior specification of particular genes to be analysed (such as would be detected by Southern blotting techniques, see V.4.5), but instead utilized methods by which DNA sequences were detected as a consequence of being reiterated. The method of in-gel renaturation was essentially followed as originally described by I.B. Roninson (1987). A diagrammatic illustration of this technique is given in Figure 25.

Solution A: 2.5M sodium hydroxide, 3M sodium chloride

Solution B: 1M sodium phosphate (pH7.0), 3.6M sodium chloride, 2mM EDTA

Solution C: deionized formamide

Solution D: 0.5M sodium acetate, 2M sodium chloride, 10mM zinc sulphate, (pH4.6)

Solution E: 10% (w/v) SDS

Genomic DNA of interest was digested with restriction enzymes as described previously (V.3.3) and 0.5μg of this digested DNA was subsequently radioisotopically labelled with ^{32}P by replacement synthesis (described in section V.5.2) using T4 DNA polymerase. This tracer DNA had to be of high specific activity (~2x 10^7 cpm/μg, or greater) for efficient in-gel renaturation. Equal amounts of tracer DNA was mixed with excess (15μg) cold driver DNA (the same restriction digested DNA but unlabelled), and loading buffer added to 1x (10x loading buffer: 0.4% bromophenol blue, 50% glycerol in TE buffer). Samples were thoroughly mixed and briefly microfuged before loading onto gel. The gel was 1% agarose in 1x electrophoresis buffer (80x electrophoresis buffer: 1.8M tris-base, 0.8M sodium acetate, 0.08M EDTA, adjusted to pH 8.3 with acetic acid) with 0.5μg/ml ethiudium bromide, final concentration. Specific dimensions of the gel were required: 390mm x 190mm x 4mm. Electrophoresis was carried out for ~30 mins at 120volts then continued for 18-20 hours at 75volts with the electrophoresis buffer continuously recirculated using a peristaltic pump.

Following electrophoresis, resolved DNA was visualized by UV transillumination and a permanent record kept as either a Polaroid photograph or a photoimage. The gel was then placed into an air-tight perspex box and incubation commenced with 350mls of pre-warmed (45°C) denaturation buffer (solution A: dH_2O, at ratios 1:4) in a rotor incubator rotating gently at ~30-40 rotations per minute and at a temperature of 45°C for 35mins. This incubation was repeated once with fresh denaturation buffer before being replaced with 350mls of pre-warmed renaturation buffer (solution B: formamide: dH_2O, at ratios 1: 2: 1) and incubation continued for 20mins. These renaturation washes were repeated 5 times and the pH of the gel measured using a pH stick. The gel required to be less than pH 8 before incubation of the gel overnight in fresh renaturation buffer. The following day, the incubation temperature was lowered to 37°C and the gel then incubated with 5 x 15min

washes with 350mls of pre-warmed S1 nuclease buffer (solution D: dH_2O, at ratios 1:9). Once the gel was equilibriated in S1 nuclease buffer, single stranded DNA was digested within the gel with 14000 units S1 nuclease in 250 mls buffer. Incubation continued at 37°C with gentle shaking for 2 hours. The second round of in-gel renaturation was then started; the incubation temperature was raised to 45°C and the gel incubated with denaturation buffer as before. Again, the renaturation steps were repeated and the S1 nuclease digestion as before. Following the second S1 nuclease digestion, the gel was incubated at 37°C with 500mls of pre-warmed elution buffer (solution B: solution E: dH_2O, at ratios 15: 1: 84) to elute digested single stranded DNA. Incubation continued for 50mins followed with a further wash in elution buffer for 1 hour 45mins. Finally the gel was removed from the sealed box and dried down on 3MM Whatman paper and the dried gel exposed to X-ray film for the appropriate times at -70°C.

V.7.2 *Electrophoretic Mobility Shift Analysis (EMSA)*

Double-stranded probes were prepared as follows:- 10μg of complementary oligonucleotides were mixed in 100μl of TE buffer and heated to 90°C before allowing to slowly cool to room temperature, thus allowing for oligonucleotide annealing. Double-stranded oligonucleotides were radiolabelled as described previously (V.5.4) and purified over a Nick column followed by PAGE. The labelled oligonucleotide was visualized by autoradiography and the appropriate band excised and eluted into 1ml of TE buffer by incubation at 37°C overnight.

The DNA:protein binding reaction was carried out as follows:- the appropriate volume of nuclear extract (typically containing 10μg protein, see section V.6.2) was incubated with 6μg poly (dI.dC).(dI.dC), 5μl gel shift buffer (final concentration 10mM Hepes, pH 8.0, 0.5M EDTA, 100mM sodium chloride, 10mM magnesium chloride, 1mM dithiothreitol, 10% (v/v) glycerol, 0.1mg BSA),

approximately 500pg labelled oligonucleotide probe and dH_2O to a total volume of 25µl. Reactions were incubated at room temperature for 20mins before loading onto a pre-run (1hr at 25mA) 6% acrylamide gel with 0.5% TBE. Running buffer was 0.5% TBE. Electrophoresis continued for approximately 3 hours at 25mA then the gel dried down and exposed to X-ray film.

V.7.3 *Dye Transfer Assessment Of Gap-Junctional Communication*

Efficiency of gap-junctional communication was assessed in cultured cells by the method of microinjection of a fluorescent dye. Cells were cultured to sub-confluence in 60mm dishes using standard techniques (see V.1.1). The culture medium was subsequently replaced with medium buffered with 25mM HEPES, and the dish placed onto a $37^{o}C$ temperature controlled microscope stage in preparation for microinjection. Individual cells were selected and iontophoretically injected with 4% lucifer yellow CH dye using microelectrodes made from "Kwik-fill" thin wall glass capillaries. Injection continued for 2 mins with a current of 10nA at 0.5sec pulses at 1Hz and was monitored on a Leitz Diavert inverted microscope with UV (epillumination) or visible (phase-contrast) light sources. The extent of dye spread to adjacent cells was photographically recorded.

RESULTS

CHAPTER VI *BIOLOGICAL CHARACTERIZATION*

VI.1 *In Vivo Confirmation Of Metastatic Phenotypes Of The CMT Model*

As described earlier (see section III.3.5), this model system for the analysis of the metastatic phenotype was founded upon two clonal murine cell lines which significantly differed in their metastatic potentials. The cell line CMT 167/C6 had a high metastatic potential and CMT 170/E9 had a low metastatic potential as assayed by the spontaneous metastasis assay (Layton & Franks, 1984). Since one of the crucial parameters of this model system was the differing metastatic potentials, the cell lines were periodically tested *in vivo* for the assessment of their metastatic abilities. In addition, to minimize phenotypic drift due to prolonged *in vitro* culture, cell lines were not maintained beyond passage 25.

Initially, the facilities at the Beatson Institute did not routinely house C57B/T animals, the original syngeneic mouse strain which is maintained at the Imperial Cancer Research Fund (ICRF) laboratories. Due to the different "in-house" viruses, simple importing of these animals to our facilities as a long term arrangement was problematic. It was concluded that alternative mouse strains may suffice for the analysis of the metastatic abilities of the cell lines and this idea was tested.

A closely related in-bred mouse strain C57B/6J and athymic nude mice were chosen for comparison, and these were already routinely housed and bred at the Beatson Institute. Thirty C57B/T animals were obtained from the ICRF and kept isolated in containment units throughout the initial experiment. The spontaneous metastasis assay was performed as described in materials and methods (section V.2). For each cell line, fifteen animals of each of the three mouse strains were subcutaneously (s.c.) injected in the right flank with 5×10^5 cells. The formation of primary tumours was closely monitored and the animals sacrificed between 3 - 5 weeks post - injection. Measurements of the primary tumour were

recorded and plotted against time (see Figure 4) and the number of metastatic deposits counted on the surface of the lungs (see Figure 5), which were prepared as described in materials and methods (section V.2).

It became clear that the tumorigenic potentials, as measured by the rate of tumour growth, and metastatic potentials of these two cell lines in the different strains of mice varied substantially. The high tumorigenicity and relatively low metastatic potentials exhibited in nude mice has been documented before by many different workers using numerous cell lines (Gioranella & Fogh, 1985; and references therein), it should therefore not have been particularly surprising that this was also the finding here (see Figures 4C and 5C). The greater metastatic potential of the CMT 167/C6 cell line was clearly evident in C57B/6J animals (see Figure 5B). However, the tumorigenic potentials of the two cell lines clearly diverged: CMT 170/E9 primary tumours largely remained below $1000mm^3$ in volume whereas CMT 167/C6 primary tumours ranged in sizes between 1000 - $3000mm^3$ (see Figure 4B). Injection into syngeneic animals, C57B/T, revealed a CMT 167/C6 cells exhibiting at least a ten-fold increase in the number of metastatic deposits found in the lungs when compared to CMT 170/E9 cells at equivalent tumour burdens (see Figure 5A) and between 4 - 5 weeks, tumour burdens were approaching equivalence (see Figure 4A).

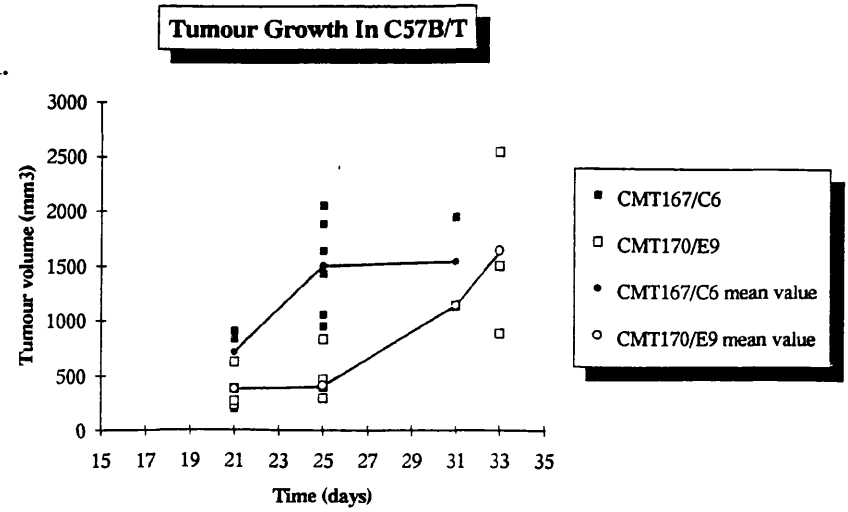
As it was considered that for a valid comparison of these two cell lines for their metastatic abilities, the tumorigenic potentials should be comparable such that the results obtained from differing metastatic abilities reflects metastatic potential and not tumorigenic potential. With this consideration, it was decided that C57B/T animals should be used for all future experiments and consequently this mouse strain was caesarian - derived and established at the Beatson Institute. This would have been a particularly vital consideration if this work had extended to the manipulation of the cell lines in an attempt to alter their metastatic abilities.

Figure 4

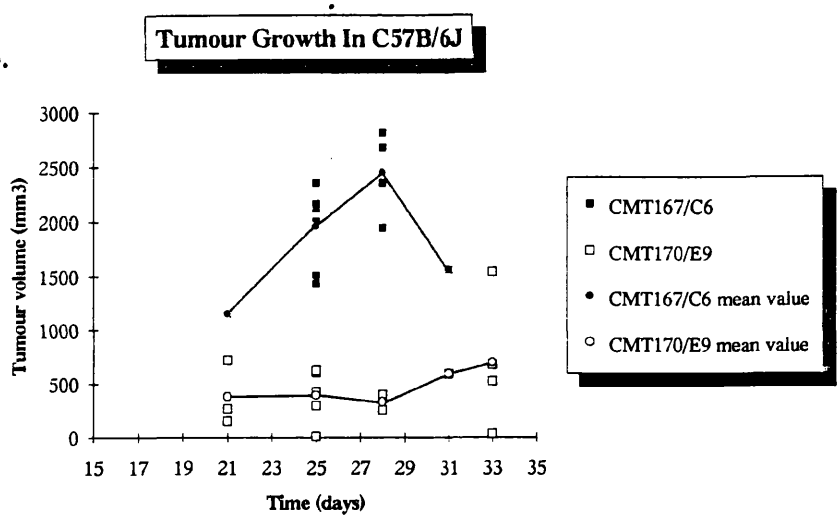
Figure 4. *Comparison Of CMT 167/C6 And CMT 170/E9 Primary Tumours In Different Mouse Strains*

Scatter graphs of primary tumour volume are plotted against time. Each point represents an individual animal sacrificed at the indicated time following subcutaneous injection of $5x10^5$ cells in 0.5mls. Graphs A, B and C represent different mouse strains as indicated. For comparison, average tumour volumes for each cell line are indicated, at the appropriate time point, as line graphs.

A.


Tumour Growth In C57B/T
Tumour volume (mm3)
3000
2500
2000
1500
1000
500
0
15 17 19 21 23 25 27 29 31 33 35
Time (days)
CMT167/C6
CMT170/E9
CMT167/C6 mean value
CMT170/E9 mean value

B.


Tumour Growth In C57B/6J
Tumour volume (mm3)
3000
2500
2000
1500
1000
500
0
15 17 19 21 23 25 27 29 31 33 35
Time (days)
CMT167/C6
CMT170/E9
CMT167/C6 mean value
CMT170/E9 mean value

C.

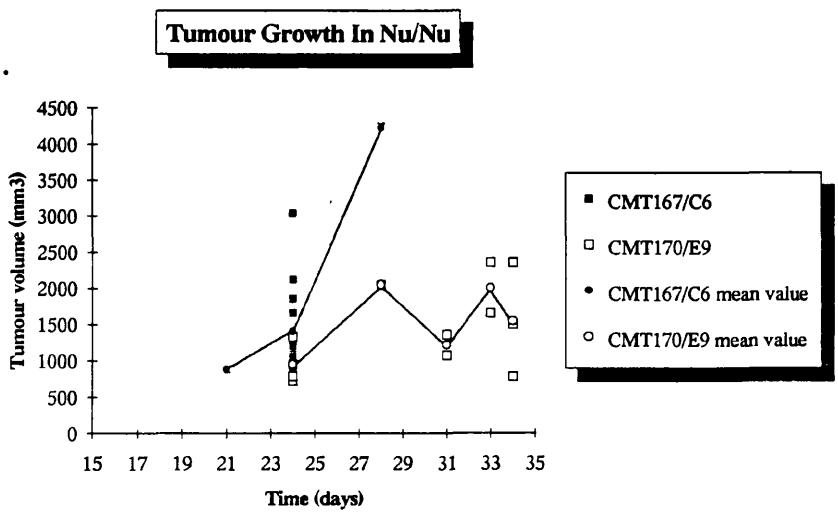
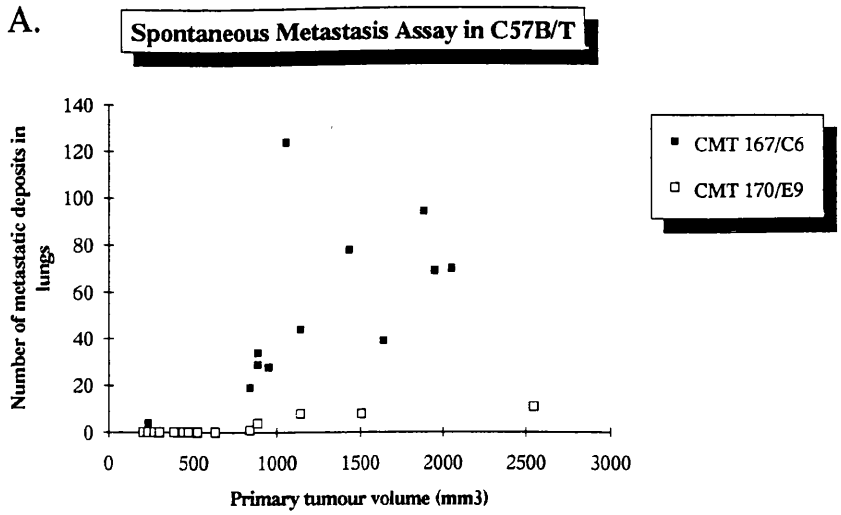

Tumour Growth In Nu/Nu
Tumour volume (mm3)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
15 17 19 21 23 25 27 29 31 33 35
Time (days)
CMT167/C6
CMT170/E9
CMT167/C6 mean value
CMT170/E9 mean value

Figure 5

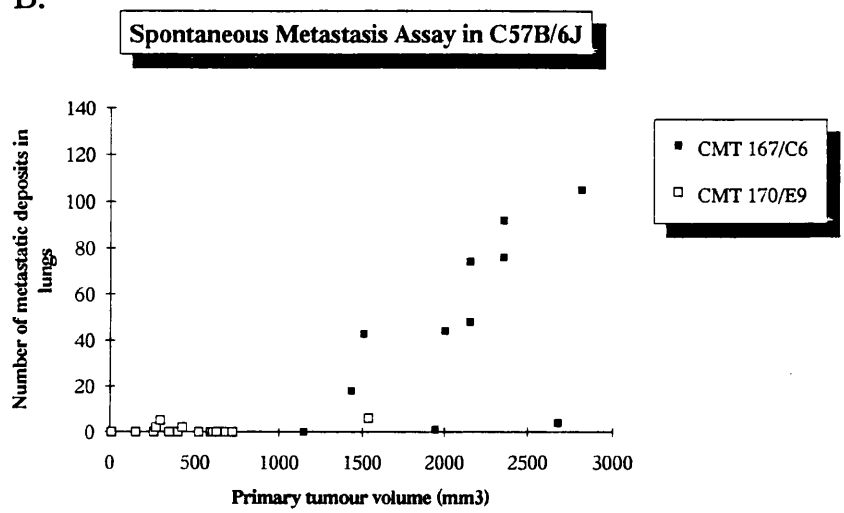
Figure 5. *Comparison Of Metastatic Potentials Of CMT 167/C6 And CMT 170/E9 Cell Lines In Different Mouse Strains*

Scatter graphs of the number of metastatic deposits counted on the surface of excised lungs plotted against the primary tumour volume at the time of sacrifice. Graphs A, B and C represent different mouse strains as indicated. Each point represents an individual animal.

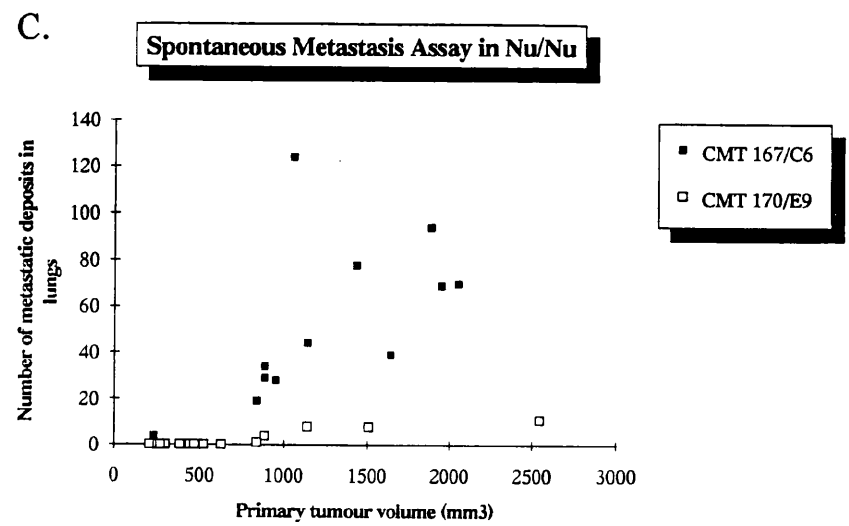
A.


Spontaneous Metastasis Assay in C57B/T
Number of metastatic deposits in lungs
0
20
40
60
80
100
120
140
0
500
1000
1500
2000
2500
3000
Primary tumour volume (mm3)
CMT 167/C6
CMT 170/E9

B.


Spontaneous Metastasis Assay in C57B/6J
Number of metastatic deposits in lungs
0
20
40
60
80
100
120
140
0
500
1000
1500
2000
2500
3000
Primary tumour volume (mm3)
CMT 167/C6
CMT 170/E9

C.


Spontaneous Metastasis Assay in Nu/Nu
Number of metastatic deposits in lungs
0
20
40
60
80
100
120
140
0
500
1000
1500
2000
2500
3000
Primary tumour volume (mm3)
CMT 167/C6
CMT 170/E9

However, the research detailed in this thesis served to characterize the cell lines in an attempt to explore and gain further insight into the metastatic phenotype as exhibited in this particular model system. The *in vivo* metastasis analyses of these cell lines confirmed previous findings about their respective phenotypes and further studies were therefore undertaken with the confidence that this model system would be effective for the analysis of the metastatic phenotype and one which would hopefully provide a unique insight.

VI.2 *In Vitro Characteristics Of The CMT Model*

The CMT cell lines CMT 167/C6 (high metastatic potential) and CMT 170/E9 (low metastatic potential) of this metastasis model system have not been previously characterized extensively, particularly with respect to the various biological characteristics associated with the metastatic phenotype (see Chapter I), such as cell adhesion, nor of genetic alterations such as inappropriate expression of particular putative metastasis-associated genes (see Chapter II). Since this is a relatively novel system, it was important to establish which factors that have been previously reported to correlate with the metastatic phenotype in other metastasis model systems, which may be contributory to the differing metastatic phenotypes of this model, and also to attempt to identify further deciding factors necessary for the metastatic phenotype. This characterization therefore serves as a foundation for future research into metastasis using this model system.

The biological characterization of the CMT murine model will be detailed in this chapter, and the following chapter (Chapter VII) will present the results from the molecular genetics analyses.

VI.2.1 *Anchorage-Independent Growth*

For many years now, it has been a recognized phenomenon that malignantly transformed cells have greater anchorage-independence than most normal cells (Folkman & Moscona, 1978). The underlying reason for this phenomenon has not been elucidated, however a recent hypothesis has evolved from evidence which suggests that anchorage-dependent cells are susceptible to apoptosis when induced to detach from matrix substrate by disruption of integrin-mediated cell adhesion with RGD peptides or by maintaining cells in suspension thereby preventing cell attachment (Meredith *et al.*, 1993; Frisch & Francis, 1994). Therefore the transformed cell may overcome the apoptotic pathway by alleviating the requirement for cell adhesion to the substratum, thus conferring anchorage-independence and initiating the malignant transformation of the cell (Ruoslahti & Reed, 1994).

Since anchorage-independent growth can be readily tested in soft agar assays (see Methods V.1.5), and is a classical test of the transformed phenotype, both CMT 167/C6 and CMT 170/E9 cell lines were assayed, together with a positive control of Swiss 3T3 cells transfected with a temperature sensitive *src* mutant, LA29 (a kind gift from M. Unlu, Beatson Institute). Following incubation at the appropriate temperatures, both CMT cell lines were seen to form tight colonies within the soft agar (see Figure 6). It was noted that both CMT cell lines took longer to establish as colonies than the control cell line, this most probably reflected the differing doubling times of the different cell lines (M. Unlu, personal communication). As expected, both cell lines exhibited anchorage-independent growth in soft agar.

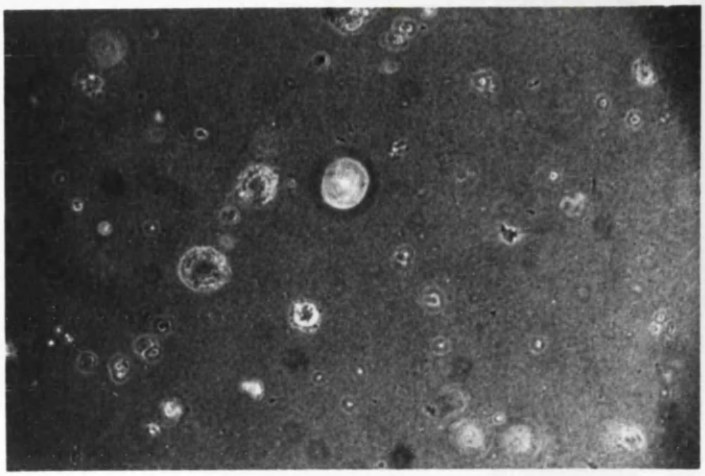
Figure 6

Figure 6. *Soft Agar Assays For Anchorage-Independent Growth*

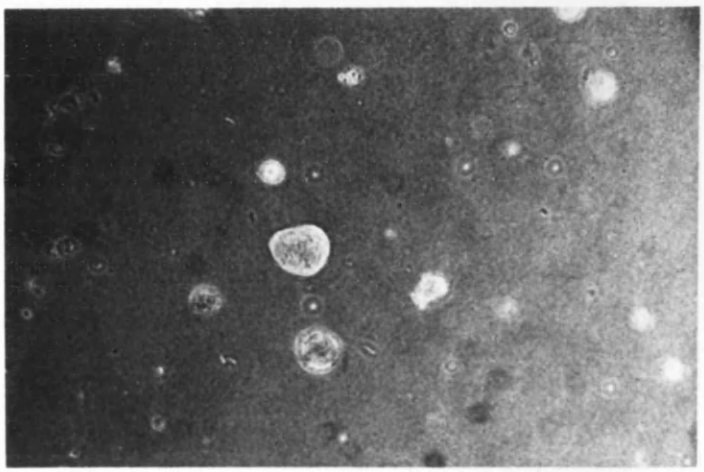
$2x10^5$ cells were plated in soft agar (0.3%(w/v) final concentration) as described in section V.1.5, and incubated for two weeks at the appropriate temperatures: CMT cell lines were incubated at 37°C, and positive control cells, Swiss 3T3 f29 cells were incubated at 35°C. Swiss 3T3 f29 cells have been transfected with a temperature sensitive *src* which is permissive at 35°C (a kind gift from M. Unlu, Beatson Institute).

Magnification x 125

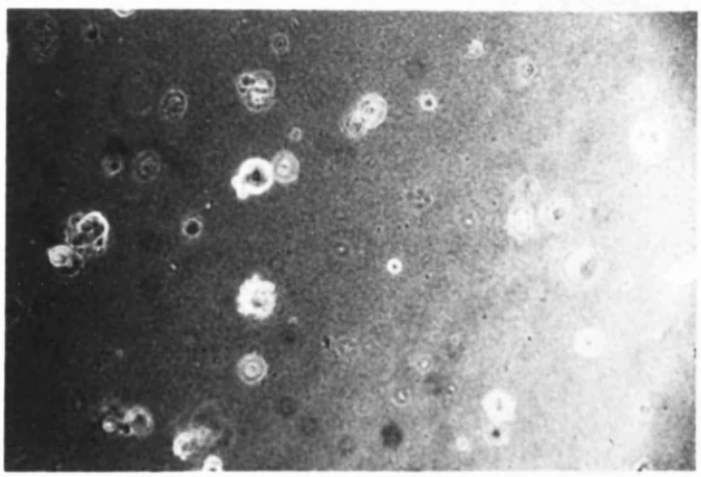
Soft Agar Assays for Anchorage-Independent Growth



CMT 167/C6



CMT 170/E9



Swiss 3T3 f29
(35°C)

VI.2.2 *In Vitro Growth Curves Of The CMT Cell Lines*

In order to address the question of whether a contributory factor to the cell lines differing in their *in vivo* characteristics was directly related to their growth characteristics *in vitro*, the cell lines were examined for their growth patterns and doubling times by MTT assay.

Standard growth curves were established for each cell line over a period of seven days using the MTT assay which estimates viable cell number, as described in section V.1.6. Replica plates for each time point were seeded simultaneously and incubated as described. Assays were performed in triplicate. Figure 7 illustrates typical growth curves of both CMT 167/C6 and CMT 170/E9 cell lines seeded at 10^3 cells / ml. From these assays, it is apparent that both cell lines have similar growth curves, although the CMT 167/C6 cell line had a slight increase in cell numbers during logarithmic growth over the CMT 170/E9 cell line. From these studies, it would appear that the minimum of a ten-fold difference in metastatic abilities observed of these cell lines *in vivo* (see section VI.1) cannot be solely attributed to any differing growth rates of the cell lines *in vitro*. This therefore substantiates the proposal that these two cell lines possess other intrinsic differences which are related to the metastatic phenotypes.

VI.2.3 *Morphology Of CMT Cell Lines Plated On Matrix Components*

During the course of this research, both CMT cell lines were observed to exhibit altered morphology when cultured on different matrix components. Previous published literature concerning these cell lines describe routine culturing of the CMT cell lines directly on tissue culture grade plastic; this was therefore a new observation. Both CMT 167/C6 and CMT 170/E9 cell lines display similar morphology on both untreated tissue culture plastic and on fibronectin-coated tissue culture plastic (see section V.1.3) with cells generally tightly packed (see

Figure 7

Figure 7. *Growth Curves Of CMT Cell Lines In Vitro*

CMT 167/C6 and CMT 170/E9 cell lines were seeded at 10^5 cells/ml in 96 well plates and incubated at 37^oC in a humidified atmosphere for 1 - 7 days before viable cell numbers were estimated by MTT assay. Assays were performed in triplicate and the average values plotted on a line graph of viable cell numbers against time.

Growth Curves Of CMT Cell Lines *In Vitro*

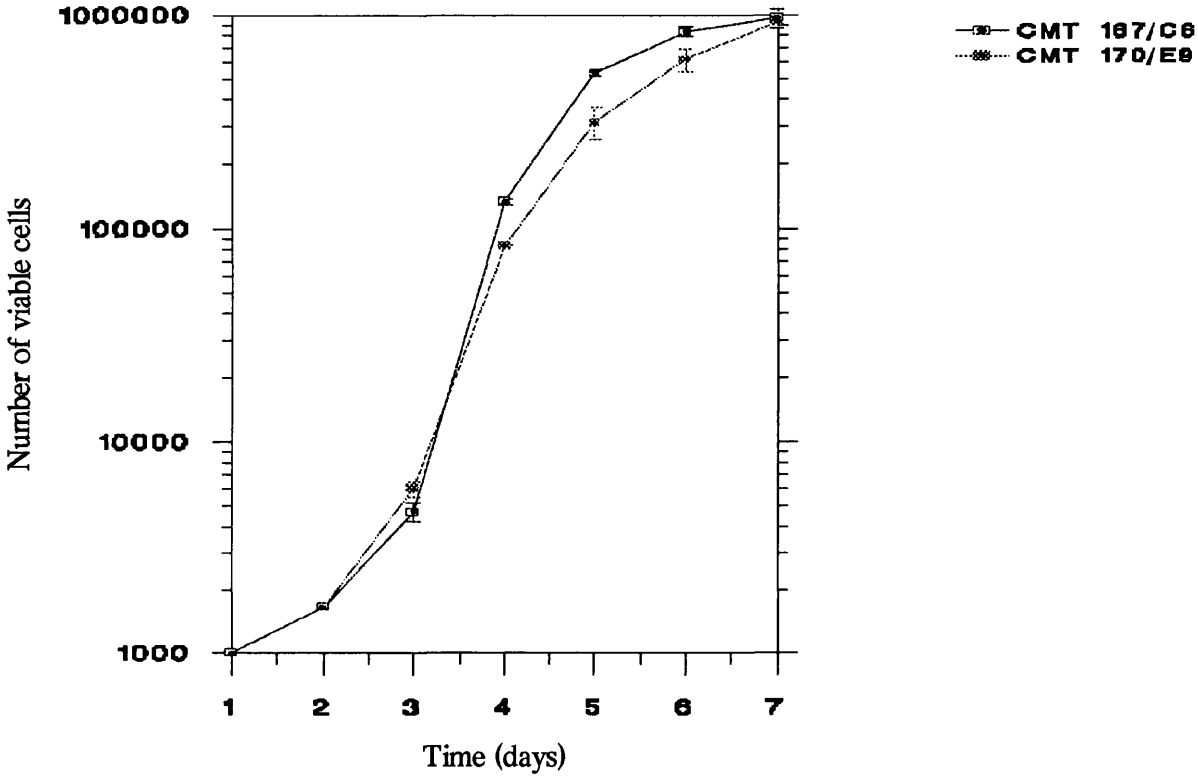


Figure 8

Figure 8. *Morphology Of CMT Cell Lines Plated Onto Matrix Components In Vitro*

Culture surfaces of plastic 6 well plates were treated with PBS alone (control), fibronectin (30μg/ml) or collagen (100μg/ml), as described in section V.1.3. CMT cell lines were subsequently seeded into the wells and cultured in normal growth medium. Photographs were taken 3 - 5 days later.

Magnification x 250

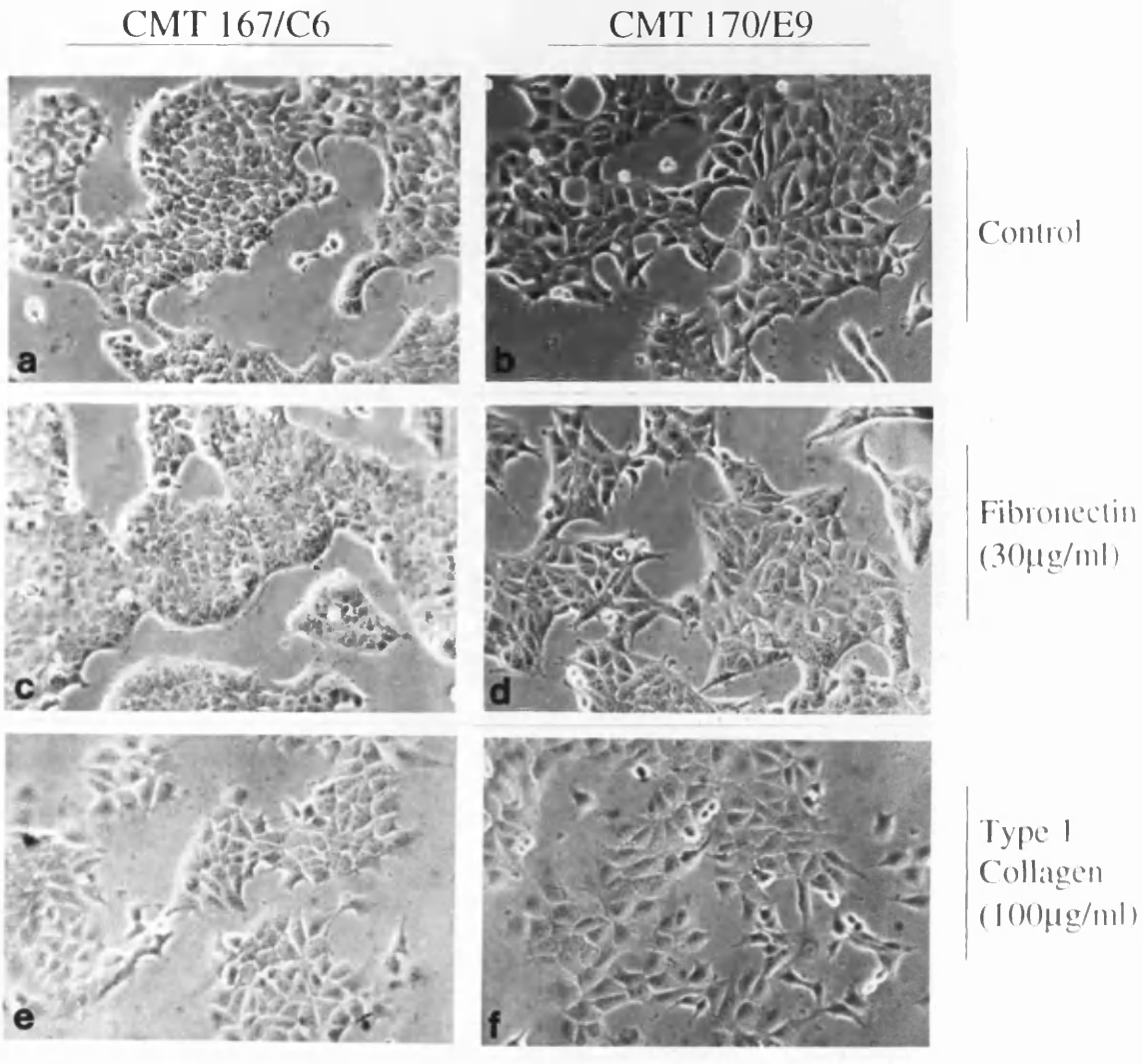

CMT 167/C6
CMT 170/E9
a
b
c
d
e
f
Control
Fibronectin
(30µg/ml)
Type 1
Collagen
(100µg/ml)

Figure 8). However, cells plated onto collagen-treated tissue culture plastic displayed subtle shape changes to a more flattened and classical epithelial morphology. Both CMT cell lines reacted in a similar fashion, and it could be speculated that these subtle shape changes upon culturing on collagen may be related to the cells expression of integrin receptors for collagen. Unfortunately, as will be discussed later (see section VI.3.3), at the time of this work, there were no suitable mouse-specific antibodies available which could be used to determine if the cells expressed either fibronectin or collagen integrin receptors.

VI.2.4 *P53 Status*

One of the most frequent genetic alterations found in human cancers is the deletion or mutation of the *TP53* gene (Nigro *et al.*, 1989). Indeed, in colorectal cancer, *TP53* is mutated in at least 50% of cases (Rodrigues *et al.*, 1990) and reported in some instances to be mutated in over 75% of colorectal cancers (Baker *et al.*, 1989). A characteristic of missense mutated *TP53* is an increase in the stability of the protein (Reich, Oren & Levine, 1983; Martinez *et al.*, 1990). Consequently, mutant p53 is found in higher concentrations in transformed cells. In order to examine the status of p53 expression in the CMT cell lines, immunocytochemistry was performed using a polyclonal antibody (CM1) capable of recognizing both wild type and mutant forms of p53. Intense cellular staining of p53 was observed in both cell lines (see Figure 9) and not of the negative controls which were identically treated cells but without incubation with the primary p53-specific antibody (see section V.6.6). Interestingly, unlike most cell lines, p53 staining was observed to be strongly cytoplasmic and not, as more commonly found, nuclear. The underlying reasons for this have not been further investigated and it could be postulated to be due to alterations in the nuclear localization signals of p53 or due to interactions with other cellular proteins. However, gross changes such as large deletions are not anticipated since polymerase chain reaction (PCR) products of

Figure 9

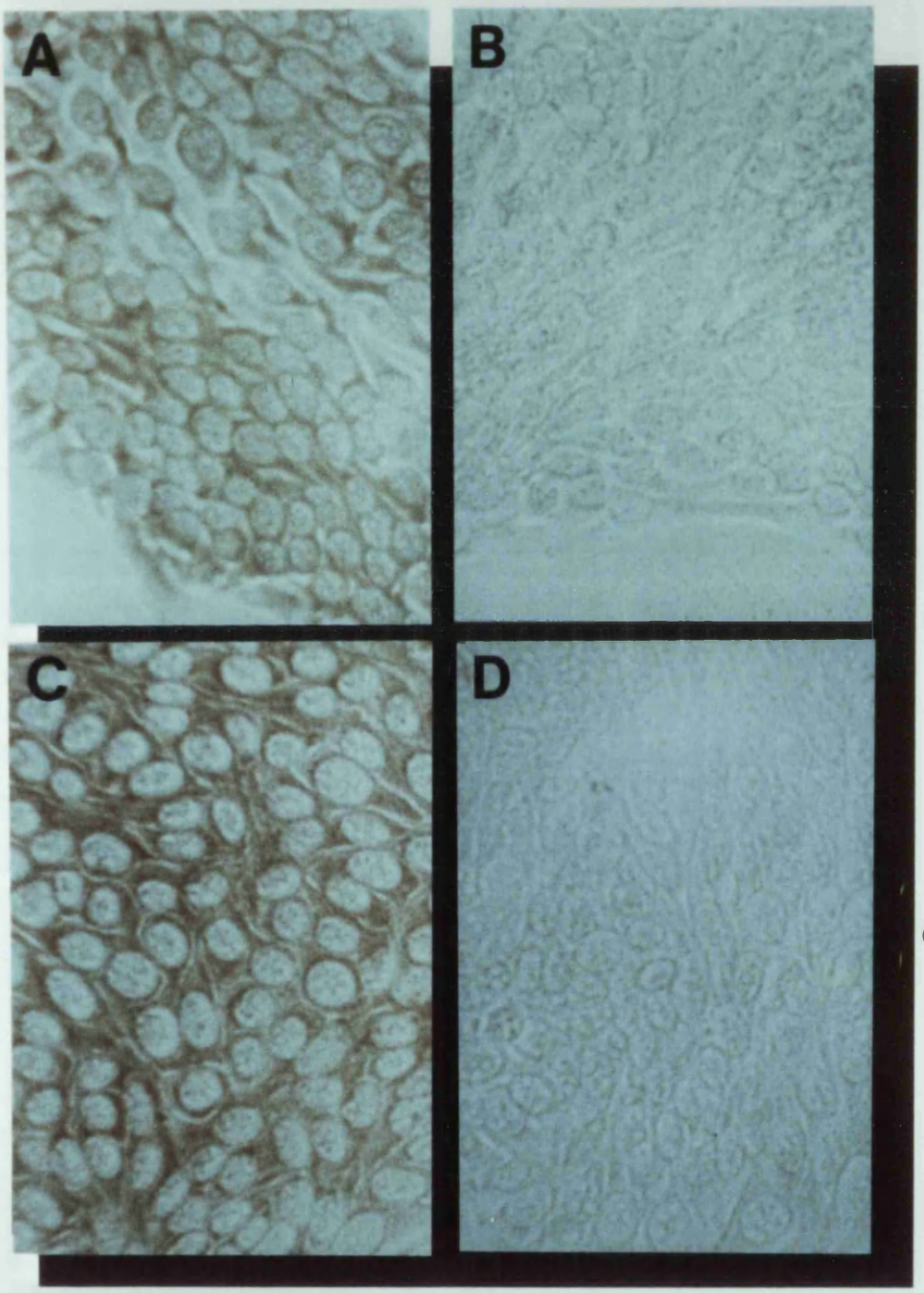
Figure 9. *p53 Immunocytochemical Analysis Of CMT 167/C6 And CMT 170/E9 Cell Lines*

CMT cell lines were cultured in 8 well chamber slides until ~90% confluency before fixing and staining as described in section V.6.6. Negative controls received identical treatment with the omission of the primary antibody which was substituted with blocking solution alone. The secondary antibody (CM1), was horseradish peroxidase (HRP)-conjugated and the substrate used was 3,3'-diaminobenzidine (DAB) hydrochloride. Positive staining is coloured brown.

As can be seen in Figures A and C, intense staining is present in the cytoplasm of the cells with scattered, granular staining in the nucleus. No background staining is detected in the negative controls (Figures B and D).

Magnification x 1250

Immunocytochemical Analysis Of p53 Expression



CMT 167/C6

CMT 170/E9

p53 immunocytochemistry

Negative control

TP53 from both CMT 167/C6 and CMT 170/E9 cell lines amplified and correlated with normal control sizes, as estimated on an agarose gel (data not shown).

VI.2.5 *Gap-Junctional Communication*

As already mentioned (see section I.2.2.6), in some instances, the reduction or loss of normal cell-cell communication *via* gap junctions has been associated with the metastatic phenotype. Since the two CMT cell lines exhibit markedly different metastatic potentials, their ability to communicate through homologous coupling of the cells was investigated. Each cell line was grown to confluency to ensure close cell-cell contact and a single cell within the monolayer selected and microinjected with lucifer yellow dye as described in section V.7.3. Examination under ultraviolet light of the monolayer reveals the extent of dye spread to adjacent cells. As can be seen in Figure 10, neither cell line exhibited dye transfer, indicating that normal gap-junctional intercellular communication has been disrupted in these cell lines.

Further investigations (in collaboration with I. Foulkes, Beatson Institute) of both CMT cell lines for their expression of connexin 43, a component implicated in gap junction formation, have revealed that although both cell lines express connexin 43 mRNA, only CMT 170/E9 (the low metastatic potential cell line) expresses the protein (I. Foulkes personal communication and unpublished data). However, this expression does not appear to represent functional gap junctions since it is not detected at regions of cell-cell contact which would indicate functional expression (I. Foulkes, personal communication & unpublished data). These results confirm the finding that both cell lines are unable to communicate *via* gap junctions as assayed by dye transfer. In addition to homologous coupling, neither cell line appears to form heterologous coupling with normal cells since both CMT cell lines are able to form foci when seeded upon a confluent fibroblast monolayer of either

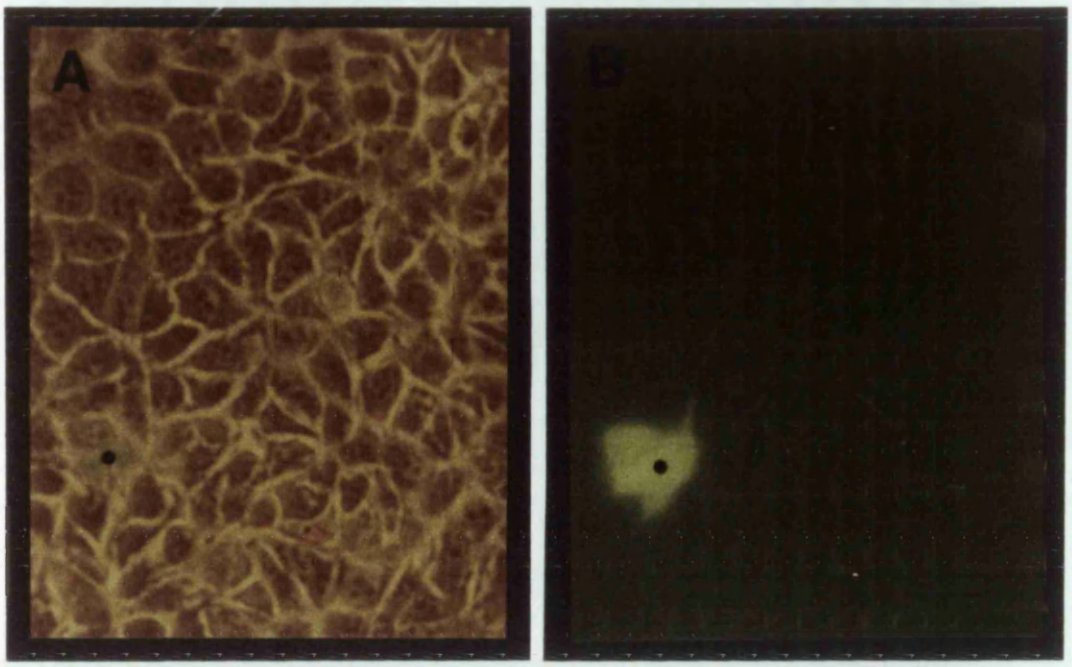
Figure 10

Figure 10. *Gap-Junction Intercellular Coupling (GJIC) Of CMT Cell Lines*

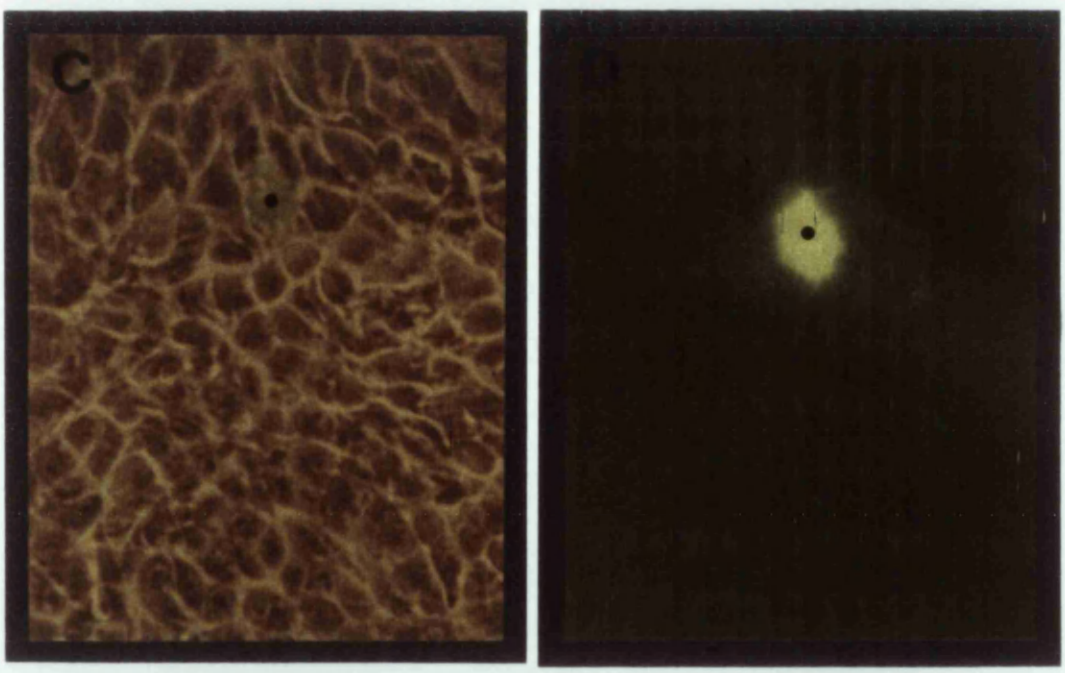
Cells were cultured to subconfluence in 60mm dishes using standard techniques. Using a Leitz Diavert inverted microscope, single cells were selected for injection with luciferase yellow CH fluorescent dye, as described in section V.7.3. The extent of dye spread, and therefore the indication of functional gap-junction intercellular communication, was visualised by epillumination. Phase contrast photographs indicate the same field of vision with the selected cell for dye injection in the confluent monolayer marked with a black dot.

Magnification x 1000

CMT 167/C6 - Lucifer Yellow Dye Transfer



CMT 170/E9 - Lucifer Yellow Dye Transfer



Phase Contrast

Fluorescence

Swiss 3T3 cells or $10T^1/_2$ cells (I. Foulkes, personal communication). If the cell lines formed functional, heterologous coupling of gap junctions, it would be expected that the cells would have been growth inhibited and unable to form foci.

These results indicate that loss of gap-junctional intercellular communication has occurred in these cell lines. However, it does appear that loss cannot be correlated directly with metastatic potentials, since both CMT cell lines have lost intercellular communication *via* gap junctions and these two cell lines have markedly differing metastatic potentials. However, because both cell lines do possess varying degrees of metastatic potential, it may be that the loss of gap-junctional communication is reflective of the capacity to metastasize rather than extent of high or low metastatic potential and perhaps the metastatic potential of CMT 170/E9 cells, although low, still requires the loss of gap-junctional communication. Experiments are presently being undertaken to establish whether or not transfection of connexin 43 is sufficient to restore functional gap-junction intercellular communication and abrogate the metastatic potential and / or affect the tumorigenic potentials of these cell lines *in vivo* (I. Foulkes, personal communication).

VI.2.6 *Matrix Metalloproteinase Expression*

A characteristic feature of invasive carcinomas is their association with the expression of matrix metalloproteinases (see section I.2.3). Therefore, it can be suggested that differing expressions of these enzymes may be correlated with the metastatic potentials, and upon this speculation, the expression and secretion of the type IV collagenases was investigated of these cell lines. Early zymography experiments, which assay for enzymatic degradation of substrate impregnated within the gel by enzymes separated according to molecular weight by non-denaturing gel electrophoresis (see section V.6.5), did not reveal any type IV collagenase activities in conditioned medium collected from either cell line. The

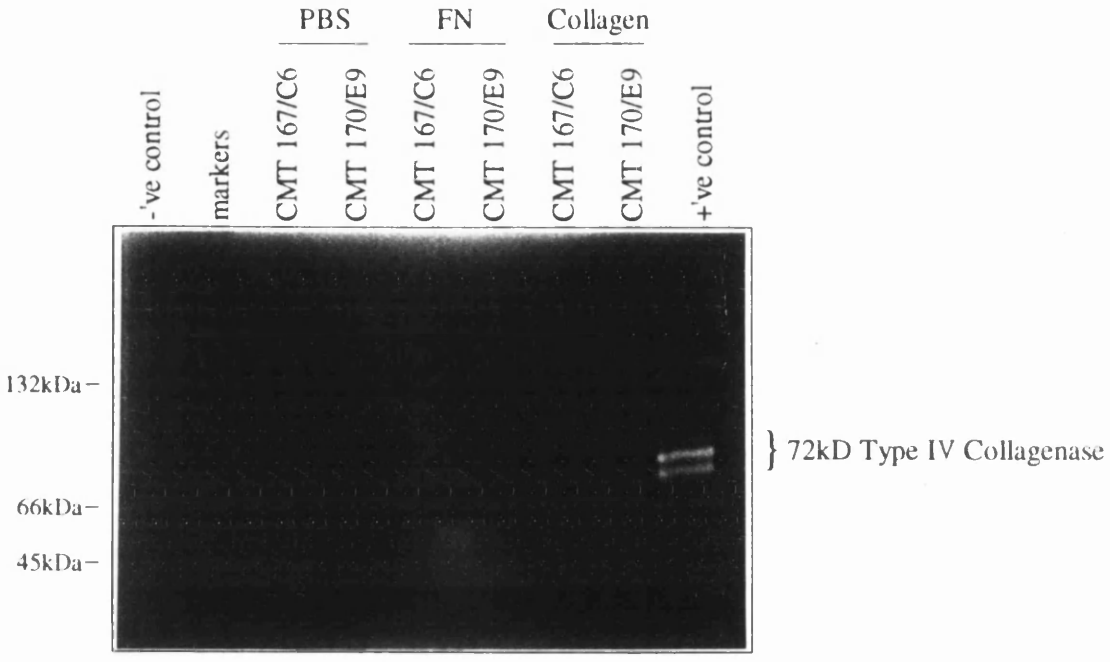
Figure 11

Figure 11. *Zymography Of Gelatinase Activity In Conditioned Media Activated With APMA*

Non-denaturing SDS-polyacrylamide gels can be impregnated with gelatin substrate to assay for the presence of enzymatic activity. CMT cell lines were cultured on untreated or fibronectin or collagen-coated surfaces as described in section V.6.5. Conditioned media were collected at 24 hours and treated with 4-aminophenylmercuric acetate (APMA) to activate any latent enzymes present before analysis by zymography (section V.6.5).

Enzymatic activity is visualized as zones of clear areas against a Coomassie blue-stained background. The negative control was medium incubated without cells and the positive control was conditioned medium from a *fos*-transformed rat fibroblast cell line, FBR (a kind gift from B. Hennigan). As can be seen in Figure 11, no gelatinase activity can be detected in either cell line.

Zymography of gelatinase activity in conditioned media activated with APMA



absence of activity was not due to the enzyme being present in its inactive proenzyme form because samples were routinely treated with an organomercurial, 4-aminophenylmercuric acetate (APMA), which activates the pro-collagenases. In addition, enzymes complexed with TIMPs are separated during electrophoresis, therefore only the active free enzyme should be present at the appropriate molecular weight position as separated by electrophoresis.

It was then considered that the expression and secretion of these enzymes may be dependent on the cells being cultured in the presence of components of the extracellular matrix, with the reasoning that the induction of the matrix metalloproteinase expression may be ligand dependent. However, even upon culturing of cells in the presence of either collagen or fibronectin (see section V.1.3), type IV collagenases were not secreted into the medium (see Figure 11). It can be noted here that the three bands of 72kD type IV collagenase present in the control lane represent the two intermediate cleavage products of the proenzyme, in addition to the fully active enzyme product (Strongin *et al.*, 1993).

Since both 92kD and 72kD type IV collagenases can be detected using gelatin as the substrate in this assay, it can be concluded that neither cell line express either type IV collagenases. Similar experiments were attempted using casein as the substrate to assay for stromelysin enzyme expression, however these were unsuccessful.

VI.3 *Cell Adhesion Analysis*

As mentioned earlier, previous published literature concerning these two CMT cell lines of this metastasis model provide minimal information on their characterization in relation to their metastatic phenotypes. Since it is becoming increasingly apparent that cell adhesion can markedly influence the processes of invasion and metastasis (Vleminckx *et al.*, 1991; Birchmeier & Behrens, 1994;

Qian *et al.*, 1994), potential alterations in quantitative or qualitative expressions of different cell adhesion molecules was investigated in these two cell lines.

VI.3.1 *Cadherins*

The cadherin superfamily of cell adhesion molecules (see section I.2.2.1) is often affected in both squamous tumours and adenocarcinomas (reviewed in Birchmeier & Behrens, 1994), with the loss or down-regulation of E-cadherin expression frequently associated with poorly differentiated tumours and tumour progression to lymph node metastasis. Expression patterns of E, N and P-cadherins, at the protein level, were examined by western analysis of both cell lines. In agreement with the general correlation between loss of E-cadherin expression and tumour dissemination, the cell line CMT 167/C6, which exhibits high metastatic potential, was observed to have reduced E-cadherin expression relative to CMT 170/E9 (low metastatic potential cell line), see Figure 12.

Interestingly, expression levels in equivalent amounts of whole cell lysate proteins extracted both from *in vitro* cultured cell lines and from *in vivo* cell line-induced primary tumours, as estimated by coomassie blue staining of parallel gels, reproducibly differed. Expression levels consistently correlated inversely with metastatic potential, however overall expression levels appeared to be down-regulated *in vivo* (see Figure 12). This could be explained by at least two possibilities: (1) the proteins extracted from the primary tumours contained a high proportion of degraded material or (2) there is a selective suppression of E-cadherin *in vivo*. The former possibility can probably be discounted since the degradation products of E-cadherin are usually detected as lower molecular weight products on a western blot. Since these degradation products are not apparent, certainly not to any extent which could explain the reduction in signal of normal E-cadherin which migrates at 120-125kD size, it may be assumed that there is a

Figure 12

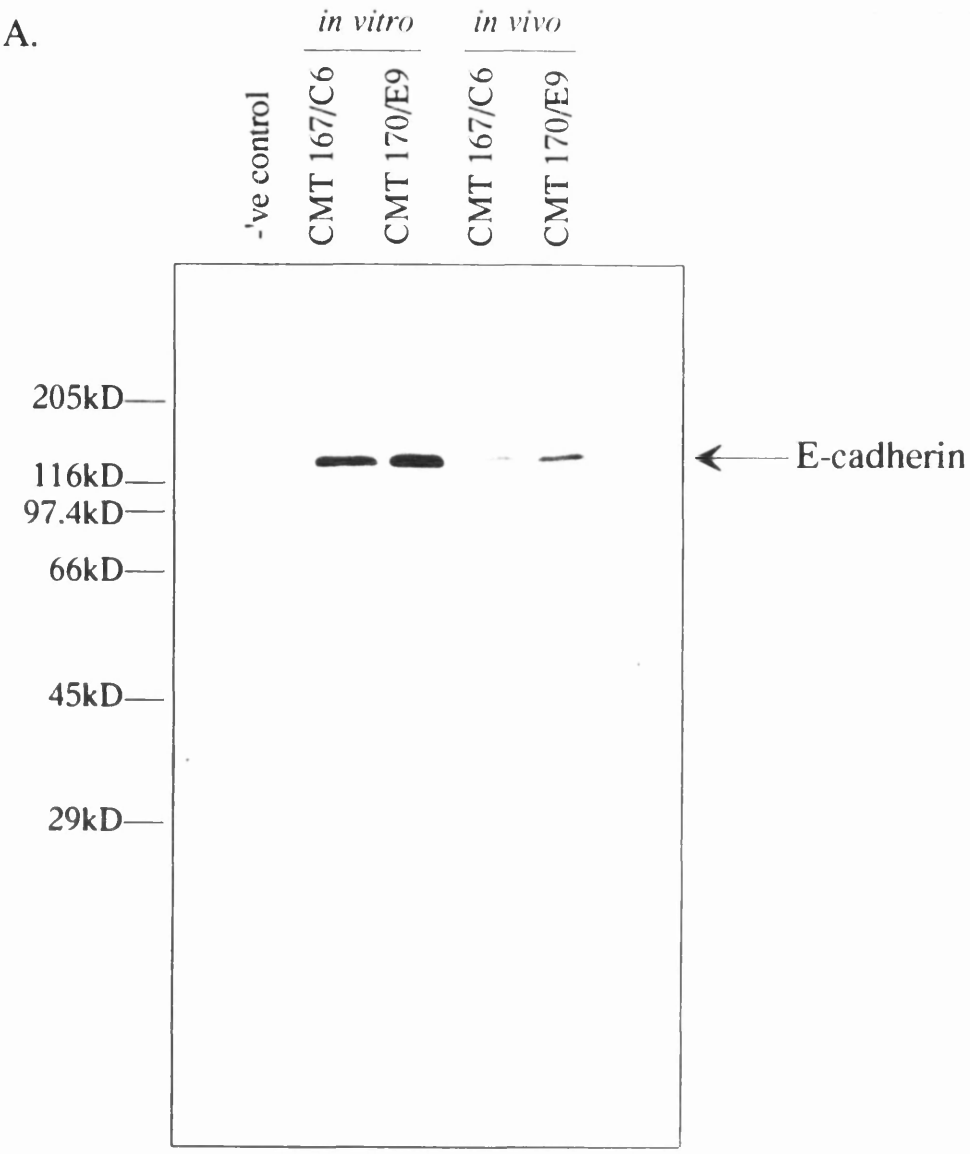
Figure 12. *ECL Western Analysis Of E-Cadherin Expression*

Protein extracts from CMT cell lines cultured *in vitro* and from primary tumours were analysed by Western blotting and detected with ECL (kit marketed by Amersham Int. plc). Equal concentrations of protein were loaded onto each lane (~15μg using ATTO mini-gel apparatus). Equal loading was confirmed by Coomassie blue staining of a parallel gel (not shown). The primary antibody, ECCD-2, was used at 1:100 dilution.

Figure 12A. Specific detection of E-cadherin can be seen in all lanes except the negative control (SDS-lysis buffer without protein extracts) at ~120-125kD. Molecular weight markers are shown to the left.

Figure 12B. Densitometric quantitation of E-cadherin expression.

A.



B.

		Optical Density (OD) x Band Area (mm^2)	Ratio $\frac{\text{CMT 170/E9}}{\text{CMT 167/C6}}$
in vitro	CMT 167/C6	2.271	1.5
	CMT 170/E9	3.299	
in vivo	CMT 167/C6	0.165	2.3
	CMT 170/E9	0.378	

Figure 13

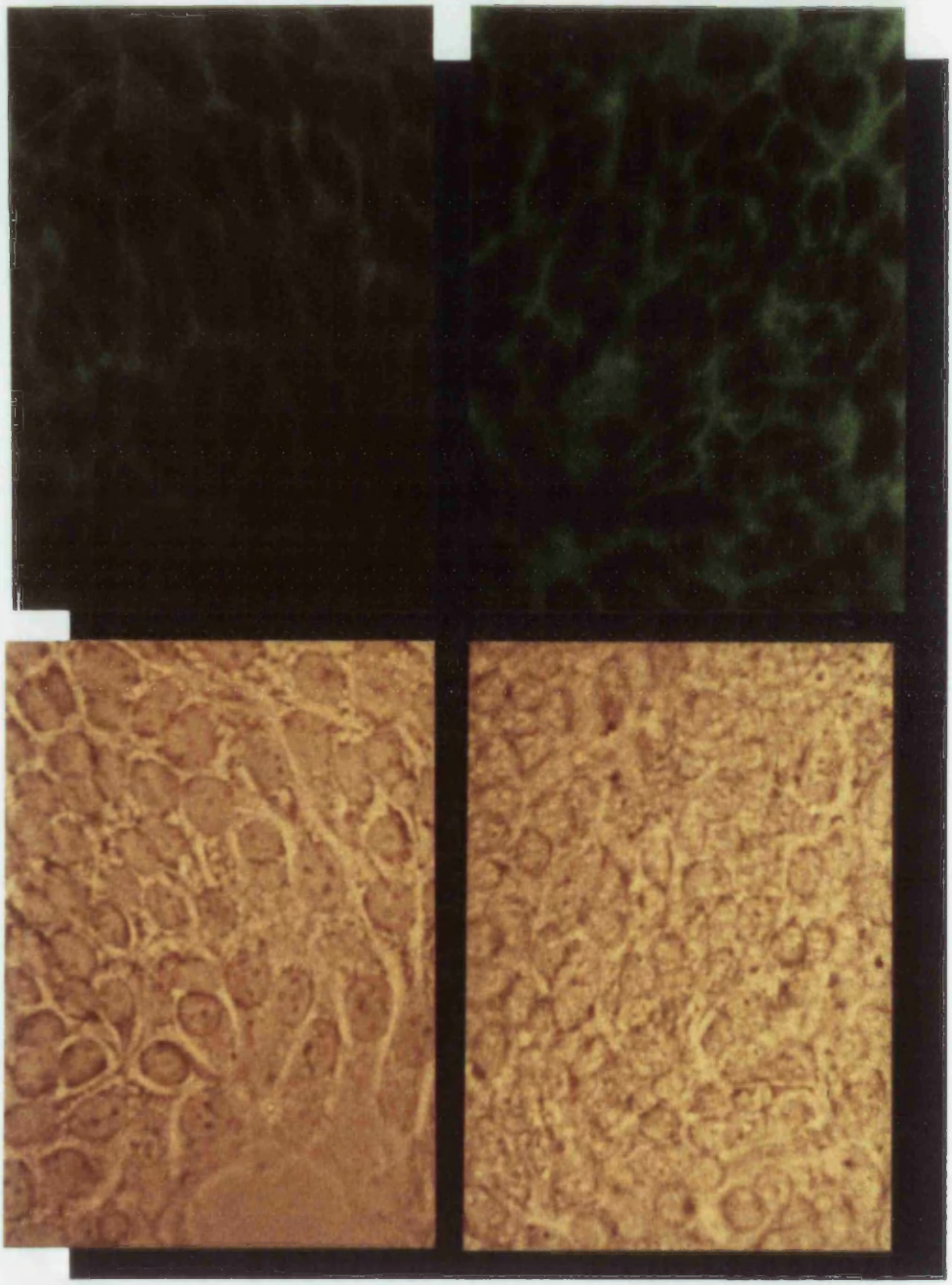
Figure 13. *Immunofluoresence Analysis Of E-Cadherin Expression*

CMT cell lines were grown to subconfluence in 8 well chamber slides before fixing and staining for E-cadherin expression with the primary antibody ECCD-2 (1:500 dilution), as described in section V.6.7.

Fluorescent staining can be seen at sites of cell-cell contact in both cell lines, thus indicating normal cellular localization and probably normal function. Slightly brighter staining is seen in CMT 170/E9 cells probably reflecting increased expression of E-cadherin in these cells as detected by Western analysis (Figure 12A). Phase contrast photographs depicts the area of cells visualized by epillumination.

Magnification x 1250

Immunofluorescence Analysis Of E-Cadherin Expression



Fluorescence

Phase contrast

CMT 167/C6

CMT 170/E9

Figure 14

Figure 14. *ECL Western Analysis Of N-Cadherin Expression*

Protein extracts from CMT cell lines cultured *in vitro* were analysed by Western blotting and detected with ECL (kit marketed by Amersham Int. plc). Equal concentrations of protein were loaded onto each lane (~15mg using ATTO mini-gel apparatus). Equal loading was confirmed by Coomassie blue staining of a parallel gel (not shown). The primary antibody, GC-4, was used at 1:50 dilution, as manufacturer's recommended instructions (Sigma).

Specific detection of N-cadherin was only detected in the positive control (murine heart extract, a kind gift from D. Prowse). The lower molecular weight bands are non-specific bands which are detected by the primary antibody (D. Prowse, personal communication). Negative control was SDS buffer without protein extracts.

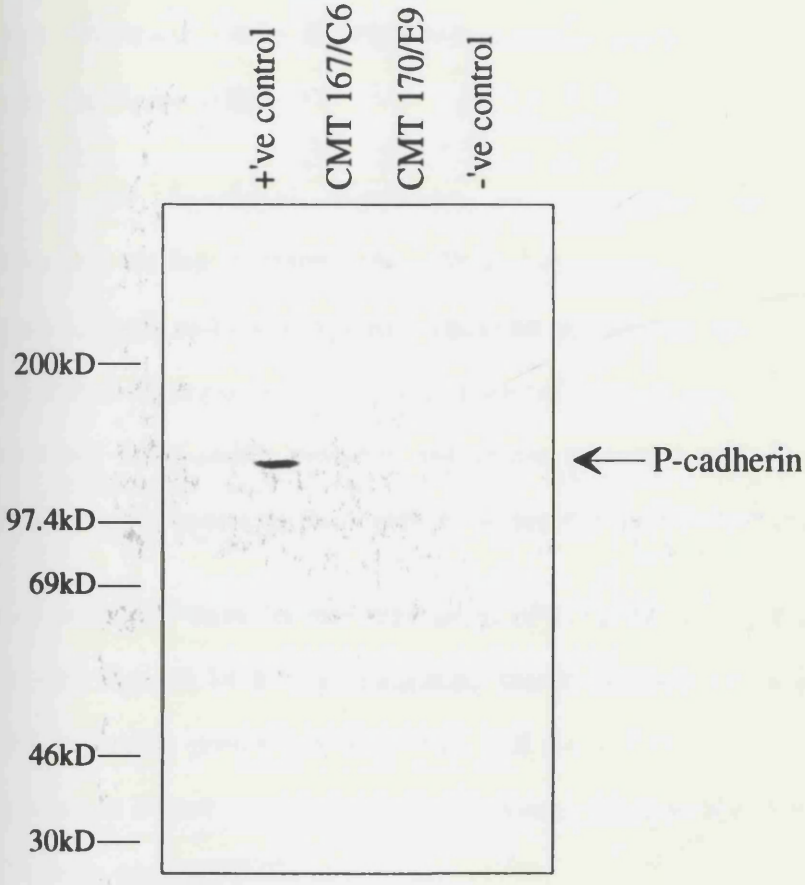
Figure 15

Figure 15. *ECL Western Analysis Of P-Cadherin Expression*

Protein extracts from CMT cell lines cultured *in vitro* were analysed by Western blotting and detected with ECL (kit marketed by Amersham Int. plc). Equal concentrations of protein were loaded onto each lane (~15μg using ATTO mini-gel apparatus). Equal loading was confirmed by Coomassie blue staining of a parallel gel (not shown). The primary antibody, PCD-1, was used at 1:50 dilution (D. Prowse, personal communication).

Specific detection of P-cadherin was only detected in the positive control (protein extract from the cell line BRL, buffalo rat liver, a kind gift from D. Prowse). The negative control was SDS-lysis buffer without protein extracts.

ECL Western Analysis of P-cadherin Expression



suppression *in vivo* of E-cadherin expression, or alternatively, there is an up-regulation *in vitro*. Despite this *in vivo* / *in vitro* discrepancy, the relative expression levels of E-cadherin in both cell lines consistently correlate inversely with metastatic potential, although the *in vivo* relative difference appears to be marginally greater as measured by densitometric scanning analysis of the original autoradiograph (see Figure 12B).

Confirmation of the functional expression of E-cadherin resulted from immunofluorescence studies examining the cellular localization of E-cadherin. As shown in Figure 13, both cell lines express E-cadherin at points of cell-cell contact thus indicating that E-cadherin is functional in both cell lines. It may be therefore speculated that the relationship between metastatic potential and E-cadherin expression is, in part, dependent on the threshold levels of E-cadherin expression.

Examination of both cell lines for the expression of N-cadherin and P-cadherin were negative (see Figures 14 & 15), suggesting that E-cadherin is the principal member of the cadherins present on these two cell lines. The lower molecular weight bands seen in Figure 14 are non-specific bands detected by the primary antibody (D. Prowse, personal communication).

VI.3.2 *NCAM*

In addition to the cadherin cell adhesion molecules, expression of the similarly Ca^{2+}-dependent cell adhesion molecule NCAM was examined. NCAM, as already described in section I.2.2.4.1, mediates homophilic cell-cell adhesion and could therefore be a contributory factor in invasion of tumour cells when altered in its expression and/or function. Expression of NCAM in both CMT cell lines was examined by western analysis and the transmembrane isoform of NCAM was detected in both cell lines. CMT 167/C6 cells consistently exhibited reduced expression of NCAM relative to CMT 170/E9 cells. A representative western blot

is shown in Figure 16A. Points to note about these results include the consistent relative reduction of NCAM expression in CMT 167/C6 (high metastatic potential) cells in comparison to CMT 170/E9 (low metastatic potential) cells. This is demonstrated both in cells cultured *in vitro* and in primary tumours formed from subcutaneous injection of both cell lines. However, it is noteworthy that the relative differences in quantitative expression appears to be dramatically affected between *in vivo* and *in vitro* cultures. As estimated by densitometric scanning analysis, the relative difference in expression levels is greater than 30-fold *in vitro*, but less than 3-fold *in vivo* (see Figure 16B). Further still, overall expression levels are increased *in vivo*. From these results, it appears that expression levels of NCAM *in vivo* are high, but with the maintenance of a 3-fold difference between the low and high metastatic cell lines. This 3-fold quantitative difference may significantly affect the physiological strength of cell adhesion (Hoffman & Edelman, 1983) and in this model system it may be postulated that the reduction in NCAM expression in CMT 167/C6 cells enhances the cells ability to disseminate from the primary tumour.

Curiously though, the inverse relationship between NCAM expression and the metastatic potential observed in this CMT model is in contrast to the clinical correlations documented between NCAM expression and poor prognosis (see section I.2.2.4.1). It is possible that this clinical correlation is significant only in particular tumour types, and it appears that this correlation is indeed restricted to mostly small cell lung carcinomas according to present literature (Jaques *et al.*, 1993; Kibbelaar *et al.*, 1991). It may be postulated that particular cell types respond differently to NCAM and it would therefore be conceivable that expression of NCAM may promote tumour outgrowth, comparable to the normal stimulation of neurite outgrowth stimulated by expression of the NCAM isoform

Figure 16

Figure 16. *ECL Western Analysis Of NCAM Expression*

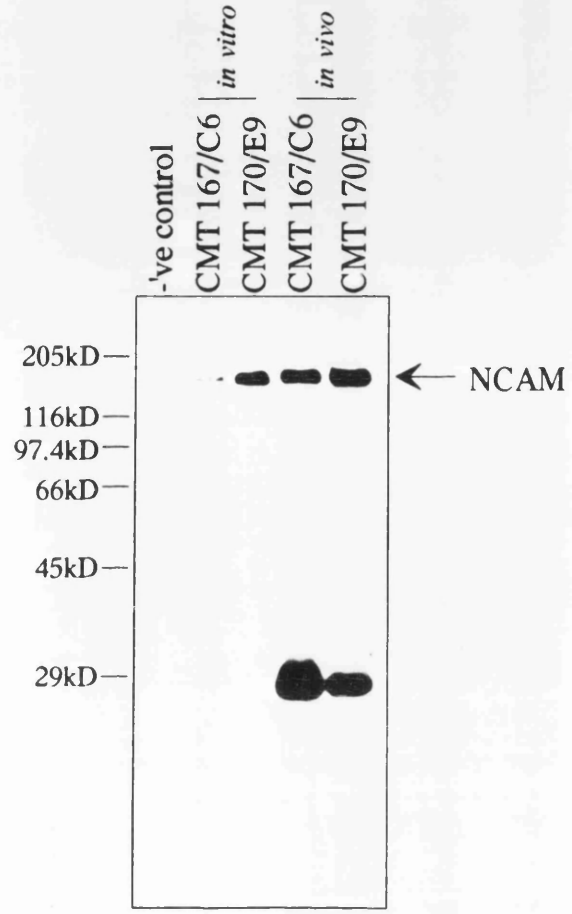
Protein extracts from CMT cell lines cultured *in vitro* and from primary tumours were analysed by Western blotting and detected with ECL (kit marketed by Amersham Int. plc). Equal concentrations of protein were loaded onto each lane (~15μg using ATTO mini-gel apparatus). Equal loading was confirmed by Coomassie blue staining of a parallel gel (not shown). The primary antibody, OB-11, was used at 1:100 dilution, as manufacturer's recommended instructions (Sigma).

Figure 16A. Specific detection of NCAM can be seen in all lanes except the negative control (SDS-lysis buffer without protein extracts) at ~140-160kD. The lower molecular weight bands seen in extracts from primary tumours appear to be specific to samples with some normal blood cell infiltration; this is suggested from the cross reaction also seen in the molecular weight standard, carbonic anhydrase extracted from bovine erythrocytes. Cross reaction is due to the secondary antibody.

Figure 16B. Densitometric quantitation of NCAM expression

ECL Western Analysis of NCAM Expression

A.



B.

		Optical Density (OD) x Band Area (mm^2)	Ratio $\frac{\text{CMT 170/E9}}{\text{CMT 167/C6}}$
in vitro	CMT 167/C6 CMT 170/E9	0.102 3.764	36.9
in vivo	CMT 167/C6 CMT 170/E9	3.931 9.776	2.5

Figure 17

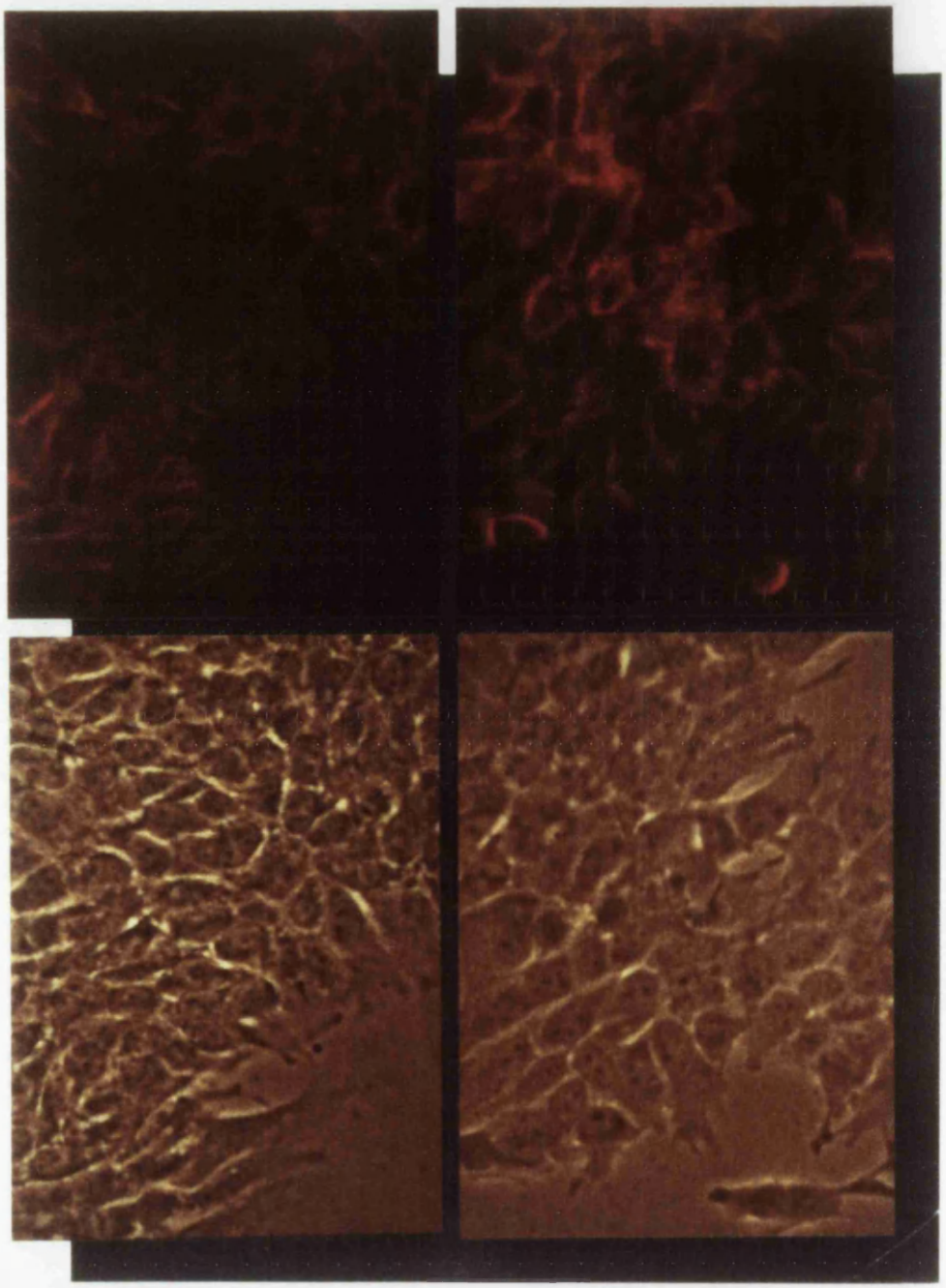
Figure 17. *Immunofluorescence Analysis of NCAM Expression*

CMT cell lines were grown to subconfluence in 8 well chamber slides before fixing and staining for NCAM expression with the primary antibody OB-11 (1:50 dilution), as described in section V.2.6.

Fluorescent staining can be seen at sites of cell-cell contacts in both cell lines, thus indicating functional expression of NCAM. Staining appears to be brighter in CMT 170/E9 cell lines which reflects the increased expression of NCAM detected by Western analysis (Figure 16). Phase contrast photographs depicts the area of cells visualized by epillumination.

Magnification x 1250

Immunofluorescence Analysis Of NCAM Expression



Fluorescence

Phase contrast

CMT 167/C6

CMT 170/E9

lacking the VASE exon (Saffell, Walsh & Doherty, 1994; Doherty *et al.*, 1992b). However, in other cell types such as epithelial cells, including the CMT cell lines, expression of NCAM may not stimulate outgrowth of these cells and functions solely as a cell adhesion molecule. Therefore increased expression of NCAM would be predicted to increase cell-cell adhesiveness and *vice versa*, and as such, in the tumour, decreased expression would be expected to be associated with increased invasive / metastatic potential.

The cellular localization of NCAM expression was examined in these two CMT cell lines by immunofluorescence (see Figure 17). In both cell lines, NCAM was found to be localized to the cell surface at points of cell-to-cell contact and thus, as predicted of E-cadherin, NCAM is expected to be functional in cell adhesion.

VI.3.3 *Integrins*

Attempts were made to examine the expression of different integrin subunits in the two CMT cell lines. However, the commercially available antibodies tested were not cross-reactive with murine samples, and therefore these expression studies did not yield results which could begin to assess the functional contribution of cell-to-matrix interactions in the phenotypes of these cell lines.

VI.4 *Two-Dimensional Gel Analysis Of Total Cellular Proteins*

Preliminary two-dimensional (2-D) analysis of whole cell protein extracts was tried in order to assess the possibility that the phenotypic differences exhibited by the CMT cell lines was due to one or more dramatic alterations of specific proteins within the cell. Large differences in protein turnover, or up-regulation / down-regulation, or the presence / absence of proteins should be detected by 2-D gel analysis. The technique of 2-D gel analysis was expertly carried out by L. McGarry (Beatson Institute). Cells of both cell lines were labelled with

^{35}S methionine for four hours prior to protein extraction and 2-D gel analysis (see section V.6.9).

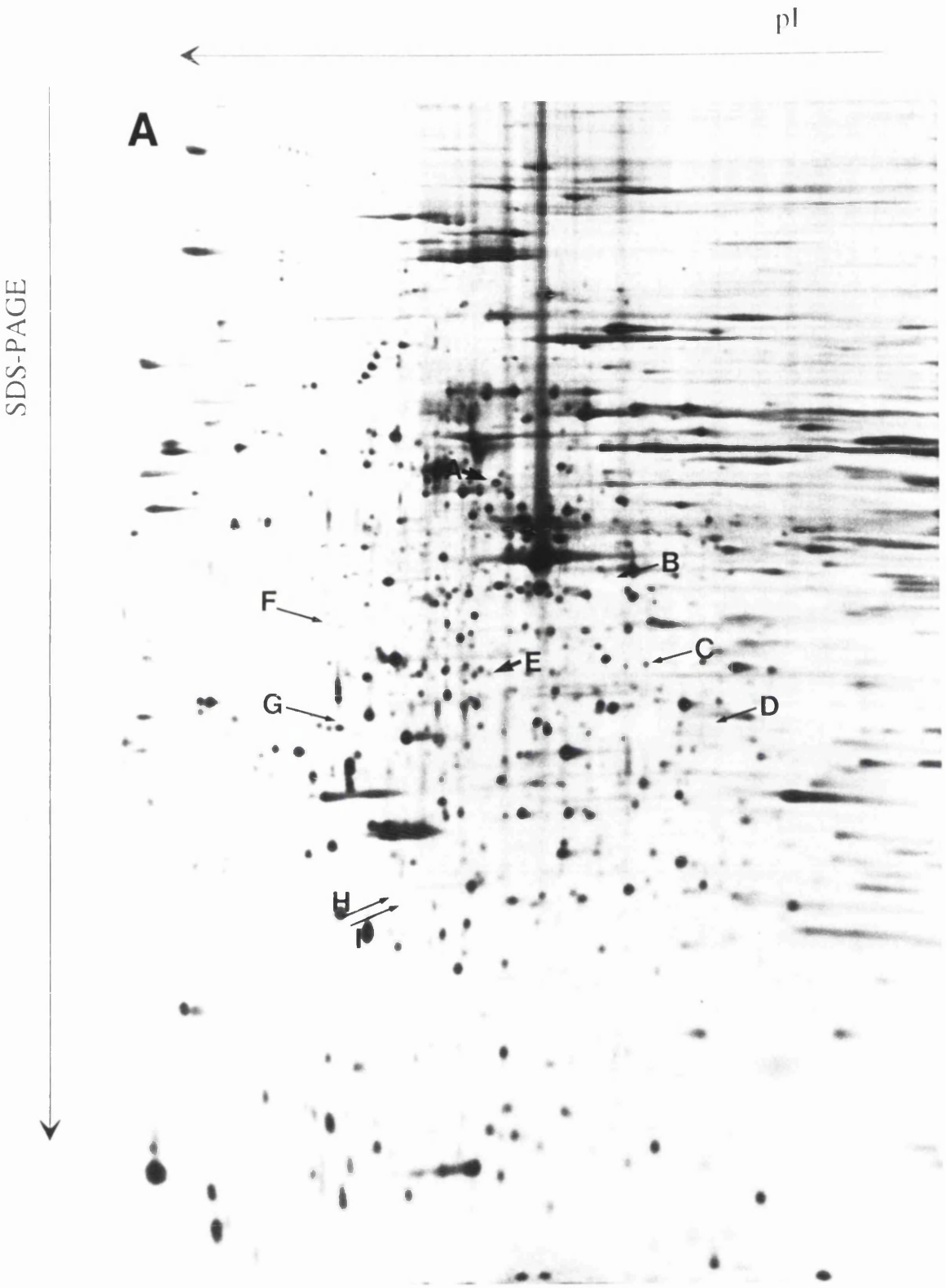
This preliminary work surprizingly revealed very few differences in the resolved proteins between the two cell lines. Altogether, nine quantitative differences were detected and no qualitative differences were found (see Figure 18). Interestingly, seven of the qualitative differences were increased in their expression levels in the low metastatic potential cell line, CMT 170/E9 and only two were increased in their relative expressions in the high metastatic potential cell line CMT 167/C6. Table 1 gives details of the estimated molecular weights and isoelectric points of these identified proteins.

Since this is only preliminary work, extrapolations should not be taken from these results inferring that the identified proteins which differ between the 2-D gels of each cell line are *specifically* metastasis-related. However, this result does lend evidence to the suggestion that metastasis may not be dependent on a single "metastasis-gene", but indeed may be the outcome of multiple subtle alterations within the cell, all of which require to attain a certain threshold level in order for metastasis to successfully occur. This would correlate the apparently low frequency with which tumour cells shed from the primary tumour actually proceed to form a metastatic deposit (Weiss, 1986; Glaves, 1983). However, it is also apparent that there must be key events which are important in metastasis which do occur, since many tumour types can be predicted to have metastasized at the time of diagnosis when the tumour has reached an advanced clinical stage, and as such, there must be events in particular cell types which predispose for the procurement of the metastatic phenotype.

Figure 18

Figure 18. *2-D Gel Analysis Of Total Cellular Proteins*

CMT cells were labelled with ^{35}S for four hours prior to protein extraction and 2-D gel analysis (see section V.6.9). Figure 18A is the 2-D gel analysis of CMT 167/C6 extracts and Figure 18B is the 2-D gel analysis of CMT 170/E9 cell extracts. From this preliminary analysis, a total of nine resolved proteins were detected which differed in intensity between the two CMT cell lines on the autoradiographs. These proteins, labelled A-I have been estimated for their approximate molecular weights and pI values (see Table 1).


pI
SDS-PAGE
A
B
C
D
E
F
G
H
I

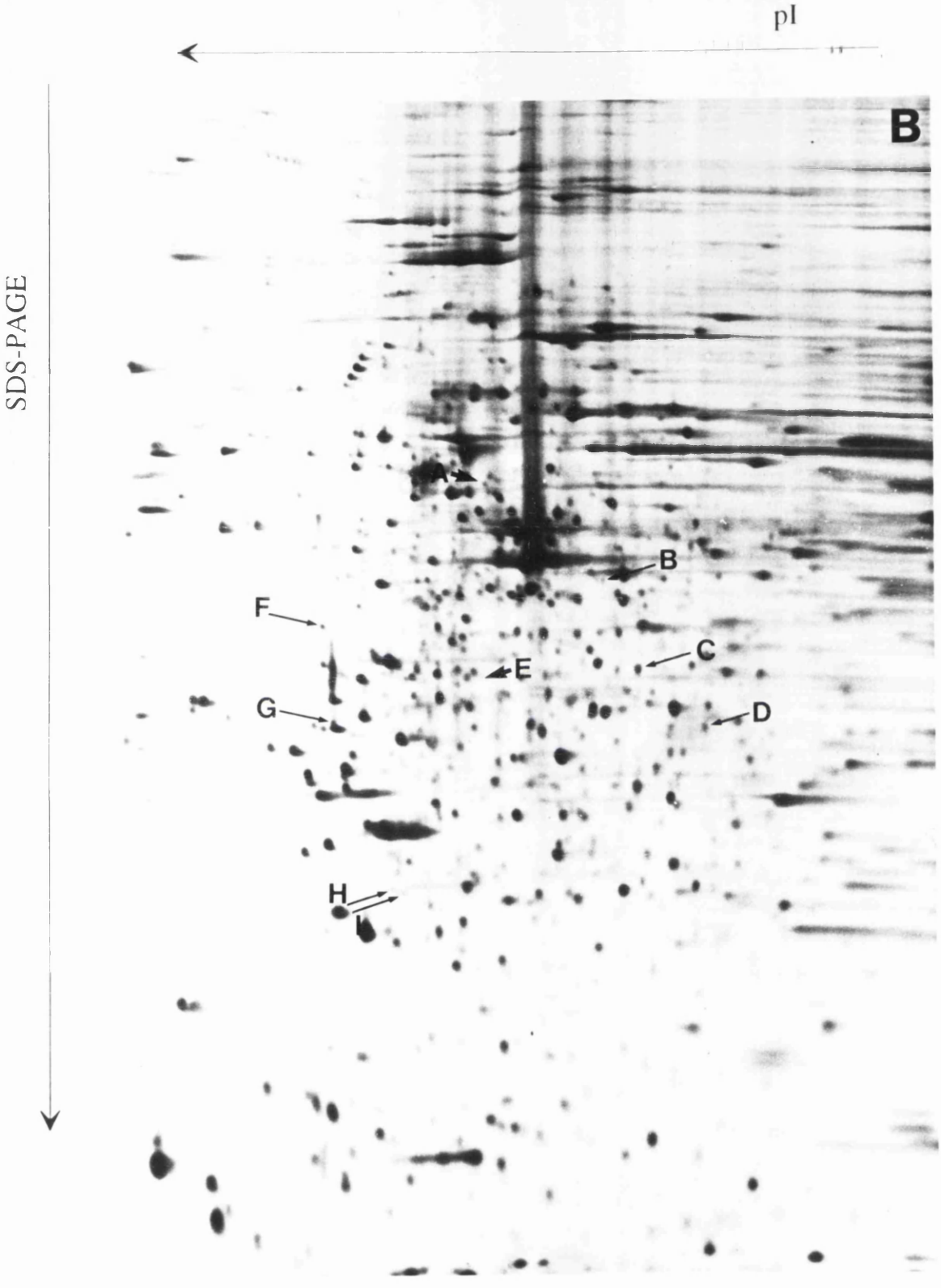

pI
SDS-PAGE
B
A
B
C
D
E
F
G
H
I

Table 1 *Estimated isoelectric points and molecular weights of proteins identified in 2-D gel analysis*

Protein	Apparent M.W. (kDa)	Approximate pI
A	51	5.1
B	41	5.35
C	38	5.45
D	35	5.65
E	37	5.05
F	40	4.55
G	34	4.55
H	29	4.6
I	28.5	4.6

By comparison, proteins A and E are increased in CMT 167/C6 cells relative to CMT 170/E9 cells (see Figure 18, marked with short arrows). The other proteins noted are increased in CMT 170/E9 cells relative to CMT 167/C6 cells (see Figure 18, marked with long, thin arrows).

VI.5 *Phosphoamino Acid Analysis Of Total Cellular Protein*

Many of the oncogenes associated with cellular transformation encode for proteins with intrinsic tyrosine kinase activity (reviewed by Hunter, 1989; and references therein). Consequently, these transformed cells have higher levels of phosphotyrosine-containing proteins, reflecting the unregulated tyrosine kinase activity. This increase in activity can be measured by phosphoamino acid analysis of protein extracts from ^{32}P-labelled cells (see section V.6.8). It was speculated that cells with differing metastatic phenotypes might be exhibiting altered signal transduction pathways affected by tyrosine kinases and their reciprocal partners, tyrosine phosphatases. Therefore, as a preliminary study, the relative phosphotyrosine content was examined in the two CMT cell lines by phosphoamino acid analysis of total cellular protein. As described in Methods V.6.8, cells were labelled with carrier-free ^{32}P overnight before protein extraction, acid hydrolysis and thin layer separation by 2-D electrophoresis. The resulting autoradiographs are shown in Figure 19a & b. Relative abundances of phosphoserine, phosphothreonine and phosphotyrosine in each cell line was calculated by scraping the appropriate areas of cellulose from the plates, in addition to suitable background control areas, and recording the levels of radioactive incorporation. The relative abundance of each of the three phosphohydroxyamino acids is expressed as a percentage of the total radioactivity incorporated (see Figure 19d).

Early studies by Hunter & Sefton (1980) demonstrated that normal chick cells contain ~0.03% phosphotyrosine and infection with Rous Sarcoma Virus (RSV) - the *src* gene product has tyrosine kinase activity - increased the cellular levels of phosphotyrosine 7-8 fold. Similar increases could be anticipated if the CMT cell lines exhibited altered constitutive tyrosine kinase activity correlating with the metastatic phenotypes. However, as can be seen in Figure 19d, the relative

Figure 19

Figure 19. *Phosphoamino Acid Analysis Of Total Cellular Protein*

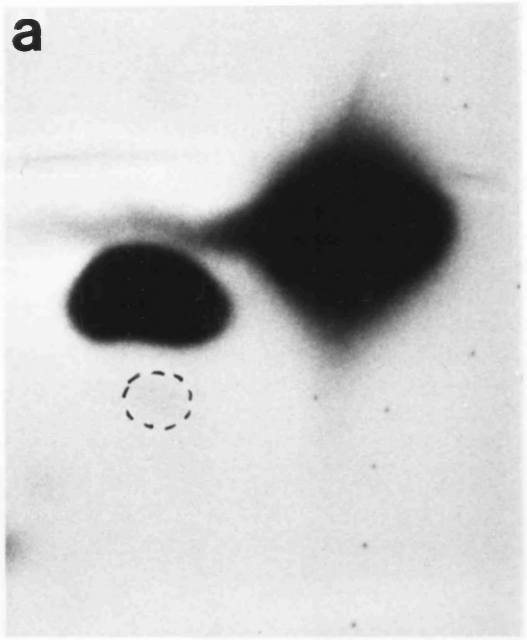
Phosphoamino acid analysis was performed as described (see section V.6.8). Cells were labelled with ^{32}P for 18-20 hours before protein extraction, acid hydrolysis and thin layer separation by 2-D electrophoresis.

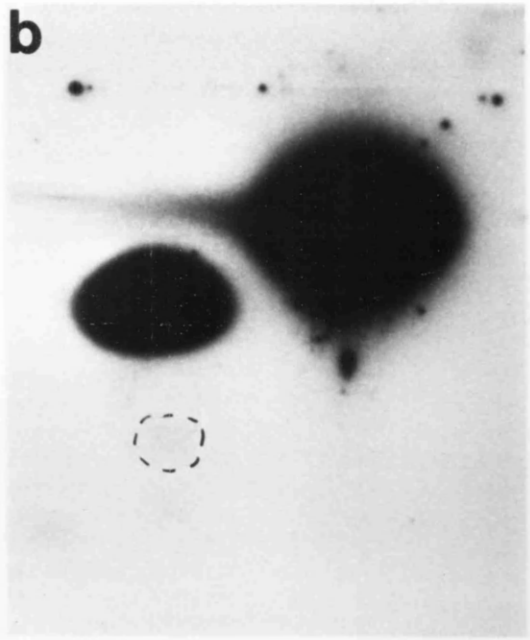
Figure 19a. Phosphoamino acid analysis of CMT 167/C6 cells

Figure 19b. Phosphoamino acid analysis of CMT 170/E9 cells

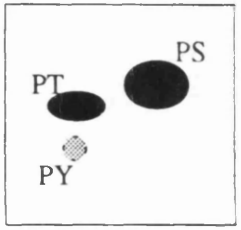
Figure 19c. Relative positions of phosphoserine (PS), phosphothreonine (PT) and phosphotyrosine (PY)

Figure 19d. Relative abundance of phosphoserine, phosphothreonine and phosphotyrosine content in each cell line expressed as a percentage of the total radioactivity incorporated.





c



d

	CMT 167/C6	CMT 170/E9
Phosphoserine	92.03%	92.11%
Phosphothreonine	7.95%	7.86%
Phosphotyrosine	0.02%	0.03%

abundance of phosphotyrosine in both cell lines was 0.03% or less. It therefore appears that tyrosine kinase activity is not a significant parameter, in this model, of the metastatic phenotype, and tyrosine kinase activity is still apparently under the normal negative regulatory controlling mechanisms.

CHAPTER VII *MOLECULAR GENETICS ANALYSIS*

Tumour progression has been associated with a number of genetic alterations, some of which were summarized in Chapter II. However, as already mentioned, few genetic alterations have been specifically associated with progression to the metastatic phenotype, although there are some genes which have been reported to be putatively metastasis-associated. Gene amplification has also, in some instances, been clinically associated with increased malignancy, for example amplification of N-*myc* in neuroblastomas (Brodeur *et al.*, 1984).

The work described in this chapter details the analysis of the putatively metastasis-associated genes *CD44* and *mts-1*. In addition, gene amplification was analysed, specifically of the N-*myc* gene and also non-specifically using the technique of in-gel renaturation (Roninson, 1987) to examine for reiterated DNA sequences with the aim of determining genes amplified in association with the metastatic phenotype.

VII.1 *Putative Metastasis-Associated Genes*

In an attempt to understand the complicated process of metastasis, many researchers have attempted to identify specific "metastasis genes". Comparative studies between metastatic and non-metastatic tumorigenic cells have lead to the identification of candidate metastasis-specific genes. These candidate genes include *CD44*, *mts-1* and *nm23* (see section II.3). The expression of both *CD44* and *mts-1* were examined in this model system, and the results of these studies will be presented in the following sections (VII.1.1 & VII.1.2).

VII.1.1 *CD44*

There is strong evidence substantiating the relationship of specific, alternatively spliced, variant isoforms of CD44 with metastatic potential in certain metastasis

model systems and in particular tumour types (Gunthert *et al.*, 1991; Rudy *et al.*, 1993; Weilenga *et al.*, 1993 & see sections I.2.2.5 and II.3.3). To assess whether CD44 was also expressed in this CMT model, and to examine the possible correlation of variant isoform expression with metastatic potential, an analysis of both cell lines was carried out using a PCR-based approach.

At the time of this particular investigation, alternative splicing of murine CD44 had not been reported. However, by comparison of the murine *CD44* cDNA sequence (also referred to as Pgp-1) with human and rat *CD44* cDNA sequences, it was possible to predict the potential site of alternative splicing within the extracellular domain (see Figure 20). Primers were designed for reverse transcription of extracted and purified RNA for cDNA synthesis (as described in section V.4.4), followed by PCR amplification (see Table 2 for oligonucleotide sequences & Figure 20 for a schematic representation of primer positions). The RNA samples were extracted from both cell lines cultured *in vitro*, in addition to RNAs extracted from CMT 167/C6 and CMT 170/E9 primary tumours *in vivo*. The PCR products were analysed by agarose gel electrophoresis and Southern blot hybridization (see sections V.3.4 and V.3.5). Hybridization of the nitrocellulose blot employed another oligonucleotide which was specific to a conserved region of *CD44* (see Figure 20). The presence of alternatively spliced exons within the *CD44* mRNAs should be detected as higher molecular weight products over the predicted molecular weight size of murine *CD44*, according to the positions of the oligonucleotide primers used to amplify by PCR. Since the oligonucleotide used to probe was specific to a conserved region of *CD44*, all variant isoforms should be detected in addition to the standard isoform which contains no alternatively spliced exons. As can be seen in Figure 21, a single *CD44*-specfic PCR product was detected in all samples. Furthermore, the estimated molecular weight of these products corresponds to the standard form of *CD44*, without any alternatively

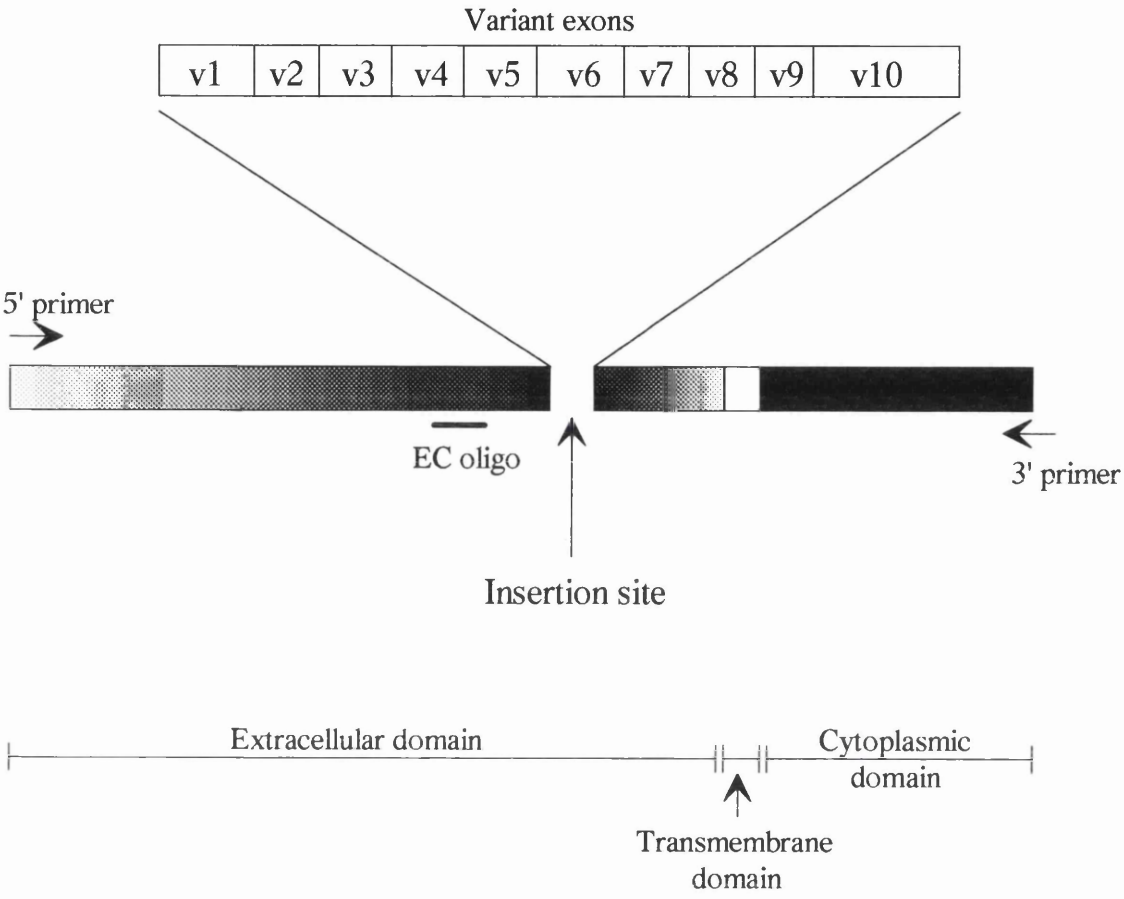
Figure 20

Figure 20. *Schematic Diagram Of CD44, Alternative Splice Sites And Primer Positions*

The proposed positions of alternative splicing within the murine CD44 gene structure are indicated. Since this work was completed, these positions have been confirmed (Screaton *et al*, 1993). Variant exons v4-v7 have been found in a rat system to confer metastatic potential (see text for details, section I.2.2.5). The standard form of CD44 has no alternatively spliced exons (also known as murine *pgp-1*).

Oligonucleotide primers were designed to span the extracellular matrix domain to the cytoplasmic domain, as represented in the diagram. An internal (EC) oligonucleotide was also designed to detect a conserved region of the extracellular domain by Southern hybridization. See Table 2 for oligonucleotide sequences.

Schematic Diagram Of CD44, Alternative Splice Sites And Primer Positions



Adapted from Screaton et al., 1993

Table 2 *Oligonucleotide sequences of PCR primers and probes for CD44 analysis*

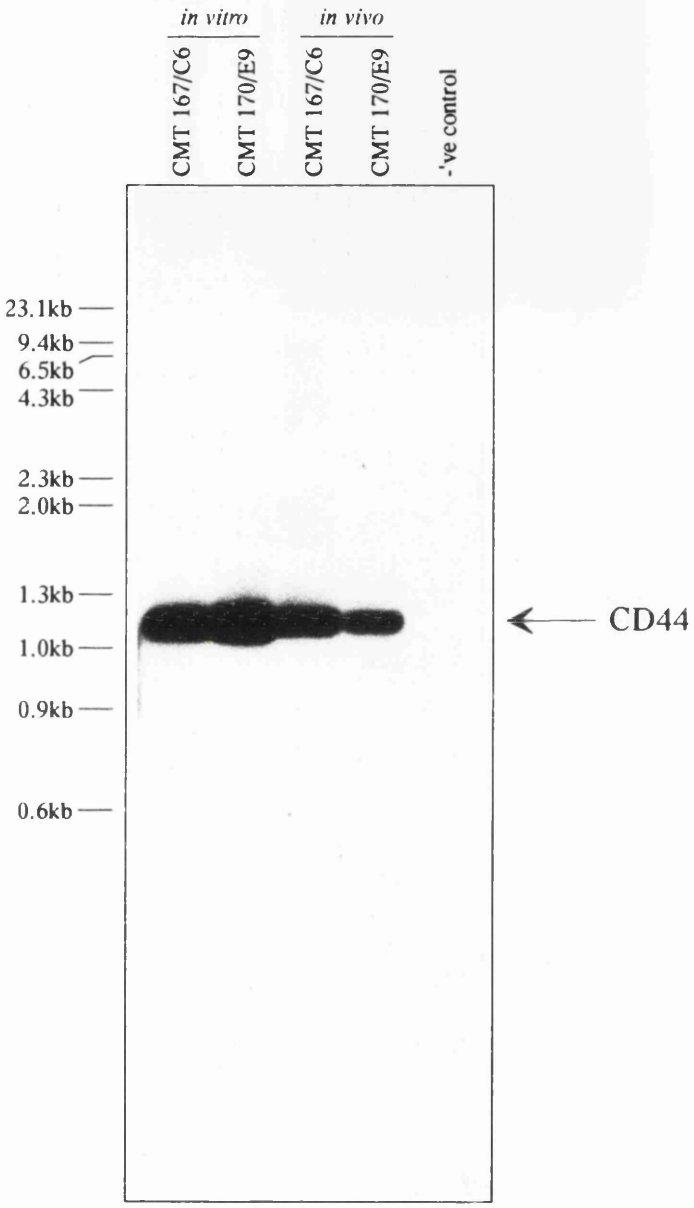
Oligonucleotide	**Sequence**
5' primer	5' TTG CCT CTT GCA GTT GAG CCT G 3'
3' primer	5' GTC AGT AGC ACA TTG CAT CTG TG 3'
EC probe	5' GAC TGT ACA TCA GTC ACA GAC CTA CCC AAT TCC 3'

Figure 21

Figure 21. *Southern Hybridization Of CD44 RT-PCR Products*

RNAs were extracted from CMT cell lines cultured *in vitro* and from primary tumours. Following RT-PCR, using the primers described in Table 2, the PCR products were electrophoresed on an agarose gel, Southern transferred and hybridized with an oligonucleotide (EC) specific to a conserved region of the extracellular domain (sequence detailed in Table 2). This EC oligonucleotide, due to its specificity to a conserved region, should detect all possible alternatively-spliced isoforms of CD44. A single CD44-specific PCR product was detected in all samples which corresponded in molecular weight to the predicted size of the standard form of murine CD44. No other CD44 products were detected which could correspond with alternatively spliced variants of CD44.

Southern Hybridization Of CD44 RT-PCR Products



spliced exons. It therefore appears that the variant CD44 isoforms are not significant in the metastatic phenotypes of this model system. Recent literature has provided evidence for the expression of *CD44* variant exons in murine tissues (Screaton *et al.*, 1993), therefore the lack of these variant isoforms in this model system is not due to inability of *CD44* to undergo alternative splicing in the murine system, but is instead because *CD44* in this CMT model is not subject to alternative splicing.

VII.1.2 *Mts-1*

Another molecule reported most notably by Lukanidin and colleagues to be putatively metastasis-associated is *mts-1* (Ebralidze *et al.*, 1989). As outlined in section II.3.2, Mts-1 appears to be related to the S100 family of calcium-binding proteins. However, the relationship of *mts-1* expression with the metastatic phenotype remains tentative, although it is presently regarded as a candidate metastasis-associated gene.

The mRNA expression of *mts-1* was examined in both CMT cell lines. Northern analysis utilized as a probe the whole *mts-1* insert from the pUC19-based construct, p271 (a kind gift of E. Lukanidin, Fibiger Institute, Copenhagen) excised with the restriction enzymes *Kpn*I and *Bam*HI, see Figure 22. To confirm this insert encoded *mts-1*, the *mts-1* insert was sequenced (data not shown) and was found to correspond to the published cDNA sequence.

Northern analysis of both CMT cell lines did not reveal any expression of *mts-1* in either cell line (see Figure 23A). Since Ebralidze *et al.* (1989) reported the association of *mts-1* expression with metastatic potential in various cell lines, including a low metastatic potential B16 melanoma cell line exhibiting a corresponding low expression of *mts-1*, another pair of cell lines were analysed. Sublines B16 F10 (high colonization / experimental metastasis potential) and

Figure 22

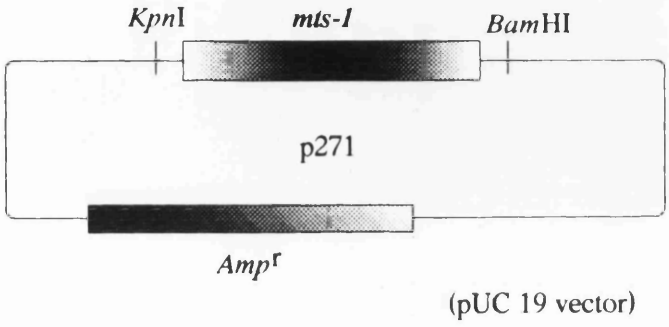
Figure 22. *Map Of Mts-1 Plasmid, p271 And Gel Purified Mts-1 Insert*

Figure 22A. Plasmid map of p271 containing the *mts-1* insert and ampicillin resistance gene. Restriction sites used to excise the *mts-1* insert are shown.

Figure 22B. Ethidium bromide stained agarose gel containing purified mts-1 insert.

Lane 1: HaeIII digested ϕX174
Lane 2: 1μg of *mts-1* insert
Lane 3: 2μg of *mts-1* insert.

A.



(pUC 19 vector)

B.

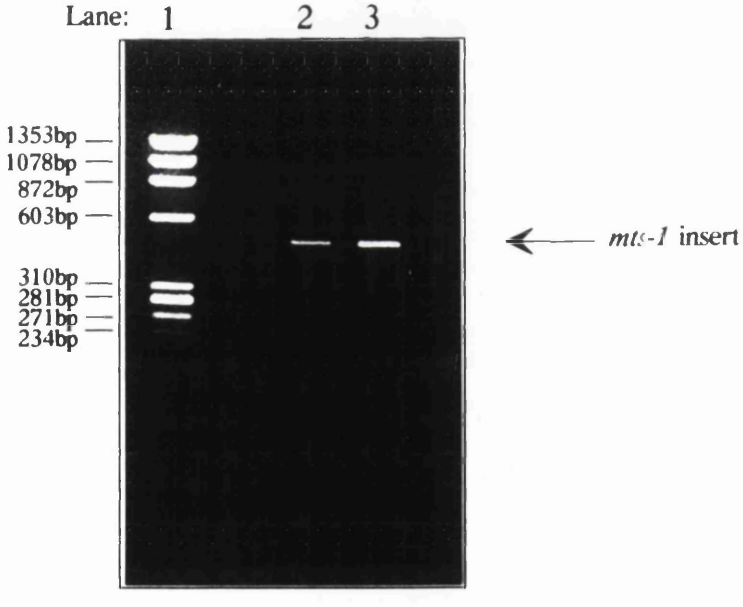


Figure 23

Figure 23. *Northern Analysis Of Mts-1 And Actin mRNA Expressions*

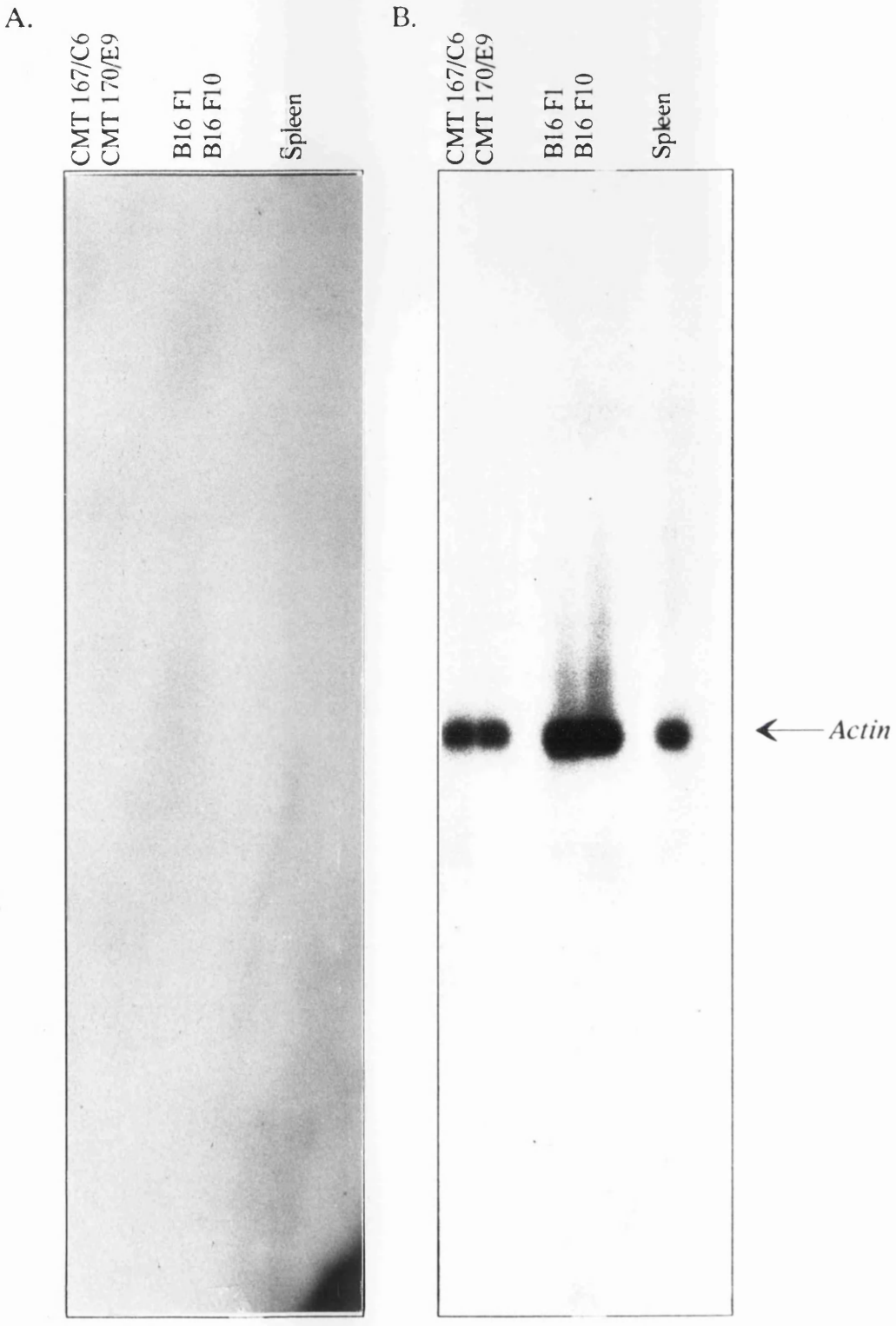
Figure 23A. Northern analysis of *mts-1* mRNA expression. 20μg of purified total cellular RNAs were electrophoresed and transferred as described (see section V.4.1, V.4.2, V.4.3). Whole *mts-1* insert (see Figure 22) was radiolabelled and used to hybridize the Northern blot, then washed under increasingly stringent conditions of:-

2x 20mins at room temperature with 2x SSC. 0.1% (w/v) SDS
1x 30-45 mins at 65^{O}C with 0.1x SSC, 0.1% (w/v) SDS

Autoradiograph exposed for 2 weeks.

Figure 23B. Northern analysis of *actin* mRNA expression. The previous blot was stripped and re-hybridized with a murine-specific *actin* probe (a kind gift of R. Nibbs). Washes were repeated as above. Autoradiograph exposed for ~12 hours.

Northern Analysis Of *Mts-1* And *Actin* mRNA Expression



B16 F1 (low colonization potential) were examined for their *mts-1* expression (RNAs a kind gift from P. Elvin, Zeneca Pharmaceuticals). Again, no expression of *mts-1* was found in these Northern analyses (see Figure 23A). Furthermore, Northern analysis of lipopolysaccharide (LPS)-stimulated bone marrow-derived macrophages, which was reported to be modulated in response to LPS stimulation in an abstract by Grigorian *et al.* (1992), did not exhibit *mts-1* expression (data not shown). To examine the possibility that these negative results were due to technical difficulties, and also to check for equal loading of samples, the Northern blots were stripped and reprobed for actin expression. As can be seen in Figure 23B, expression of actin was readily detectable and samples were approximately equal in RNA loading.

Since it was apparent that the methodology of Northern analysis was not failing, the *mts-1* probe was checked for its ability to hybridize specifically to a nitrocellulose blot. Southern analysis of *Hind*III restriction digested genomic DNA using the same *mts-1* insert to probe, hybridized to a single specific band identical in both CMT cell lines and corresponded with *Hind*III restriction digested genomic DNA extracted from normal kidney tissue of a syngeneic, C57B/T, animal (Figure 24).

Therefore, from these results it appears that the evidence presented here does not support the observations by Lukanidin and co-workers of the association between *mts-1* expression and the metastatic phenotype. As already mentioned in section II.3.2, *mts-1* may not have a direct role in the metastatic phenotype. However, since it is related to the calcium-binding proteins and may be inferred to be associated with cellular aspects such as shape change, it may therefore be seen, in certain circumstances, to be associated with the metastatic phenotype. However, it is apparent that further studies are necessary to clarify the role, if any, of *mts-1* in metastasis.

Figure 24

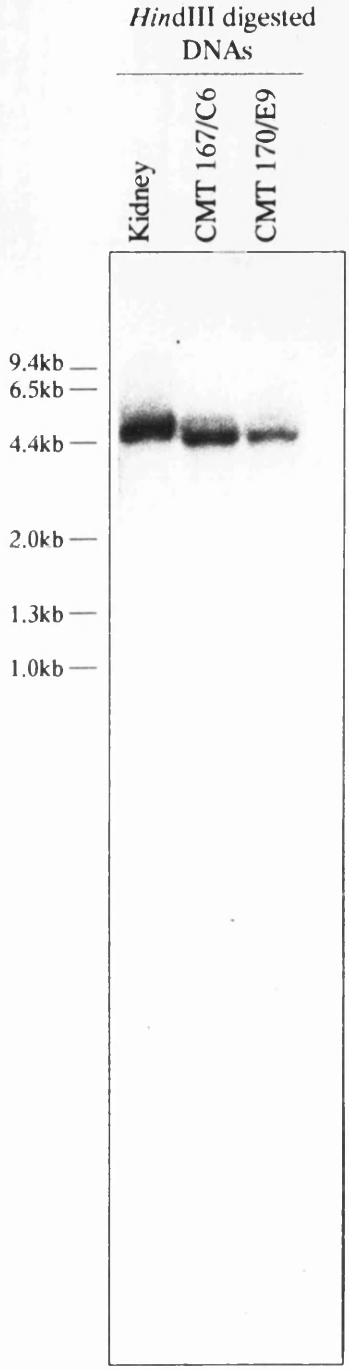
Figure 24. *Southern Analysis Of Mts-1*

Genomic DNAs were extracted from both CMT cell lines and normal C57B/T kidney tissue and subsequently restriction digested with *Hin*dIII. 10μg of each restriction digested DNAs were electrophoresed on a 1%(w/v) agarose gel and Southern transferred, as described in sections V.3.1, V.3.3, V.3.4, V.3.5. Hybridization was with radiolabelled, whole *mts-1* insert (see Figure 22). Washes were under the following increasingly stringent conditions:-

2x 15mins at room temperature with 2x SSC, 0.1% (w/v) SDS
2x 30mins at 65^{o}C with 0.5x SSC, 0.1% (w/v) SDS
1x 30-45mins at 65^{o}C with 0.1% SSC, 0.1%(w/v) SDS

Autoradiograph was exposed for ~8 hours.

Southern Analysis Of *Mts-1*



VII.2 *Gene Amplification*

Gene amplification has been described in association with malignancy (see section II.2) and may be involved in the acquisition of the metastatic phenotype. The search for amplified genes in association with the metastatic phenotype is hampered by the enormous task of examining individual candidate genes (often oncogenes) unless an alternative approach is attempted. Searching for the amplification of DNA sequences without the specific selection of genes has been made possible by the technique of in-gel renaturation (Roninson, 1987). Due to the potential of this technique to enable the identification of reiterated DNA sequences without the prior knowledge of the encoded DNA sequence, this technique could prove powerful in the discovery of previously unidentified amplified genes which may have significance in the metastatic phenotype. Both CMT cell lines of this metastasis model were examined and compared by the technique of in-gel renaturation.

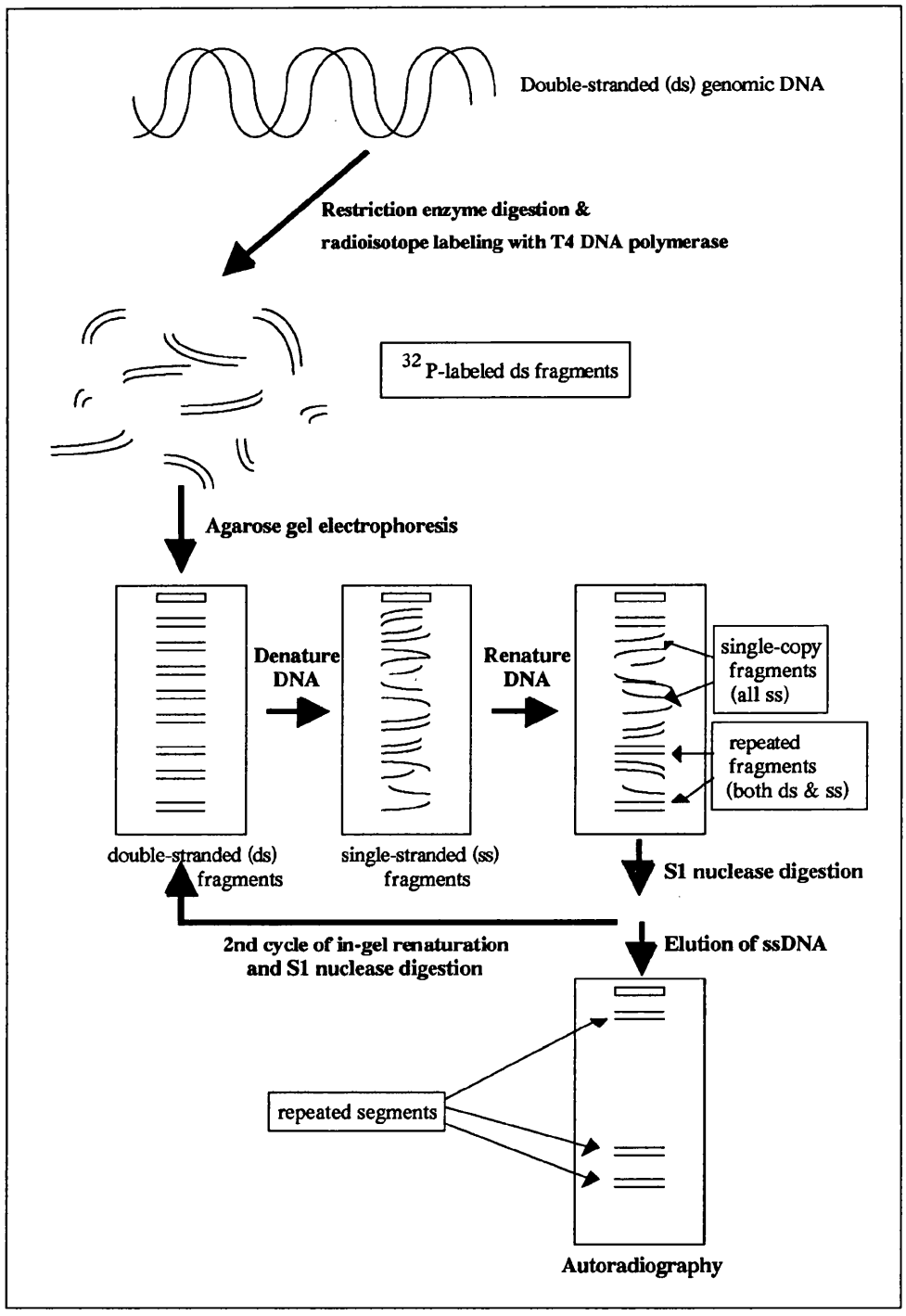
VII.2.1 *In-Gel Renaturation*

The technique of in-gel renaturation, as already mentioned, allows for the detection of reiterated sequences without the prior knowledge of the DNA sequence (Roninson, 1987 & see Figure 25). Details of this technique are given in section V.7.1.1. Briefly, whole genomic DNA was extracted from each cell line and from tissue samples of C57B/T animals, subjected to restriction endonuclease digestion and a portion of these digested DNAs were radioactively labelled with ^{32}P using the T4 DNA polymerase replacement synthesis method (see section V.5.2), which allows for the incorporation of radiolabelled nucleotides whilst retaining the original size of the restriction fragment, this being a critical factor of in-gel renaturation. A 30-fold minimum excess of unlabelled driver DNA and labelled tracer DNA were mixed together and electrophoresed on a large agarose gel. After

Figure 25

Figure 25. *Diagrammatic Representation Of The In-Gel Renaturation Procedure*

The technique of in-gel renaturation for the detection of amplified sequences without the prior knowledge of the sequence was developed by Roninson (1987). The critical features of this technique require that amplified sequences must be of the same molecular weight, as separated by agarose gel electrophoresis, and are present at a high enough copy number to enable renaturation of DNA contained in the agarose gel following denaturation (see section VII.2.1 for details).



Diagrammatic representation of in-gel renaturation
(adapted from Roninson, 1987)

electrophoretic separation of fragments according to molecular weight size, the DNA whilst still retained in the gel, was subjected to two cycles of DNA denaturation, renaturation and S1 nuclease hydrolysis. These treatments were carried out under stringent conditions whereby only restriction DNA fragments which are present in multiple copies would be retained in the gel after elution since the higher local concentration of repeated fragments results in their higher efficiency of reassociation as double-stranded DNA. The gel was then dried down and an autoradiograph exposed. The resulting autoradiograph reveals a series of bands corresponding to reiterated DNA sequences separated according to their varying molecular weights. By comparison of the two cell lines it was hoped that differences would be revealed between these cell lines which may then be subsequently isolated and cloned and hopefully identified to be associated with the metastatic phenotype.

The detection limit of in-gel renaturation for reiterated DNA sequences was estimated by Roninson to be 40 - 50 copies for the mouse genome (Roninson, 1987). Estimation of in-gel renaturation sensitivity was determined by the minimum amount of *Hin*dIII-digested λ phage which could be detected in mixtures with mouse genomic DNA and expressed as the number of copies per haploid genome. Since the size of λ DNA is 49 kb and the approximate size of the mammalian haploid genome is 2.9 x 10^6 kb, then a mixture of 100μg of mammalian DNA and 170ng of λ DNA would correspond to approximately 100 copies of λ per mammalian genome. Based on these calculations, the sensitivity of the in-gel renaturation technique was determined for the experiments presented in this thesis. At varying ratios from 500 λ copies per mouse genome to 30 copies per mouse genome, this sensitivity experiment was repeated as described by Roninson (Roninson, 1983; Roninson, 1987). However, a number of experiments following the described protocol of radioactively labelling premixed λ and CMT 167/C6 or

CMT 170/E9 DNAs and running the in-gel renaturation experiment as standard, did not reveal any bands on the resulting autoradiograph which corresponded to the expected sizes of λ fragments (see Figure 26 for an example).

Since it did not appear reasonable that the sensitivity of this experiment was less than 500 copies, another strategy was attempted to estimate the sensitivity of this technique. Instead of radioactively labelling premixed λ DNA with either CMT cell line DNAs, λ DNA was radioactively labelled separately and subsequently aliquoted to appropriate concentrations of "cold" λ DNA and CMT DNA to give the relative ratios of λ DNA to *Hin*dIII-digested CMT DNA varying between 100:1 to 20:1. Subsequent analysis of these samples by in-gel renaturation demonstrated that λ DNA fragments could readily be detected at 20-fold reiteration for high molecular weight fragments and at least 30 - 40 fold for lower molecular weights, such as the 500bp λ *Hin*dIII DNA fragment (see Figure 27). The reasons why the original experiments designed to test the sensitivity of in-gel renaturation were unsuccessful remain elusive. Perhaps in a large mixture of fragments there is preferential radioactive labelling by T4 DNA polymerase of particular fragments. This point should be noted for subsequent experiments, since the results may not be representative of total genomic DNA, but may have some preferential selection. However, this point cannot be readily proven. It can be concluded, however, that the sensitivity of in-gel renaturation here is at least as efficient as reported by Roninson (1987).

Comparative in-gel renaturation analysis of the two CMT cell lines, in addition to normal syngeneic DNA, was carried out using several different restriction endonucleases since these would yield different results according to their restriction site within potentially amplified sequences. An example of such an experiment is illustrated in Figure 28. This experiment used a double restriction digest of *Bam*HI and *Eco*RI to increase the number of lower molecular weight

Figure 26

Figure 26. *In-Gel Renaturation Sensitivity Experiment (1)*

Attempts to estimate the limit of in-gel renaturation for the detection of known copy numbers of *Hin*dIII digested λ DNA in CMT genomic DNA digested with *Eco*RI and *Hin*dIII following the protocol described by Roninson (see section VII.2.1 for details) were unsuccessful. No bands corresponding to the sizes of *Hin*dIII restriction digested λ DNA can be detected, only the bands corresponding with in-gel renaturation-treated CMT 170/E9 restriction digested DNAs are seen.

Autoradiograph was exposed for 18 hours.

In-Gel Renaturation Sensitivity Experiment (1)

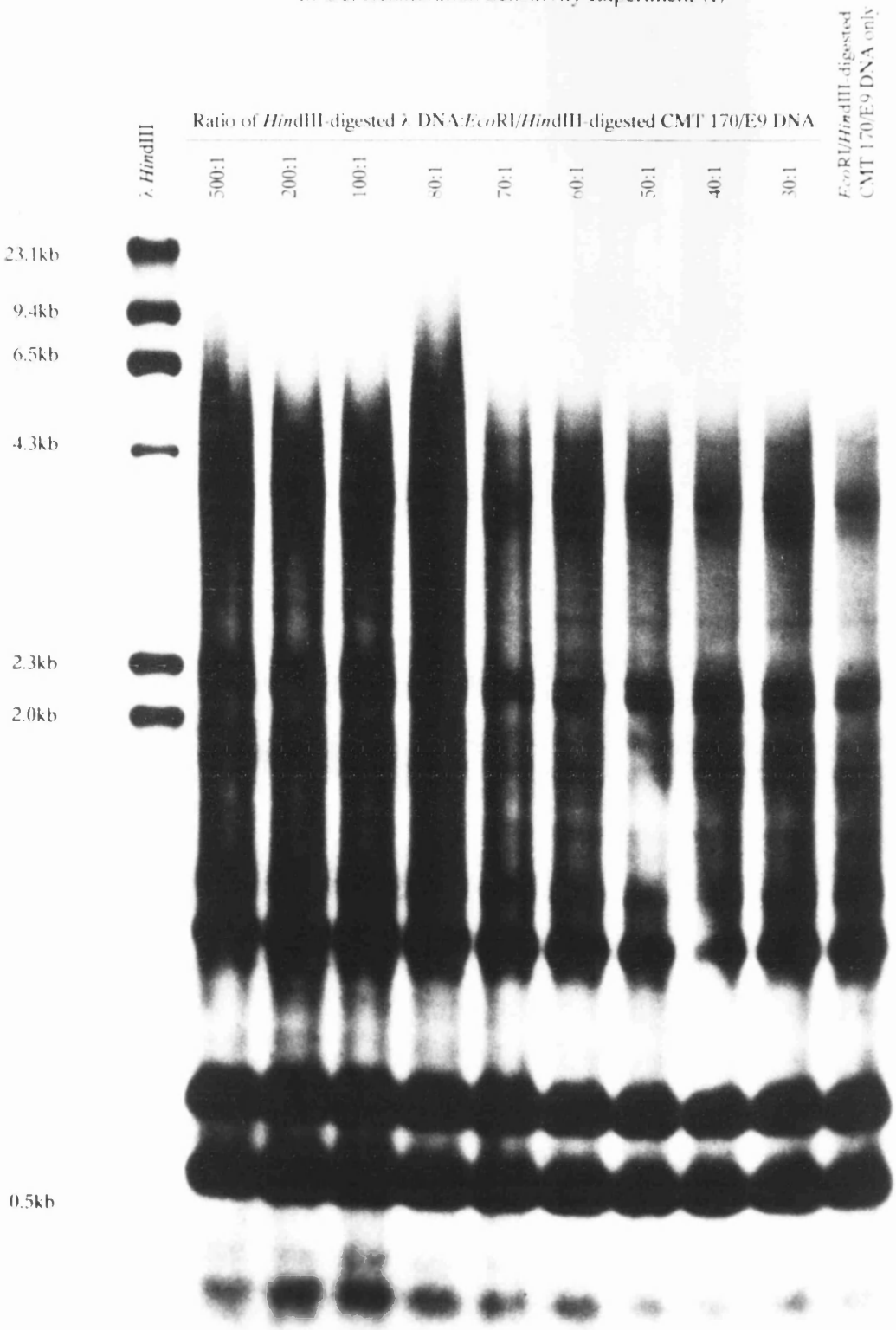


Figure 27

Figure 27. *In-Gel Renaturation Sensitivity Experiment (2)*

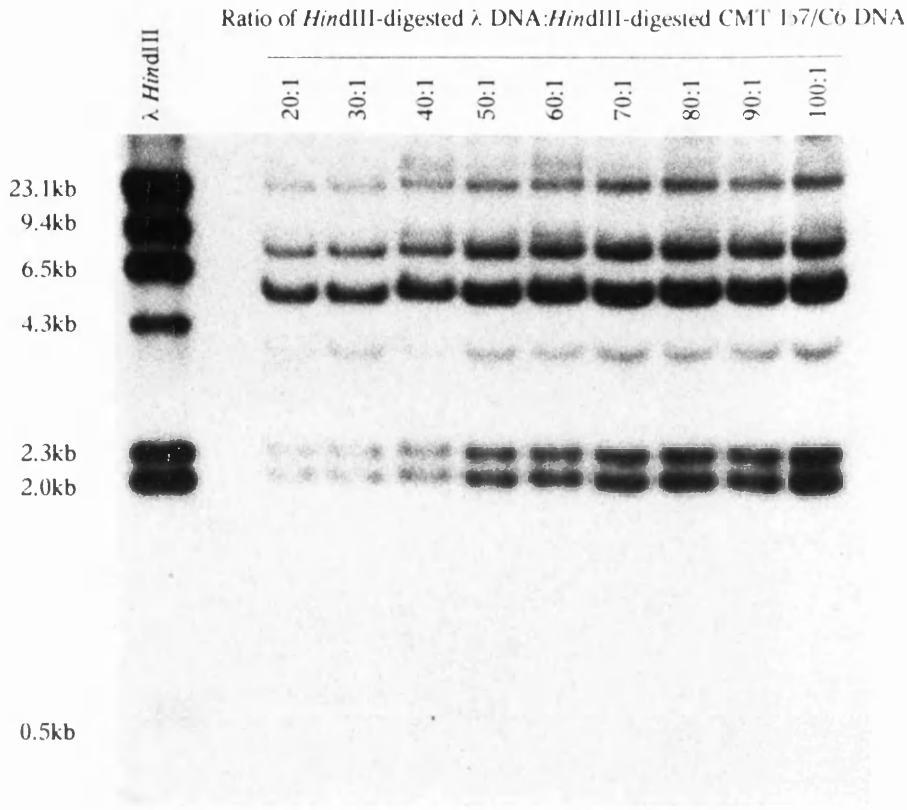
Modification of the technique to estimate the limit of detection of amplified sequences by in-gel renaturation followed essentially the same protocol as described by Roninson, except only the *Hin*dIII λ DNA was radioactively labelled, which was then mixed with restriction digested genomic DNA extracted from CMT 167/C6 cells. Mixtures were at known copy numbers of λ DNA:genomic DNA ratios, ranging between 100:1 to 20:1 (see Figure 27A). Autoradiograph was exposed for 12 hours.

Figure 27B. Longer exposure of the 0.5kb fragment (5 days).

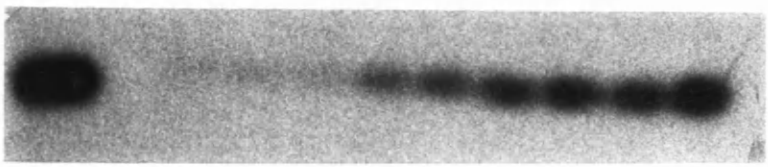
Figure 27C. Ethidium bromide stained gel following electrophoresis, but prior to in-gel renaturation demonstrating the presence of equal quantities of restriction digested genomic DNA.

In-Gel Renaturation Sensitivity Experiment (2)

A.



B.



C.

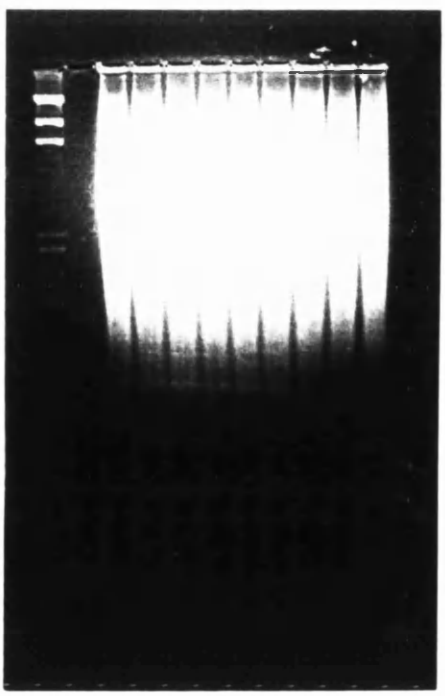


Figure 28

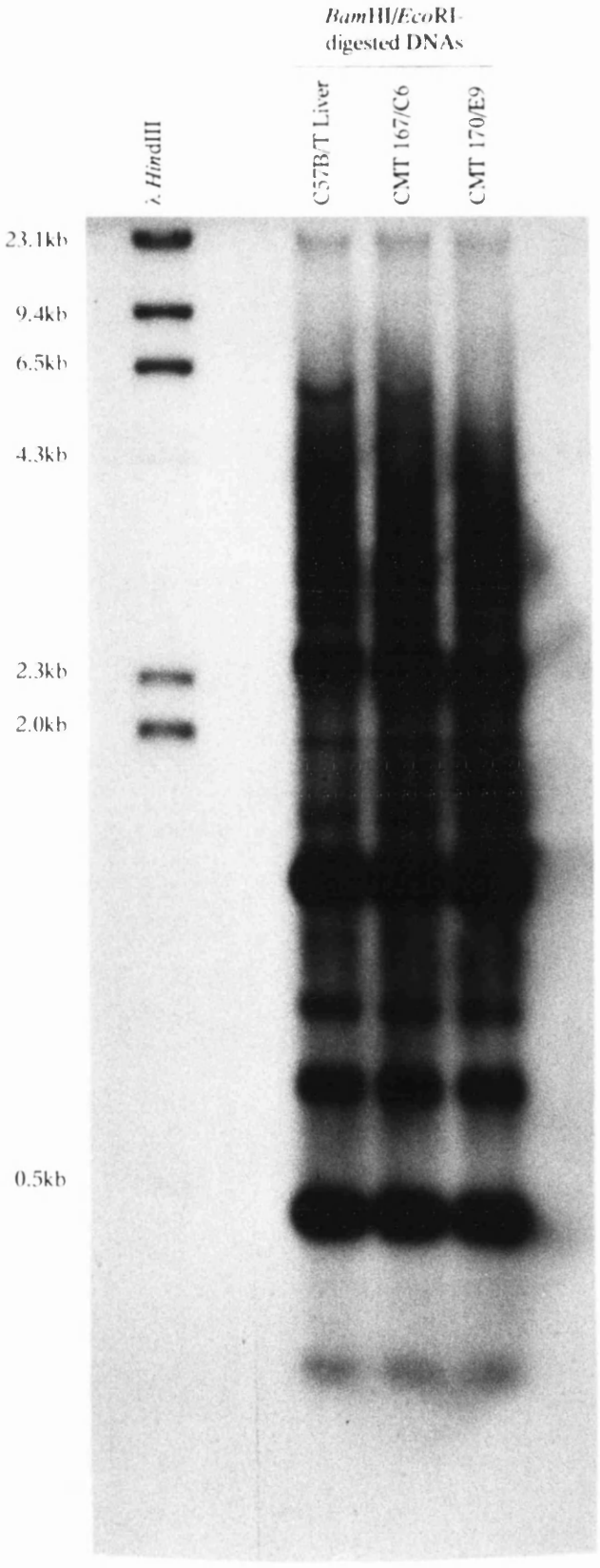
Figure 28. *Example Of An In-Gel Renaturation Experiment Comparing Both CMT Cell Lines And Normal Syngeneic DNA*

In-gel renaturation proceeded as protocol comparing *Bam*HI/*Eco*RI double restriction digested genomics DNAs extracted from *in vitro* cultured cell lines CMT 167/C6 and CMT 170/E9. Comparison was also with normal, syngeneic DNA extracted from a C57B/T liver and similarly restriction digested.

λ *Hin*dIII markers were also radiolabelled and electrophoresed in parallel to approximate molecular weights. Each lane contained radioactive tracer DNAs of 9×10^6 cpm. Comparison of these restriction digested DNAs by in-gel renaturation did not reveal any differences in bands corresponding with differences in reiterated sequences present in either of the cell lines, nor of normal syngeneic DNA.

Autoradiograph was exposed for 18 hours.

*Bam*HI/*Eco*RI In-Gel Renaturation Experiment



fragments which are resolved more efficiently in in-gel renaturation. However, this and other similar experiments did not yield qualitative differences in bands representative of different repeated DNA sequences between the cell lines.

Interestingly though, comparison of *Hin*dIII digested normal syngeneic DNA with both CMT DNAs revealed a quantitative difference in a single band which was consistently seen in repeat experiments (see Figure 29). This band is seen in both CMT cell line DNAs, and therefore does not appear to be a repeat fragment which has been entirely lost in both cell lines, however it does appear that there is a marked reduction when the relative intensities of the bands are compared. It may be postulated that this sequence has been altered as a consequence of the artificial maintenance of cells in tissue culture *in vitro*, or alternatively it may be possible that this sequence is tumorigenicity-related as opposed to metastatically-related. As the major aim of this project was to identify potentially metastasis-specific alterations causative of the phenotypes displayed by the CMT cell lines, and the possibility that the difference noted here between normal cellular DNA and the DNA of both cell lines may only be a tissue culture artefact, this finding was not followed through during this research.

It can be concluded from these results that at the level of detection of reiterated sequences by the technique of in-gel renaturation, gene amplification does not appear to have a significant role in the metastatic phenotype of this model system. However, this does not preclude the possibility that gene amplification is important in metastasis since the detection level of in-gel renaturation is not sensitive to detect small amplifications of 20-fold or less. As gene amplification may have a significant effect even if only as little as 2-3 fold, this remains a mechanism which may be contributory to metastasis, but obviously the technique of in-gel renaturation is not sensitive to detect such small amplifications.

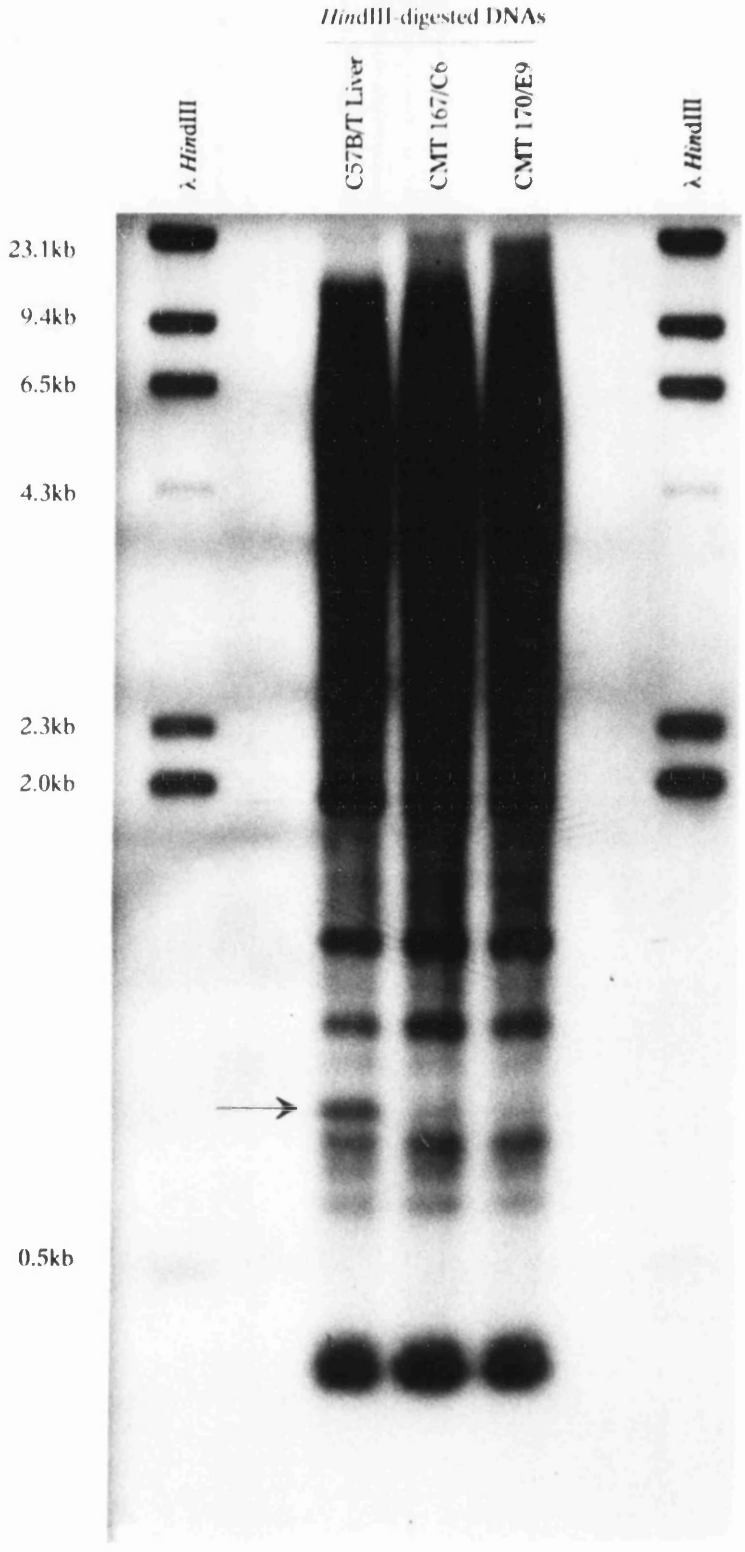
Figure 29

Figure 29. *HindIII In-Gel Renaturation Experiment Comparing Both CMT Cell Lines And Normal Syngeneic DNA*

In-gel renaturation proceeded as protocol comparing *Hin*dIII restriction digested genomic DNAs extracted from *in vitro* cultured cell lines CMT 167/C6 and CMT 170/E9. Comparison was also with normal syngeneic DNA extracted from a C57B/T liver and similarly *Hin*dIII restriction digested.

Comparison between the two cell lines did not reveal any differences in reiterated DNA sequences. However, a single band was noted to be consistently of greater intensity in normal syngeneic DNA, as compared with either CMT cell lines (band marked with an arrow in Figure 29). The band is not absent in the two cell lines, but is of lower intensity, suggesting fewer copies of this sequence(s). Alternatively this may be a polymorphism differing between the cell lines and C57B/T DNA.

*Hin*dIII In-Gel Renaturation Experiment



VII.2.2 *N-myc*

Consideration was given to the possibility that gene amplification of N-*myc* may have occurred in correlation with the altered expression of NCAM in this model system (see section VI.3.2). Previous evidence has linked the down-regulation of NCAM expression with the transfection and expression of N-*myc* in a rat neuroblastoma cell line, which also correlated with increased metastatic capacity (Akeson & Bernards, 1990). N-*myc* is known to be frequently amplified in advanced stage neuroblastomas (Brodeur *et al.*, 1984), therefore the suggestion that N-*myc* amplification may be in direct association with decreased NCAM expression exhibited in this model was examined.

Southern analysis of N-*myc* copy number was assessed of both CMT cell lines and compared with normal syngeneic DNA (see Figure 30). From densitometric analysis, N-*myc* appears to be present in both cell lines at twice the normal copy number (see Figure 30C). The differences in intensities of the upper and lower bands between the cell lines and the normal syngeneic DNA is due to partial retsriction digest. Amplification of N-*myc* does appear to be correlated with the altered NCAM expression observed in these cell lines (see section VI.3.2), however, it is possible that N-*myc* is differentially expressed in the two cell lines which may correlate with NCAM expression, but this possibility was not examined.

Figure 30

Figure 30. *Southern Analysis Of N-Myc In Both CMT Cell Lines And Compared With Normal Syngeneic DNA*

Figure 30A. Ethidium bromide staining of electrophoresed DNA samples. Each sample contained 10μg of restriction digested DNA.

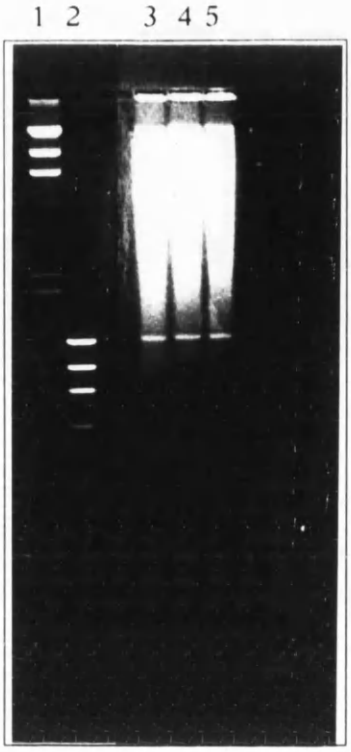
Lane 1: *Hin*dIII λ DNA markers
Lane 2: *Hae*III ϕX174 DNA markers
Lane 3: *Hin*dIII digested normal, C57B/T kidney DNA
Lane 4: *Hin*dIII digested CMT 167/C6 DNA
Lane 5: *Hin*dIII digested CMT 170/E9 DNA

Figure 30B. Southern hybridization with an N-*myc* specific probe (a kind gift from K. Ryan).

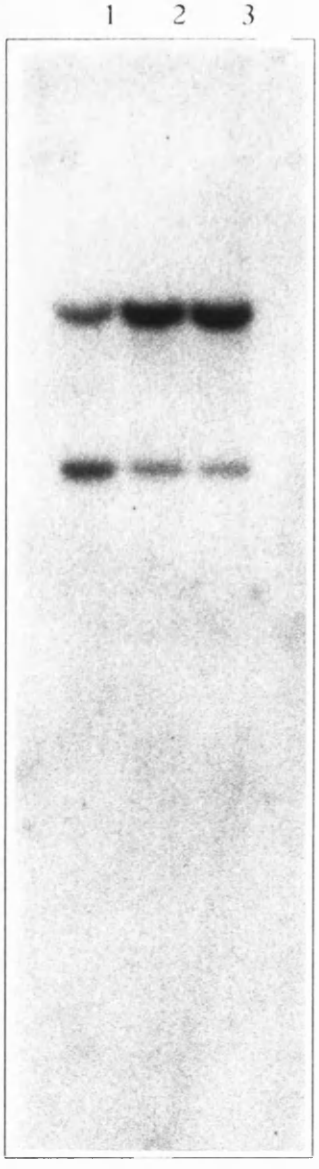
Lane 1: Normal kidney
Lane 2: CMT 167/C6
Lane 3: CMT 170/E9

Figure 30C. Densitometric analysis of cumulative values of N-*myc* specific band intensities for each sample.

A.



B.



C.

	CMT 170/E9	CMT 167/C6	C57B/T kidney
Upper band	7.317	7.251	2.308
Lower band	0.842	0.877	1.308
Total Value	8.159	8.128	3.616

Interestingly, the promoter region of the NCAM gene has been characterized and found to contain a number of species-conserved regulatory elements including a homeodomain binding sequence, an SP-1 element and an AP-1 element (Colwell *et al.*, 1992). Preliminary studies of *Hox* gene expression in these two cell lines (M. Jackson, Beatson Institute, personal communication) prompted further investigation into transcription factor DNA binding activities present in these two CMT cell lines (see chapter VIII).

CHAPTER VIII *TRANSCRIPTION FACTORS IN THE CMT MODEL*

Transcription is of key importance in the regulation of cell growth and differentiation through the control of gene expression. Various reports have suggested that particular DNA recognition sequence motifs (*cis*-acting elements), which are recognized and bound by corresponding transcription factors (*trans*-acting factors), are important in the regulation of certain cell adhesion molecules and extracellular matrix degrading proteases. Therefore, the divergent metastatic phenotypes of the two CMT cell lines described in this work may be expected to display differential regulation of transcriptional activities which may account for the observed phenotypes.

As a preliminary and general analysis for DNA:protein interactions of different transcription factors with sequence-specific oligonucleotides, electrophoretic mobility shift assays (EMSAs) were performed with nuclear protein extracts from both CMT cell lines (see sections V.6.2. & V.7.2 for methods). EMSA double stranded DNA probes are typically 20-40 bp in length (see Table 3 for oligonucleotide sequences used in EMSAs described). These probes were radioactively labelled at the ends of both strands using T4 polynucleotide kinase (see section V.5.4). Following gel purification, the 5' end-labelled probes were incubated with crude nuclear extracts from each cell line in the presence of a large excess of non-specific competitor poly(dI.dC).poly(dI.dC). Transcription factors bind specifically and with high affinity to the short recognition sequences of DNA. However, they can also weakly bind non-specifically to random DNA sequences, therefore the incorporation of large quantities of poly(dI.dC).poly(dI.dC) in the incubation reaction will compete for any non-specific binding thus allowing for the specific, high affinity binding of transcription factors to the labelled probe. The presence of non-specific DNA binding proteins which do not readily bind poly(dI.dC).poly(dI.dC) can be distinguished from specific binding by

Table 3 *Oligonucleotides for electrophoretic mobility shift assays (EMSAs)*

Name	Sequence	Reference
NFκB	5' CAA CGG CAG GGG AAT TCC CCT CTT 3'	Ballard *et al.*, 1990
E3-AP-1	5' CCG AAG TTC AGA TGA CTA ACT CAG GG 3'	Hurst *et al.*, 1987
AP-2	5' CCG GCC CCA GGC GT 3'	J. O'Prey, unpublished data
PEA3	5' GAT CCT CGA GCA GGA AGT TCG AGA C 3'	Wasylyk *et al.*, 1990
ERG	5' GAT CTC TAG CTG AAT AAC CGG AAG TAA CTC ATC CTA GGA TC 3'	Reddy & Rao, 1991
C/EBP	5' GCT GCA GAT TGC GCA ATC TGC AGC 3'	Landschulz *et al.*, 1988
c-myb	5' TTC GGC ATA ACG GTT CCG TAG CC 3'	Guehmann *et al.*, 1992

transcription factors through the inclusion in a competition EMSA assay of either an excess of unlabelled probe which should compete for binding, or competition with another unrelated DNA sequence which, if the DNA binding is specific, should not compete for binding with the labelled probe. The DNA:protein complexes are resolved by low ionic strength, non-denaturing PAGE according to their size, charge and conformation.

VIII.1 *NF-κB*

NF-κB is composed of two subunits designated p65 and p50. Specific inhibition of p65, using anti-sense phosphorothio oligonucleotides, correlates with inhibition of cell adhesion of murine embryonic stem cells (Narayanan *et al.*, 1993). Furthermore, stable transfection of a murine fibrosarcoma cell line with antisense p65 resulted in a reduced capacity to form tumours *in vivo* (Higgins *et al.*, 1993). Indeed, antisense p65 constructs caused the regression of established primary tumours following induction of antisense p65 expression (Higgins *et al.*, 1993).

NF-κB sites have also been identified in the promoter regions of the cell adhesion molecules E-selectin and P-selectin (Smith *et al.*, 1993; Shu *et al.*, 1993; Pan & McEver, 1993). Furthermore, NF-κB has been demonstrated to be involved in the transcription of the serine protease, urokinase plasminogen activator (Hansen *et al.*, 1992). Potentially, NF–κB may be differentially regulating the expression of genes directly involved with the metastatic phenotype, therefore, NF-κB was one of the candidate transcription factors examined by EMSA. As illustrated in Figure 31A, nuclear extracts from both cell lines demonstrated similar banding patterns corresponding with radioactively labelled double-stranded oligonucleotide sequence (probe) incorporating the consensus *NF-κB* element complexed with NF–κB subunits. DNA binding was not competed out with non-specific probes to Myb binding sites or AP-2 sites but was competed with the same unlabelled

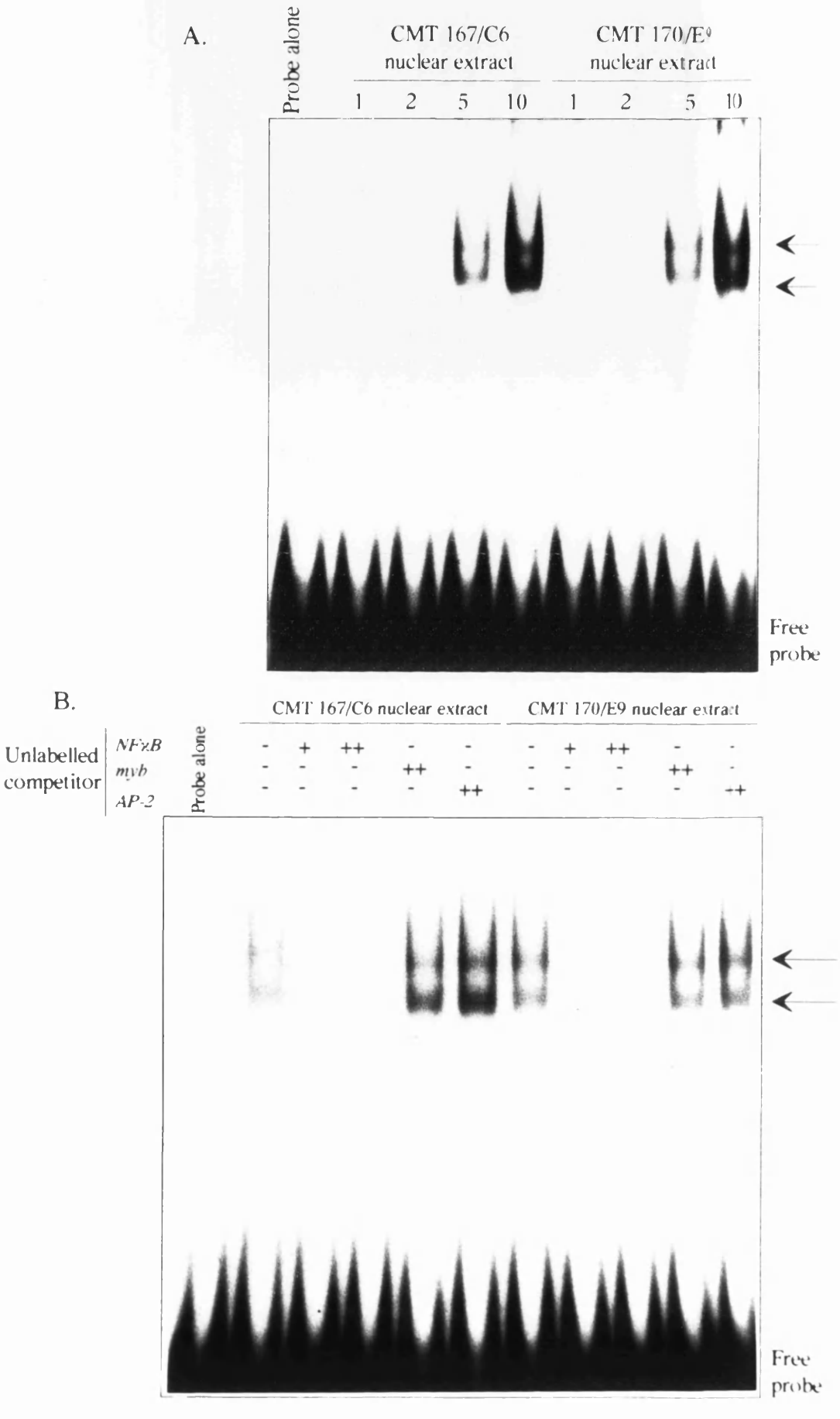
Figure 31

Figure 31. *EMSA Demonstrating The Presence Of NFκB Oligonucleotide Binding Activity In Nuclear Protein Extracts From Both CMT Cell Lines*

Figure 31A. Nuclear protein extracts were prepared from both CMT cell lines as described in section V.6.2. Double-stranded, end-labelled *NFκB*-specific oligonucleotides were incubated with 1, 2, 5 and 10μg of nuclear extracts of each CMT 167/C6 and CMT 170/E9 cell lines, as described in section V.7.2. Following non-denaturing polyacrylamide gel electrophoresis, the gel was dried down and exposed to X-ray film for ~12 hours. Two complexes can be seen in extracts from both cell lines, marked with arrows. Uncomplexed, free oligonucleotide is labelled as "free probe".

Figure 31B. NFκB competition EMSA. Cold competitor *NFκB* oligonucleotide was added to the binding reactions simultaneously with the labelled oligonucleotide at 50-fold excess (+) or 100-fold excess (++). Non-specific competitors were added to reactions at 100-fold excess (++). No competitor is labelled (-). Oligonucleotide sequences are as detailed in Table 3.

Specific complexes are indicated with arrows.


A.
Probe alone
CMT 167/C6
nuclear extract
CMT 170/E9
nuclear extract
1
2
5
10
1
2
5
10
Free
probe
B.
Unlabelled
competitor
NFκB
myb
AP-2
CMT 167/C6 nuclear extract
CMT 170/E9 nuclear extract
Probe alone
Free
probe

NF-κB probe (see Figure 31B), thus indicating the protein interactions with the *NF-κB* oligonucleotide were specific. However, it is also apparent that no striking differences, qualitatively or quantitatively, exist between the nuclear extracts from the cell lines in this analysis.

VIII.2 *PEA3 & AP-1*

The *PEA3* DNA binding motif, first recognized in the polyomavirus enhancer, can complex with members of the Ets family of transcription factors *in vitro*, including: c-Ets-1, c-Ets-2, Elk-1 and the PEA3 protein (Wasylyk *et al.*, 1990; Rao & Reddy, 1992; Xin *et al.*, 1992). The *AP-1* motif can complex with the transcription factor AP-1, is a composite factor consisting of heterodimers between the protein products of c-*fos* and c-*jun* gene family members (Curran & Franza, 1988). Fos:jun heterodimers are stable complexes which bind strongly to *AP-1* sites and activate transcription (Smeal *et al.*, 1989). Whereas jun can form homodimers which only weakly bind *AP-1* sites, fos is unable to homodimerize and therefore does not activate transcription (Smeal *et al.*, 1989).

Adjacent *PEA3*-like and *AP-1* motifs have been identified in the promoters of various genes including the extracellular degrading enzymes matrilysin, collagenase and urokinase (Gaire *et al.*, 1994; Gutman & Wasylyk, 1990; Nerlov *et al.*, 1992). Furthermore, an *AP-1* site is also present in the promoter region of stromelysin, in addition to two c-Ets-1 binding sites, although these elements do not appear to act synergistically in stromelysin transcription (Wasylyk *et al.*, 1991). It is apparent that the transcription factors which complex with the *PEA3* and *AP-1* motifs may significantly influence the metastatic phenotype through the control of the expression of extracellular matrix degradative enzymes which are characteristic of invasive and metastatic tumours (see section I.2.3). *PEA3* has also been found to be overexpressed in *neu*-induced mammary carcinomas which had

metastasized to the lung in a mouse model (Trimble *et al.*, 1993). Therefore, nuclear proteins prepared from both CMT cell lines were examined for their DNA:protein interactions with oligonucleotides encompassing the recognition sequences of *PEA3* and *AP-1* by EMSA.

Protein interactions with the *PEA3* oligonucleotide of both CMT cell lines gave virtually identical band shifts (see Figure 32A). The three major bands which can be seen on the gel are competed out with increasing concentrations of non-radioactive *PEA3* oligonucleotide, indicating that these DNA:protein interactions are specific (see Figure 32B). The lower complex, which is of greatest mobility, is relatively broad and may be indicative of more than one complex present in close proximity to each other. Alternatively, it may be possible that this broad band represents some degradation products. However, it appears that there are no major alterations in the presence or quantity of different Ets-related proteins which can bind with the *PEA3* recognition sequence present in either CMT cell lines. This may be interpreted as a slightly surprising result since both cell lines possess dramatically different metastatic phenotypes and it is realised that PEA3, together with AP-1, contributes to the regulation of different extracellular matrix degrading enzymes. On the other hand, earlier experiments assaying for the production and secretion of collagenase matrix metalloproteinases by zymography (see section VI.2.6) revealed that neither cell line apparently secretes either inactive procollagenase or active collagenases. Therefore, the matrix degrading enzymes may not be a significant factor in the metastatic phenotype in this model system.

Examination of the DNA : protein interactions with an *AP-1*-specific oligonucleotide revealed subtle differences between nuclear extracts prepared form CMT 167/C6 and CMT 170/E9 cell lines which was repeated in three different experiments. A predominant AP-1 band shift is seen in CMT 167/C6 extracts which is also seen in CMT 170/E9 band shifts, however, the intensity of the band

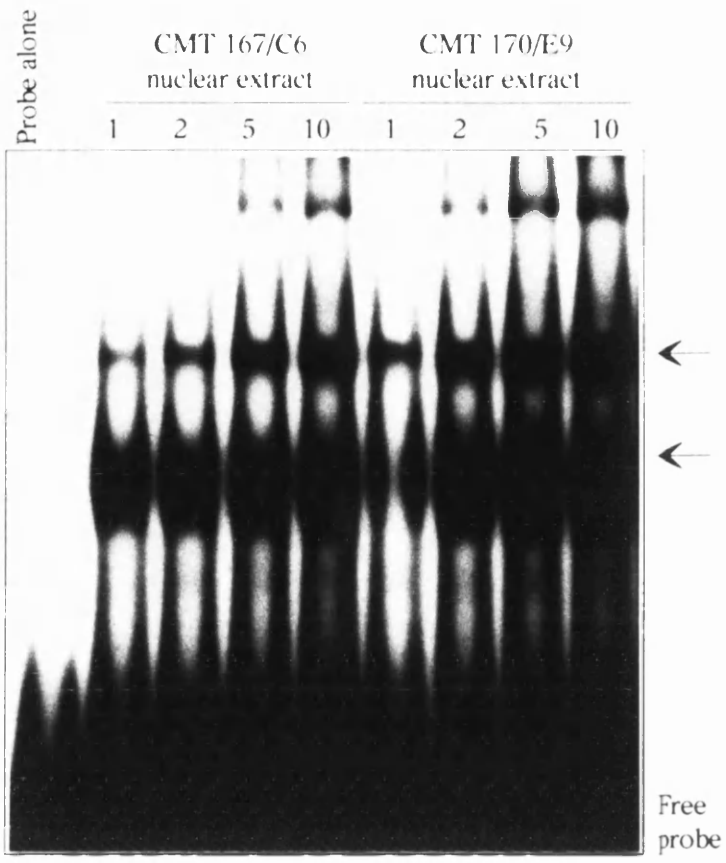
Figure 32

Figure 32. *EMSA Demonstrating The Presence Of PEA3 Oligonucleotide Binding Activity In Nuclear Extracts From Both CMT Cell Lines*

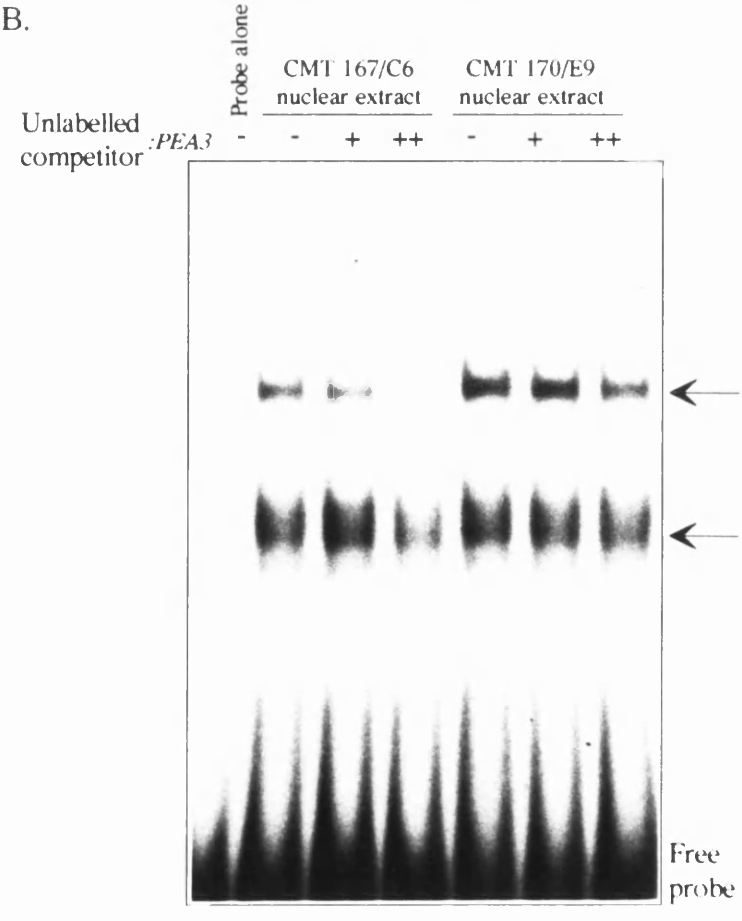
Figure 32A. Nuclear protein extracts were prepared from both CMT cell lines as described in section V.6.2. Double-stranded, end-labelled *PEA3*-specific oligonucleotides were incubated with 1, 2, 5 and 10μg of nuclear extracts of each CMT 167/C6 and CMT 170/E9 cell lines, as described in section V.7.2. Following non-denaturing polyacrylamide gel electrophoresis, the gel was dried down and exposed to X-ray film for ~12 hours. Two major complexes can be seen in extracts from both cell lines, marked with arrow. Uncomplexed, free oligonucleotide is labelled as "free probe". The *PEA3* oligonucleotide sequence is detailed in Table 3.

Figure 32B. PEA3 competition EMSA. Cold competitor *PEA3* oligonucleotide was added simultaneously with the labelled oligonucleoide at equal concentrations (+) or 10-fold excess (++). No competitor is labelled (-). Specific complexes are labelled with arrows.

A.


Probe alone
CMT 167/C6
nuclear extract
CMT 170/E9
nuclear extract
1
2
5
10
1
2
5
10
Free
probe

B.


Probe alone
CMT 167/C6
nuclear extract
CMT 170/E9
nuclear extract
Unlabelled
competitor :PEA3
-
-
+
++
-
+
++
Free
probe

shift appears greater in CMT 167/C6 extracts in comparison with CMT 170/E9 extracts (see Figure 33A). This result indicates increased AP-1 transcriptional activity in CMT 167/C6 cells in comparison with CMT 170/E9 cells. However, it should be taken into account that CMT 167/C6 cells grow marginally faster than CMT 170/E9 cells (see section VI.2.2), and the nuclear extracts were prepared from logarithmically growing cells in the presence of 10% serum. Therefore, this result may be a reflection of the slightly differing growth rates. A further experiment to examine this point would be to assay nuclear extracts prepared from cells cultured in low (0.5%) serum.

Another complex of higher mobility (labelled "X" in Figure 33A) is seen in extracts of CMT 170/E9 which is present to a much lesser extent in CMT 167/C6 extracts. It is not clear whether this is a specific AP-1 complex or not, although it does seem to be competed with cold probe, but its presence appears to be variable between experiments. However, the strong AP-1 complex is specifically competed with the same, non-radioactively labelled oligonucleotide and is not competed in the presence of other unrelated oligonucleotides (see Figure 33B).

VIII.3 *Erg*

Another member of the Ets-superfamily of transcription factors is Erg (Reddy, Rao & Papas, 1987). Different Erg isoforms are encoded in a single *erg* gene through alternative sites of splicing and polyadenylation, together with alternative sites of translation initiation (Rao, Papas & Reddy, 1987; Duterque-Coquillaud *et al.*, 1993).

Analysis of the DNA:protein interactions with an Erg-specific oligonucleotide and nuclear protein extracts prepared from both CMT cell lines revealed a single predominant band shift in an EMSA (see Figure 34A). This band shift displays no quantitative difference between the two cell lines which could correlate with the

Figure 33

Figure 33. *EMSA Demonstrating The Presence Of AP-1 Oligonucleotide Binding Activity In Nuclear Protein Extracts From Both CMT Cell Lines*

Figure 33A. Nuclear protein extracts were prepared from both CMT cell lines as described in section V.6.2. Double-stranded, end-labelled *E3-AP-1*-specific oligonucleotides were incubated with 1, 2, 5 and 10μg of nuclear extracts of each CMT 167/C6 and CMT 170/E9 cell lines, as described in section V.7.2. Following non-denaturing polyacrylamide gel electrophoresis, the gel was dried down and exposed to X-ray film for ~12 hours. An intense complex can be seen in extracts from both cell lines, although more intense in CMT 167/C6 extracts (marked with an arrow). A higher mobility complex is also faintly seen, which is labelled "X". Uncomplexed, free oligonucleotide is labelled as "free probe".

Figure 33B. AP-1 competition EMSA. Cold competitor *E3-AP-1* oligonucleotide was added to the binding reactions simultaneously with the labelled oligonucleotide at 50-fold excess(+) or 100-fold excess (++). Non-specific competitor oligonucleotides were added to 100-fold excess (++). No competitor is labelled (-). Oligonucleotide sequences are detailed in Table 3. Specific AP-1 complex is indicated.

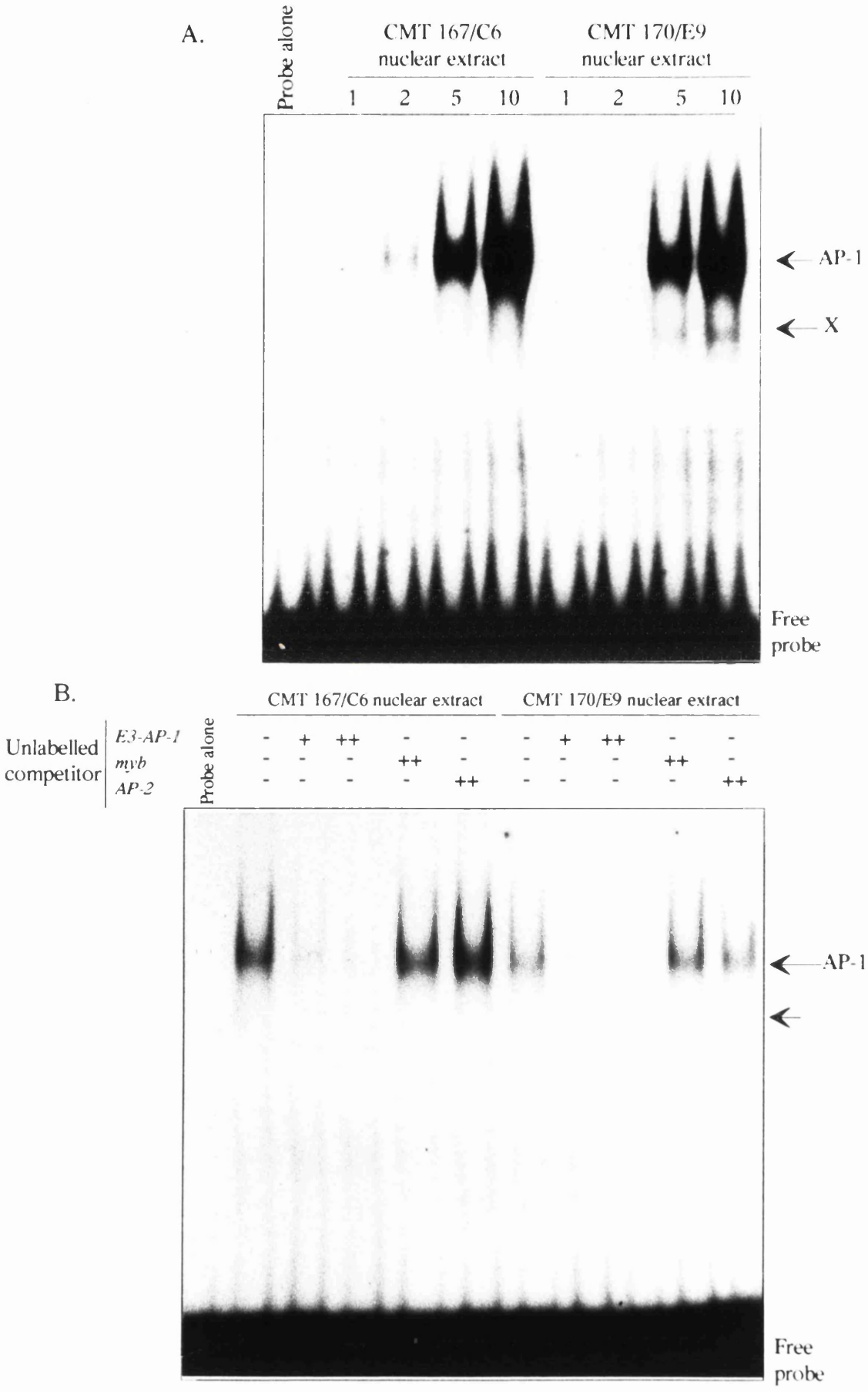

A.
Probe alone
CMT 167/C6
nuclear extract
CMT 170/E9
nuclear extract
1
2
5
10
1
2
5
10
AP-1
X
Free
probe
B.
CMT 167/C6 nuclear extract
CMT 170/E9 nuclear extract
Unlabelled
competitor
E3-AP-1
myb
AP-2
Probe alone
AP-1
Free
probe

Figure 34

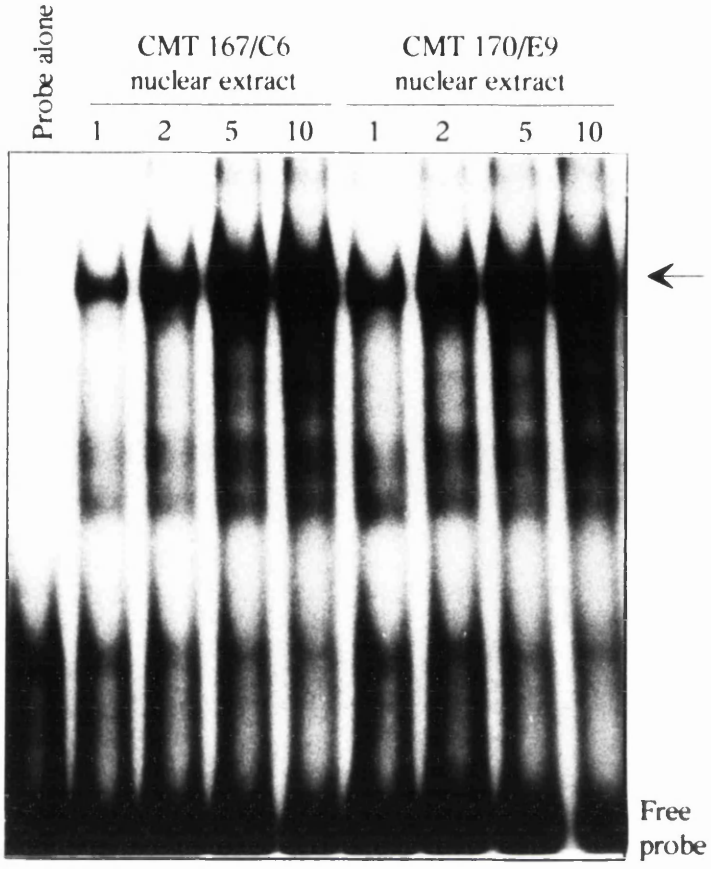
Figure 34. *EMSA Demonstrating The Presence Of Erg Oligonucleotide Binding Activity In Nuclear Extracts From Both CMT Cell Lines*

Figure 34A. Nuclear protein extracts were prepared from both CMT cell lines as described in section V.6.2. Double-stranded, end-labelled *erg*-specific oligonucleotides were incubated with 1, 2, 5 and 10μg of nuclear extracts from both CMT 167/C6 and CMT 170/E9 cell lines, as described in section V.7.2. Following non-denaturing polyacrylamide gel electrophoresis, the gel was dreid down and exposed to X-ray film for ~12 hours. One major complex can be seen in extracts from both cell lines, marked with an arrow. Uncomplexed, free oligonucleotide is labelled as "free probe".

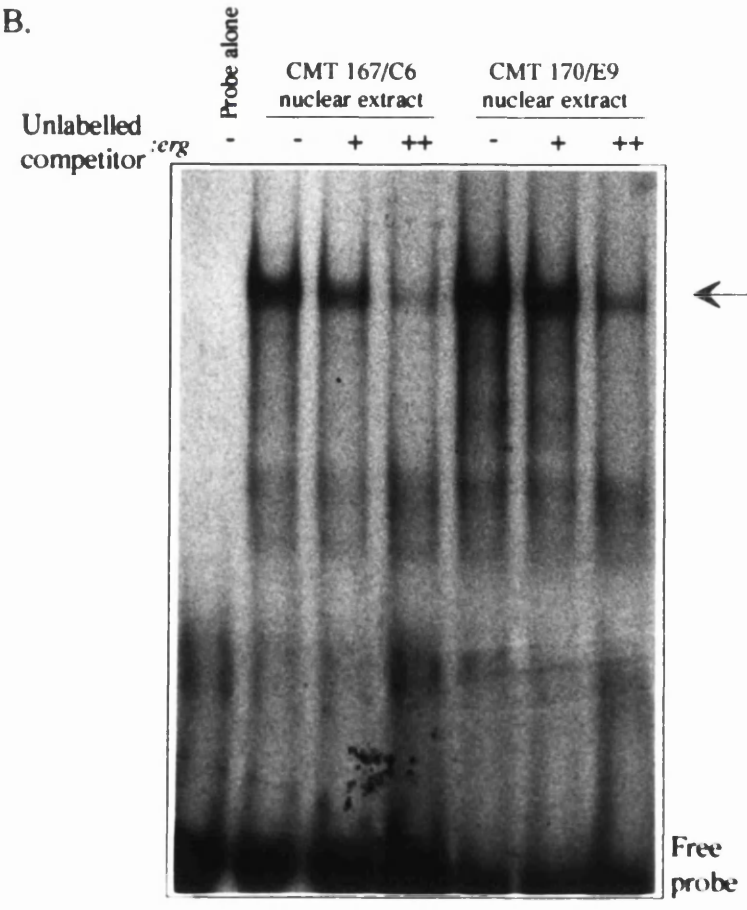
Figure 34B. Erg competition EMSA. Cold competitor *erg* oligonucleotide was added simultaneously to the binding reactions with labelled oligonucleotide at equal concentrations (+) or 10-fold excess (++). No competitor is labelled (-).

erg-specific oligonucleotide sequence is detailed in Table 3.

A.


Probe alone
CMT 167/C6
nuclear extract
CMT 170/E9
nuclear extract
1
2
5
10
1
2
5
10
Free
probe

B.


Probe alone
CMT 167/C6
nuclear extract
CMT 170/E9
nuclear extract
Unlabelled
competitor :erg
-
-
+
++
-
+
++
Free
probe

different metastatic phenotypes. The specificity of this complex was confirmed through the competitive binding of the band shift with the same unlabelled oligonucleotide (see Figure 34B).

VIII.4 *C/EBP*

The family of C/EBP transcription factors, the term being derived from CCAAT/enhancer-binding protein, has been associated with the cellular process of terminal differentiation (Birkenmeier *et al.*, 1989; Friedman, Landschulz & McKnight, 1989; Christy *et al.*, 1989). Since malignancy is frequently associated with loss of differentiation, the presence or absence of C/EBP transcription factors was assayed by EMSA.

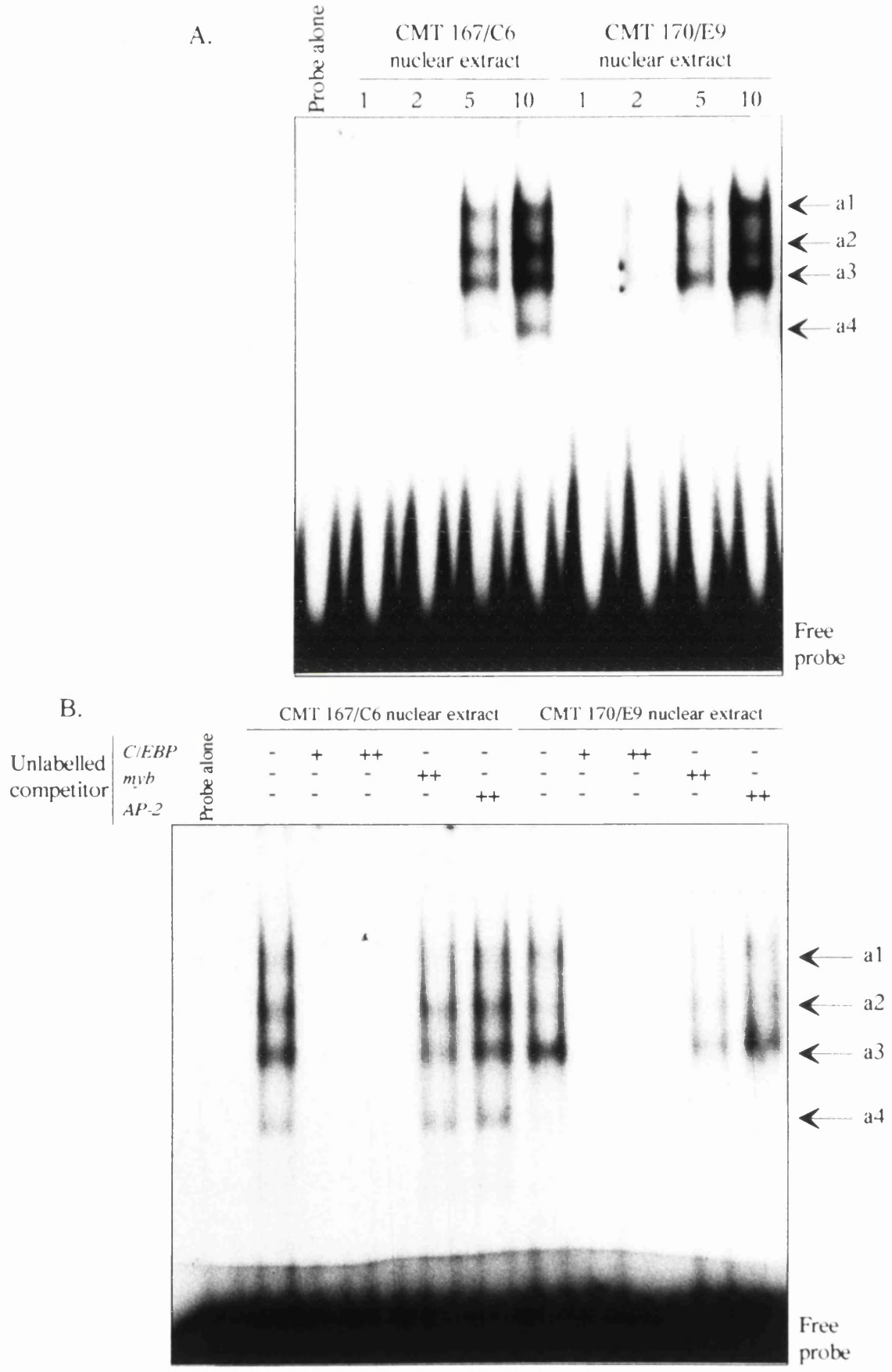
From repeat experiments with a *C/EBP*-specific oligonucleotide, four distinct complexes were found (see Figure 35A). Each of these complexes are present in both CMT cell line extracts, however, it appears that the complex termed a4 is less abundant in CMT 170/E9 nuclear extracts in comparison with CMT 167/C6 nuclear extracts. The other complexes termed a1, a2 and a3, appear to be at comparable levels in the two cell lines. Further analyses were not carried out to examine which components were present in the a4 complex since these experiments were only of a preliminary nature. However, it appears that complexes a1-a4 are all specific since they are efficiently competed with cold probe and not affected by competition with unrelated oligonucleotides (see Figure 35B). The conflicting expectation of poorer differentiation to be associated with the high metastatic potential cell line, CMT 167/C6 and the result presented here with CMT 170/E9 displaying less apparent C/EBP abundance of complex a4 is addressed in the Discussion (see section IX.3).

Figure 35

Figure 35. *EMSA Demonstrating The Presence Of C/EBP Oligonucleotide Binding Activity In Nuclear Extracts From Both CMT Cell Lines*

Figure 35A. Nuclear protein extracts were prepared from both CMT cell lines as described in section V.6.2. Double-stranded, end-labelled *C/EBP* specific oligonucleotides were incubated with 1, 2, 5 and 10μg of nuclear extracts of each CMT 167/C6 and CMT 170/E9 cell lines, as described in section V.7.2. Following non-denaturing polyacrylamide gel electrophoresis, the gel was dried down and exposed to X-ray film for ~12 hours. Four complexes can be seen in extracts from both cell lines, labelled a1, a2, a3 and a4. Uncomplexed, free oligonucleotide is labelled as "free probe".

Figure 35B. C/EBP competition EMSA. Cold competitor *C/EBP* oligonucleotide was added to the binding reactions simultaneously with labelled oligonucleotide at 50-fold excess (+) or 100-fold excess (++). Non-specific competitors were added to reactions at 100-fold excess (++). No competitor is labelled (-). Oligonucleotide sequences are detailed in Table 3.


A.
Probe alone
CMT 167/C6
nuclear extract
CMT 170/E9
nuclear extract
1
2
5
10
1
2
5
10
a1
a2
a3
a4
Free
probe
B.
CMT 167/C6 nuclear extract
CMT 170/E9 nuclear extract
Unlabelled
competitor
C/EBP
myb
AP-2
Probe alone
a1
a2
a3
a4
Free
probe

DISCUSSION

CHAPTER IX *GENERAL DISCUSSION*

Fundamental questions about metastasis can be expressed simply. For example, why are particular tumour cells metastatic and therefore what characterizes a metastatic cell? How does metastasis arise and which step(s) is(are) crucial to the metastatic process? Unfortunately, these questions, as to be expected, are not so simply answered.

Metastasis appears to be a highly complicated process with multiple stages through which the potentially metastatic cell must apparently progress successfully (see Chapter I). It can be envisaged that for primary tumour establishment there is loss of various regulatory mechanisms, such as loss of checkpoints in the cell cycle involving for example, pRb or p53 (reviewed by Hinds & Weinberg, 1994) which prevent inappropriate cell proliferation, or loss of the cell's ability to undergo programmed cell death or apoptosis (reviewed by Stewart, 1994). Metastasis could be envisaged to arise from *further* losses in normal cellular regulation. One such normally controlled regulation is the maintenance of the integrity of the organ tissue: once differentiated, cells are confined within the structured organ environment (an exception being the haematopoetic system), breakdown of this integrity would lead to cellular disorganization, as is essentially seen in invasion and metastasis. Further still, it may be also considered that metastasis is a transient or cyclical event since secondary tumours must re-establish in a distant organ and induce angiogenesis for example (see section I.2.6), which is also necessary in the establishment of the primary tumour. Thus the metastatic phenotype may not be constitutively expressed, but induced under appropriate conditions. Additionally, metastasis may not be an entirely "programmed" event, but instead may contain a degree of randomness. This can arise from the chance events of certain tumour cells which are beginning to invade the normal surrounding stroma encountering a blood vessel or lymphatic channel thus allowing for these tumour cells to gain

access to the circulation leading to their spread. Furthermore, tumour cells which gain access to the circulation require to survive such indiscriminate factors as shear stress and mechanical forces before depositing in a secondary site. Therefore, due to the complexity of metastasis and our limited understanding of the disease, clinical applications to the treatment of patients with cancer and metastasis remains poor. However, present research has led to some insight into the various steps of metastasis and a large proportion of this work has arisen through the use of models.

The study of the metastatic phenotype described in the previous sections (Chapters VI, VII & VIII) was founded upon a model system which was essentially two variant cell lines derived from the same spontaneous tumour and possessing different metastatic phenotypes. This CMT model of metastasis had not been previously studied to any great extent, therefore, a large proportion of this work examined the essential characteristics of the CMT cell lines, with some speculative research attempting to discover some possible clues to the nature of the metastatic phenotype in this model. By comparison of the two cell lines which differ in their metastatic capacities, it was hoped that some differences would be detected by the methods employed which may be implicated with the metastatic phenotype. These alterations, if found, would have been examined to determine their significance in metastasis using transfection experiments.

IX.1 *Biological Characterization Of The CMT Model*

A vital consideration of the CMT model of metastasis was the maintenance of the phenotypes originally described by Layton & Franks (1984). Periodical assessment of the phenotypes of the cell lines CMT 167/C6 (high metastatic potential) and CMT 170/E9 (low metastatic potential) were routinely carried out using the

spontaneous metastasis assay in immunocompetent syngeneic animals (see section VI.1). Both cell lines were confirmed to have retained their respective differences in metastatic potential throughout the course of this work. This assurance was critical as phenotypic drift is recognized to occur as a consequence of the culture of cell lines under artificial conditions *in vitro*, which would therefore affect the outcome of any studies relating to the metastasis-associated characteristics (see chapters VI and VII).

Additionally, the possibility that the metastatic potentials of both cell lines merely correlated with proliferative capacity was also discounted following studies of *in vitro* growth rates. The two cell lines exhibited almost identical growth curves *in vitro*, in contrast to their substantially different metastatic potentials *in vivo* as assayed in the spontaneous metastasis assay in C57B/T animals. The average number of metastatic deposits from CMT 167/C6 tumours was 53 in comparison to CMT 170/E9 tumours which had an average of 2 metastatic deposits. However, to take into account differing growth rates of tumours *in vivo*, the number of metastatic deposits were counted and compared of tumours at equivalent tumour volumes and metastatic potentials were found to be a minimum of a ten-fold difference. Although an initial lag phase in tumour growth was noted of CMT 170/E9 cells in comparison with CMT 167/C6 cells in C57B/T animals (see Figure 4), at four weeks post-injection, tumour burdens were of equivalent sizes. The differing metastatic phenotypes of these two CMT cell lines does not appear to be simply a consequence of differing proliferation rates, but is indeed a result of some other fundamental difference between the two cell lines.

Expression studies of NCAM and E, N and P-cadherins in the CMT metastasis model (see section VI.3) have revealed that both E-cadherin and NCAM are expressed at quantitatively higher levels in the low metastatic potential cell line, CMT 170/E9, than in the related high metastatic potential cell line CMT 167/C6.

The inverse correlation between the expression of these cell adhesion molecules and the metastatic potentials agrees with the hypothesis that reduced intercellular adhesion contributes to the overall invasive and metastatic behaviour of tumorigenic cells by allowing cell detachment from the primary tumour mass. Appropriate studies to further examine this hypothesis in the CMT model would be transfection experiments examining the effect of increasing the expression levels of one or both cell adhesion molecules, E-cadherin and NCAM, in the cell line CMT 167/C6. Following stable transfection, the metastatic potential of the transfected CMT 167/C6 cell line could be compared with non-transfected CMT 167/C6 cells and mock-transfected CMT 170/E9 cells. The expected result, if decreased cell-cell adhesion confers an advantage for cell detachment from the primary tumour prior to tumour dissemination to secondary sites, would be a suppression in the observed metastatic capacity of transfected cells. Conversely, transfection of the low metastatic potential cell line CMT 170/E9 with anti-sense constructs to either cell adhesion molecule and comparison of metastatic potentials would be predicted to enhance metastatic potential. Similar experiments to these have successfully demonstrated transfection of each E-cadherin and the α4 integrin subunit to form functional α4β1 integrin, suppress the invasive properties of tumorigenic cells which express low protein levels of these cell adhesion molecules (Vleminckx *et al.*, 1991; Qian *et al.*, 1994). Therefore, these experiments substantiate the hypothesis that cell adhesion is a critical determinant in invasion of tumour cells *in vivo* and this conclusion would be expected to be supported from the above described experiments in this CMT metastasis model.

Unfortunately, the corresponding predicted increase in cell-matrix interactions, primarily through integrin-mediated adhesion, allowing for active dissemination into the extracellular matrix during invasion and extravasation processes of the different integrin protein subunits, could not be investigated with the materials

available at the time of this work. Analysis of the mRNA expression levels of the different integrin subunits could have been examined; however, the ultimate criteria is the expression of a functional integrin receptor which influences the cells behaviour, and measurement of mRNA abundances is much less informative than that of cell surface protein levels.

Noteworthy of these cell adhesion studies (see section VI.3) is the modulatory nature of the expression of the different cell adhesion molecules. There were no instances discovered of one cell line expressing a particular cell adhesion molecule and the other cell line without the expression of the same cell adhesion molecule. This indicates that metastasis may not be strictly dependent on an "all-or-nothing" expression of a single specific metastasis gene or inactivation of a metastasis-suppressor gene. Instead, metastasis may involve a number of modulatory changes of a quantitative nature.

Preliminary results which also suggest this conclusion arise from the 2-D gel analysis of total cellular proteins prepared from both cell lines (see section VI.4). Surprisingly, the two related CMT cell lines, although markedly different in their metastatic capacities, have apparently remarkably few differences in expressed proteins as resolved by 2-D gel analysis. Indeed, from the 2-D gel analysis, it appears that no qualitative differences can be readily associated with either the low or high metastatic properties. This suggests that metastatic potential may be consequence of a number of subtle alterations in the expression of normal cellular proteins. Another possibility is that metastasis may be a transient property which is induced only in responsive cells under appropriate conditions *in vivo*. Therefore, transient alterations which may occur within the cell concomitant with the onset of the metastatic process are unlikely to be detected either by 2-D gel analysis or, if signal transduction pathways involving the tyrosine kinases are affected, by

phosphoamino acid analysis. Indeed, quantification of protein phosphotyrosine content by phosphoamino acid analysis revealed approximately equal phosphotyrosine content in both CMT cell lines.

The qualitative differences noted in the 2-D gel analysis, if found to be consistent in repeat experiments, could be very interesting to follow up. However, due to the present lack of a database for information regarding 2-D gel analyses of murine epithelial cells, which is available for rat fibroblasts and human keratinocytes (Celis *et al.*, 1990), the identification of the protein(s) of interest would require microsequencing and / or possible screening of immunoblotted 2-D gels with antibodies specific to candidate proteins. These proteins would be chosen because of their similarity in estimated pH values and molecular weight sizes to the resolved proteins of interest. However, further experiments obviously require to be carried out before such experiments are attempted.

IX.2 *Molecular Genetics Analyses Of The CMT Model*

Associated with metastatic phenotype in some model systems are a few putatively metastasis-associated genes. These include *CD44*, *mts-1* and *nm23* (see section II.3). The expression of both *CD44* and *mts-1* were examined in this model system, however *nm23* was not chosen to be studied for the following reasons: firstly, *nm23* mRNA expression and its nucleoside diphosphate kinase activity have not been consistently correlated with the proposed metastasis suppressor activity in tumour samples (Myeroff & Markowitz 1993; Sastre-Garau *et al.*, 1992; Haut *et al.*, 1991); secondly, in the experimental model system reported by Leone *et al.* (1991), transfection of *nm23* into the TK cell lines did not, in my opinion, convincingly alter metastatic potential, but did appear to affect cell viability after transfection, in comparison with control transfections, and inhibit the *in vivo* tumour forming capacities and growth rates of the transfected cells. These results

suggest that *nm23* may not be directly causing altered metastatic potential, but instead may be affecting cell survival / proliferation. Since *nm23* has not been convincingly demonstrated as a metastasis-specific gene and it would be impossible to analyse every candidate metastasis gene in this study, *nm23* mRNA expression was not examined in this model.

IX.2.1 *CD44 Expression*

Expression of variant isoforms of CD44 has been associated with the activation of T cells (Arch *et al.*, 1992; Haegel *et al.*, 1993) and has been postulated to be involved in homing of these T cells to lymph nodes (Wu *et al.*, 1993, Arch *et al.*, 1992). Similarities can be suggested between homing of activated T cells and metastatic cells which spread through the lymphatic system. Therefore, expression of variant isoforms of CD44 could be expected to be involved in metastasis. This is also apparent from recent studies of a rat metastasis model in which expression of particular CD44 variant exons appear to be strongly correlated with metastatic capacity (Gunthert *et al.*, 1991; Rudy *et al.*, 1993). Expression of CD44 and possible variant isoforms was also examined in this model.

Examination of *CD44* expression adopted an RT-PCR / Southern hybridization approach (see section VII.1.1). This method allowed for the detection of all possible alternatively spliced transcripts of *CD44* expressed in either of the two CMT cell lines. Although at the time of this study, alternative splicing of murine *CD44* had not been reported as had been reported of rat *CD44* (Gunthert *et al.*, 1993; Rudy *et al.*, 1993) and of human *CD44* (Screaton *et al.*, 1992; Screaton *et al.*, 1993), it was predicted that murine *CD44* would be similarly capable of undergoing alternative splicing as was later demonstrated to be the case (Screaton *et al.*, 1993). However, as found in both CMT cell lines, no alternatively spliced isoforms of *CD44* were discovered. Only the expected standard form, also known

as *pgp-1*, was detected by Southern hybridization. This was found in both RNAs extracted from cells grown in culture and from RNAs extracted from primary tumours. Therefore, alternative splicing was not observed in cells growing under artificial conditions nor are other isoforms induced *in vivo*. Since alternative splicing of *CD44* is still not understood, although it is recognised to occur transiently in such cells as T cells upon their activation (Arch *et al.*, 1992; Haegel *et al.*, 1993), the significance of the expression of alternative *CD44* isoforms for example, in the rat metastatic model system, remains to be elucidated. Apparently it is not a significant factor in the CMT model system since it would be anticipated that with the sensitivity of PCR methodology, that the presence of any alternative transcripts of *CD44* would be readily detected.

IX.2.2 *Mts-1 Expression*

Another gene reported to be metastasis-associated was *mts-1* (Ebralidze *et al.*, 1989). However, although this gene has been reported to be expressed in association with various metastatic cell lines and in lipopolysaccharide (LPS)-stimulated macrophages, expression was not detectable in either CMT cell line, nor was it detected in putatively-positive controls of B16 melanoma cells or LPS-stimulated bone marrow-derived macrophages. Considerable effort was given to determine if the cause of this negative result was due to technical failure or due to other reasons. Northern analysis of actin expression was readily detectable, therefore the technique of northern analysis was not at fault. The *mts-1* plasmid insert was sequenced and found to encode the correct sequence (Tulchinsky *et al.*, 1990) and therefore the appropriate probe was being used (*mts*-1 plasmid a kind gift from Lukanidin). In addition, this insert was successfully used to probe a Southern blot and therefore was fully capable of hybridizing to DNA on nitrocellulose in a specific manner. Together, these experiments appear to confirm that *mts-1* expression is undetectable in either of the CMT cell lines. The link

between *mts-1* expression and metastasis still remains tenuous since no further evidence has been reported to further substantiate the putative association between *mts-1* and metastasis. Since it appears to be related to the S100 family of calcium-binding proteins (reviewed by Kligman & Hilt, 1988), the association seen may be related to cytoskeletal reorganization associated with active migration seen in cells such as activated macrophages and invasive cells. *Mts-1* expression may not therefore be causative of the metastatic phenotype, but is instead modulated in response to the invasive behaviour of metastatic cells.

IX.2.3 *Gene Amplification*

Earlier when this study was attempted, it was postulated that gene amplification may have a role in the acquisition of the metastatic phenotype. Amplification of N-*myc*, for example, has been recognised to occur in, and correlate with, advanced stage neuroblastomas (Brodeur *et al.*, 1984). However, although the presence of cytogenetically identified elements of gene amplification (homogeneously staining regions and double minutes) are often recognised in association with malignant tumours, the genes contained in these elements often elude identification. In an attempt to screen for gene amplification present in the CMT cell lines which may be involved in the metastatic phenotypes, without prior selection of candidate genes which could be analysed by Southern hybridization techniques, the technique of in-gel renaturation was tested.

This technique as explained earlier (see section VII.2.1) can detect amplified sequences without previous knowledge of the encoded sequence. Roninson had estimated the limit of detection for the mouse genome of reiterated DNA sequences to be ~40 copies (Roninson, 1987). The experiment described by Roninson to determine this detection limit was repeated in order to confirm that the experiments carried out in this work was at least as sensitive as expected.

Curiously, using the same protocol as described for the detection of known copy numbers of λ DNA, up to 500 copies were "undetectable". This was obviously not an accurate result since the technique itself was operational and bands representing reiterated sequences contained in the murine genome were readily detectable and not all, if any, of these would be present at a greater than 500-fold copy number. Subsequent experiments using a modified protocol allowed for the determination of repeated sequences to be readily detected at 20 copies per murine genome (see section VII.2.1). The ensuing analysis of the two CMT cell lines for the presence of reiterated sequences in comparison with normal syngeneic DNA revealed no differences between the two CMT cell lines subsequent. Surprisingly, a consistent reiterated sequence was found to be present at a higher copy number in the normal syngeneic DNA as compared with both cell lines, as estimated by the relative intensities of the corresponding bands. This reiterated sequence may be related to the establishment and maintenance of the cell lines *in vitro*, possibly with relation to the tumorigenic nature of the cell lines, or alternatively this may represent a polymorphism which has occurred subsequent to the formation of the original tumour. This polymorphism would be retained in the CMT cell lines and would thus differ from normal syngeneic DNA. Since the difference identified was not between the two cell lines and therefore most likely not to be directly related with the metastatic phenotypes of these two CMT cell lines, this reiterated sequence was not pursued during this work. However, as already noted earlier, although no differences were detected between the two cell lines as analysed by in-gel renaturation, this does not preclude the possibility that gene amplification is an important phenomena in metastasis. Gene amplification may be of significance at less than 20-fold amplification of a particular gene and therefore the technique of in-gel renaturation would not be sensitive enough for their detection.

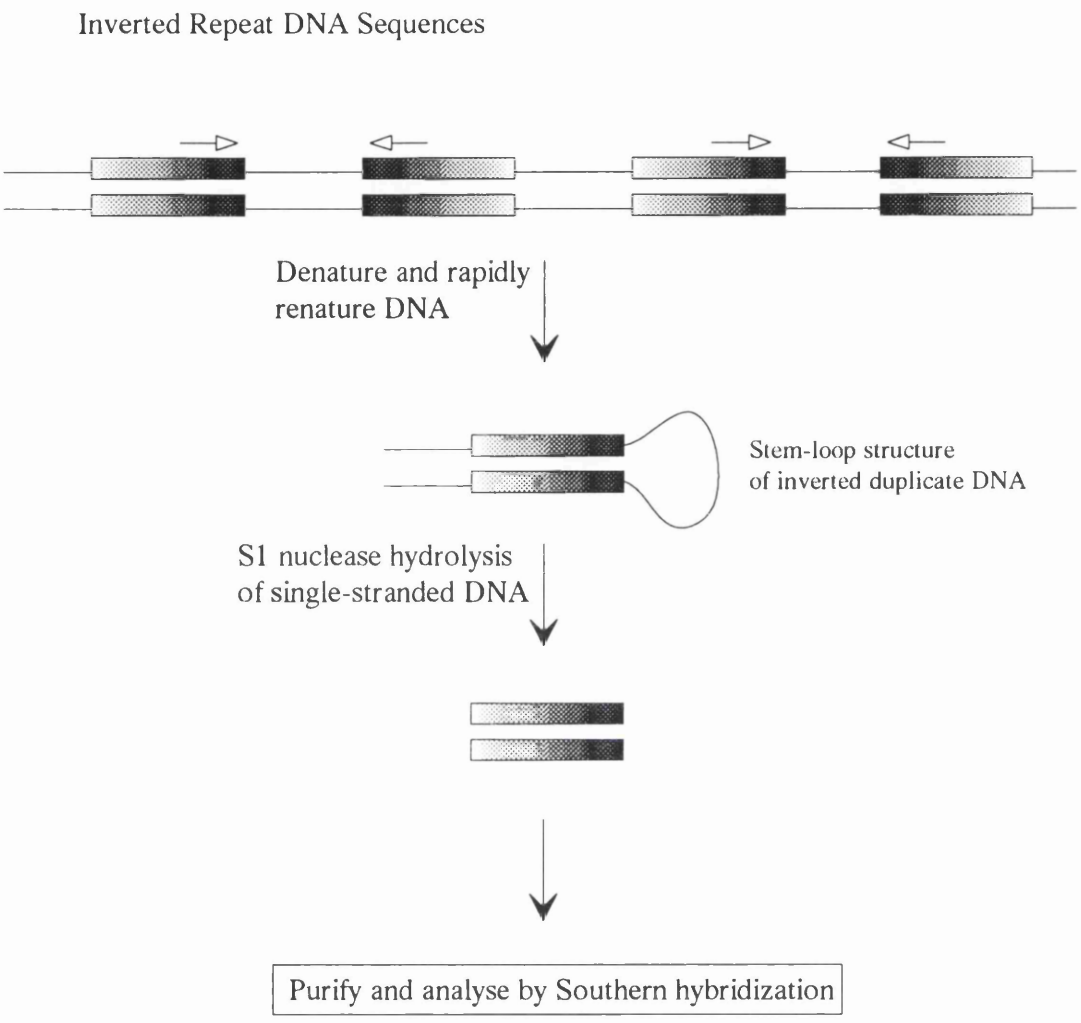
The presence of amplified sequences in the conformation of inverted repeat duplications was also briefly analysed. The technique used was described by Ford & Fried (1986) and was a relatively simple procedure of intrastrand duplex formation and analysis (see Figure 36 for a simplified representation). Purified genomic DNA was subjected to denaturation followed by rapid renaturation and S1 nuclease hydrolysis. The rapid renaturation step favours the annealing between only homologous sequences containing inverted duplications which are in direct proximity, thus forming intrastrand duplexes ("snap-backs") which are S1 nuclease resistant. Purified intrastrand duplexes, after S1 nuclease treatment, were either electrophoresed on an agarose gel and probed specifically for a candidate gene or non-specifically with restriction digested whole genomic DNA for a non-specific analysis following Southern transfer. Alternatively, purified intrastrand duplexes were used to probe a Southern blot of restriction digested genomic DNA. These analyses did not yield the specific detection of amplified sequences which were present in either cell line as inverted DNA sequences which may have been related with the metastatic phenotypes (data not shown).

The analysis of gene amplification by non-specific methods did not yield results pertaining to the metastatic phenotype, however, the specific analysis of one particular gene was examined. Consequent to the finding that *NCAM* had increased expression in the low metastatic cell line, CMT 170/E9, in comparison with the related cell line CMT 167/C6 (see section VI.3.2), and the realization that in another system, the transfection of N-*myc* into a rat neuroblastoma cell line caused down-regulation of *NCAM* expression and increased metastatic capacity (Akeson & Bernards, 1990), N-*myc* copy number was examined by Southern analysis. However, as estimated from the Southern blot, both cell lines possess equal copy numbers of N-*myc*. Therefore, in this model system of metastasis which does exhibit altered expression levels of NCAM correlating inversely with the

Figure 36

Figure 36. *Schematic Illustration Of Inverted Repeat DNA Analysis*

This technique was described by Ford & Fried (1986) to detect the presence of repeated sequences which are in the inverted conformation. The rapid renaturation step favours the annealing between only homologous sequences which contain inverted duplications and are in direct proximity. These intrastrand duplexes can be specifically enriched by S1 nuclease hydrolysis of single-stranded DNA before analysis by Southern transfer techniques (details in section IX.2.3).



Diagrammatic illustration of the technique for the detection of inverted repeat DNA sequences

metastatic phenotype (see section VI.3.2), no correlation was observed with N-*myc* copy number, unlike the report by Akeson & Bernards (1990). It may be that alterations of N-*myc* are present at the level of mRNA expression or the protein in this model system, but these possibilities were not examined due to time constraints. In addition, this model system is not neuroblastoma-derived unlike Akeson & Bernards', and therefore there may be tumour specificity in the effect of N-*myc* on *NCAM* expression. Indeed, the mechanism by which N-*myc* alters NCAM expression may not be a direct effect, but a secondary event. Therefore, in the CMT model, N-*myc* itself may not be targeted but instead a component downstream of N-*myc* is altered which in turn modulates NCAM expression.

IX.3 *DNA Binding Activities Of Transcription Factors In The CMT Model*

Interestingly, the promoter region of the *NCAM* gene has been characterized and found to contain a number of species-conserved regulatory elements including a homeodomain binding sequence, an SP-1 element and an AP-1 element (Colwell *et al.*, 1992). Further still, in transfection studies using NIH 3T3 cells and *Xenopus laevis Hox* expression vectors, strong induction of *NCAM* expression by *Hox-2.5* was demonstrated, an effect which was abolished by *Hox-2.4* in co-transfections (Jones *et al.*, 1992). It is possible that these *Hox* genes, which can regulate the transcription of *NCAM*, may be involved in the differential expression of NCAM seen in the CMT model. Indeed, preliminary evidence has indicated the cell line CMT 170/E9 (low metastatic potential) which displays high NCAM expression in comparison with CMT 167/C6 (high metastatic potential), also exhibits expression of *Hox-3.2* (M. Jackson, Beatson Institute, personal communication). *Hox-3.2* is a paralogue of *Hox-2.5* and it may be postulated that *Hox-3.2* has a similar regulatory role of cell adhesion molecule expression as *Hox-2.5*, thus this gene could also influence the metastatic phenotype.

Further to this finding, preliminary analysis was carried out to determine the transcription factor DNA binding activities of crude nuclear protein extracts prepared from both CMT cell lines. The transcription factors analysed have been previously shown to be important in the control of the expression of a number of genes important in metastasis. These include many of the matrix-degrading enzymes and cell adhesion molecules. Curiously though, differences in binding activity to an oligonucleotide specific for NFκB and related Rel proteins were not apparent between the cell lines. This was surprising since NFκB has been strongly implicated in the process of cell adhesion (Narayanan *et al.*, 1993; Smith *et al.*, 1993; Pan & McEver, 1993; Shu *et al.*, 1993). However, it may be that the cell adhesion molecules which are subject to regulation by NFκB are not the specific molecules which are altered in their expression in this CMT model of metastasis. Indeed the cell adhesion molecules known to contain *NFκB* recognition sequences in their promoter regions include E- and P-selectin and ELAM-1, which have not been examined in this model and it is unknown if their expressions are altered in either of the cell lines.

Again, no significant differences in DNA:protein interactions were noted for a *PEA3*-specific oligonucleotide. *PEA3* together with *AP-1* have been shown to be adjacent motifs in the promoter regions of the matrix-degrading enzymes matrilysin, collagenase and urokinase (Gaire *et al.*, 1994; Gutman & Wasylyk, 1990; Nerlov *et al.*, 1991). However, earlier experiments analysing the expression of Type IV collagenases by zymography (section VI.2.6) did not detect the expression in either cell line of active or inactive proenzymmatic forms, and therefore it may not be entirely surprising that differences in *PEA3* DNA binding activity were not observed.

Subtle differences were noted in AP-1 DNA binding activity. Increased AP-1 activity was apparent in CMT 167/C6 nuclear extracts. However, because these

experiments were performed with crude nuclear extracts prepared from logarithmically growing cells, this slight quantitative change in DNA binding activity may be due to subtly different proliferation rates. To examine this question, further experiments need to be attempted with nuclear extracts prepared from cells maintained in 0.5% serum to eliminate the possible interference of serum stimulation in the interpretation of these results.

Another family of transcription factors subjected to preliminary analysis in this EMSA study was C/EBP (Umek, Friedman & McKnight, 1991). This large family of transcription factors is associated with terminal differentiation processes, therefore, it might be predicted that alterations may occur in the differentiation pathways corresponding with progression of tumorigenic cells towards a more aggressive state, *i.e.*, the metastatic phenotype. From this hypothesis, it would be expected that the high metastatic potential cell line, CMT 167/C6, would exhibit a less differentiated phenotype in comparison with the low metastatic potential cell line CMT 170/E9. However, the result obtained from this EMSA experiment and repeat experiments gave the opposite result. Complexes a1, a2 and a3 all appear to be equal between the two cell lines, but complex a4 appears to be weaker in CMT 170/E9 nuclear extracts. It is difficult to reconcile this conflicting result. However, since both cell lines were previously reported to form well differentiated tumours (Franks & Layton, 1984), it may be that the normal differentiation programme in these cells is retained to a certain degree thus the interpretation of an EMSA with a C/EBP differentiation-associated binding site is obscure. Further experiments are required to eliminate the possibility that this alteration is a consequence of the culture conditions of the cells thus relating to their growth status. In addition to these experiments examining DNA binding activities of nuclear protein extracts of cells maintained in 0.5% serum, experiments such as supershift assays, whereby specific antibodies are included in the assay to displace

band shifts containing the recognized protein, could be tried. Alternatively, nuclear extracts can be pre-treated with antibodies to deplete the nuclear extract of specific proteins before incubation with the probe. Since the EMSA experiments described in this work were only of a preliminary nature, and through time constraints, these experiments have still to be attempted.

IX.4 *Conclusions*

Metastasis is a particularly complex process, of which the mechanisms involved at the various steps have still to be comprehended. The study of the metastatic phenotype described in this work utilized a murine model system which was based on two closely related cell lines, each of which were derived from the same spontaneous tumour. The CMT model of metastasis has not been used extensively before for the study of the metastatic phenotype and this work represents largely a characterization of the model with some studies aimed at hopefully providing an insight into the differing phenotypes. Unlike many other model systems, the CMT cell lines are assayed for their metastatic potential by a *spontaneous* metastasis assay which allows for the whole process of invasion through to secondary site colonization to be examined simultaneously. By these same criteria, dissection of the metastatic phenotype may be complicated due to the complex, multistep nature of metastasis which may hinder the isolation of particular traits associated with particular steps in the overall metastatic process. Unfortunately, it is widely accepted that at this stage in the research into metastasis, no ideal model system exists to date, and may not ever exist. As such, various approaches to the study of the metastatic phenotype have been adopted by different researchers with the hope that the next model will lead to a further understanding of metastasis. Since this model has not been in existence for any great length of time, particularly in comparison with some other models, it was hoped that various spurious traits, which were a consequence of prolonged culture under artificial conditions *in vitro*

and which are irrelevant to the metastatic nature of the model, would not have been incorporated into this system.

An understanding of the metastatic phenotype has proved to be an extremely elusive goal. It would appear from many studies that the nature of metastasis is particularly intricate and multifaceted, as such it may well be proven that metastasis arises through a multitude of subtle alterations of normal cellular behaviour and the outcome will dependent on numerous qualities. *How* metastasis occurs may be an identifiable question with knowledge of the probable steps in the progression of a benign tumour to a fully disseminated malignant tumour. However, such questions as to *why* metastasis occurs may remain unanswered. Importantly, if we can understand how metastasis occurs and what characterizes a metastatic cell, then the treatment of patients with tumours, circulating tumour cells and metastatic disease can be given the most appropriate therapy to prevent metastasis or contain the disease and ultimately cause tumour regression.

IX.5 *Future Prospects*

Further strategies for the study of metastasis in this model system and in other suitable model systems include the techniques of differential mRNA display and cDNA subtractive hybridization analysis. The advent of more advanced and efficient procedures allows for these avenues of research to be ventured into, however, each have their own intrinsic difficulties and disadvantages but without a "more ideal" model to examine metastasis, these may provide the best insight into metastasis.

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