TRANSGLUTAMINASE APOPTOSIS AND TUMOUR PROGRESSION

by

TIMOTHY SCOTT JOHNSON BSc

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Declaration

This work has not been excepted in substance for any other degree and is not concurrently being submitted candidature for any other degree.

This is to certify that the work here was carried out by the candidate himself. Due acknowledgement has been made of all assistance received.

Signature:.....
(Candidate)

Signature:.....
(Director of studies)

This thesis is dedicated to the 3 people I hold most dear:

My father, who died of lymphatic cancer on the 22nd March 1971. I hope the work within this thesis helps in some small way in helping to find a cure to this cruel, indiscriminate disease.

My Mother, who was left to bring up two small babies alone, for her immense personal sacrifices to give both my brother and I the best possible childhood and supporting us both through university education. She, more than anyone made this work possible.

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ABSTRACT

Reduced expression of the tissue transglutaminase in both murine and human tumours has been consistently associated with tumour growth and progression. This study confirms that this reduction in transglutaminase activity may inpart, be due to the expression of an inactive transglutaminase that results from perturbations at the translational or post translational level.

To investigate the functional effects of transglutaminase expression, the pharmacological agents all-trans retinoic acid and dexamethasone were initially used to modulate transglutaminase activity in hamster fibrosarcoma and 'normal' cell lines.

Retinoic acid causes *de novo* synthesis of transglutaminase resulting in increased activity in both the highly metastatic hamster fibrosarcoma cell line Met B and the 'normal' cell line BHK-21. In addition, retinoic acid causes an increase in the number of detergent insoluble bodies (apoptotic bodies) within a cell population.

A range of hamster fibrosarcoma cells (Met B, D and E), BHK-21 hamster fibroblast cells and the human malignant melanoma cell line B16 were treated with dexamethasone. This resulted in a powerful, dose dependent, but mRNA independent increase in transglutaminase activity that could be correlated to dexamethasone responsive receptor numbers in each cell line. In addition, increasing the number of dexamethasone responsive receptors by transfection of the HG1 glucocorticoid receptor protein caused increases in transglutaminase activity that was proportional to the level of transfected receptor. In all experiments levels of detergent insoluble bodies were measured to demonstrate cellular increases in transglutaminase product. Correlation between transglutaminase activity and apoptotic bodies was noted.

To provide a more permanent increase in transglutaminase expression without the non specific effects of pharmacological agents a constitutive human tissue transglutaminase expression construct was transfected into a highly malignant hamster fibrosarcoma cell line Met B. Transglutaminase transfected clones exhibited no significant differences in anchorage dependent growth rate *in vitro*, cell morphology or levels of spontaneous apoptosis measured by the determination of detergent insoluble apoptotic envelopes, but showed an increased adherence to tissue culture plastic and fibronectin coated surfaces when compared to transfected and non transfected control cells.

When transglutaminase transfected fibrosarcoma cells were returned to the *in vivo* situation by subcutaneous injection, these cells exhibited both an increased time for tumour development and a reduced incidence of primary tumour formation, but no

alteration in tumour growth rate was observed. Assay of developing tumours indicated loss of transglutaminase expression at the mRNA level, although the transglutaminase cDNA insert remained within the tumour cell genome. A gradual increase in transglutaminase expression was observed compared to controls on return of the tumour cells to the *in vitro* environment. When transglutaminase transfected fibrosarcoma cells were subcutaneously injected into Balb C (nu^+ \ nu^+) mice, no differences in primary tumour formation or tumour development time compared to controls was observed. In addition, injection of transglutaminase transfected fibrosarcoma cells into hamsters pre immunised with irradiated transglutaminase transfected cells demonstrated an increases in tumour development time compared to controls. These results suggest transglutaminase expression in the tumour cell may have an effect on tumour cell immunogenicity.

The data clearly demonstrates a suppressive effect of tissue transglutaminase on tumour growth and confirms its importance in the phenotypic changes associated with the cancer process.

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Publications.

I

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Contents

CHAPTER 1.

INTRODUCTION

1.1: Transglutaminases.

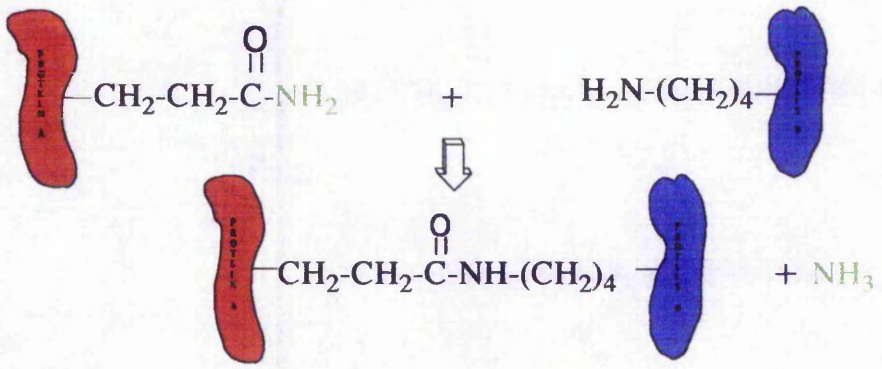
1.1.1. Reaction mechanism

Transglutaminases [EC 2.3.2.13] are calcium dependent enzymes which catalyse the crosslinking of polypeptide chains both intramolecular and intermolecular leading to the formation of protein polymers that are insoluble in detergents and chaotropic agents (Folk et al 1980, Lorand and Conrad 1984). In transglutaminase catalysed reactions the carboxamide groups of peptide bound glutamine residues act as acyl donors while primary amine groups of a large number of compounds may function as acyl acceptors (Figure 1.1). Transglutaminases are very selective with respect to glutamine residues, only reacting with those which are protein bound. This is the one feature that separates transglutaminases from other enzymes of glutamine metabolism (Folk 1980a, Lorand and Conrad 1984).

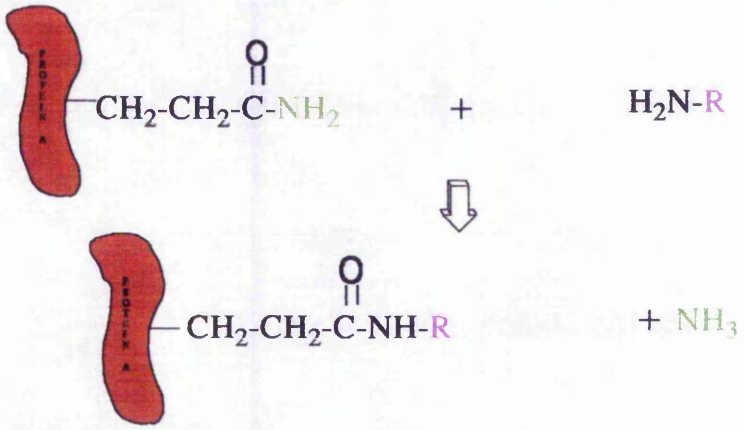
When the amine group of a protein bound lysine residue acts as an acyl acceptor, the resulting reaction product is the inter- or intra- isopeptide ε(γ-glutamyl) lysine bond. While free lysine is a poor substrate for transglutaminase, polyamines (putrescine, spermidine and spermine) are excellent substrates and are covalently incorporated into proteins both *in vivo* (Folk et al 1980b, Beninati et al 1985) and *in vitro* (Beninati et al 1985, Hand et al 1987a, Piacentini et al 1988a). Natural polyamines once incorporated into protein as the corresponding mono-γ-glutamyl derivative may further act as transglutaminase substrates reacting with a second protein bound γ-glutamyl residue. This latter reaction results in the formation of the corresponding N,N'-bis (γ-glutamyl) derivatives, which are polyamine crosslinks (Folk et al 1980b, Beinati et al 1985a,b, 1988, Piacentini et al 1988a,b) .

Figure 1.1.: Transglutaminase reaction mechanisms.

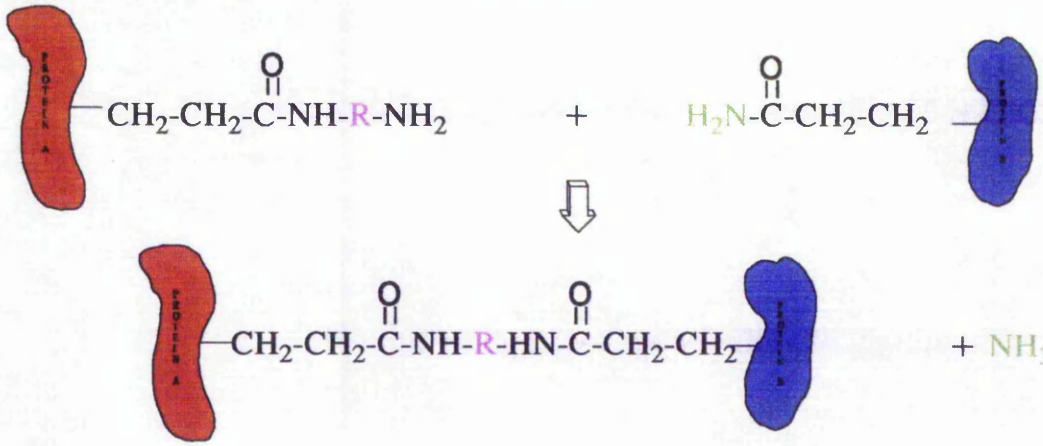
a. The formation of the ε(γ-glutamyl) lysine bond.



b: The incorporation of amines into proteins, forming an N'(γ-glutamyl) amine bond.



c: Formation of an N',N'-bis(γ-glutamyl) polyamine linkage. Following the reaction in b, a free amine on the R group is linked to another γ-glutamyl group on a second protein.



A biological role for transglutaminases is thought to be in the protection of cells (from physical stresses) and specifically cell integrity (Thomazy and Fesus 1989). This occurs due to the transglutaminases catalysing the formation of exceptionally strong protein crosslinks leading to the formation of protein polymers very resistant to breakage and chemical attack. Degradation is only possible by exhaustive proteolytic digestion of the protein polymers.

1.1.2. The Transglutaminase family

Transglutaminase is a term which encompasses a family of biochemically and immunologically distinct enzymes present both intracellularly and extracellularly. Three distinct forms of the enzyme were initially characterised. The plasma factor XIIIa which catalyses the polymerisation of gamma and alpha chains of fibrin in the final stage of coagulation (Chung 1975, Lorand and Conrad 1984). The intracellular associated keratinocyte transglutaminase which determines the assembly of the cornified envelope beneath the plasma membrane of terminally differentiated keratinocytes (Petersson and Wuepperl 1984, Thacher and Rice 1985). The third transglutaminase type is termed the tissue enzyme and a definitive physiological role for this enzyme has not been identified (Chung 1975, Adany et al 1985, Byrd and Lichti 1987, Piacentini et al 1986, Hand et al 1988). In addition a prostate enzyme, hair follicle enzyme and an epidermal enzyme have been identified. If the primary structure of these transglutaminases is investigated, the structure of human factor XIIIa, human keratinocyte transglutaminase and soluble tissue transglutaminase (guinea pig liver) share several regions of strong homology, specifically at the active sites (Amino acid sequence [Y-G-Q-C-W-V]), but all are encoded for by separate genes (Ikura et al 1988, Chiocca et al 1988, Gentile et al 1991).

1.1.2.1. Plasma transglutaminase (Factor XIII)

Human factor XIII is a heterotetramer of two **a** chains and two **b** chains. It is found in plasma as a proenzyme that is activated by the protease action of thrombin during blood coagulation. The activated form is termed Factor XIII**a** (Ichinose and Davie 1988), and has both **a** chains cleaved near the amino acid terminus. In the presence of calcium ions the **b** chain dimer dissociates from the **a** chain dimer, but only in the presence of fibrinogen (Credo et al 1978). Fibrin monomers produced by the thrombolytic cleavage of fibrinogen are then crosslinked by factor XIIIa into a highly stable matrix which is rendered resistant to plasmin degradation by the incorporation of α2-antiplasmin by factor XIIIa (Tamaki and Aoki 1985). Crosslinked fibrin polymer further enhances the rate of thrombin cleavage of factor XIII by providing binding sites for both factor XIII and thrombin (Greenberg et al 1987a). The importance of factor XIII can be seen in patients with a factor XIII deficiency who experience bleeding disorders due to the mechanically weak clots unable to resist degradation by plasmin and in women who are prone to spontaneous abortion (Lorand et al 1980).

The factor XIII **a** chain has been found in human monocytes, macrophages and heptatocytes (Weisenberg et al 1987), while the **b** chain in the hepatocyte (Wolpl et al 1987). The extracellular nature of factor XIII, its ability to crosslink other plasma and extracellular matrix proteins such as fibronectin (Barry and Mosherl 1990), collagen and thrombospondin (Ichinose et al 1990) and its binding to the surface of a number of cells including fibroblasts suggest it may also play an important role both in cell adhesion and cell migration.

1.1.2.2. Keratinocyte transglutaminase

Keratinocyte transglutaminase is found in the keratinocytes of the stratified squamous epithelia. Immunohistochemical evidence suggests that it is mainly expressed in the granular layer of the epidermis where its function is to crosslink proteins such as involucrin (Simon and Green 1988), loricrin (Hohl et al 1991) and cornifin (Marvin et al 1992) to form an insoluble cell envelope in the terminally differentiating keratinocyte. These highly crosslinked envelopes form an intricate part of the outermost (Callus) layer of the epidermis which mainly consist of disulphide bonded, hydrophobic, keratin intermediate filament proteins. Terminally differentiated cells, surrounded by the crosslinked envelope are highly cohesive and together form the major extra-pulmonary protective barrier to the environment (Greenberg et al 1991). In cultured keratinocytes the majority of keratinocyte transglutaminase is membrane bound which is brought about by post translational modification of the enzyme through fatty acid acylation with palmitate and myristate. This fatty acid mediated anchorage to the plasma membrane can be reversed in cell extracts by a Ca^{2+} activated protease (Rice et al 1990) which reduces the protein in size by 10 kDa to 80 kDa. It is therefore possible that in the final stages of keratinocyte differentiation influx of Ca^{2+} into the cell results in activation of the protease and partial release of the keratinocyte enzyme into the cytoplasm, a process which may be required for the final stages of envelope formation.

Regulation of keratinocyte transglutaminase (synthesis and activity) has been studied both *in vitro* and *in vivo*. In culture the enzyme is suppressed with all-*trans* retinoic acid (Saunders et al 1993) but raised with phorbol esters implicating involvement of the C-kinase pathway. In skin, retinoic acid causes increased, non mRNA dependent expression of keratinocyte transglutaminase (Rosenthal et al 1992). Keratinocyte transglutaminase synthesis is low in low calcium environments (Floyd and Jetten 1989), with further regulation by phosphorylation of enzyme serine residues.

Keratinocyte transglutaminase has been implicated in epidermal diseases. In psoriasis the expression of keratinocyte transglutaminase is moved to an earlier stage in the epidermal layer, and is found associated with the supra-basal spinous layer rather than the granular layer (Nonomura et al 1993). There is also evidence to indicate that peptides inhibitory to the protease elastase may be crosslinked to cell envelope proteins in psoriasis by the keratinocyte transglutaminase, and it is deactivation of elastase that leads to the formation of the psoriatic plaque (Molhuizen et al 1993).

In ichthyosis, changes in the physiology of the keratinocyte transglutaminase occur. Patients with non-erythrodermic autosomal recessive lamellar ichthyosis have raised keratinocyte transglutaminase in their skin scales (Van Hooijdonk et al 1991). In non bullous ichthyosiform erythroderma altered membrane anchorage of the enzyme has been observed and implicated in the aberrant cornified envelope formation observed in these individuals (Hohl et al 1993). The degree of dysplasia in pre-malignant oral tumours can also be related to a reduced keratinocyte transglutaminase expression (Ta et al 1990)

1.1.2.3. Epidermal transglutaminase

The epidermal transglutaminase is a second transglutaminase found in epidermal tissue (Kim et al 1991) which is involved in the crosslinking of keratinocyte proteins during terminal differentiation. This is a 72 kDa proenzyme that is activated by chaotropic agents or proteolysis (Negi et al 1985) to give a 50 kDa active product.

The epidermal transglutaminase in squamous epithelia (Thacher and Rice 1985, Petersson and Wuepper 1984, Floyd and Jetten 1989) provides a good example of the cellular role of transglutaminases in general. Expression of the enzyme occurs during

the transit of terminally differentiating cells to the stratum corneum at the level of the stratum granulosum. Activation of the enzyme leads to the crosslinking of specific proteins resulting in the laying down of a rigid protein matrix underneath the cell membrane (Green 1979, Thacher and Rice 1985). The stability of this envelope essential for skin integrity is conveyed by the high content of the transglutaminase catalysed ε(γ-glutamyl) lysine bonds and the N,N'-bis (γ-glutamyl) polyamine crosslinks (Piacentini et al 1988a, Peterson and Wuepper 1984, Rice and Green 1977, Rothnagal and Rogers 1984).

Epidermal transglutaminase is not found in cultured keratinocytes. The relationship between epidermal transglutaminase to the transglutaminase found in the inner and outer root of sheath cells of hair follicles (Martinet et al 1988) is also unclear. Both have the same molecular mass, but are considered to be distinct as they are immunologically different.

1.1.2.4. Tissue transglutaminase

The physiological role of the major transglutaminase, tissue transglutaminase (Folk and Finlayson 1977), remains elusive, although the highly conserved homology between the amino acid sequence between species (80% homology between mouse, human and guinea pig) suggests an important physiological role. It has generally been assumed to be an ubiquitous enzyme present in all mammalian tissues (Lorand and Conrad 1984, Folk et al 1980b, Fesus et al 1988a). However, a recent immunohistochemical study has shown that this widespread biochemical occurrence was largely due to its presence in ubiquitous cell types such as smooth muscle and endothelial cells in arteries, veins and capillaries (Folk 1980, Folk et al 1980, Fesus et al 1987). The enzyme may be induced or activated in other cell types by a variety of stimuli (Fesus et al 1988a, Davies et al 1985, 1988a, Melino et al 1988, Birckbichler et al 1983,

Dadaby and Pike 1987) that will be discussed later, suggesting that *in vivo* the tissue transglutaminase may be under the control of both external and/or intracellular factors coupled to a specific cell function.

Various factors have been shown to modulate the cytosolic form of tissue transglutaminase expression in different cell systems. For example retinoic acid has been shown to induce cytosolic tissue transglutaminase expression and differentiation in human promyelocytic leukaemia cells (Davies et al 1985) and in mouse peritoneal macrophages (Moore et al 1984, Chiocca et al 1988) In Balb C 3T3 cells increased cytosolic tissue transglutaminase expression appears to correlate with the presence of retinoic acid receptors (RAR), RARβ and RARγ (Davies et al 1993), while in HL-60 cells regulation involves the RXR α receptor Sodium butyrate induces cytosolic tissue transglutaminase in human lung fibroblast cells (Birckbichler et al 1983). Dimethyl sulphoxide (DMSO) and n-butyric acid increase cytosolic tissue transglutaminase activity in Friend erythroleukemia cell line GM979 (Hsu and Friedman 1984). In addition TGFβ1 has been reported to increase cytosolic tissue transglutaminase in rabbit tracheal epithelial cells (Jetten and Shirley 1986) and human epidermal keratinocytes (George et al 1990). Dexamethasone induction of transglutaminase activity was reported in mouse and rat mononuclear phagocytes, and human and murine myeloid leukaemia cell lines by Goldman 1987.

At the enzyme level the cytosolic tissue transglutaminase binds GTP and has GTPase activity (Lee et al 1989). GTP binding to the enzyme is reported to inhibit enzyme activity while high concentrations of Ca^{2+} can inhibit GTP binding (Achyuthan and Greenberg 1987). In the absence of GTP the enzyme is activated at physiological levels of Ca^{2+} (Hand et al 1985). It is therefore possible that the local concentrations of Ca^{2+} and GTP may regulate enzyme activity *in vivo* (Achyuthan and Greenberg

1987). The presence of an endogenous inhibitory factor has also been proposed to regulate cytosolic tissue transglutaminase in endothelial cells (Korner et al 1993).

Various physiological functions have been associated with tissue transglutaminase. It has been suggested that tissue transglutaminase, like factor XIII can work extracellularly, thus the presence of transglutaminase in the extracellular matrix could have a role in cell adhesion (Juprelle-Soret et al 1988, Gentile et al 1992), or stabilisation of the extracellular matrix by the crosslinking of proteins such as fibronectin, collagen, nidogen and laminin (Juprelle-Soret et al 1988, Barsigian et al 1988, Aeschlimann and Paulsson 1991). In addition the enzyme has been shown to be involved in the crosslinking of fibronectin and fibrinogen to the surface of certain cells (Barsigian et al 1988). The mechanism by which the enzyme interacts with the cell surface is unclear. Studies show that it is not via glycosylation (Ikura et al 1988). Tissue transglutaminase also bears no classical secretory signal sequence (Ikura et al 1988), although a non classical secretory route has been suggested (Muesch et al 1990).

The involvement of transglutaminase with extracellular components is reinforced with its implication in diseases effecting the extracellular matrix. Raised tissue transglutaminase has been noted in fibrotic lesions including pulmonary fibrosis (Griffin et al 1979) and atherelerosclerotic plaques (Bowness and Tarr 1990). In addition raised expression has been detected in cataract tissue (Lorand 1988), erythrocyte membrane fraction of patients with Koln's disease (Lorand 1988) and in Alzheimer's disease in the formation of neurofibrillary tangles of neurofilaments (Selkoe et al 1988) and tau containing polymers (Dudek and Johnson 1993).

Other potential roles for tissue transglutaminase are in membrane / cytoskeletal events associated with stimulus / secretion coupling (Bungay et al 1986), receptor

mediated endocytosis (Davies et al 1980) and in a terminal differentiation process comparable to that found in the differentiating keratinocyte.

The role for tissue transglutaminase that is currently receiving the most investigation is in the formation of the insoluble apoptotic envelope found in cells undergoing physiological cell death (Fesus et al 1989). The tissue transglutaminase is highly expressed in cells undergoing terminal differentiation and ageing (Rice and Green 1977, Wyllie 1980, Rothnagel and Rogers 1984, Bursh et al 1984, Thacher and Rice 1985, Maccioni and Seeds 1986, Schindler 1986, Kerr et al 1987, Fesus et al 1987, 1988a) and can demonstrate elevated expression in tissues that are subject to high apoptotic rates, such as liver, thymus and mammary tissue (Wyllie 1980a, Kerr et al 1987, Thomazy and Fesus 1989). More recent investigations using immunohistochemistry also indicate a strong positive staining for the enzyme in cells undergoing apoptosis (Fesus et al 1987). All this evidence suggests a role for tissue transglutaminase in final cell maturation and cell death.

To try and confirm the role of the enzyme in apoptosis it has recently been shown that an increased level of transglutaminase mRNA and protein are expressed in coincidence with lead nitrate induced liver hyperplasia. The enzyme is accumulated in the apoptotic hepatocytes and activated during the wave of involution resulting in a large increase in transglutaminase activity and enzyme protein (Fesus et al 1987, 1989, Piacentini et al 1990). The activation of the enzyme in the hepatocyte leads to the assembly, probably below the plasma membrane of the apoptotic cell, of a crosslinked protein shell (Fesus et al 1989, Piacentini et al 1990). This envelope is rich in $\varepsilon(\gamma$-glutamyl) lysine crosslinks and renders the cells insoluble in detergents and chaotropic agents, conveying the same properties as protein polymers produced by transglutaminase action. The highly crosslinked envelope can be isolated from a number of cell lines undergoing apoptosis (Piacentini et al 1991a,b, Knight et al 1991,1993). The envelope

serves an important physiological role in the removal of dead cells, sealing in all cell contents to prevent the release of factors which could trigger inflammation until the apoptotic body is phagocytosed by surrounding cells or macrophages. Indeed it is believed that the apoptotic envelope actually promotes phagocytosis by surrounding cells (Fesus et al 1989). This envelope is similar in character to the cornified envelopes found in the epidermis (Fesus et al 1989, Piacentini et al 1990, Knight et al 1993).

Additional *in vitro* evidence for the role of transglutaminase in apoptosis can be found using histochemical staining. *In vitro* cells which can be identified as undergoing apoptosis show a strong staining for tissue transglutaminase as well as the protein polymer product of the enzyme (Fesus et al 1987, Roch et al 1991).

Further evidence for the involvement of tissue transglutaminase in cell death processes comes from it's involvement in the ageing process of the human erythrocyte, where crosslinking of membrane proteins leads to a reduction in membrane deformability (Lorand 1988); in the human lens where β-crystallin subunits are substrates for the enzyme (Lorand 1988) and in the terminal differentiation of chondrocytes where expression correlates with chondrocyte differentiation, proceeds cartilage calcification and is thought to be involved in the crosslinking of the mineralisation matrix (Aeschlimann et al 1993).

Thus, tissue transglutaminase may play a key role in the regulation of the apoptotic program by its ability to produce protein crosslinks via $\varepsilon(\gamma$-glutamyl) lysine bridges (Lorand and Conrad 1984, Fesus et al 1987, Hand and Griffin 1990, Knight et al 1990b,d, Roch et al 1991). The enzyme is thought to be involved in the assembly and stabilisation of the apoptotic envelope which is laid down beneath the plasma membrane in the dying cell. Crosslinking of this envelope by tissue transglutaminase through the $\varepsilon($

γ-glutamyl) lysine bridges leads to its stabilisation and shrinkage resulting in structures commonly referred to as apoptotic bodies.

However, with this increasing amount of evidence for the role of tissue transglutaminase in apoptosis it is important to point out that certain cells undergoing apoptosis neither form crosslinked envelopes nor show raised expression of tissue transglutaminase (Szende et al 1991). This should not be viewed as disproving the role of transglutaminase in apoptosis, as virtually every functional protein that has been associated with apoptosis has a cell type where its action is not required. This is discussed later in this chapter.

1.1.3. The inactive tissue transglutaminase, apoptosis and the malignant phenotype

As apoptosis (Chapter 1.2) plays a complimentary but opposite role to cell proliferation in the regulation of cell populations and hence tissue size (Wyllie et al 1980b), it is an obvious link to associate the development of malignant neoplasms (Chapter 1.3) with a failure of the apoptotic program. Since transglutaminase is currently believed to play such a fundamental role in apoptosis, then a link between apoptosis, transglutaminase and tumour progression is a possibility (Birckbichler et al 1976, 1977a,b, Barnes et al 1984, 1985, Knight et al 1990a,d).

The possibility that normal and malignant cell growth can be connected to transglutaminase action was first raised by Laki in 1966 and examined in a series of following publications (Laki et al 1966, Laki and Yancy 1968, Yancy and Laki 1972, Laki 1972, Laki et al 1976, Laki et al 1977). His basic ideas and logic were that wound healing is impaired in Factor XIII deficient patients, and that neoplastic growth can be considered as a form of over healing such that fibrin and some other unrecognised protein might be covalently crosslinked by transglutaminase, Factor XIII from plasma,

or fibrin stabilising enzymes from the malignant cells. This crosslinked matrix around the malignant cells of a growing tumour mass, as found around proliferating fibroblasts during wound healing, would serve as a supportive matrix for tissue proliferation and vascularisation. Laki's theories were supported by his findings that animals carrying tumours with raised transglutaminase activity were able to survive for shorter periods of time than those with low transglutaminase activity following implantation of malignant cells. Furthermore, organs with high transglutaminase activity such as the liver showed greater tumour growth rates than tissues such as spleen with lower transglutaminase activity. Laki's final conclusions was that tumour development and transglutaminase activity are directly proportional.

Laki's interpretations have been contradicted by more recent work on transglutaminase activity in proliferating and resting cells, and in normal and transformed cells. It has been claimed that transglutaminase is part of a negative control mechanism (Birckbichler et al 1981). Studies with human fibroblasts and the transformed variants indicated that low transglutaminase activity and crosslink levels facilitate cell cycling, and that the resting cell is in a highly crosslinked state with high transglutaminase activity (Birckbichler and Patterson 1978). Low transglutaminase in malignant cells compared to their normal counterparts has been noted in several cell lines, A431(epidermal carcinoma) (Dadabay et al 1989), adenocarcinoma HeLa-TV and neuroblastoma SK-N-BE-2 (Piacentini et al 1991), Chinese hamster ovary cells (Milhaud et al 1980), Yoshida ascites (Vanella et al 1983) and hepatocarcinomas (Hand et al 1987a,b).

Using a number of malignant neoplasms from both animal and human sources it has been shown that tumour progression and development of the metastatic phenotype is accompanied by a corresponding reduction in tissue transglutaminase activity (Delcros et al 1986, Hand et al 1987a,b, Griffin et al 1989). In some metastatic tumours, this

reduction in activity has been hypothesised to be a result of the expression of an inactive tissue transglutaminase in the malignant cell (Knight et al 1990a,b,d).

In a normal cell, tissue transglutaminase is found in two cellular compartments. Approximately eighty percent of the transglutaminase is found in the cytoplasm and termed the cytosolic form (molecular weight 75-80 kDa), the remainder is found bound to the plasma membrane and termed the particulate form (molecular weight 95-100 kDa) which has now been reported in a number of cell lines (Chang and Chung 1986, Chung et al 1988a,b, Hand et al 1988, Knight et al 1990c,d). Under native conditions the particulate and cytosolic forms of tissue transglutaminase are immunologically distinct and can be resolved by ion exchange chromatography and gel electrophoresis (Knight et al 1990a). Under denaturing conditions cytosolic and particulate tissue transglutaminase show common immunoreactivity and molecular mass. In the malignant cell, the levels of the particulate form remain constant, whereas there appears to be a reduction in the cytosolic form as the inactive form increases (Griffin et al 1989, Knight et al 1990a,d). Study of a series of breast carcinomas of known clinical staging has demonstrated that the greater the clinical advancement of the tumour, then the greater the level of the inactive enzyme present (Knight et al 1990a,d). Moreover, investigation of a series of cell lines of differing metastatic potential confirms the finding that a raised metastatic potential is accompanied by a raised level of the inactive enzyme and a corresponding reduction in the cytosolic form (Griffin et al 1989, Knight et al 1990a,d).

Production of the inactive tissue transglutaminase in the metastatic tumour cell is most likely to be a result of inappropriate expression of the cytosolic form since expression of the two forms is inversely related. Furthermore, both forms of enzymes are found exclusively in the cytoplasm. The inactive enzyme has a molecular weight of 120 kDa, much larger than either the cytosolic or particulate forms and hence is unlikely to be a proteolytic product. Additional evidence comes from the fact that limited

proteolysis of the inactive form with trypsin or thrombin results in an active transglutaminase with biophysical properties comparable to the cytosolic enzyme (Knight et al 1990a,d).

The ability of limited proteolysis to form active enzyme suggests inappropriate post-translational processing, however sequence studies on cDNA suggest this to be unlikely since primary extension studies on the 5' end of transglutaminase mRNA indicate only 110 nucleotide residues upstream from the translational start signal. Thus translation of this mRNA could not lead to a proenzyme 40 kDa greater than the active cytosolic enzyme unless transcription extended into the 3' 1000 bp non coding region (Gentile et al 1991). This read through is again unlikely as there are repeated stop codons throughout the non coding region (personal communication P.J. Davies). Correspondingly Northern blots using mRNA isolated from normal liver cells indicate only one stable form of mRNA corresponding to 3.7-3.8 kb (Ikura et al 1987).

This suggests that the large inactive enzyme found only in metastatic tumour cells is likely to be a result of an inappropriate gene expression occurring either at the transcriptional or post transcriptional level, possibly during mRNA splicing (Knight et al 1990a,d), although it can not be ruled out that the inactive enzyme results from a translational or post translational perturbation / modification.

The increase in the inactive enzyme found with increasing metastatic potential is also accompanied, as suspected, by a reduction in apoptotic rate of between 70 - 80 percent in highly metastatic cells when determined by numbers of detergent insoluble bodies (Knight et al 1990a,d, Hand et al 1990a). Thus perturbation of the regulatory events leading to programmed cell death resulting in expression of the inactive transglutaminase may be an essential feature of the developing metastatic phenotype.

Such a phenotypic feature of the malignant cell would favour a number of important events in tumour progression. The imbalance in cell proliferation and apoptosis would result in an increase in tumour mass. This imbalance would result in the selection of a cell population that is less able to undergo programmed cell death, and which incurs an increased immunity to natural killer cells and cytotoxic T cells (cells which kill by inducing apoptosis) (review Walker et al 1993). This would facilitate dissemination, survival and recolonisation.

These findings confer two very attractive future possibilities for the medical field, initially in the area of markers for malignancy and metastasis, and ultimately as an attractive route of tumour therapy.

1.2. Cell Death.

1.2.1. Necrosis

Cell death can occur by two processes; necrosis or apoptosis. Necrosis is the classical form of cell death (Trump et al 1981), typically effecting large cell numbers in a concentrated area. It occurs when the cell is confronted with extreme non physiological conditions and the cell consequently losses control of ion-flux. This results in calcium entering the mitochondria while continued metabolism makes the cell hypertonic to its environment. Morphologically it is characterised by high amplitude swelling of the mitochondria, nuclear flocculation and uncontrolled lysis of the cell leading to inflammation and damage of surrounding cells. Necrosis is a response to trauma, either mechanical, chemical or physical, whereas apoptosis is a programmed, controlled sequence of events.

1.2.2. Apoptosis

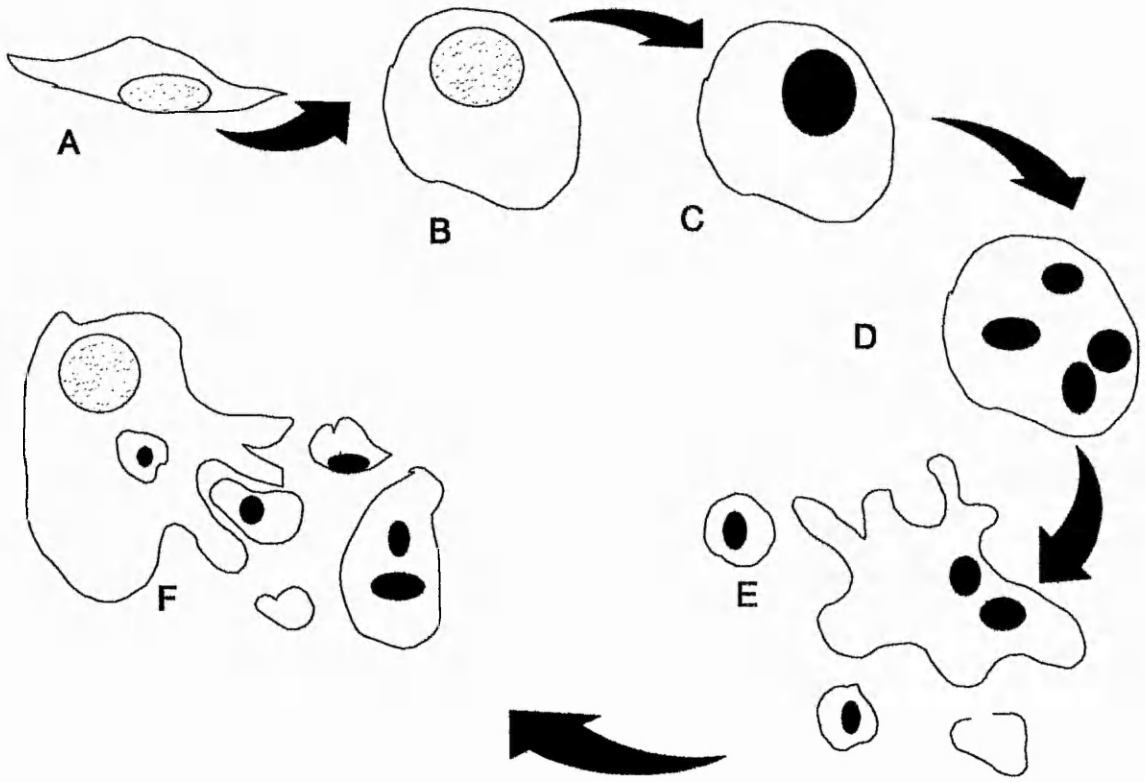
The term apoptosis was initially used by Kerr (Kerr 1971) to describe a type of cell death exhibiting a distinct set of morphological features. Apoptosis has become synomonous with the term programmed cell death with the two being interchanged frequently. This interchange of terms is actually incorrect as programmed cell death is a functional term used to describe cell death that is a normal part of the life of a multicellular organism following a predetermined sequence of events including initiation. Apoptosis on the other hand, while in most cases is true programmed cell death, can be induced by a variety of toxic drugs and physical stimuli and is therefore not programmed in these instances. Subsequently a better all encompassing term is physiological cell death. For the purpose of this thesis the term apoptosis will be used to cover both programmed and physiological cell death. Apoptosis is a programmed mode of cell death, which actively balances cell proliferation in controlling cell numbers within the tissues of multicellular organisms (Wyllie 1980a,b, Bursh et al 1984, Kerr et al 1987, Fesus et al 1987) and during embryological development (Kerr 1971).

1.2.2.1. The Morphology of Apoptosis

Cell death by apoptosis typically affects scattered cells in large tissue areas and is not accompanied by exudative inflammation. First signs of the onset of apoptosis is the loss of water from cells without loss of macromolecules, with the cells at this stage showing no increases in cell permeability as determined by vital dye exclusion (Kerr 1971). As the cells loose water shrinkage occurs as a result of the loss in volume. The shrinkage corresponds well to cell death determined biochemically (Sorenson et al 1990).

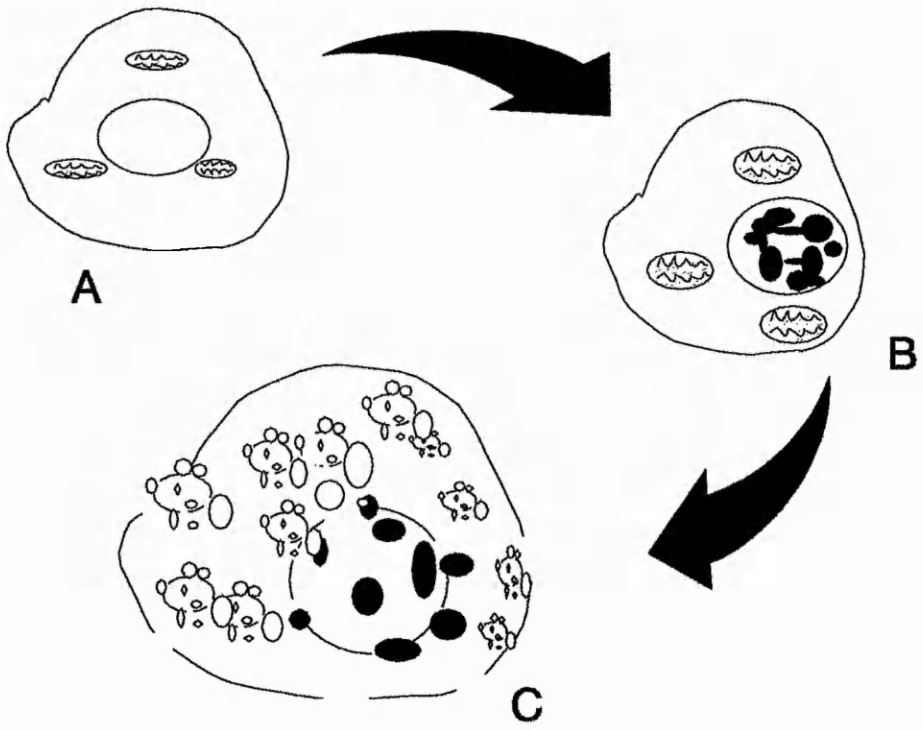
Figure 1.2.1.: Diagram illustrating the sequences of ultrastructure changes during A. apoptosis and B. necrosis

A: Apoptosis



A. A normal cell receives the signal to undergo apoptosis. **B.** The cell rounds up. **C.** The cell rapidly condenses its DNA as a result of chromatin condensation, cytoplasm condensation occurs and the cell surface smoothes loosing micro villi. **D.** The nucleus separates into discrete masses of condensed chromatin. **E.** The cell fragments into several membrane bound vesicles apoptotic bodies. **F.** Apoptotic bodies are rapidly recognised and phagocytosed by neighbouring cells and macrophages and digested by lysosomes. Process complete in approximately 15 minutes.

B: Necrosis.



A. A normal cell receives a physiological injury. **B.** Irregular clumping of the chromatin and marked swelling of the organelles occurs. **C.** Focal disruption of membranes occurs leading to membrane disintegration. The cell maintains its overall shape until removed by mononuclear phagocytes. Cell contents leak from the cell causing inflammation. Process can take from 1 hour to several days.

Apoptotic cells then loose contact with neighbouring cells and subsequently show chromatin condensation and compaction of cytoplasmic organelles which remain intact. At a later stage these appear as membrane bound fragments known as apoptotic bodies (Wyllie et al 1980).

Chromatin gradually becomes granular and extends as a band underneath the inner lamina of the nuclear membrane. This subsequently disintegrates as small osmiophilic granules. These chromatin granules become anchored on the protein matrix of the nucleus (Wyllie and Morris 1982).

Morphological and chemical changes in the plasma membrane of the apoptotic cells permit their recognition by adjacent cells and by phagocytes (Hedgecock et al 1983). Changes in charge density of the plasma membrane via the loss of N-acetyl neuraminic acid groups, alterations in surface carbohydrates and lectins and changes in phospholipid asymmetry resulting in phosphatidylserine exposure are all noted (Duvall et al 1985). Dilated endoplasmic reticulums burst and fuse with the plasma membrane (Yamada et al 1980). The plasma membrane loses its characteristic structure and projections causing a smoothing of the cellular surface which ultimately shows blebbing. The plasma membrane remains intact excluding vital dyes and preventing the release of lysosomal enzymes. The blebbing extends into large protuberances into which intact organelles and nuclear chromatin fragments are packed. These membrane bound extrusions enlarge and detach into the extracellular milieu forming the so called 'apoptotic bodies'. The apoptotic bodies dispersed in the extracellular matrix are phagocytosed by neighbouring cells, whereas in some epithelia they are released from the surface into the intercellular space (Wyllie 1980, Bursh et al 1984, Kerr et al 1987, Fesus et al 1987). Alternatively they are engulfed by phagocytes.

1.2.2.2. Biochemical events.

The primary biochemical event seen in the vast majority of cells is the increase in the intracellular level of free Ca^{2+}either via influx or intracellular release that can be caused by a vast array of agents (Bellamo et al 1992). Moreover Ca^{2+} ionophores are potent induces of apoptosis suggesting that in some cells Ca^{2+} may itself be a trigger.

Ca^{2+} has many potential sites of action, but there are at least 2 effector enzymes that are commonly activated during apoptosis; the endonuclease that cleaves at internucleosomal sites and the tissue transglutaminase (See section 1.1).

The endonuclease activation leads to cleavage of the double stranded linker DNA between nucleosomes. This cleavage occurs at regularly spaced internucleosomal sites giving rise to DNA fragments the length of which represent the length of nucleosomes (180-200 base pairs) (Wyllie and Morris 1982, Manes and Menzel 1982). Cleavage of DNA generates 2 classes of chromatin fragments: 70% of the DNA exists as long H1 rich oligonucleosomes bound to the nucleus and 30% is comprised of short oligosomes and mononucleosomes which are depleted in H1 but enriched in high mobility group proteins 1 and 2. It is this endonuclease activity that leads to the typical DNA ladder seen on a polyacrylamide gel that is used as a qualitative determination of apoptosis in many cell lines and tissues.

The specific endonuclease has yet to be identified, but it is Ca^{2+}, Mg^{2+} sensitive and is inhibited by Zn^{2+}. It is also greatly reduced if protein synthesis is inhibited. DNase I (Peitsch et al 1993) and DNase II (Barry and Eastman 1993) have been implicated in this endonuclease activity with the cofactor protein NUC 18 (Gaido and Cidlowski 1991).

Apoptotic cell death requires RNA and protein synthesis in the majority of cells as demonstrated by the prevention of apoptosis using actinomycin D and cyclohexamide (Wyllie et al 1984), but in certain cell lines these agents actually induce apoptosis (Martin et al 1990). Increase in mRNA expression for several genes during apoptosis has been reported but their actual roles remain elusive. These include mRNA for transglutaminase (Piacentini et al 1991b), ribonuclease (Engel et al 1980), Cathepsin D (Tanabe et al 1982) and γ-glutamyl transpeptidase (Shulte-Hermann et al 1990). Cyclic adenosine monophosphate kinase 1 (Lanotte et al 1991) and plasminogen activator (Rennie et al 1984) become active.

1.2.2.3. Induction of apoptosis

Many factors have been seen to induce apoptosis in a variety of cell types, but not all cells within a population will respond to the inducing factor in the same way thus suggesting the existence of a complex regulatory network. Examples of induction of apoptosis include: Cell killing by the immune systems natural killer cells (NK cells) and the cytotoxic T cells is caused by the induction of apoptosis in the target cell (Liu et al 1989, Knight et al 1993); temperature changes; radiation; hormones and growth factors; antibodies: anti cancer chemotheraputic agents; retinoids and many others (Sen 1992).

Figure 1.2.2.4.: Summary diagram of suppressors and induces of programmed cell death.

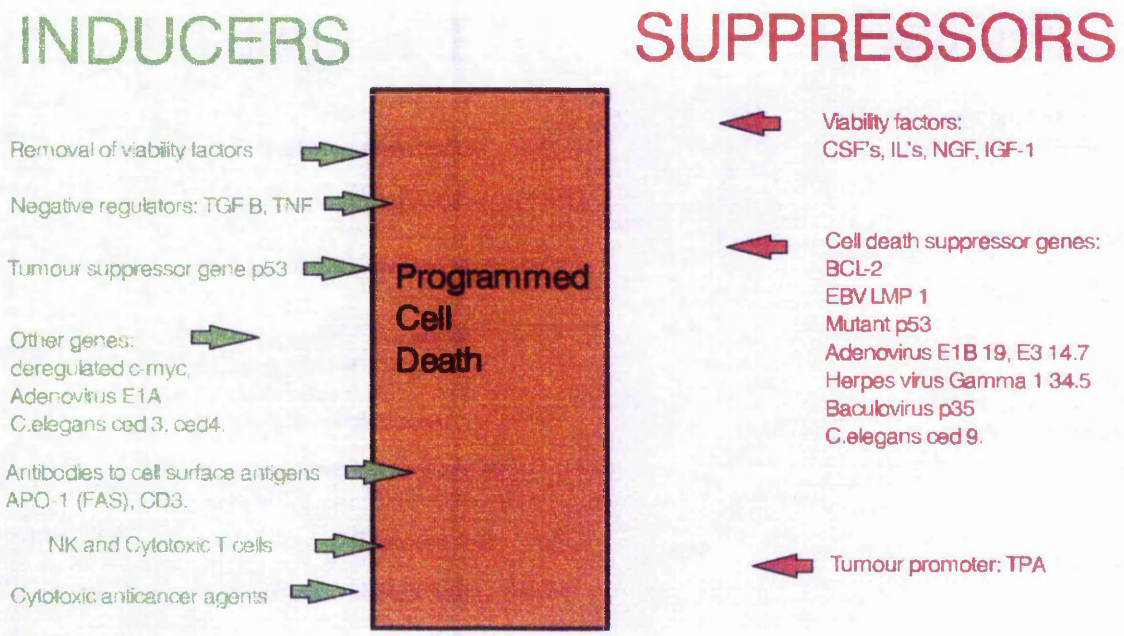


Diagram represents a traffic light scenario. Cell is constantly waiting in limbo between life and death (amber). Signals to go into apoptosis (Green) and to stop apoptosis (red).

1.2.2.4. The regulation of Apoptosis

There are genes that induce or suppress apoptosis and it is these that regulate the action of the apoptotic inducing agents. Wild type p53 (Yournish-Rouach et al 1991), adenovirus E1A (Rao et al 1992) and ced 3 and ced 4 in the nematode (Ellis et al 1991) can induce apoptosis. Deregulated c-myc can enhance apoptosis under certain conditions (Lotem and Sachs 1993). Genes for cell surface antigens such as APO-1 (FAS) and CD3 (Trauth et al 1989, Oehm et al 1992) mediate apoptosis when cells expressing these antigens are incubated with the appropriate antibody. Other genes including bcl-2 (Vaux et al 1988), mutant p53 (Lotem and Sachs 1993), adenovirus E1B 19 kDa (White et al 1991) and E3 14.7 kDa (Gooding et al 1988), herpes simplex virus $1_{\gamma 1}$ 34.5 (Chou and Roizman 1992), baculovirus p35 (Clem et al 1991) v-abC and c-abC (Cotter et al 1992) and C elegans ced 9 (Ellis et al 1991) can suppress apoptosis. Of these the most prominent regulators in mammalian tissues are p53, c-myc, bcl-2 and APO-1. The interactions of these are summarised in figure 1.2.2.4..

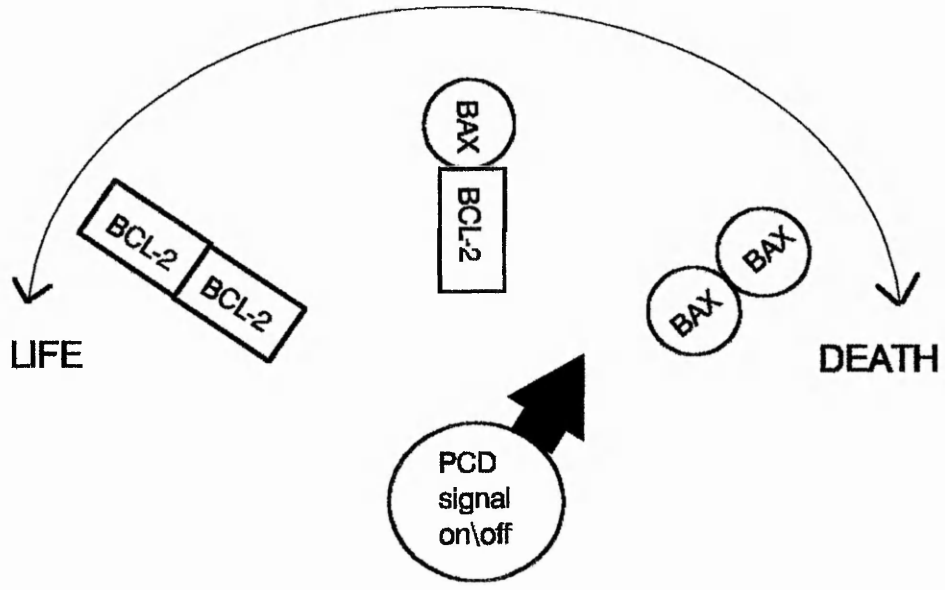
1.2.2.4.1. Bcl-2

Bcl-2 is a potent repressor of apoptosis in many cell lines (Korsymeyer 1992) with the major exception of when apoptosis is induced via the action of cytotoxic T cells. Subsequently cells expressing raised bcl-2 are prone to longevity and suggests that bcl-2 is a potential oncogene. The bcl-2 gene was identified at the chromosomal breakpoint of t(14;18) bearing B cell lymphomas (Fukuhara et al 1979). Bcl-2 expression is found in the mitochondria, the nuclear envelope and the endoplasmic reticulumn (Jacobson et al 1993). The mechanism of bcl-2 action in suppressing apoptosis is still not clear, although its subcellular localisation does provide clues. Bcl-2 has a C-terminal hydrophobic region that functions as a signal-anchor sequence responsible for the integral membrane position of the 25 kDa bcl-2α product (Nguyen et al 1993) with its

NH_2 terminus in the cytosol. The ability of bcl-2 to block γ-irradiation induced cell death (Sentman et al 1991) is notable in that ionising radiation produces hydroxyl radicals via radiolytic attack on water (Blok and Lohman 1986) and bcl-2 is positioned at the sites of oxygen free radical generation (mitochondria, nuclear membranes and endoplasmic reticulum). Many of the effects of oxygen free radicals including DNA strand breaks and membrane blebbing match features of apoptosis and thus promote an examination of the ability of bcl-2 to counter other oxidative cell death. Bcl-2 protects cells from hydrogen peroxide in a dose dependent manner and it protects from menadione that forms superoxides (Hockenberry et al 1993). Importantly bcl-2 does not interfere with normal electron transport nor production of peroxides (Hockenberry et al 1993). Thus, bcl-2 functions to completely suppress vital damage to cells including lipid membrane peroxidation and suggests that reactive oxygen species are involved in the induction of cell death. How bcl-2 performs this task remains elusive.

As bcl-2 lacks any conserved functional motifs it was thought highly likely that bcl-2 was a member of a multicomponent complex. This was verified by immunoprecipitation experiments (Lin et al 1993) that revealed that bcl-2 heterodimerises with a 21 kDa partner termed bax (bcl-2 associated X protein) that shared regions of homology with bcl-2 at 2 highly conserved domains. Overexpression studies indicated that excess bax countered bcl-2 and accelerated death but only following a death signal. When bcl-2 is in excess bcl-2 homodimers dominate and the cells are protected from apoptosis, when bax is in excess bax homodimers dominate and the cells are susceptible to apoptosis. This is shown in figure 1.2.2.4.1.

Figure 1.2.2.4.1.: A systematic diagram showing how the pre set rheostat of the ratio of bcl-2 / bax determines the life or death response of a cell following a programmed cell death signal.



Bcl-2 dimers indicate complete suppression of the apoptotic program. Bax dimers allow entry into the apoptotic programme. Bcl-2 / bax dimer indicates indecision and the cell could live or die depending on signals the cell receives.

Recent studies indicate that bcl-2 and c-myc intercommunicate in determining a cells fate (Bisonnette et al 1992).

1.2.2.4.2. c-myc

c-myc is one member of a family of genes termed the myc family, which also contains n-myc, l-myc, b-myc and s-myc. All these proteins encode for related, but distinct nuclear proteins, with each gene displaying a unique activation pattern both in normal and tumourogenic development. c-myc is located on human chromosome 8 and is encoded in a 3 exon structure (Crew et al 1982, Bernard et al 1983). All myc genes demonstrate high evolutionary conservation of the untranslated regions suggesting that they may play an important role in regulating the differential expression of the myc family (Bernard et al 1983, Depinho et al 1987). Transcription of the c-myc gene is initiated at two evolutionary conserved promoters termed P1 and P2 (Bernard et al 1983, Stanton et al 1984). These two promoters are separated by approximately 160 nucleotides. A third promoter termed P0 has been identified 550-650 base pairs upstream of P1 but this accounts for less than 5% of c-myc mRNA (Bernard et al 1983). The majority of mature c-myc transcript is either 2.2 or 2.4 kb depending on which promoter transcription was initiated at, this leads to 2 c-myc proteins, p64 (64 kDa) and p67 (67 kDa) that are N-terminally distinct and differential expression of these has been linked to c-myc functions both in tumourogenesis and apoptosis (Persson and Leder 1984, Ramsey et al 1984). Elevated or inappropriate expression of all the myc genes has been associated with tumourogenisis (Hann et al 1988), with c-myc being the most widely characterised thus far.

Expression of the c-myc gene was normally thought to be simply a promoter of cell proliferation, but in some cell lines it is an important inducer of apoptosis (Evan et al 1992) but only under reduced levels of growth factors and bcl-2. The mechanism of

action of c-myc protein, which is thought to be a nuclear phosphoprotein transcription factor, is unclear. The suggestion of c-myc being a DNA-binding transcriptional regulatory protein is primarily due to it sharing two regions of homology with DNA-binding transcriptional regulatory proteins. The first region termed the leucine zipper domain shares homology with a group of proteins including those originating from the **fos** and **jun** proto oncogenes, the enhancer binding C/EBP protein and the yeast GCN4 protein (Landschultz et al 1988). Mutation analysis suggest the leucine zipper domain is important for the activity of the c-myc protein, possibly functioning in multimer formation (Dang et al 1989). A second, basic amino acid rich region N terminal to the leucine zipper region and termed the helix loop helix domain has shared homology with cell determination genes including the myogenic determination genes myoD1 and myogenin (Davis et al 1987, Edmundson and Olson 1989) and immunoglobin enhancer binding proteins E12 and E47 (Murre et al 1989a). Deletion studies indicate the helix loop helix domain regulates DNA binding and transcriptional activation (Murre et al 1989a,b, Lasser et al 1989).

Cellular genes whose expression is thought to be altered by c-myc are poorly characterised. Two dimensional gel electrophoresis shows that c-myc expression is associated with increased expression of eight cellular proteins while 5 are down regulated (Schweinfest et al 1988). Also a G0 / G1 specific cDNA is apparently affected by c-myc (Schweinfest et al 1988). Another target candidate is the mr1 gene, which encodes plasminogen activator inhibitor 1, a regulator of extracellular proteolysis which is implicated in tissue remodelling, tumour invasion and metastasis (Prendergast et al 1989,1990). Two H1 histone genes are negatively regulated in association with c-myc expression and the timing of their induction suggests they may play an important role in commitment to terminal differentiation (Cheng and Skoultchi 1989). Another putative target of the c-myc protein is the ornithine decarboxylase gene (Dean et al 1987), which

encodes for a highly regulated enzyme of the polyamine biosynthetic pathway in mammalian cells.

As described previously, the major sites of transcriptional initiation are the P1 and P2 promoters. Minimal flanking of sequence is necessary for correct transcriptional initiation of either of these promoters (Asselin et al 1989). However a number of additional positive and negative regulatory elements have been identified in 5' flanking and first exon sequences, suggesting the regulation of c-myc is a complex process (Chung et al 1986). Recently a fos encoding protein complex, probably the fos and jun containing AP-1 complex, and an octamer binding protein have been shown to bind to a negative regulatory element upstream of the c-myc gene (Hay et al 1989). Transcriptional attenuation of the c-myc gene was first shown using nuclear 'run on' assays which showed that a block to transcriptional elongation exists at the 3' end of the fist exon (Eick and Bornkamm 1986, Bently and Groudine 1986). Modulation of the transcriptional block accounts for both increases (Eick et al 1987) and decreases (Bently and Groudine 1986) on c-myc expression. Studies indicate that the P2 promoter is responsible for attenuation regulation (Miller et al 1989). In many cell lines, changes in steady state levels of c-myc expression are not accompanied by changes in the rate of c-myc transcription (Blanched et al 1987). In these cases, post transcriptional control is invoked as the mechanism responsible for the modulation of c-myc RNA levels. The c-myc mRNA molecule has a short half life of approximately 15 minutes (Dani et al 1984). The increased stability of truncated c-myc transcripts suggest that fist exon sequences missing from these transcripts may mediate transcript destabilisation (Rabbitts et al 1985). The increased stability of c-myc mRNA following cyclohexamide treatment suggests that destabilisation may be mediated by labile protein factors (Dani et al 1984).

The noncoding regions of all the myc genes are highly evolutionary conserved suggesting that they have an important function in regulating expression of myc proteins.

Expression of the c-myc gene is regulated by a number of mechanisms including transcriptional initiation, transcriptional attenuation and post transcriptional processes including variations in mRNA stability (Levine et al 1986, Mechti et al 1986, Siebenlist et al 1988). Control of transcriptional initiation contributes to both positive and negative effects upon c-myc steady state expression. Transcription of c-myc is induced in the response of resting cells to certain mitogenic stimuli (Greenberg and Ziff 1984, Levine et al 1986).

The apparent dual role of c-myc in promoting both cell proliferation and death is connected to the relative abundance of appropriate growth factors. Cells placed into growth arrest by withdrawal of growth factors are susceptible to apoptosis if c-myc is activated (Evan et al 1992). In addition certain cells are more susceptible to entering apoptosis in general when c-myc is in abundance. The c-myc protein is involved in the transduction of extracellular growth signals to the genetic level, with mitogenic stimuli inducing signal transduction pathways that flow into the induction of c-myc. The expression of c-myc plays an important role in the cells decision to proceed through the G1 state into a new cell cycle, or enter the G0 resting state or to pass from G1/ G0 into a cycle that terminates in cell death (Schonthal 1990, Berges et al 1993). The action of the c-myc protein is mostly likely to result from its ability to bind to promoter regions of several inducible genes thus altering gene expression and c-mycs short half life requiring continual c-myc expression to cause gene suppression. The action of c-myc can be suppressed via negative feedback and binding to its own promoter region and the concomitant induction of pathways repressing gene expression (Littlewood and Evans 1990, Zelenka 1990).

1.2.2.4.3. APO-1 (FAS)

APO-1 is a transmembrane member of the tumour necrosis factor receptor family. Crosslinking of APO-1 (Experimentally by monoclonal antibody binding) leads to programmed cell death of activated T and B cells as well as lymphoid tumours (Itoh et al 1991). It has recently been suggested that APO-1 expression is necessary for cells to be killed by cytotoxic T cells in the absence of Ca^{2+} (Rouvier et al 1993). APO-1 is unusual amongst other cell surface molecules able to trigger cell death in that its perturbation does not induce lymphoid effector functions such as proliferation and lymphokine secretion. The binding of antibodies to the APO-1 receptor induces apoptosis but causes no increase in PI hydrolysis, intracellular calcium or tyrosine phosphorylation (Mercep et al 1994). Unlike many types of apoptosis APO-1 induced death does not require *de novo* gene expression indicating this system has a discrete and distinct mechanism.

1.2.2.4.4. p53

The cellular phosphoprotein p53 is a negative regulator of cell growth (Barker et al 1990a, Isaacs et al 1991) is commonly termed the tumour suppresser gene and is a potent inducer of apoptosis. Mutations in the wild type p53 gene are the most common genetic error in human cancers (Volgestein 1990, Hollstein et al 1991, Caron de Fromentel et al 1992). Mutations of the p53 gene are mainly localised in phylogenetical highly conserved regions of the p53 polypeptide chain (Masuda et al 1987, Ahuja et al 1989). In addition tumours reveal loss of the short arm of chromosome 17 carrying the p53 locus (Barker et al 1990b). Genetic changes affecting p53 alter or abolish growth control regulation of p53, while wild type p53 expression in tumours results in inhibition of cell proliferation (Diller et al 1990, Mercer et al 1990).

P53 is classified as a DNA binding protein (Collins et al 1991, Takano et al 1991). A DNA structural motif has been identified that contains binding sites for p53 protein (MacLeod 1993). The DNA binding activities of p53 influence DNA replication (Gannon and Lane 1987) and transcription (Forster et al 1992). p53 protein represses multiple viral and cellular promoters including the SV40 promoter element, the cytomegalovirus promoter and the Rous sarcoma virus long terminal repeat (Jackson et al 1993). Among the cellular targets influenced by wild type p53 are the promoters of c-myc (Moberg et al 1992), retinoblastoma susceptibility gene Rb (Shilo et al 1992), the multi drug resistance gene MDR-1 (Chin et al 1992), the proliferating cell nuclear antigen PCNA (Subler et al 1992), c-fos, interleukin 6, β-actin (Ginsberg et al 1991), c-jun and the promoter of hsc70 (Heat shock protein) (Agoff et al 1993). Furthermore there is evidence that p53 regulates its own transcription (Dieffe et al 1993). In contrast the MCK promoter (Weintraub et al 1991) and the enhancer element of HTLV-1 (Aoyama et al 1992) are activated by p53. Loss of wild type p53 function might therefore transcriptionally activate genes normally repressed by p53 or alternatively deactivate genes that would normally be induced by p53. In addition the binding of viral gene products may also inhibit the regulation properties of p53, for example the complex formation between p53 and the SV40 T antigen results in stabilisation of p53 protein (Lane and Crawford 1979) and the binding of the E6 proteins of human papilloma virus induces an enhanced degradation of the p53 protein (Band et al 1993) and abolishes the p53 mediated transcriptional regression (Lechner et al 1992). It is important to note that the phosphorylation of p53 by protein kinase p34 influences the regulation of DNA replication (Sturzbecher et al 1990).

p53 is a nuclear transcriptional regulator that acts to arrest DNA damaged cells at G1 of the cell cycle, this block allows additional time for appropriate DNA repair and prevents fixation of potentially harmful mutations (Kastan et al 1991). In experiments using DNA damaging agents, it has been shown that p53 protein accumulates in the

nucleus via increased stability and not expression. (Fritsche et al 1993). If cells are exposed to similar DNA damaging agents but have no endogenous p53 then growth arrest in G1 does not occur. If DNA damage cannot be repaired then p53 induces the apoptotic programme (Yonish - Rouach et al 1991, Shaw et al 1992). During the cell cycle the content of p53 increases from G1 to S phase and further to G2 and M phase (Danova et al 1990). Interestingly p53 in quiesent cells can not be detected (Milner and McCormick 1980). It has been postulated that p53 may be critical for the G0/G1 to S phase transition (Mercer et al 1982), while further work has shown that p53 may control the transition of cells through two restriction points, that is from G0 to G1 and G1 to S phase (Deppert et al 1990). Changes in the subcellular localisation of p53 during the cell cycle have been reported. p53 is cytoplasmic in G1, shifts into the nucleus at the G1 / S transition and remains there until the completion of the G2/M phase (Shaulsky et al 1990) This may reflect the presence of specific targets for p53 during different phases of the cell cycle or cell cycle dependent alterations in post translational modifications. p53 is unphosphorylated in G0 / G1 phase when compared to S phase (Bishcoff et al 1990) and thus it is possible that the under phosphorylated form may exert the G1 control.

The role of p53 as a global damper of cellular growth signals may be via regulation of the guanosine tri phosphate (GTP) production since it was found that p53 protein may be implicated in the regulation of guanosine nucleotide biosynthesis (Sherly 1991). Wild type p53 may limit the growth capacity of cells by regulating the availability of GTP for growth factor signal transduction.

1.2.3. Apoptosis Summary

Biochemically, the understanding of apoptosis is still very much in its infancy. Although it is known to generally require active protein synthesis and is a calcium dependent process, these are by no means exclusive and simply serve to demonstrate the

many differing way physiological cell death can occur. Apoptosis is thought to be regulated by several gene products with the proteins c-myc (Evans et al 1992), p53 (Shaw et al 1992) and Apo-1 (Itoh et al 1991, Trauth et al 1989) thought to be instrumental in increasing apoptosis when their expression is raised whilst bcl-2 is involved in the suppression of apoptosis. The regulation of these influential proteins (plus many others that are continually being added to a long list) and their effect on apoptosis is poorly understood, but it would seem likely that the regulation of apoptosis is a result of co-operative interactions between all of these proteins.

1.3. Tumour progression

The initial events by which a normal cell becomes a tumour cell are poorly understood. It is still not clear whether the development of a tumour results from a perpetual proliferative signal such as that seen from oncogenes for example ras and erb B, or a failure of the cell to enter the death program which as suggested earlier may be a result of the action of the oncogenes p53, bcl-2, c-myc or APO-1. What seems certain is that the conversion from normal to a malignant tumour cell is likely to be the result of the activation of a number of oncogenes resulting from either gene mutation or over expression or both. The development of the malignant cell is therefore undoubtedly a highly complex event. Once the malignant cell is established the chain of events that follow are well characterised.

1.3.1. Initial development and vascularisation

As the primary tumour cell divides and develops a tumour mass, the growth is limited by the availability of nutrients. As long as the tumour remains in this non vascularised state the tumour remains benign and small. The action of vascularisation results from the secretion by the tumour cells of Tumour Angiogenesis Factors (TAF).

These are a collection of polypeptides that resemble endothelial growth factors such as α -endothelium (αENDF) or β- endothelium (βENDF). The most widely characterised is angiogenin, a 14 kDa protein isolated from colon cancers. Secretion of TAF's results in capillary endothelium cells detaching from capillaries and growing towards the developing tumour mass. These cells then divide and differentiate to produce capillaries that enter the tumour. Antagonists to TAF such as heparin / cortisone combinations have been used to inhibit vascularisation of a selected number of tumours.

As the tumour mass becomes vascularised interactions between leukocytes and tumour cells increase and continue through all progression stages. Lymphocytes and monocyte / macrophages infiltrate the tumour mass and modulate tumour cell migration and proliferation by means of release of cytokines and growth factors PDGF (Ross et al 1986), TGFβ1 (Sporn and Roberts 1992), and chemotactic peptides such as platelet factor 4 (chemoattractant) (Zucker et al 1989), thrombospondin (adhesion) (Tuszynski et al 1987), GMP-140 (aggregation) (Stenberg et al 1985). Tumour cells themselves can modulate their own proliferation through the release of growth factors (reviewed by Goustin et al 1986, Heldin and Westermark 1989 and Moses et al 1990).

Chemoattractants direct the migration of tumour cells of both primary and secondary tumours towards target tissues (Nicolson 1991) and modulate the angiogenic reaction (Weiss et al 1989, McCormick and Zetter 1992).

1.3.2. Invasion.

For the tumour to develop and progress it must invade surrounding tissues, this includes not only its immediate surrounding tissue but body cavities and vessels (blood and lymphatic). Many of the events occurring during invasion also occur at the later stage of reinvasion (discussed later).

The extracellular matrix is composed of several large macro molecules such as collagen, laminin, fibronectin, thrombospondin, proteoglycans and elastin which all play a role in promoting cell adhesion, migration and proliferation. The deposition of extracellular matrix is stimulated by growth factors and cytokines released by platelets, leukocytes, fibroblasts, endothelial cells and tumour cells, whereas its dissolution is caused by proteolytic enzymes and endoglycosides released by the same cells (Liotta and Stettler - Stevenson 1991). A dynamic equilibrium exists between deposition and dissolution. This equilibrium is destroyed by tumour cells (Herlyn and Malkowicz 1991, Duffy 1992). Destruction of this equilibrium allows the growing tumour to impregnate surrounding tissues due to the reduction in adhesion between cells of that tissue.

Proteoglycans are important components of the extracellular matrix (Ruoslahti and Yamaguchi 1991). Endoglycosidases, such as heparanase, also present in platelets, can digest proteoglycans of the extracellular matrix surrounding the tumour or the basement membrane (Oldberg et al 1980). A positive relation between heparanase activity and metastatic potential has been established on the basis of the ability of heparanase to release growth factors such as basic fibrogenic growth factor (bFGF), associated with the extracellular matrix (Ishai-Michaeli et al 1992).

Tumour cells show increased proteolytic properties that are relevant for the remodelling of extracellular matrix and basement membranes. As an example, cysteine proteases such as cancer procoagulant activity and cathepsin B are released by tumour cells (Donati and Semeraro 1984, Slone 1990). Considerable interest has developed in the metalloproteinases and their inhibitors, a class of serine proteases secreted by several tumour cells and endowed with the ability to digest any kind of collagen (Matrisian et al 1992). Metalloproteinases play a crucial role in the dissolution of extracellular matrix and are in constant equilibrium with specific tissue inhibitors. Tumour cell derived

growth factors such as bFGF and TGF β, modulate the activation/inhibition of metalloproteinases and their release from blood or endothelial cells (Herlyn and Malkowicz 1991). A relevant role in tumour cell invasion seems to be played by plasminogen activators and inhibitors. Plasminogen is a ubiquitous pro-enzyme, present in body fluids, that may be activated to form plasmin. Plasmin dissolves fibrin, degrades many proteoglycans of the extracellular matrix and activates collagen degrading metalloproteinases (Duffy 1992). Release of plasminogen activators has long been associated with malignant transformation; two species of plasminogen activators are known, tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) (Dano et al 1985, Kwaan 1992).

As well as proteolytic secretions reducing the adherence of surrounding cells, tumour cells also show reduced adherence which not only helps in penetrating tissues in the primary growth, but also is important for the next step, release. At this stage of tumour development, reduced glycosylation of the tumour cell surface is observed, cAMP levels decrease and the cells on the tumour periphery round and express increases in microvilli and lectin agglutinability (Hart et al 1983).

1.3.3. Release

As the tumour invades it will break into capillaries and/or the lymphatic system. When a tumour penetrates a vessel, the physical stress of blood flow, coupled with reduced adhesiveness due to the proteolytic secretions results in cells being washed from the tumour surface. The changes in cell shape, microvilli and lectin agglutination cause the cells to become round, but 'rough'. This increases the resistance of the cell to flow and increases the releases of cells from the tumour mass. Oncogenes such as c-src that affect involucrin may be important at this stage of development (Hart et al 1983).

1.3.4. Distribution and Embolism.

The vast majority of cells released from the tumour surface are detected and destroyed by the immune system. The few that survive to form secondaries may do so for one of a number of reasons. Primarily thromboplastic activity may be important. Thromboplastic activity causes factor V to be converted to factor Xa which then acts to convert prothrombin to thrombin which then acts to convert fibrinogen to fibrin which is the crosslinked by the plasma transglutaminase factor XIIIa. The end result of all this is aggregation of platelets round the tumour cell forming a thrombus (Embolism) that moves in the blood. The thrombus protects the tumour cell from immune attack (Gasic et al 1973). An alternative to thromboplastic activity in platelet aggregation may be the shedding of microvesicles by the tumour cell that contain lipoproteins and Sialic acid (Hart et al 1983) or the release of ADP (Lampugnani and Crawford 1987). In addition cytoskeleton integrity and the presence of a membrane glycoprotein immunologically related to platelet glycoprotein IIb/IIIa on tumour cells are important prerequisites of the tumour cells ability to induce platelet aggregation (Chopra et al 1992).

The ability of cells to induce *in vitro* platelet aggregation was correlated to their metastatic potential in experimental tumour models (reviewed Ordinas et al 1990).

1.3.5. Arrest and Adherence.

For the development of a secondary tumour (a metastasis) the travelling embolism must be arrested in a capillary bed. There would appear to be some chemical interaction / attraction between particular tumour types and particular tissues such that metastasis released from one tumour type have a preference for colonisation of a certain tissue. This may depend on endothelial cell heterogenicity, i.e. growth factors / adherence receptors or chemoattractants (M^{c}Carthy et al 1991). The adhesion of tumour cells to

the endothelium or subendothelial matrix is one of the initial steps in reinvasion and is stimulated by activated platelets (Abecassis et al 1987) and receptors on the platelets surface (Menter et al 1987). Platelet induced tumour cell adhesion to the vessel wall seems to be influenced by cycloxygenase and lipoxygenase products derived from platelets, endothelium and tumour cells (Buchanan et al 1990).

Various families of cell adhesive receptors regulating cell-cell and cell-matrix interactions have been identified. The primary ones involved in attachment are: selectins including endothelial leukocyte adhesion molecule 1 (ELAM-1) (Bevilacqua et al 1986) and GMP-140 (Stenberg et al 1985); integrins including $\alpha_2\beta_1$, $\alpha_4\beta_1$, $\alpha_6\beta_1$ (Hynes 1992), Lymphocyte function associated antigen-1 (LFA-1) (Peri et al 1990), $\alpha_{IIb}\beta_3$ (Honn et al 1992b) and $\alpha_v\beta_3$ (Gehlsen et al 1992); immunoglobins including intracellular adhesion molecule 1 (ICAM-1) , intracellular adhesion molecule-2 (ICAM-2) (Dustin and Springer 1988), and vascular cellular adhesion molecule-1 (VCAM-1) (Rice and Bevilacqua 1989) and various others including Cadherins (Takeichi 1990), CD44 (Birch et al 1991) and 67 kDa laminin receptor (Ywamoto et al 1987).

The arg-gly-asp (RGD) sequence has been identified as the primary cell adhesion site of several attachment molecules such as fibronectin, vitronectin, collagen I, fibrinogen, Von Willebrand factor and laminin (Ruoslahti 1988). Synthetic peptides with the RGD sequence have been shown to cause reduced colonies in experimental metastasis (Humphries et al 1986). Disintigrins, a class of peptides containing the RGD sequence that are isolated from snake venom have been shown to reduce experimental metastasis (Soszka et al 1991).

1.3.6. Reinvasion.

The Thrombin activity and the activity of the proteolytic enzymes used in the primary invasion help the arrested tumour cell embolism to break through the basal lamina of the capillary endothelium and reinvade the tissue (Poste and Fidler 1980). Platelet and neutrophiles that help make up the thrombus secrete cytokines / growth factors under the influence of the tumour cell, for example, the tumour cell may release platelet growth releasing factor that acts on platelets to release PDGF. Many of the characteristics seen in the tissue invasion of the primary tumour are replicated here.

Activated platelets in the embolism release the contents of their αgranules such as peptides with mitogenic and chemotactic activity, adhesive proteins and enzymes (Niewiarowski and Varma 1982). Some of these products are involved in progression and invasion, for example PDGF (Ross et al 1986), TGFβ1 (Sporn and Roberts 1992), platelet factor 4 (Zucker et al 1989), thrombospondin (Tuszynski et al 1987), GMP-140 (Stenberg et al 1985), thrombin (Nierodzik et al 1991), 12(s)-HETE and 13(S)-HODE (Chen et al 1992), TXA_2, (Donati et al 1982) and PGI_2 (Honn et al 1981).

The importance of platelets in tumour progression has led to the use of anti platelet drugs in the treatment of experimental metastasis on the basis of reduced lung colony formation in thrombocytopenic mice (Gaesic et al 1968, Pearlstein et al 1972). The first drug to be used was aspirin (Gasic et al 1972). Other inhibitors of platelet aggregation such as ticlopidine gave conflicting results in spontaneous models of dissemination and even potentiated metastasis in the artificial model (Karpatkin et al 1988). Honn et al 1987 showed the effect of prostacyclin on experimental tumours. Prostacyclin analogues, thromboxane synthetase and receptor antagonists have been used successfully since (Honn et al 1992a).

The ability of tumour cells to adhere to basal lamina and to extravate seems to be correlated with the tumour cell ability to digest basement membrane by activation of specific type (IV) collagenase (Timpl 1989).

1.3.7. Tumour progression summary.

Tumour cell progression and dissemination is a complex process depending on the ability of the malignant cell to grow, invade tissues and vessels and escape from the primary tumour. Circulating tumour cells can adhere to the vessel wall at distant sites, dissolve the basal lamina and extravaste giving origin to metastases. Interactions between tumour cells, blood platelets and leukocytes favour tumour cell adhesion to the vessel wall, migration in extra vascular space and growth in secondary sites. Tumour and blood cells release growth factors and inflammatory proteins, such as cytokines and chemokines that are involved in tumour cell migration and proliferation. Tumour cells and cells of surrounding tissue possess procoagulant and fibrinolytic properties that are important in modulating the extracellular matrix around the tumour cells thus facilitating tumour cell invasion and progression.

CHAPTER 2

AIMS

The aim of this study is to examine whether modulation of cytosolic tissue transglutaminase in a neoplastic cell can influence its ability to either undergo apoptosis and / or its malignant phenotype.

There are two potential ways to modulate transglutaminase in malignant cells. Firstly there are known pharmacological agents such as trans-retinoic acid, sodium butyrate and DMSO / trans-retinoic acid and physiological agents such as TNFα and TGFß which result in increased expression of the tissue enzyme (Byrd and Lichti 1987, Murtaugh et al 1983, Davies et al 1990). Interestingly, trans retinoic acid has recently been shown to be highly successful as a differentiation therapy for acute promyelocytic leukaemia (Castaigne et al 1990). Secondly, by transfection of metastatic cells with expression vectors carrying the full coding sequence for tissue transglutaminase. At the onset of this work vectors were available in the constitutive eukaryotic expression vector pSG5 and in the inducible expression vector pMAMneo.

Stably transfected malignant cell lines in which transglutaminase expression is modulated can then be studied for changes in their apoptotic index, their ability to form primary tumours when subcutaneously injected and for any change in metastatic potential when injected either intravenously or subcutaneously.

If either of the above protocols demonstrate that malignancy of a tumour cell can be modified by alteration of its transglutaminase activity / apoptotic rate, then this opens up a number of potential therapeutic routes. Pharmacological agents may be found that specifically increase transglutaminase activity / apoptosis in tumour cells either via an increased expression of the active enzyme or through activation of the inactive enzyme protein without additional harmful effects. It is therefore important to understand what factors govern the regulation of transglutaminase in cells and what events lead to perturbed expression of the enzyme in the malignant cell.

CHAPTER 3

METHODS AND MATERIALS

3.1.: Cell Culture.

3.1.1.: Cell lines and growth medium.

a: The Met Cell lines.

The Met cell line was originally developed by Teale and Rees (1987) from a HSV-2-transformed cell line. Briefly, hamster embryo fibroblasts were transformed *in vitro* with inactivated HSV-2. One of the resulting clones (HSV-2-333-3-2-26) (Parent) was used to induce a primary tumour in hamster. The resulting fibrosarcoma was resected after metastasis had occurred. Lung nodules were excised and placed *in vitro* to give cloned cell lines which were named by the prefix Met (metastatic variant) and then either A to G, which were subsequently characterised by there own metastatic potential. In this study the variants Met B, Met D and Met E were used. Met B and E have a high metastatic potential (20/20 and 19/20 respectively) were as Met D has a low potential (1/20). All of the met cells grow as a monolayer.

b: BHK-21(Baby hamster kidney) cell line.

This a commercially available Hamster fibroblast cell line sold by flow laboratories (Macpherson 1963). Briefly, it was derived from a hamster kidneys from five, 1 day old animals. The kidneys were fine chopped, trypsinised and introduced to culture. The resulting cell type was undifferentiated, consisting exclusively of fibroblasts after 12 generations. This cell line was used as a 'normal' control to the Met cell lines. This cell line grows as a monolayer.

c: K562 cell line

K562 is a human erythroleukemia cell line derived from a resected tumour which grows in suspension. It was kindly supplied by R.C. Rees (Institute for Cancer Studies, Sheffield).

d: Growth medium and conditions

All cell lines in this study were cultured in the same growth medium. This had a two fold advantage. Primarily it was intended to reduce any variation in response that may have resulted from different factors present in the incubating solutions or differing concentrations of salts or growth factors present. Secondly it simplified and reduced the cost of the daily tissue culture routine.

Medium consisted of a base of Dulbecco's modified eagles medium (DMEM) containing 5% (v/v) foetal calf serum (FCS) (Imperial Laboratories) which was supplemented with L-glutamine to a concentration of 2 mM (added fresh) (Sigma Chemicals). As a prophylactic against infection the medium was routinely supplemented with 200 units ml^{-1} Penicillin and 200 mg l^{-1} streptomycin. If the cells showed signs of bacterial infection the medium was further supplemented with one or more of the following : 10 mg l^{-1} ampicillin, 5 mg l^{-1} chloramphenicol, 50 mg l^{-1} polymixin B, 40 mg l^{-1} ciprofloxacin, 10mg l^{-1} tetracycline depending on sensitivity tests. Fungal infection was treated with 2.5 mg l^{-1} amphotericin B. Cells where not used for experiments while supplemented by microbial agents. All antibiotics and fungicides were supplied by Sigma Chemicals. Cells were grown at 37°C in a 5% (v/v) CO_2, humidified environment.

All monolayer cultures were routinely harvested by incubation in 0.025% (w/v) trypsin at 37°C for as long as was required to release the cells and the cells recovered by centrifugation at 400 x g. Cells were then washed in complete medium before further use. If experimental protocol dictated cells were released by the use of 5 mM Tris, 1 mM EDTA or a cell scrapper.

3.1.2.: Treatment of cells with pharmacological agents.

a: Dexamethasone

Dexamethasone treatment was performed in complete medium. A 10 mM Dexamethasone solution in 100% (v/v) ethanol was prepared and stored at -20°C for up to a month. Cells were grown to approximately 50-70% confluency prior to commencing the experiment. Confluency was varied depending on the duration of the dexamethasone exposure required. The medium was replaced and dexamethasone added to give the required final concentration. Concentrations used ranged from 1 nM to 10 μM, although for most experimental procedures the concentration for dexamethasone was 1 μM. Dexamethasone incubation times were either 24 or 48 hours. Near maximum transglutaminase response was achieved after 24 hours.

b: All-*trans* retinoic acid

All-*trans* retinoic acid treatment was performed in complete medium. A 10 mM all-*trans* retinoic acid solution in sterile distilled water was prepared and stored at 4°C in the dark for several months. Cells were grown to approximately 80 % confluency, the medium was then replaced and all-*trans* retinoic acid added to give a final concentration of 1 μM. Retinoic incubation was carried out for 15 hours. All cells were kept in the dark.

c: Tumour Necrosis Factor Alpha (TNFα)

TNFα treatment had to be performed in a serum free medium, thus all work required a serum free medium control. A 50 000 units per ml stock in sterile PBS was prepared and stored indefinitely at -70°C in aliquots. Cells were allowed to grow to 80% confluency and then the medium replaced with complete medium lacking serum. TNFα was added to a concentration of 500 units ml^{-1} and incubated for 24 hours.

3.1.3.: Transfection procedure.

The artificial introduction of cDNA into mammalian cells is termed transfection. For this process the cDNA sequence of interest was inserted into an expression vector, i.e. a plasmid with a promoter sequence that can inserted itself into the cells genome at various sites and then control transcription of the cDNA sequence in a mammalian cell. Typically these promoter sequences are of viral origin.

There are several chemical transfection methods available e.g. DNA / calcium phosphate coprecipitation, DEAE-dextran and lipid micelle transfer (Gorman et al 1982, Felger et al 1984, Chang and Brenner 1988). Each is designed to aid the transfer of the expression vector through the cell membrane. Additionally physical techniques can be applied such as electro permeabilisation of the cell membrane to allow passage of the cDNA (Traas et al 1987, Leonard and Sedivy 1990). The transfection method chosen for these experiments is a chemical one, employing the reagent Lipofectin (Gibco BRL). Basically this consists of the molecule N-(1-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride or DOTMA for short. This molecule forms lipid micelles that surround DNA and thus facilitate the transfer of DNA through the cell membrane (Felger et al 1984). Once inside the cell the expression vector facilitates the random incorporation of the cDNA into the genome. The lipofection technique was chosen for 3 main reasons. It is a highly efficient and reproducible process especially in fibroblasts, the method is simple, the cost is relatively low and requires no expensive equipment.

The method described below was refined to allow maximum incorporation of cDNA into the MET cell lines. It was essential to asses the levels of Lipofectin and DNA as well as incubation times to create the greatest efficiency of transfection for each cell line to be transfected. Sterile reagents and materials were used at all times and the process was performed in a sterile environment.

a: Cells were grown in a 10 cm Petri dish until the confluency reached 65 to 70% of total plate area. The complete growth medium was removed and the

cells washed with DMEM containing 2 mM L-glutamine (no serum or antibiotics). A fresh 10 ml of this serum free DMEM was added to the plate and the cells incubated at 37°C in a 5% (v/v) CO_2 environment for 5 minutes.

b: Two sterile 50 ml tubes made of either glass, polypropylene or polycarbonate (not polystyrene) were required to produce the DNA-micelle. In to one of the tubes 100 μl of 1 mg ml^{-1} Lipofectin was placed, while in the other 10 μg of DNA was aliquoted. The DNA added may have consisted of more than one vector, but total concentration did not exceed 10 μg. Each tube was made up to a volume of 2.5 ml with DMEM containing 2 mM L-glutamine (no serum or antibiotics).

c: The Petri dish containing the cells incubating in DMEM was removed from the incubator and the medium removed.

d: The Lipofectin tube was poured into the tube containing the DNA. It was essential to pour the Lipofectin into the DNA and not the other way round to allow good incorporation of the DNA into the lipid micelles. The solution was poured from tube to tube 4 times, this caused maximum incorporation of the DNA into the micelles without breaking the micelles into too smaller units to carry the plasmid. The Lipofectin DNA mix was then poured onto the cells which were then returned to the 37°C, 5% (v/v) CO_2 incubator for 6 hours for the transfection to occur.

e: Five ml of DMEM containing 2 mM L-glutamine and 20% (v/v) foetal calf serum was then added to the Petri dish and thus returned to the incubator overnight. The double strength serum deactivated the transfection procedure and restarted cell proliferation.

g: Following the overnight incubation, the medium was removed and replaced with normal complete fresh medium. If the transfected vector was an inducible vector, then the inducing compound was added at this stage and the cells left for 48 hours before measurements made. If the transfected vector carried a constitutive promoter then a further 24 hours was allowed before the

cells were subjected to measurements. These time spans were incorporated to allow the cells to recover from the transfection procedure and allow the inserted DNA time to be transcribed and translated to maximum levels. If the cells were to undergo cloning then the cells were left for 24 hours before the cloning procedure was begun.

3.1.4.: Cloning of Met B cells transfected with pSG5 Tgase and the antibiotic resistance vector pSV_2neo.

The method described below required a co-transfection to be performed with the vector of interest, pSG5 Tgase, plus a antibiotic resistance vector to neomycin (pSV_2neo) to allow selection of cells that had incorporated the transfected cDNA. A co-transfection is not always necessary as there are many expression vectors available that now have a resistance gene for use in the mammalian cell already incorporated into them, but the co-transfection route was selected here as the pSG5 Tgase vector was already prepared and resulted in high transglutaminase activity, and both the vectors to be co transfected were relatively small so that co-transfection occurred easily.

The transfection procedure was performed exactly as outlined in the transfection protocol in section 3.1.3. with the exception of the points listed below.

i: Cells were grown in a 10 cm diameter Petri dish until confluency reached 30-50% of total plate area. This was less confluent than for a transient transfection due to the necessity to isolate discrete colonies at the latter stages. The cells were treated in the same way as for the transfection procedure outlined in section 3.1.3.

ii: Vectors and cDNA were prepared in the ratio of 9 μg of pSG5 Tgase to 1 μg of the antibiotic resistance vector pSV_2neo. This ratio increased the possibility that any cell that was selected out using the neomycin resistance vector was likely to carry the transglutaminase vector.

The cloning procedure was carried out as follows.

a: 48 hours post transfection from commencement of the procedure, the single Petri dish used in the transfection was split (using trypsin) into 16 Petri dishes. This heavy split of a sub confluent population allowed cells to be well spaced out, which facilitated further selection. At the same time G418 sulphate (neomycin antibiotic class) was added to a concentration of 800 units ml^{-1} to each Petri dish. The cells were maintained in complete medium and a 5% CO_2 environment. The lethal dose of G418 was determined by performing a kill curve on the non transfected cell before commencing the cloning procedure. This was done by administering different doses of the antibiotic and determining at which concentration the cells died. For Met B this is shown in figure 3.1.1.

A high concentration of G418 (800 units ml^{-1}) was used to give a 'quick kill' to prevent the plate from becoming confluent and thus making cloning difficult. A concentration of 200 units ml^{-1} was lethal after 4 weeks and this concentration was then used as a constant selection pressure following the initial rapid selection.

b: After approximately 1 week all cells that had not incorporated pSV_2neo into there genome had died. Any cells that showed resistance to the G418 and began to divide into the start of a colony were marked. In particular cells forming colonies that were very discrete such that they did not overlap with other colonies when they started to divide were noted.

c: Individual, discrete colonies that had originated from a single cell were allowed to develop. The colonies were allowed to become as large as possible without encroaching on other colonies. This took between 3 and 8 weeks from the addition of the G418.

d: When colonies of cells had developed it was necessary to remove these cells from the Petri dish and place them into a twelve well tissue culture plate.

This procedure was done in one of two ways both of which were effective in isolating clones without cross contamination of other clones.

This was initially done using cloning rings. Although available commercially, these were made by cutting a 5 mm cylindrical section off the shaft end of a 100 μl pipette tip. The rings were autoclaved in a Petri dish together with a second Petri dish which had a smear of silicon vacuum grease inside. The Petri dish containing the cells to be cloned had the medium removed and was washed with PBS. Using sterile forceps a cloning ring was selected and dipped into the sterile vacuum grease. Then using a microscope a colony was selected, the ring was placed over the colony and pushed down firmly to make a seal round the cells. A drop of trypsin solution was placed inside the ring to release the cells and the Petri dish returned to the 37 ^{o}C incubator to allow the trypsin to act for two minutes. The trypsin solution was then pipetted up and down several times to ensure all cells were released using a 100 μl pipette. All the solution containing cells was sucked out of the ring and placed into a twelve well plate. Immediately complete medium was added to the well containing double strength foetal calf serum, penicillin and streptomycin.

As an alternative to cloning rings colonies were picked off using a 100 μl pipette and tip. The medium was removed from the Petri dish containing the cells to be cloned. A sterile pipette tip was placed on to a pipette then using a microscope the pipette tip was positioned at the side of a colony touching the bottom of the dish. The pipette piston was emptied and the pipette slid into the middle of the colony, and the pipette brought to the vertical and then the colony drawn into the pipette tip. This action resulted in a large number of cells being stuck in the pipette tip. The cells trapped in the pipette tip were then released by pipetting up and down several times in a well all ready containing medium with additives as previously described.

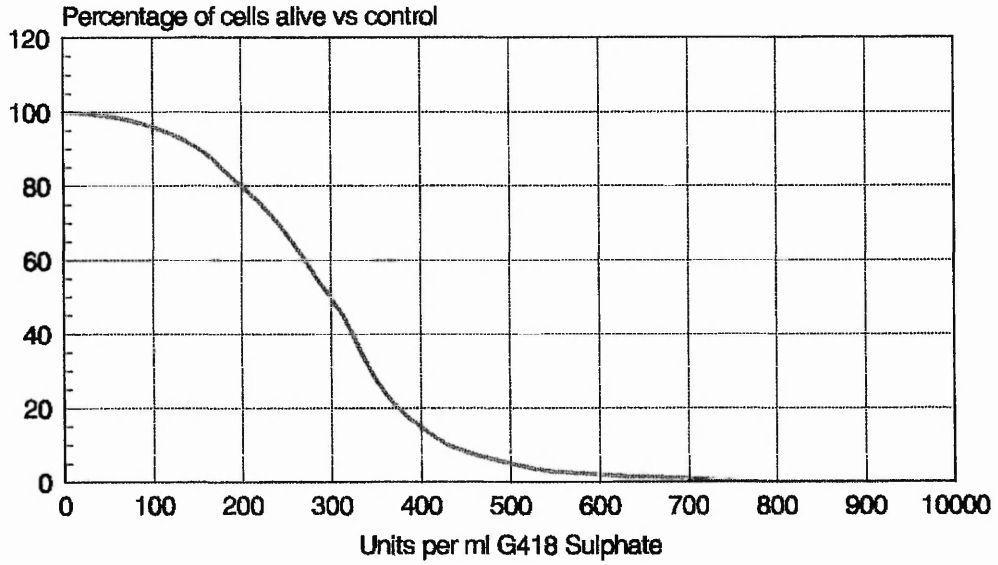
The picking tip method was faster and incurred less problems with infection and cross clone contamination, but resulted in fewer cells being captured from the colony. Therefore the pipette tip method was the method

of choice. Cloning rings allowed the entire colony to be transferred but suffered from the major problem of the vacuum grease seal failing thus contaminating the whole plate with the clone. The clones placed into the wells were allowed to become confluent, then transferred to a 25 cm^2 flask using a trypsin harvest. Once released by a minimum amount of trypsin, the trypsin solution containing cells was placed straight into flask containing complete medium. The cloned cells were allowed to become confluent in the flask, harvested and the first passage stored in liquid nitrogen. The flask was then re seeded and the second passage screened for transglutaminase activity using the ^{14}C putrescine incorporation assay. From placing the clones into the twelve well plate to actual screening took approximately 1 month. When the clones were placed into the 25 cm flask the G418 level was reduced to a selection pressure level of 200 units ml^{-1} from 800 units ml^{-1}.

Figure 3.1.1. Toxicity curve for Met B using G418 sulphate. 7 days from application of G418.

Met B cells were allowed to grow in a 10 cm Petri dish to approximately 50% confluency. At this stage G418 sulphate was added to the medium at a variety of concentrations ranging from 0 (control) to 1000 units ml^{-1}. The medium was changed every day and replaced with medium containing the same concentration of G418 sulphate. After 7 days the cells remaining attached to the plate were harvested using trypsin and counted on a haemocytometer. The cells in the G418 sulphate treated plates surviving were expressed as a percentage of those cells in the dish that had not been exposed to G418 sulphate.

Figure 3.1.1.



3.1.5.: Cell attachment and adhesion studies.

This was performed on 96 well plates that were coated with collagen type IV, fibronectin, BSA and on uncoated plastic. In addition commercially coated tissue culture grade 96 well plates were also used.

Binding of substrates to 96 well plates.

Modification of the method of DeLuca et al 1991

a: Plates were incubated for 60 minutes at room temperature with 100 μl of either 10 μg ml^{-1} fibronectin, 50 μg ml^{-1} collagen type IV, 10 mg ml^{-1} BSA or PBS for the uncoated plastic. BSA and uncoated plastic acted as controls.

b: The substrate was removed and the plate washed twice with PBS. Any remaining binding sites in the wells were blocked with 100 μl of 10 mg ml^{-1} heat denatured BSA by incubation at room temperature for 30 minutes. The heat denatured BSA was prepared by completely dissolving BSA to the required concentration in PBS and placing it in a water bath at room temperature. The water bath was brought to the boil, simmered for 1 minute and then the BSA solution left in the bath for a further 5 minutes as it cooled. This denatured the BSA, but prevented it precipitating out of solution.

c: The plates were then washed twice in PBS. It was then possible to store the plates for several months at 4°C before use.

Attachment assay.

Modification of techniques described by Hendrix et al (1990)

i: Cells were harvested from a normal tissue culture flask using trypsin (0.025% (w/v)) and washed in PBS containing 0.5 mg ml^{-1} trypsin inhibitor. The cells were then resuspend in PBS containing 0.5 mg ml^{-1} trypsin inhibitor and

incubated at 37°C for 15 minutes in a 5% (v/v) CO_2 environment. Cells were then counted on a haemocytometer and resuspended in DMEM (Dulbecco's modified Eagle's medium) containing 5% (v/v) foetal calf serum to a concentration of 2 x 10^5 cells ml^{-1}. 100 μl of this was added to each well. Each well therefore received 2 x 10^4 cells.

ii: The plate was then incubated at 37°C in a 5% (v/v) CO_2 environment for 90 minutes. After incubation any cells that had not attached to the plate were removed by gently inverting the plate and tapping to release the liquid. The plate was then washed in PBS by gently filling the plate wells using a multi channel pipette and inverting the plate to remove the wash. Only cells which had attached to the substrate remained in the wells. Unattached cells were removed by the wash.

iii: Cells attached to the substrate were then fixed and stained by adding 100 μl of 0.5 % (w/v) crystal violet in 70 % (v/v) ethanol to each well and leaving for 30 minutes at room temperature. All cells attached to the plate acquired crystal violet staining of the plasma membrane that could not be removed by PBS. Thus, all excess crystal violet stain was removed from each well by immersing the plate into a tank of PBS until no more colour was released from the plate, at which stage a purple precipitate like patch was visible at the bottom of any well in to which attachment had occurred.

iv: Attached cells were dissolved in 100 μl of 30 % (v/v) acetic acid which released the crystal violet stain into solution. The absorbance at 550 nm was measured using a 96 well plate spectrophotometer. The optical density of the solution was proportional to the amount of crystal violet which was proportional to the number of cells attached.

v: To determine cell number from the optical density it was necessary to produce a standard curve. From each batch of cells used in the attachment assay, a volume containing to 1 x 10^6 cells was placed in to a microfuge tube in duplicate. The cells were pelleted and resuspended in 100 μl of 0.5 % (w/v) crystal violet in 70 % (v/v) ethanol and left at room temperature for 30

minutes. The cells were then pelleted in a microfuge, the supernatant discarded and the cells washed in PBS until no more dye was released from the cells. The stained cells were then dissolved in 100 μl of 30 % (v/v) acetic acid to release the crystal violet stain into solution. This was then placed into a 96 well plate and serial diluted to give colouration corresponding to 5×10^5 to 1.9×10^3 cells.

Adhesion studies.

i: Cells were prepared and plated out exactly the same as in the adhesion studies as explained in section d. The only difference was that a standard curve of cell numbers was prepared on each plate ranging from the highest value of cells in each well, 2×10^4 cells, serially diluted 8 times to 312 cells.

ii: The plate was incubated overnight in a 5% (v/v) CO_2 environment at 37°C to allow attachment of the majority of the cells. The plate was washed in PBS gently. Both the plate and the washes were then carefully checked to determine if more than the odd cell had been released in the wash process. If a large majority of the cells had been washed off then the experiment should be stopped as the cells had not adhered correctly.

iii: Each cell line on the plate was then divided into 5 groups, each group consisting of at least 8 wells. To group 1 100 μl of 0.025% (w/v) trypsin was added, to group 2 100 ul of 0.0025% (w/v) trypsin was added, to group 3 100 μl of 0.00025% (w/v) trypsin was added. Group 4 acted as a control in a serum free environment with either 100 μl of PBS or serum free DMEM added and to group 5 complete medium was added again as a control. The wells used for the standard curve were returned to complete medium. The plate was then Incubated at 37°C for 30 minutes in a 5% (v/v) CO_2 atmosphere.

iv: All incubation medium was removed and each well washed in PBS gently to remove any cells released from the plate by the proteolytic digestion.

Fixation, staining, washing and dissolving the cells was performed as described in attachment studies sections f and g.

v: Determination of the number of cells remaining adhered to the substrate was via reference to the standard curve. The cells in the group incubated in the complete medium were the primary control group representing the total number of cells attached to the well. The group incubated in PBS or DMEM should contain roughly the same number of cells as the primary control, but this group took into account changes in adhesion that occurred in the absence of serum and should therefore be used as the reference when determining the percentage of cells still attached following the proteolytic digestion with differing trypsin concentrations. Obviously the greater the adhesion of the cells for a substrate then the higher percentage of cells still attached following trypsinisation.

3.1.6.: Culturing tumours *in vitro.*

Introducing a tumour to an in vitro environment had a number of technical advantages in studying its biochemistry and genetics than the same tumour *in vivo.* It allowed the tumour cell to be selected out from a heterogeneous cell population that would also contain blood cells of many varieties and endothelial cells from blood vessels thus clarifying the source of protein expression and activity. Additionally the 'cleaner preparation' also allowed better and simpler extraction of many factors, specifically in this case nucleic acids and tissue transglutaminase. Finally and most importantly it removed all physiological stimuli from the tumour cell, thus determining if changes in tumour cell function were in response to systemic factors in the animal.

a: The animal was terminated (by ether) and placed into a laminar flow cabinet. The skin area surrounding the tumour was swabbed with 70% (v/v) ethanol and using sterile instruments the complete tumour removed and placed into a sterile Petri dish.

b: The tumour was cut in half to reveal any necrotic areas and then a piece cut from the tumour corresponding to approximately 2 cm^3 in volume of healthy tissue which was placed into a 50 ml sterile centrifuge tube.

c: The aim was to release as many individual cells from the block of tissue as possible. This was most efficiently done by firstly using a pair of sterile scissors to chop the tumour mass into as many pieces as possible to increase the surface area. Next, 30 ml of 0.25% (w/v) trypsin in PBS was added and the tube shaken strongly and placed on an end to end rotator at room temperature for 20 minutes.

d: The tube was then removed from the rotator and allowed to stand for approximately 2 minutes to allow any large lumps of tissue to sediment while leaving individual cells in suspension. The supernatant was carefully decanted into a fresh tube and the cells pelleted by a 5 minute spin at 400 x g in a centrifuge.

e: Cells were resuspended in 40 ml of complete medium and split between two, 75 cm^3 tissue culture flasks and then incubated under standard tissue culture conditions overnight. After this period all of the viable cells had settled on to the plate. The medium was removed and the flask washed once with complete medium before fresh complete medium was added and the flask returned to the incubator until the cells were required or needed splitting. A variety of cells settled onto the plate during the first incubation period, but since these did not posses immortality like the tumour cells, they quickly died and the dead cells removed with the standard 48 hourly change of medium.

3.1.7: *In vitro* growth rate.

Some of the manipulations used in this study required an accurate determination of the growth rate *in vitro*, especially when cells that were reintroduced to animals began to express differing rates of growth *in vivo*. The most accurate way to assess growth rate was using large cell numbers, with

independent growth curves at different times and counting directly using a haemocytometer.

a: A healthly growing population of cells was harvested using 0.25% (w/v) trypsin in PBS. The trypsin was deactivated by adding the cell suspension into 2 volumes of complete medium. The cells were washed in PBS twice and then cell number determined by counting on a haemocytometer in duplicate.

b: The duration of the growth curve was decided on and for each day of growth 3 x 10 cm Petri dishes were selected and 10 ml of complete medium added to each dish. To each dish a volume of cell suspension containing 1 x 10^5 cells was added. This was in a volume of no greater than 1 ml to prevent over dilution of the growth medium. For the cell lines used in this study 1 x 10^5 cells in a 10 cm dish allowed for up to 8 days growth before the cells reach confluency and deviated from exponential growth. Medium was changed ever 2 days.

c: Every 24 hours 3 of the Petri dishes were selected, the medium removed and the monolayer washed twice in PBS pre warmed to 37^{o}C to remove any non attached cells. All cells still attached were assumed to be viable. Cells were harvested using 0.25 %(w/v) trypsin, pelleted and resuspend in an accurate volume to a cell concentration such that counting on a haemocytometer was within accurate limits. Cells from each Petri dish were counted individually and in duplicate. This was repeated for every day of the growth curve.

d: The growth curves were repeated for a minimum of five times. For each curve a plot of the natural log of cell number against time was made. A line correlation was performed to determine if the line was linear and thus cell growth was exponential. Then for each growth curve the equation of the line was determined using linear regression and the mean equation of the line calculated from the five individual growth curves. An accurate growth curve was then determined from this mean line equation.

3.2.: Experimental procedures using animals.

3.2.1.: Introduction of tissue cultured tumour cells in to hamster and nude mouse, tumour detection and growth rate measurements.

a: A healthly growing population of cells was harvested using 0.25% (w/v) trypsin in PBS. Deactivation of the trypsin was achieved by the addition of 2 volumes of complete medium to the cell suspension. The cells were washed in PBS twice and then the cell number determined by counting on a haemocytometer in duplicate. Cells were re-suspended in sterile PBS such that 100 μl of PBS contained the required number of cells to be implanted.

b: A 1 ml syringe was loaded with the cell suspension and attached to a 23 (blue) gauge needle. The animal was injected with 100 μl of the suspension on either of the rear flanks, sufficiently high enough up the flank to prevent tumour development causing any impairment of leg function. When injecting mice the mouse was held firmly in the hand via the skin on its back and neck while being injected. Hamsters being larger were able to twist easily, therefore it was preferable to quickly render them unconscious using ether. Following injection and for the course of the experiment the animals were checked daily for any sign of distress or discomfort. Useful signs of distress included reduction in food consumption, laboured breathing, irritability or saw skin round the developing tumour. If any of these signs occurred the animal was immediately terminated. Any cells for the preparation not injected into the animal were returned to the normal culture environment to verify viability.

c: Detection of the point when implanted cells resulted in a subcutaneous tumour was simply done by palpation. The animal was picked up by the skin at the back of the neck which pulled all the skin of the animal tort. A finger was run over the area of implantation to feel for a small lump under the skin. Extremely small imperfections could be detected by this method after a small amount of practice.

d: The measurement of tumour size was a difficult problem. The difficulty arose from the fact that without a body scanner it was extremely difficult to measure the depth of the tumour and thus difficult to calculate a true tumour volume. There are a number of mathematical models available to try and calculate the volume from surface diameter measurements, but from the irregular shape development that tumours follow then these mathematical models generally result in values that when comparing tumours by eye are obviously incorrect. Therefore we decided to use a simpler method by which we calculated an average surface diameter of the tumour and ignored the depth which appeared by eye to remain proportional to surface diameter of the tumour. To measure tumour size a pair of skin callipers was used. The smallest surface diameter of the tumour was determined together with the largest. If required a series of other diameters were measured on the tumour and an average surface diameter calculated using all measured diameters. This gave an accurate representation of the tumour size. The use of callipers was only possible when tumour diameter reached approximately 5 mm, and therefore any measurements before this size were estimated by touch.

e: Tumours were allowed to develop up to 3 cm surface diameter in hamster and 2 cm in nude mice, at which stage the animals usually showed no signs of distress. Animals were then terminated by ether asphyxiation and the tumour immediately removed for analysis.

3.2.2.: Pre immunisation of hamsters with tissue cultured cells.

Pre immunising hamsters before reintroduction of a cultured tumour cell allowed the animals immune system to be primed, thus enhancing any immune response that may occur when the cultured cells were implanted. The ideal way to pre immunise an animal to a tumour cell line was by injecting the exact cell that would be injected later, but treating the pre immunising cell in a way to prevent that cell from actually growing. This was achieved by exposing the immunising cell to a radiation source. Irradiating the immunising cell should not alter any of the cells antigenic properties as the cell should be not killed, but the

damage to the cells DNA was so great that the cells were prevented from entering mitosis by their own DNA repair systems.

a: A healthily growing population of cells was harvested using 0.25% (w/v) trypsin in PBS. The trypsin was deactivated by adding to the cell suspension 2 volumes of complete medium. Cells were washed in PBS twice and then cell number determined by counting on a haemocytometer in duplicate. Cells were resuspended to a concentration in sterile PBS such that 100 μl of PBS contained the required number of cells to be injected. The higher the number of cells the better, but practical considerations limit this to between 1×10^6 and 1×10^7 cells.

b: The cell suspension was exposed to 15000 rads of gamma radiation. This was achieved using a ^{60}Cobalt source, which required 35 minutes to deliver the required dose. 15000 rads was a high enough dose to prevent all cell mitosis (Teale and Rees 1987).

c: The irradiated cell suspension was loaded into a 1 ml syringe and a 23 (blue) gauge needle attached. The animals were injected with 100 μl of the suspension on either of the rear flanks. It was important to use the same flank for each immunisation as the other flank was used for the implantation of active cells. This ensured that any developing tumour was from the non radiated cells and the radiated cells division was completely inhibited.

d: The immunisation was repeated at 7, 14 and 24 days from the first immunisation. Active cells were injected 10 days after the last immunisation as described in methods section 3.2.1..

3.3.: Nucleic acid techniques.

3.3.1.: DNA.

3.3.1.1: Isolation of genomic DNA.

From cells grown in tissue culture.

a: Cells were isolated from the plate by trypsinisation and cells washed twice in sterile cold PBS by pelleting at 200 x g for 5 minutes. Following washing, cells were resuspended to a concentration of between 1x 10^6 and 2 x10^7 cells ml^{-1} in PBS and 1 ml of this placed into a 1.5 ml Eppendorf tube. Cells were then pelleted as before and the supernatant discarded.

b: 1 ml of lysis buffer (10 mM EDTA, 50 mM Tris pH 8.0, 0.5% n-lauroyl sarcosine) was added to the cell pellet and the cells resuspended.

From whole tissue.

a: The tissue was removed from the animal quickly, finally chopped and then frozen in liquid nitrogen. The snap frozen tissue was ground to a fine powder in a pre-chilled mortar and pestle. Alternatively the tissue was smashed to powder with a hammer in a plastic bag. 100 mg of the powdered tissue was weighed out into a 1 ml Eppendorf tube.

b: 1 ml of lysis buffer (10 mM EDTA, 50 mM Tris pH 8.0, 0.5% n-lauroyl sarcosine) was added to the powdered tissue and the tube vortexed to form a suspension.

Protein digestion and nucleic acid extraction.

For all applications from this point a 1 ml pipette tip with the end cut off to reduce shear forces on the DNA was used. All tips, containers and solutions were autoclaved.

c: 2 μl of Proteinase K (250 μg μl^{-1}) was added to the lysate to give a concentration of 0.5 mg ml^{-1}. Tissue culture cells were incubated at 37°C for 1 hour with gentle end over end rotation. For tissue it was necessary to incubate for 3 hours minimum with constant rotation. If discernible tissue remained the sample was left to incubate overnight. The proteinase K digested all cellular protein including endonucleases that may act on the DNA.

d: The resulting solution was deproteinised with 300 μl of phenol:chloroform:isoamyl alcohol (25:24:1). The phenol sevage was added and mixed with the cell lysate by gentle inversion. Separation of the phenol and aqueous phases was achieved by a 5 minute spin in a microfuge. The aqueous supernatant containing the DNA was carefully removed and placed into a clean tube. The phenol phase was then discarded. The phenol extraction was repeated twice more.

e: Residual phenol was removed from the aqueous phase by 2 ether extractions with 400 μl of ether. Phases were separated by centrifugation in a microfuge and the upper ether phase discarded. Any ether remaining following the extraction was allowed to evaporate at room temperature.

f: Contaminating RNA was removed by the addition of 2 μl of RNase A (30 mg ml^{-1}) and incubating for 1 hour at 37°C. The RNase was boiled for 2 minutes before use to destroy any DNases. RNases are heat labile. To remove the RNase following digestion the phenol and ether extractions in d and e were repeated

g: The sample was split into 2x 500 μl samples. 50 μl of 3M sodium acetate and either 1 volume of isopropanol or 2 volumes of ethanol was added to each tube. This was left to precipitate until required at -20°C. This was at least an overnight incubation.

3.3.1.2.: DNA dot blot.

a: The DNA precipitate was pelleted from either ethanol or propan-2-ol by spinning in a microfuge at maximum g force (8 - 10000 x g depending on microfuge) for 5 minutes. Salt contamination was removed by washing the pellet twice in 70% ethanol. After each wash the DNA was pelleted by a 2 minute spin in a microfuge at maximum g force.

b: The DNA was resuspended to a concentration of approximately 1 mg ml^{-1} in sterile TE (10 mM Tris pH 7.4, 1 mM EDTA). This gave a concentration such that 1 μg of DNA could be dot blotted in a volume of 2 μl following denaturation.

c: To denature the DNA an equal volume of 1 M sodium hydroxide was added to give a a final concentration of 0.5 M sodium hydroxide and 0.5 mg ml^{-1} DNA. This was left to incubate for 10 minutes at room temperature. Denaturation was increased if necessary by incubating at 37 ^{o}C.

d: 2 μl (1 μg) of DNA was spotted on to a nylon membrane. Boehringer positive charge nylon, (catalogue number 1209 299) was used. To crosslink the DNA to the membrane, the nylon was baked at 95^{o}C for 30 minutes. If nitrocellulose was used it was necessary to bake under vacuum or the nitrocellulose exploded. Alternatively DNA was crosslinked to either membrane using a Stratalinker (Stratagene), although this was not as efficient as baking under the alkali conditions used.

3.3.1.3.: Gel electrophoresis and Southern blotting.

a: Between 1 and 5 μg of DNA mixed 50:50 with 10 x loading buffer (20% (w/v) Ficoll 400, 0.1M EDTA, 10% (w/v) SDS, 0.25% (w/v) bromophenol blue) was loaded onto a 1%(w/v) agarose TAE (40 mM Tris pH8, 0.1% (v/v) acetic acid 1 mM EDTA) gel containing 0.5 μg ml^{-1} ethidium bromide. To the TAE running buffer 0.5 μg ml^{-1} of ethidium bromide was added. This gel was then run at 80 volts (constant voltage) until the bromophenol

blue front was approximately 1 cm from the end of the gel. The gel was then viewed under UV light to verify that electrophoresis had occurred correctly.

b: To southern blot the gel captured DNA to positive charged nylon, the DNA was denatured by placing the gel into 0.4M sodium hydroxide for 30 minutes, the sodium hydroxide was changed and the gel incubated for a further 30 minutes. A blot was then set up as described in methods section 3.3.2.5. using 0.4M sodium hydroxide as the transfer agent.

d: The DNA was crosslinked to the nylon filter by baking at 95°C for 30 minutes or UV crosslinking (Stratalinker, Stratagene Ltd). UV crosslinking was not particularly effective following alkaline transfer and therefore baking was the method of choice.

3.3.1.4: Labelling a cDNA probe with digoxigenin.

For use in this experiment digoxigenin is covalently linked to the dUTP nucleotide. When this is used in a random priming reaction approximately every 20-25 bases there is a U inserted instead of a T which has digoxigenin attached to it.

The cDNA templates for probes used for probing DNA filters were the same as described in cDNA probes for transglutaminase in the RNA methods section 3.3.2.6.

a: The cDNA probe was isolated from agarose gel using 'Gene Clean' (Bio 101) or 'Prep a Gene' (Biorad) as described in section 3.3.2.6. If the probe was to be used straight from a restriction enzyme digest, the cDNA was precipitated using 0.5 volumes of 3M sodium acetate and 6 volumes of ethanol and incubated for 1 hour at -20°C. The resulting precipitate was then washed twice in 70% ethanol to remove salt contamination.

b: The isolated cDNA to be used as a probe template was resuspended to a concentration of 1 μg in 15 μl. (The concentration of probe could be increased up to 3 μg or down to 100 ng. Above 1 μg non specific binding occurs as the probe sequences become short, but below 500 ng binding levels became lower and require longer exposures. These effects differ between probes and therefore need to be optimised for each probe used. 15 μl (1 μg) of the probe was placed into a clean Eppendorf tube and denatured by boiling for 3 minutes and cooled immediately on ice. Random priming was then carried out using the Boehringer Digoxigenin random prime kit. i.e.

2 μl dUTP-Digoxigenin(+ dCTP, dGTP, dATP).
2 μl random prime buffer.
1 μl Klenow fragment

This was incubated for 2-3 hours at 37^{o}C to allow priming to occur. 1 hour was the minimum time to achieve a sufficiently labelled probe. Random priming could be left to proceed for several hours if required.

3.3.1.5.: Pre-hybridisation and hybridisation of cDNA labelled digoxigenin probes to DNA crosslinked to nylon membranes.

a: Preparation of pre-hybridisation / hybridisation buffer is critical when using the digoxigenin system to achieve acceptable background levels. This buffer was therefore prepared exactly as described below.

Tween 20 (0.05g) was weighed out into a 250 ml beaker and 25 ml of Tris saline buffer (TSB) [3M sodium chloride, 300mM Tris pH 7.5] added. This was then made up to 100 ml with ultra pure water. This was stirred using a magnetic stirrer and 0.75 ml of 1 M sodium hydroxide added while stirring The beaker was then placed into a microwave and heated for 1 minute on full power bringing the temperature to just below boiling. The beaker was returned to stirrer and 0.5 g of Boehringer's blocking agent for nucleic acids added. Stirring was continued for a further 5 minutes. The solution was then reheated for 1 minute in the microwave on full power and

then returned to the stirrer for an additional 5 minutes. All the blocking agent had dissolved by this stage.

The buffer was allowed to cool to room temperature and denatured salmon sperm DNA added to a concentration of 100 μg ml^{-1}. i.e. 1 ml of 5 mg ml^{-1} stock into 50 ml of pre-hybridisation buffer.

The buffer was prepared up to stage of adding denatured DNA and stored for several weeks at room temperature. The salmon sperm DNA was denatured by boiling for 3 minutes and added fresh just before use.

b: The nylon filter was prehybridised for 1 hour at 55^{o}C in 25 ml of buffer. It was important to ensure that the filter was free floating in the buffer, i.e. not stuck to the bottom of the tank. Prehybridisation was reduced to 15 minutes or left for several hours depending on time restrictions with no variation in the final result.

c: Hybridisation was commenced by the addition of the digoxigenin labelled cDNA probe which was denatured prior to addition by boiling and cooling on ice. The probe was added to the pre-hybridisation buffer to a final concentration of 5-10 mg ml^{-1} i.e. 5 μl of the random prime solution containing 1 μg of cDNA.

d: Incubation was allowed to proceed at 55^{o}C overnight.

3.3.1.6.: Post hybridisation stringency washes for digoxigenin labelled probes.

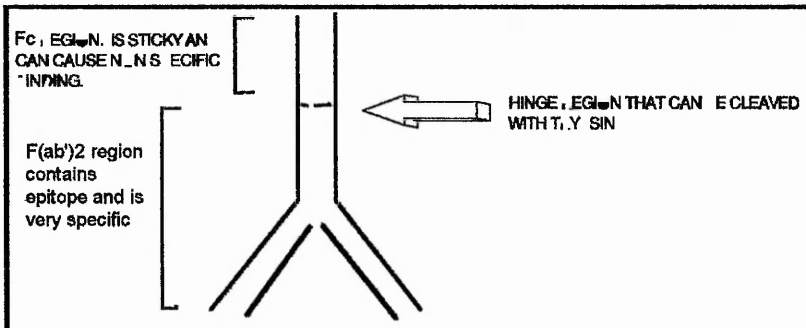
Following hybridisation it was very important to remove the non specifically bound probe, but under conditions that would leave the specifically bound probe attached. This was done by using a series of stringency washes. The filter was washed twice in 50 ml of 2 x TSB at 60^{o}C, followed by a second wash twice in 50 ml of 0.1 x TSB, 0.1% (w/v) SDS at 60^{o}C.

3.3.1.7.: Detection of hybridised digoxigenin labelled probe.

a: Before adding the anti Digoxigenin antibody conjugated to alkaline phosphatase, it was necessary to completely block the filter to reduce non specific binding to the nylon. This was done by washing the filter quickly 2 x in buffer 1 [150 mM sodium chloride, 100 mM Tris HCl pH 7.5.] and then incubating the filter for 30 minutes at room temperature in 50 ml of blocker reagent. The filter could be left to block overnight if required, but was blocked for a minimum of 10 minutes to achieve a passable level of blocking. The preparation of the blocker was critical and therefore was prepared as follows.

0.05g of Tween 20 was weighed into a 250 ml beaker and 100 ml of Buffer 1. [150 mM sodium chloride, 100 mM Tris HCl pH 7.5.] added. To this 0.75 ml of 1M sodium hydroxide was added and the solution heated to nearly boiling i.e. 1 minute in a microwave set to high. 0.5 g of Boehringer blocking reagent was added while stirring. Stirring was continued for 5 minutes at which stage the solution was reheated until just boiling.

b: The anti Digoxigenin antibody was conjugated to alkaline phosphatase. Only the $F(ab')_2$ fragment of the antibody was used to increase specificity.



If using a second antibody it was important to ensure that the $F(ab')_2$ region is the antigen for the second antibody and not the Fc region.

Application of the antibody was proceeded by spinning the antibody for 1 minute in a microfuge. This pelleted down any clumps of antibody that had formed and could have resulted in non specific spot binding. 5 μl of the antibody was added to the blocker reagent already bathing the filter. This resulted in a 1 in 10000 dilution of the anti Digoxigenin. This was then incubated at room temperature for 30 minutes.

c: The filter was then rinsed in buffer 1, and then left to wash twice in a clean buffer 1 for 15 minutes at room temperature. The filter was then rinsed quickly with buffer 3 [100 mM sodium chloride, 100 mM Tris-HCl pH 9.5, 1 mM magnesium chloride] and then washed for 30 seconds in the same buffer. Buffer 3 was filtered through a Watman 3 MM filter immediately before use to removes 'clumps' of magnesium chloride that form in the solution that are not visible. If these clumps are not removed they lead to spotted non specific binding. When making up buffer 3, the sodium chloride and Tris were made up in water first, pH corrected and then the magnesium chloride added.

d: All the buffer 3 was removed and then a fresh 1 ml of buffer 3 added. To this 10 μl of AMPDD solution (Boehringer catalogue number 1357 328) was added. This was a 1 in 100 dilution of the AMPDD solution. This was run over the blot so the entire filter was evenly covered with the substrate.

e: An acetate sheet was folded along the centre (Gibco) and wiped over with 100% ethanol to clean the acetate and reduced static. The filter was placed between the two halves of the folded acetate taking care to remove all air bubbles. The acetate and filter were placed into a film cassette and warmed at 37°C for 5 minutes to initiate reaction of the alkaline phosphatase mediated light emitting reaction with AMPDD substrate.

f: Preflashed film was placed into the cassette and exposed at 37°C. It was advisable to allow the reaction to proceed initially for 2 hours before developing the film, since at this point the reaction would have reached its maximum. From the resulting plate it was assessed if a longer or shorter exposure was required.

Reaction kinetics with the alkaline phosphatase and AMPDD solution show that the reaction reaches its maximum in 2 hours and maintains this for 2 days (personal communication M. Sharred).

3.3.1.8.: Probe production, pre-hybridisation, hybridisation, post hybridisation stringency washes and detection of hybridised cDNA labelled ^{32}P probes to DNA crosslinked to nylon membranes.

The use of the digoxigenin labelling and detection system in probing for nucleic acid sequences as described above has several advantages over the traditional ^{32}P detection system in the areas of speed and the obvious problems associated with higher energy radionucleotides. The digoxigenin system gives excellent results with a 'clean' preparation such as that arising from PCR, and gives passable results from cell culture homogenates where there is a strong signal to noise ratio. The major problem with the system comes from its use on nucleic acids isolated from culture and to a greater extent from tissue where the signal is lower and the exposure times increased to allow for detection of the lower signal. The greater exposure times cause a major problem with background which increases to such an extent that any signal is completely masked. In addition even on clean preparations the signal achieved from a probed sequence appears to be much weaker than that attained with the ^{32}P

system on the same filter. The action of horse radish peroxidase on AMPDD solution to provide a light emitting reaction is impressive, therefore one can only conclude that this weaker signal can only occur either as a result of lower probe binding and that the digoxigenin molecule attached to the sequence results in adverse binding to the nylon bound sequence with could account for the high background seen or that the cDNA probe contains a low level of digoxigenin. Subsequently the use of the ^{32}P labelling and detection system was preferred.

a: All methods used for the production of probes, hybridisation, stringency washes and detection of ^{32}P labelled probes used in conjunction with DNA fixed to nylon membranes were performed exactly as described in the RNA methods section.

3.3.2.: RNA

3.3.2.1.: Isolation of total RNA from cells grown *in vitro.*

Two methods were used for the isolation of total RNA. The method used initially was based on the separation of RNA by centrifugation through a caesium chloride gradient, but this was later replaced by a method utilising the properties of RNA to form complexes with guanidium using RNAzol B™ system (Cinna Biotex). The second method was preferred due to it providing much more reproducible results and taking less time to carry out as a result of fewer manipulations.

In both methods extreme care was taken to minimise the degrading effects of RNases. Unless stated all chemicals were of molecular grade, diethyl pyrocarbonate (DEPC) treated and autoclaved. All solutions were prepared in sterile DEPC treated containers. Fresh gloves were worn for all manipulations.

Method 1. Isolation of Total RNA using a Caesium Chloride Gradient.

a: Cells were grown to a density of approximately $3x10^6$ in a 10 cm diameter Petri dish. The growth medium was removed and the monolayer washed in sterile PBS twice. The monolayer was then lysed directly in 2 ml of guanidium solution (4M guanidium isothiocyanate, 10 mM sodium citrate, 10 mM EDTA, 150 mM β mercaptoethanol, 0.5% (v/v) sarkosyl, 0.1% (v/v) antifoam A.), and the lysate placed into a fresh tube. The Petri dish was then rinsed in a further 1 ml and combined with the rest of the lysate. When cells were grown in suspension, the cells were pelleted into a sterile tube, washed twice in sterile PBS and then lysed in 3 ml of guanidium solution. During the lysing process it was important to shear the DNA. This was most effectively done by forcing the homogenate through a 23 gauge needle 4 times.

b: The homogenate was loaded onto a caesium chloride gradient in a Beckman 10 ml quick seal centrifuge tube. The gradient consisted of a 1.5 ml base of 5.7 M caesium chloride solution (5.7M caesium chloride, 25 mM sodium citrate, 10 mM EDTA pH 7.5) on to which a 0.5 ml layer of caesium chloride / guanidium solution was added (1 g of caesium chloride to 2.5 ml of guanidium solution.). Finally the homogenate was layered on to the gradient and the tube filled completely with molecular grade mineral oil before sealing to prevent collapse.

c: The total RNA was isolated by centrifugation at 150000 x g in a Beckman ultracentrifuge using a 70.1 TI rotor. 'Slow acceleration' and 'no brake' were selected. Following centrifugation the top from the tube was removed and the upper layers removed very carefully using a hypodermic syringe and needle, taking care not to dislodge the invisible RNA pellet. Carefully, 500 μl of 70% ethanol at room temperature was used to wash the walls of the tube to remove salt deposits. All liquid was removed using a pipette and the tubes inverted to drain on sterile new Kimwipes on ice. The RNA pellet was visualised by the addition of ice cold 100 % ethanol and leaving this to incubate for thirty minutes on ice to allow the RNA to fully precipitate. All

the liquid was removed and again the tubes drained on iced Kimwipes to remove as much liquid as possible. 100 μl of DEPC treated water was used to dissolve the RNA. This was left for 30 minutes at 4^{o}C for the RNA to completely dissolve. The sample was pipetted several times to ensure the RNA had completely dissolved.

d: The RNA was transferred to a sterile Eppendorf tube and the centrifuge tube rinsed with a further 100 μl of water. To remove contaminating protein the RNA solution was extracted with 100 μl of cold phenol-chloroform-isoamylalcohol (25:24:1) with vigorous shaking. Aqueous / phenol phases were separated by centrifugation for 1 minute in a microfuge. The top aqueous phase was transferred to a clean Eppendorf tube and extracted again using sevage (24:1, chloroform to isoamylalcohol) to remove residual protein. The top layer (aqueous phase) was placed into a clean Eppendorf tube and extracted twice more with ether to remove any contaminating sevage. The top ether layer was removed and discarded. Any residual ether was removed using a 5 minute spin in a rotary evaporator. The purified RNA was precipitated by the addition of 0.1 volumes of sodium acetate and 3 volumes of 100% cold ethanol and incubating overnight at -20^{o}C.

e: The RNA precipitate was spun down using a 15 minutes spin at 4^{o}C in a microfuge (13400 RPM), the supernatant was discarded and the pellet washed twice in cold 70% ethanol to remove any salt contamination. All liquid was removed and the pellet dried in a rotary evaporator (5 minutes). It was important not to completely dry the pellet as it became near impossible to re-dissolve the RNA if it was completely dried. The pellet was resuspended in 100 μl of DEPC treated water and then the quantity and purity assessed by optical density at 260 and 280 nm. If possible it was preferable to optically scan the sample to check a characteristic profile was present.

On a 1 cm light path

Quantity = $OD_{260\ nm}$ x 0.04 = μg / μl.

Purity = $OD_{260\ nm}$ / $OD_{280\ nm}$. > 1.9 = pure RNA.
< 1.9 = protein / phenol contamination.

Method 2. Extraction of total RNA using the RNAzol B reagent.

(RNAzol B™ is a reagent supplied by Cinna / Biotex and distributed by Biogenesis limited, Yeoman's Way, Bournemouth, England.)

a: Cells were grown to a density of approximately $5x10^6$ in a 10 cm diameter Petri dish. The growth medium was removed and the monolayer washed in sterile PBS twice. The monolayer was then lysed directly in 2 ml RNAzol B and the lysate fully solubilised by passing the lysate through a pipette a few times. The homogenate was placed into 2 Eppendorf tubes and 100 μl of chloroform added to each. The tubes were shaken vigorously for 15 seconds and then placed at 4°C for 5 minutes. The samples were then centrifuged for 15 minutes at 12000g at 4°C to form 2 phases. The lower phase contained phenol and chloroform, DNA and protein were trapped in the interphase and lower organic phase. The upper aqueous phase from both tubes was placed into 2 fresh tubes and an equal volume of isopropanol added followed by a 15 minute incubation at 4°C to allow the RNA to precipitate. The RNA was pelleted by centrifugation at 12000g for 15 minutes at 4°C, the supernatant removed and the pellets combined into 1 tube. The pellets were washed in 75% ethanol to remove salt contamination, dried and dissolved in 200 μl of DEPC treated water. Quantity and quality of RNA was determined as described in section 3.3.2.1 part 1d.

3.3.2.2.: Isolation of total RNA from tissue.

Tissue samples were removed as quickly as possible from the animal and snap frozen in liquid nitrogen to minimise the degrading effect of endonucleases

which are found in much higher levels in tissue than *in vitro* material. Approximately 100 mg of the tissue samples was placed into a clean 7 ml disposable tube and 2 ml of RNAzol B solution added to the still frozen material. This was then homogenised using 3 x 5 second bursts of a Ultra Turrax homogeniser.

The homogenate was then placed into 2 Eppendorf tubes and 100 μl of chloroform added to each. The tubes are then shaken vigorously for 15 seconds and then placed at 4^{o}C for 5 minutes. The samples were then centrifuged for 15 minutes at 12000g at 4^{o}C to form 2 phases. The lower phase contained phenol chloroform. DNA and protein were trapped in the interphase and the organic phase. The upper aqueous phase from both tubes was placed into 2 fresh tubes and an equal volume of isopropanol added followed by a 15 minute incubation at 4^{o}C to allow the RNA to precipitate. The RNA precipitate was then pelleted by centrifugation at 12000g for 15 minutes at 4^{o}C and the supernatant discarded. The two pellets were combined into 1 tube and washed 75% ethanol to remove salt contamination. The pellet was dried in a rotary evaporator and then dissolved in 200 μl of DEPC treated water. Quantity and quality of RNA was determined as described in section 3.3.2.1. part 1 d.

3.3.2.3.: Isolation of mRNA from cells grown *in vitro.*

Two types of commercially available kits were used for this purpose. Initially the Micro Fast Track kit produced by Invitrogen and supplied by British Biotechnology was used, and secondly the Pharmacia mRNA isolation kit from Pharmacia was used. Both of these kits were based on the same idea of separating the mRNA using it's polyadenylated tail sequence to bind to oligo (dT)-cellulose in a spin column to isolate it from all other cell components. Both kits provided mRNA of undegraded and pure quality, but as the Pharmacia kit was designed to be used on higher numbers of cells it subsequently yielded higher levels of mRNA which made quantitation much easier and since the recovery of more mRNA meant larger amounts could be run on gels to provide a greater signal following hybridisation. The methods for both kits were very

similar, but discrepancies in solutions could not be ascertained due to the amount of information a company will forward regarding its kits.

Procedure

a: Cells were harvested using trypsin, washed in PBS and counted on a haemocytometer and up to $5x10^6$ cells (Fast Track) or $1x10^8$ cells (Pharmacia) aliquoted into a microfuge tube and pelleted by a 2 minute spin at maximum g force in a microfuge. The cell pellet was then lysed in 1 ml of lysis buffer containing RNase / protein degrader (composition not disclosed) followed by a 20 minute incubation at 45°C to allow complete digestion of all proteins. Insoluble material was removed by a 4000 x g spin for 10 minutes.

b: The supernatant NaCl concentration was adjusted by the addition of 63μl of 5M NaCl. A single oligo (dT) cellulose tablet was added to the lysate, allowed to swell for 2 minutes and then crushed with a pipette tip against the tube wall. This was incubated at room temperature for 20 minutes with gentle shaking to allow the mRNA to bind to the oligo (dT) cellulose.

c: The oligo (dT) cellulose was pelleted by a 4000 x g spin for 8 minutes at room temperature, the supernatant discarded and the oligo (dT) cellulose washed with 1.3 ml of binding buffer (composition not disclosed) repeatedly until the washes became clear.

d: The oligo (dT) cellulose was resuspended in 300 μl of binding buffer, transferred to a spin column and spun at 5000 x g for 10 seconds to pellet the oligo (dT) cellulose in the column. This was then washed a further 3 times with binding buffer. Ribosomal RNA was removed by 2 washes in a low salt buffer (composition not disclosed).

e: The spin column was then placed into a fresh microfuge tube and the mRNA released by resuspending the oligo (dT) cellulose in 100μl of elution buffer (Composition not disclosed) and the eluent collected by 5000 x g spin for 10 seconds. This was repeated for maximum mRNA recovery.

f: The RNA was then precipitated by the addition of 10μl of glycogen carrier (composition not disclosed), 30 μl of 2M sodium acetate and 600μl of 100% ethanol and freezing at -70^{o}C. The RNA was recovered by centrifugation at 16000 x g at 4^{o}C and resuspending the pellet in a suitable volume of elution buffer (1-10 μl).

3.3.2.4.: Formaldehyde gel electrophoresis for RNA.

Two methods of RNA gel electrophoresis were used. Initially a borate buffer based system was utilised, but this was succeeded by a MOPS based technique that was not only quicker to run, but produced much clearer and better resolved gels. Endonuclease free techniques were maintained throughout both procedures. Gel tanks and beds were thoroughly washed with 1M sodium hydroxide and rinsed in DEPC treated water before use to remove endonucleases.

Method 1. Borate / Agarose RNA gel electrophoresis..

a: An Agarose gel was prepared that consisted of 1% (w/v) Agarose, 1X Borate buffer (this was diluted from a 12 x stock: 620 mM boric acid, 62 mM sodium borate (borax), 125 mM sodium citrate pH 8.2.) and 2% (v/v) formaldehyde (from a 37% stock). The agarose and borate were mixed with water brought to the boil and allowed to simmer for 2 minutes to allow the agarose to dissolve completely. This was then heat cooled to 60^{o}C before addition of the formaldehyde to a concentration of 2% (v/v) after which the gel was poured and allowed to harden for at least 1 hour.

b: Samples of RNA were prepared containing between 5 and 30 μg of total RNA in 10 μl of DEPC treated water. To this an equal volume of loading buffer was added (5 mM methyl mercuric hydroxide, 30% (v/v) glycerol, 0.12% (w/v) bromophenol blue, 1.5% (w/v) sodium dodecyl sulphate, 1 x borate). This was pipetted into the wells in the gel and electrophoresis commenced as quickly as possible using a borate, formaldehyde running buffer (1 x borate, 2% (v/v) formaldehyde). The gel was run at constant voltage ranging between

45 and 60 volts. It was important not to let the current rise approve 2.5 mA per cm^3 of gel as this resulted in warming of the gel which caused poor resolution and breakdown of the matrix. A typical mini gel would be run at 60 volts for 4 hours (240 volt hours) or a 15 cm gel at 50 volts for 15 hours (750 volt hours). The gel was run until the bromophenol blue dye front was approximately 1 cm short of the end of the gel.

c: After electrophoresis the gel was removed from the tank and washed 3 times in DEPC treated water, and stained for 20 minutes at room temperature with 1 μg ml^{-1} ethidium bromide. The gel was destained overnight with several changes of DEPC treated water. The gel was viewed under UV light. A undegraded total RNA sample exhibited 2 strong bands at 5 kb (28s rRNA) and 2 kb (18s rRNA) connected by a smear extending the length of the gel which was the mRNA. On occasion the 45s and 32s pre-ribosomal subunits were also visible at the top of the gel.

Method 2. MOPS / Agarose RNA gel electrophoresis.

a: Agarose gels were prepared containing 1% (w/v) agarose and 1 x MOPS buffer (diluted down from 10 x MOPS which consists of 200 mM MOPS, 50 mM sodium acetate, 10 mM EDTA) . The mixture was brought to the boil and allowed to simmer for 2 minutes to allow the agarose to completely dissolve. This was then heat cooled to 50°C and formaldehyde add to a concentration of 2% (v/v) after which the gel was poured and allowed to harden for 1 hour.

b: Total RNA samples were prepared in 5 μl of DEPC treated water containing between 5 and 30 μg of total RNA. To this 25 μl of sample buffer (50% (v/v) deionised formamide, 1 x MOPS, 6% (v/v) formaldehyde, 3.5% (w/v) ficoll 400, 1.2 mM EDTA, 0.005% (w/v) bromophenol blue) and 1 μg of ethidium bromide were added. The sample was then loaded into the gel wells using a pipette and electrophoresed using a 1 x MOPS running buffer. The gel was run at constant voltage ranging between 50 and 100 volts depending on gel size and current available from the power supply. It was important not to let

the current rise approve 2.5 mA per cm^3 of gel as this resulted in warming of the gel which caused poor resolution and breakdown of the matrix. A typical mini gel would be run at 80 volts for 3 hours (240 volt hours) or a 15 cm gel at 90 volts for 8 hours (720 volt hours). The gel was run until the bromophenol blue dye front was approximately 1 cm short of the end of the gel. On completion there was no need to stain the gel as the ethidium bromide was added to the sample buffer. The gel was viewed immediately under UV light on a transilluminator. A undegraded total RNA sample exhibited 2 strong bands at 5 kb (28s rRNA) and 2 kb (18s rRNA) connected by a smear extending the full length of the gel which was mRNA. On occasion the 45s and 32s pre-ribosomal subunits were visible at the top of the gel.

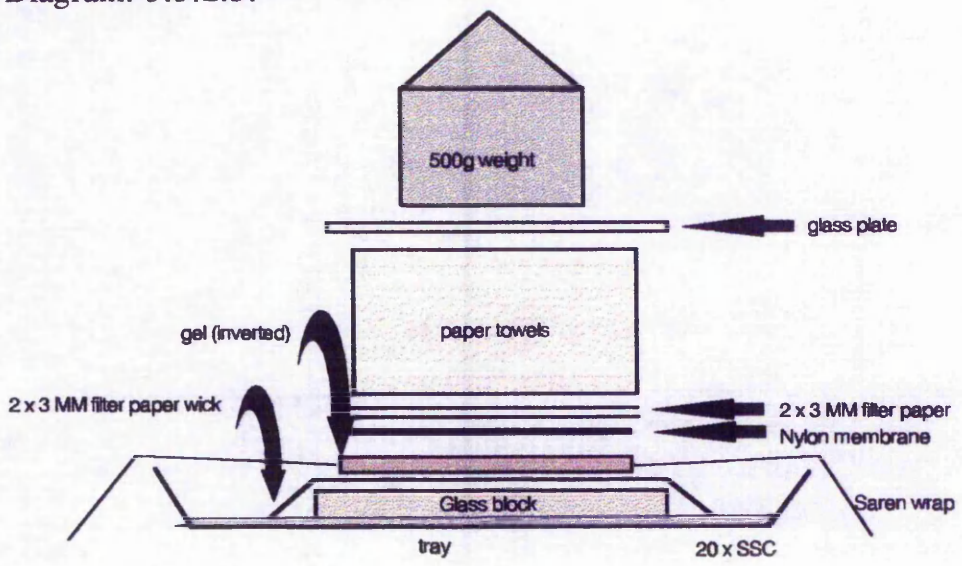
3.3.2.5: Northern transfer.

In line with current opinion gels were not denatured / neutralised or pre-soaked in transfer buffer as these steps were found not to effect the transfer procedure.

a: The gel was washed in 20 x SSC and inverted onto a wick of 3 MM filter paper which was fed from a pool of transfer buffer in a tray (1 litre for a 10 x 6 cm gel). The apparatus was set up as in Diagram 3.3.2.5..

b: After placing the gel onto the wick the gel was surrounded with saran wrap to prevent any short circuiting of the transfer buffer to the absorbent layers and thus reducing transfer of the RNA. A nylon membrane which was pre wetted in 2 x SSC was placed onto the gel ensuring all air bubbles were removed. Two types of nylon were used. Initially Amersham's hybond N was used which gave good transfer and a high level of crosslinking with little loss of sample after several strippings of the membrane. Secondly Boehringer's positively charged nylon membrane was used which produced all round better results especially in the areas of transfer and non specific binding when the membrane was probed.

Diagram. 3.3.2.5.



c: On top of the membrane were placed two sheets of 3 MM filter paper which were pre-soaked in 2 x SSC. Above these were approximately 15 cm depth of paper towels which acted as the absorbent material. Finally a 500 g weight was placed on the top to hold components together and aid capillary action. This weight was supported on a glass sheet to evenly dissipate the weight over the surface of the gel. To much or two little weight resulted in poor capillary action as it effected the absorbance of the towels.

d: Transfer was allowed to proceed overnight. To check the transfer had taken place, the gel and membrane were viewed under UV light. If transfer was successful, the gel should have no staining while the membrane should have a mirror image of the initial banding seen on the gel. Banding appeared strongest on the side of the membrane not adjacent to the gel.

e: The nucleic acid was crosslinked to the membrane either via baking for 2 hours at 80^{o}C, or more efficiently via UV crosslinking in a Stratalinker (Stratagene Inc.) using autocrosslink. Membranes were air dried and stored at room temperature under vacuum until required.

3.3.2.6.: Production of cDNA probes for tissue transglutaminase.

Four probes were used, all produced from the 3 vectors pBluescript Tgase.H (human transglutaminase) and pGEM 3-Z Tgase.M (mouse transglutaminase) kindly supplied by P.J.A. Davies of the University of Texas and pSG5 Bal 1 TGase sense (Bal 1 deletion of the coding region of human transglutaminase) supplied by S. Mian (Nottingham Trent University). From the pBluescript vector, containing the human transglutaminase (section 3.3.3.4.) two probes were produced; the complete 3.3 kb transglutaminase gene isolated using restriction enzymes *Eco R1* to excise and Dra 1 to linearise the plasmid, and a more specific probe of the 2.2 kb coding region isolated using *Cvn 1* and *Nco 1.* The complete mouse transglutaminase cDNA insert was isolated from pGEM 3-Z (section 3.3.3.4) using *Eco R1.* The pSG5 Bal 1 Tgase sense vector contained a construct inserted into the multiple cloning site in pSG5 using *EcoR1* that consisted of a Bal 1 deletion (Figure 3.3.3.4.). This was removed from the pSG5 plasmid by digestion with *Eco R1.* All restriction enzyme digestions were allowed to proceed for 2 hours at 37°C.

3.3 kb complete human transglutaminase cDNA.

The restriction digest mixture consisted of 8 units of Dra 1(Gibco), 10 units of *EcoR1*(Gibco), 6 μg of pBluescript H.Tgase which was made up to a 20 μl volume using Gibco react buffer 3 at 1 x concentration. This digestion resulted in a 200 kb, 700 kb, 1000 kb and a 1100 kb fragments of the plasmid linearised by Dra 1 and a 3.4 kb transglutaminase cDNA fragment excised by *EcoR1.*

2.2 kb human transglutaminase coding region.

The restriction digest mixture consisted of 8 units of *Cvn 1*(Gibco), 10 units of *Nco 1*(Gibco), 6 μg of pBluescript H.Tgase which was made up to 20 μl with Gibco react buffer 4 at 1 x concentration. This digestion resulted in a 3.4 kb fragment which consisted of the plasmid plus the first 26 bp of the insert and the last 500 bp of the insert, a 700 bp fragment which was the non coding sequence of the insert and a 2.2 kb fragment which was the coding sequence.

3.5 kb complete mouse transglutaminase cDNA.

The restriction digest mixture consists of 12 units of *EcoR1* and 6 μg of pGEM-3Z which is made up to 20 μl with Amersham Buffer 4. Two fragments resulted, the 3.2 kb plasmid and the 3.5 kb insert.

Bal 1 deletion probe.

The restriction digest mixture consisted of 10 units of *EcoR1*(Gibco), 6 μg of pSG5 Bal 1 Tgase which was made up to a 20 μl volume using Gibco react buffer 4 at 1 x concentration. The digestion resulted in a 3.2 kb linearised plasmid fragment and a 1 kb transglutaminase Bal 1 deletion fragment .

Isolation of the cDNA probe fragment from restriction digest.

This was achieved by separation of digested nucleic acids on an agarose TAE gel and then purification of the required band from the gel using the Geneclean™ kit.

a: A 1% (w/v) agarose TAE gel was prepared as described in DNA methods (section 3.3.1.3.). An equal volume of loading buffer was added to the restriction digest and loaded on to the gel splitting the sample into 2 lanes. The gel was run until the Bromophenol blue front was three quarters of the way down the gel, stopped and the ethidium bromide stained DNA viewed under UV light. The required band was selected and then cut from the gel using a new, sterile scalpel blade.

b: The isolated gel containing the DNA had three volumes of 6M sodium iodide added and incubated at 55°C for 5 minutes to dissolve the agarose. This was allowed to cool to room temperature and 1 μl of 'glass milk' per μg of DNA added (glassmilk is a silica matrix with a high binding efficiency for DNA under these ionic conditions). This was incubated for 15 minutes at room temperature, mixing every two minutes to allow the DNA to bind. The

glassmilk / DNA complex was pelleted by centrifugation and washed three times in "new wash" (50% ethanol, sodium chloride, Tris, EDTA (concentrations unknown)). This removed all agarose contamination from the pellet. The DNA was eluted from the glassmilk by washing pellet 2 times in DEPC treated water. Two 10 μl washes were usually sufficient. Recovery was normally around 80%.

c: A second agarose TAE gel was run to check quantity and quality of recovered DNA. 5 μl of the purified fragment was normally used for this. The DNA was stored at -20^{o}C until required for probe labelling.

^{32}P labelling of the cDNA template

Two types of commercially available kit were used. The Amersham "multiprime kit" and the Promega "prime a gene kit". Both worked on the same principle as developed by Feinberg and Volgestein 1983a,b. The Promega kit was generally superior incorporating on average 45-60 % of the radio labelled nucleotide compared to 30-40% for the Amersham kit. The radio labelled base used for both systems was cysteine triphosphate carrying ^{32}P on the α phosphate.

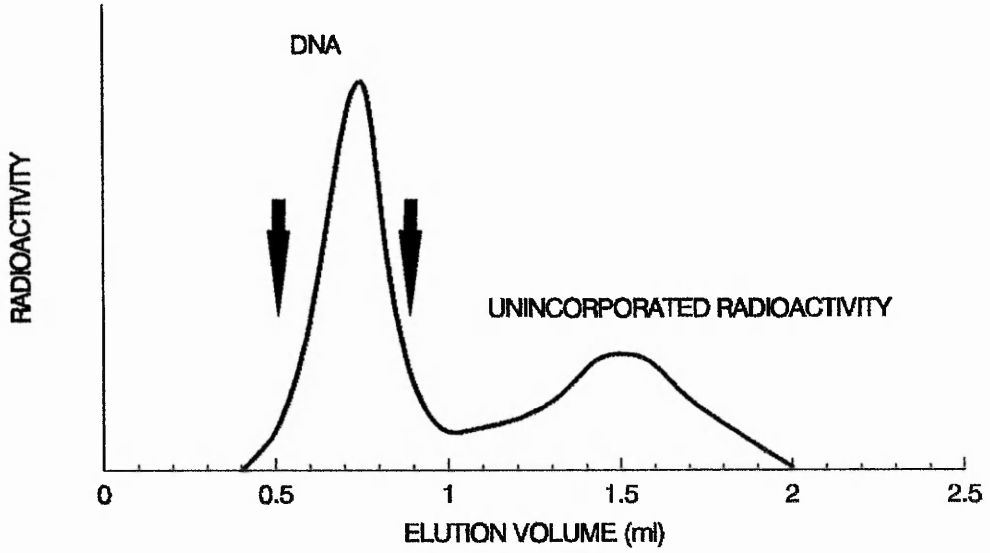
a: A volume of cDNA probe sequence corresponding to 25 ng nucleic acid was aliquoted in to an Eppendorf tube in a volume not greater than 30 μl. This was boiled for 5 minutes to denature the cDNA and then cooled rapidly on ice. Accurate determination of 25 ng of template was essential as greater levels of template resulted in short copy sequences resulting in non-specific binding where as lower levels required a long incubation period to attain good incorporation.

b: To the denatured template 10 μl of 5X Labelling buffer (50 mM Tris / HCl pH 8.0, 5 mM magnesium chloride, 2 mM dithiothreitol, 0.2M HEPES pH 6.6), 5.2 Absorbance units ml^{-1} (260 nm) of random hexadeoxyribonucleotides) was added. Labelling buffer maintained reaction conditions while the hexadeoxyribonucleotides provided a primer for the polymerase enzyme. Non labelled nucleotides, adenosine triphosphate,

guanidine triphosphate and thymidine triphosphate were added to a concentration of 20 μM each, followed the addition of nuclease free BSA to a concentration of 400 μg ml^{-1}. Radio labelled ^{32}P dCTP containing 50 μCi of activity (3000 Ci $mmole^{-1}$) was added. This gave a final concentration of around 333 ηM of CTP. Optimum probe length occurred when the level of the labelled dNTP was 10-25 pmoles. However the highest incorporation level occurred when less than 30 pmoles of labelled dNTP was present, an important consideration if high background was a problem. Finally Klenow was added to a concentration of 100 units ml^{-1} to start the reaction. Klenow is a fragment of DNA polymerase 1 that contains the exonuclease activity, but lacks endonuclease action. The reaction was allowed to proceed for a minimum of one hour after which the majority of incorporation had occurred.

c: To minimise non specific background during probing, any non incorporated labelled nucleotide was removed. This was done using the Pharmacia Nick column which is a disposal, 0.9 x 1.9 cm, G50 sephadex column. The column was equilibrated with 3 ml of TE (10 mM Tris pH 7.4, 1 mM EDTA), after which the random prime reaction mixture in a volume not exceeding 100 μl was pipetted on to the top of the gel bed and allowed to enter the gel bed. This was washed into the sephadex bed with 400 μl of TE. A collection tube was placed under the column and the sample eluted by a second 400 μl volume of TE. Non incorporated nucleotides were able to enter the interstitial spaces in the Sephadex and thus remained on the column, while the DNA passes straight through the column with little retention. The elution profile is shown in figure 3.3.2.6.

figure 3.3.2.6.



d: The DNA fraction was eluted between 400 and 800 μl and collected accordingly. The ^{32}P labelled cDNA probe was then ready to be used in a hybridisation reaction.

3.3.2.7.: cDNA probe hybridisation to DNA / RNA attached to nylon filters.

Two hybridisation systems were used, one using SSC and Tris as the buffer system, the other using SSPE as the buffer.

SSC. Tris system.

The hybridisation reaction mixture contained: cDNA probe labelled with 50 μ Ci ^{32}P dCTP, 50% (v/v) deionised formamide, 5 x SSC, 50 mM Tris pH 7.4, 0.1% (w/v) disodium orthophosphate, 1% (w/v) SDS, 4 mM EDTA, 0.2% (w/v) polyvinylpyrolidine, 0.2% (w/v) Ficoll, 1% (w/v) denatured salmon sperm.).

SSPE system.

The hybridisation reaction mixture contained: cDNA probe labelled with 50 μ Ci ^{32}P dCTP, 50% (v/v) deionised formamide, 5 x SSPE, 0.2% (w/v) polyvinylpyrolidine, 0.2% (w/v) Ficoll, 1% (w/v) denatured salmon sperm.

Both techniques used the same methodology.

a: All components of the hybridisation buffer minus the DNA components were prepared and preheated to 42 ^{o}C. The salmon sperm DNA was denatured by boiling for 5 minutes, cooled on ice and added to the hybridisation solution to give pre-hybridisation buffer. 10 ml of this was placed into a suitable vessel that allowed the nylon filter to be continually wetted. Flat glass bottles, perspex hybridisation chambers and sealed plastic bags were all used for this purpose. The filter was immediately placed into the prehybridisation solution left to prehybridise for 1 hour at 42^{o}C.

b: A second volume of hybridisation buffer minus the DNA components was prepared as above. The ^{32}P labelled cDNA probe and salmon sperm DNA were denatured by boiling for 5 minutes and cooled rapidly on ice. These were added to the hybridisation buffer solution and mixed thoroughly. The pre-hybridisation buffer was immediately replaced with the complete hybridisation solution and incubated overnight at 42^{o}C in a shaking or rotating environment.

c: The filter was removed from the hybridisation solution and washed twice for 15 minutes at room temperature in 2 x SSC, 0.1% (w/v) SDS and then once for 30 minutes at 65^{o}C in 0.1 x SSC, 0.1%(w/v) SDS. The SSC was replaced with SSPE if the SSPE hybridisation system was used. The washes were flexible and subsequently adjusted to suit background levels and strength of binding to the RNA sample for each blot probed. Usually the room temperature washes were sufficient. An intermediate wash was occasionally used using 0.1 x SSC, 0.1%(w/v) SDS at 42^{o}C which was

excellent for removing background noise with little loss of signal. It was possible to expose the membrane to film, assess signal and background and then select which wash to use after the initial wash as long as the filter was not allowed to dry. Variation in wash stringency was important as if a weak attachment had occurred (poor hybridisation or low homology) then the signal may be lost with any of the higher stringency washes.

d: The filter was then air dried and exposed to Kodak X-OMAT RP film or the more sensitive Kodak X OMAT AR film for up to 12 days using intensifying screens.

3.3.3.: Plasmids.

3.3.3.1.: Transformation of bacteria with plasmids.

All plasmids used in this study for transfections, to produce probe sequences or as reporter genes were originally supplied as gifts mainly from Dr. P.J.A. Davies of the University of Texas and are described in detail in section 3.3.3.4.. Unfortunately these were supplied in very small amounts, which were not large enough to carry out a fraction of the experiments required. Therefore it was necessary to amplify these plasmids before any experimental work could be performed. The plasmids firstly were transformed into bacteria and the incorporation of the plasmid checked using a small scale plasmid preparation. A large scale preparation was then used to produce large quantities of the plasmid.

The first stage was to transform bacteria with the plasmid in question. The transformation method used was based on the Hanahan method (Hanahan 1985) using E.coli, JM109 (Yanish-Perron et al 1985). Reagents used consisted of:

SO = 2% (w/v) Bactotryptone, 0.5% (w/v) yeast extract, 10 mM sodium chloride, 2.5 mM potassium chloride. Autoclave.

SOB = SO + 1M magnesium chloride (hexahydrate), 1M magnesium sulphate (septahydrate). Sterile filter.

SOC = SOB + 2M glucose.

Transformation buffer = 10 mM MES (2-[Morpholino] ethanesulphonic acid) pH 6.3 with potassium hydroxide, 100 mM potassium chloride, 45 mM magnesium chloride (quadhydrate), calcium chloride, 3 mM hexamine cobalt 3 trichloride. sterile filter.

Procedure

a: 5 ml of SOC was inoculated with the JM109 strain of E. coli bacteria and left stationary at 37°C overnight. 40 ml of SOC was inoculated with 0.4 ml of the overnight culture of JM109 in a 250 ml conical flask. This was incubated at 37°C in an orbital shaker rotating at 200 - 300 r.p.m. until the optical density (550 nm) of the culture reached 0.45 - 0.55. This required approximately 2 hours.

b: The culture was poured into a 50 ml Oakridge centrifuge tube and cooled on ice for 15 minutes and centrifuged at 5000 x g (MSE 24 pre cooled 8 x 50 ml rotor) for 12 minutes. The supernatant was discarded and the bacterial pellet resuspend in 13 ml of transformation buffer and placed on ice for a further 15 minutes. The previous centrifugation was repeated, but this time the pellet resuspend in 3 ml of transformation buffer.

c: 112 μl of dimethyl formamide (DMF) was added. The solution was then mixed and incubated on ice for 5 minutes. 112 μl of dithiothreitol (sterile filtered) was then added and incubated on ice for a further 10 minutes. Finally another 112 μl of dimethyl formamide was added, the solution mixed and incubated for 5 minutes on ice. 200 μl aliquots of these 'competent' cells were aliquoted into Eppendorf tubes on ice. At this stage some of the competent cells were frozen for later use, and as many tubes selected as required for the transformation and controls. Usually 1 tube was used as a control and 2 tubes for the transformation procedure.

d: The plasmid to be used in the transformation was prepared at a concentration of 20 ng ml^{-1} in sterile distilled water. 5 μl (100 pg) of this was added to each transformation tube, mixed and incubated on ice for 30 minutes. The bacteria were then heat shocked at 42°C for 90 seconds by placing the tube into a water bath. The tube was returned to ice for 2 minutes. This rapid change in temperature coupled with the chemical manipulations resulted in changes in the bacteria's cell membrane wall permeability and thus allowed the plasmid to pass through the cell wall membrane and into the cytoplasm.

e: 800 μl of SOC was added at room temperature, and the tube shaken at 200 r.p.m. for 60 minutes at 37°C.

f: 1 μl, 10 μl and 100 μl of the transformed bacteria were plated onto nutrient agar containing the selection agent carried by the plasmid (usually ampicillin at 50 μg ml^{-1}). Any bacterium that had undergone transformation and accepted the plasmid grew on the agar that contained the selection agent. Discrete colonies were then isolated from the plates and then screened to verify they contained the intact plasmid. This was done using a small scale plasmid preparation.

3.3.3.2.: Small scale plasmid preparation.

An alkaline lysis based method was used which was a modification of the method of Birnboim and Doly (1979). It provided a rapid method of checking the insertion of plasmids into bacteria, providing enough DNA of sufficient purity to allow restriction enzyme digestion to verify inserted plasmid integrity and identity.

a: 5 ml of medium containing the appropriate antibiotic was inoculated with a single bacterial colony and incubated at 37°C overnight with vigorous shaking (300 r.p.m.). The medium used in all preparations was LB (1% (w/v) Bacto-tryptone, 0.5%(w/v), 1% (w/v) sodium chloride, 0.5%(v/v) 1M sodium hydroxide) containing 100 μg ml^{-1} ampicillin. 1.5 ml of the culture

was pipetted into an Eppendorf tube and centrifuge for 1 minute in a microfuge on maximum g force to pellet the bacteria. The remainder of the overnight culture was stored at 4°C until required as an innoculum. The supernatant was removed by inversion and the tube left inverted for several minutes to remove as much liquid as possible.

b: The bacterial pellet was resuspended in 100 μl of an ice cold solution of 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8.0, 4 mg ml^{-1} lysozyme (added fresh). This was incubated at room temperature for 5 minutes and then 200 μl of a freshly prepared solution of 0.2M sodium hydroxide, 1% (w/v) SDS added. The contents of the tube were mixed by inverting the tube rapidly two or three times (not vortexed) and the tube incubated on ice for 5 minutes.

c: 150 μl of an ice cold solution of potassium acetate (pH 4.8) was added to the lysate that was prepared as follows: To 60 ml of 5 M potassium acetate 11.5 ml of glacial acetic acid and 28.5 ml of distilled water was added. The resulting solution was 3M with respect to potassium and 5 M with respect to acetate. This was mixed by vigorous inversion of the tube and incubated on ice for 5 minutes. Vortexing resulted in breaking the plasmid. At this stage a precipitate of chromosomal DNA became visible. This was pelleted by centrifuging for 10 minutes in a microfuge on high at 4°C. The supernatant contained plasmid DNA and soluble proteins. The supernatant was transferred to a fresh tube.

d: Proteins were removed using a phenol extraction. An equal volume of phenol / chloroform (1:1) was added to the supernatant and mixed by inversion. The resulting phenol and aqueous phases were separated by centrifugation in a microfuge for 2 minutes at room temperature. The top aqueous phase was transferred to fresh tube. Two volumes of ethanol at room temperature was then added and mixed by inversion. This was left to stand at room temperature for 10 minutes to allow the plasmid DNA to precipitate. The plasmid DNA was pelleted by centrifugation in a microfuge set to maximum

g force for 10 minutes at room temperature, the supernatant removed and the tube drained in an inverted position.

e: The pellet was washed in 1 ml of 70% (v/v) ethanol to remove salt contamination, the DNA repelleted and the supernatant discarded. The pellet was then dried in a vacuum desiccater for 10 minutes or a rotary evaporator for 5 minutes.

f: The pellet was resuspended 30 μl of 10 mM Tris pH 8.0, 1 mM EDTA containing DNase free pancreatic RNase (60 μg ml^{-1}) and incubated at 37°C for 10 minutes.

g: 10 μl of the solution was used in a restriction digest using the specific restriction enzymes that gave a characteristic banding pattern for the plasmid of interest when the DNA fragments are analysed by gel electrophoresis (Agarose TAE gel for DNA analysis, methods section 3.3.1.3). This confirmed the identity and presence of the desired plasmid.

Once the cloned bacteria had been positively identified as containing the plasmid of interest then it was necessary to carry out a large scale plasmid preparation to provide large quantities of the DNA for experimental use.

3.3.3.3: Large scale plasmid preparation.

This method should provided between 1 and 5 mg of the plasmid.

a: On to an ampicillin (100 μg ml^{-1}) nutrient agar plate a fresh streak of the transformed bacteria was made. This was done from the remainder of the culture in the small scale plasmid preparation or from stored bacteria in glycerol, slopes or stab cultures. This was incubated overnight at 37°C. 10 ml of LB medium containing 50 μg ml^{-1} ampicillin in a 50 ml flask was inoculated with a single colony and incubated overnight at 37°C with vigorous shaking (300 r.p.m.). 125 ml of medium containing 50 μg ml^{-1} ampicillin in a 500 ml flask was then inoculated with 0.5 ml of the overnight

culture. This was grown at 37°C with shaking until optical density at 550 nm = 0.7. This took about 3 hours. Meanwhile, four 2 litre flasks were prewarmed, each containing 500 ml of LB medium containing 50 μg ml^{-1} ampicillin. After the required absorbance had been reached, each of the 2 litre flasks were inoculated with 25 ml of the culture and incubated at 37°C for 16-18 hours with vigorous shaking.

The following steps were performed at 4°C.

b: The cultures were poured into 500 ml centrifuge bottles and centrifuged at 4000 x g for 15 minutes. The supernatant was discarded and each of the four pellets washed with 100 ml of STE (10 mM Tris pH 8.0, 1 mM EDTA, 100 mM sodium chloride) and centrifuged again at 4000 x g for 30 minutes.

c: Each pellet was resuspended in 5 ml of 25% (w/v) sucrose, 50 mM Tris pH 8.0 and the pellets combined into 2 bottles prior to the addition of 1 ml of lysozyme solution (15 mg ml^{-1} lysozyme, 25 mM Tris pH 8.0). This was incubated on ice for 7 minutes. 2 ml of 0.5 M EDTA was added to each bottle and incubated on ice for 8 minutes. 15 ml of Triton lysis solution (50 mM Tris pH 8.0, 0.15% (v/v) triton X100, 50 mM EDTA) was added to both bottles which were further incubated on ice for another 10 minutes. By this stage all cells had lysed.

d: The lysed pellets were transferred into 50 ml polypropylene Oakridge type centrifuge tubes and spun at 40 000 x g for 1 hour at 4°C. This pelleted down all the cell debris leaving the soluble protein and nucleic acids in the supernatant. The supernatant was collected in a graduated measuring cylinder and 1 g of caesium chloride for each ml of supernatant added. At this point the DNA was relatively stable and was stored at 4°C for several days if required.

e: 0.8 ml of 1% (w/v) ethidium bromide was added for each ml of caesium chloride / supernatant. The mixture was transferred to quick seal polyallomer tubes and any excess space at the top of the tube filled with

paraffin or mineral oil and the tube sealed. This was centrifuged at 150000 x g for 24 hours at 20°C.

All following procedures were carried out at room temperature.

f: The tubes were viewed under UV light. Two bands were evident in the top half of each tube. The upper band contained chromosomal DNA. The lower band contained plasmid DNA. The lower band was removed by pushing a 16 gauge hypodermic needle into the top of the tube. A 16-19 gauge needle was pushed through the tube wall just below the plasmid band and a syringe attached to this. The needle was tilted so it touched the bottom of the plasmid band and suction applied to the syringe. Approximately 4 ml or less of the band was removed from each tube and combined in a 15 ml disposable centrifuge tube. The collected band was mixed with 22.2 g caesium chloride, 23.2. ml of TE pH 7.5 and 0.76 ml of 1 % (w/v) ethidium bromide. This was loaded into 1 quick seal tube, rebanded and the plasmid band collected as before.

g: It was important to extract the ethidium bromide from the plasmid fraction. This was done by mixing it with an equal volume of isopropanol saturated with 5M sodium chloride, shaking the plasmid band / isopropanol mixture and then leaving it to stand until the phases to separated. The upper phase was the isopropanol phase and was pipetted of and discarded. 5 or 6 extractions were needed to remove the ethidium bromide. The 5M sodium chloride saturated isopropanol was prepared by adding isopropanol to 5M sodium chloride, shaking and leaving the phases to separate overnight. The upper layer contained the isopropanol.

h: The plasmid solution was transferred to 30 ml corex tubes, placing 2 ml into each tube. This was diluted with 3 volumes of water. 2 volumes (8 x original) of cold ethanol was added and left overnight at -20°C to allow the plasmid to precipitate. If stored longer than overnight then the caesium chloride precipitated. This was then centrifuged for 10 minutes at 8000 x g at 4°C to pellet the plasmid DNA. The pellet was resuspended in 4 ml of

200 mM sodium acetate and 8 ml of cold ethanol added and placed overnight at -20°C to re precipitate the plasmid DNA which was pelleted as before. At this stage prior to the second ethanol precipitation it was an advisable option to extract protein with a phenol / chloroform extraction followed by a chloroform clean up.

i: The ethanol precipitate was centrifuged at 8000 x g for 20 minutes at 4°C to pellet plasmid DNA. The pellets were resuspended in a small volume of TE pH 7.6 or sterile distilled water. The nucleic acid concentration was determined by optical density determination and a small amount digested with restriction enzymes that give a known banding pattern for the plasmid when run on an agarose TAE gel (methods section 3.3.1.3) to check plasmid integrity. The plasmid was then stored frozen at -20°C until required.

3.3.3.4.: Plasmid maps.

a: pSG5 Tgase.

The vector includes the SV40 early gene enhancer and promoter, intron II of the rabbit beta globin gene, the T7 bacteriophage promoter, a 3.3 kb human tissue transglutaminase cDNA cloned into the *EcoR1* site of the vector, the SV40 polyadenylation signal sequence and the ampicillin resistance gene. (figure 3.3.3.4a)

b: pSG5 HG1.

The vector includes the SV40 early gene enhancer and promoter, intron II of the rabbit beta globin gene, the T7 bacteriophage promoter, the HG1 glucocorticoid receptor protein cDNA, the SV40 polyadenylation signal sequence and the ampicillin resistance gene. (figure 3.3.3.4.b)

c: pBluescript Tgase.

The phagemid contains the 3.3 kb human transglutaminase cDNA cloned into the multiple cloning site in the centre of the LacZ promoter (Blue/white colour selection). The vector has a f1 phage origin of replication, a colE1 origin and contains the β lactamase ampicillin resistance gene for bacterial selection. (figure 3.3.3.4.c)

d: pGem3Z Tgase.

The vector contains the 3487 bp mouse tissue transglutaminase cDNA cloned into the *EcoR1* site of the multiple cloning site situated in the Lac operon, the T7 and SP6 RNA polymerase transcription initiation sites, the f1 phage origin and the colE1 origin and the β lactamase ampicillin resistance sequence. (figure 3.3.3.4.d)

e: pBluescript Bal-1 Tgase.

The phagemid contains the 1 kb *Bal-1* deletion fragment of the human transglutaminase cDNA cloned into the multiple cloning site in the centre of the LacZ promoter (Blue/white colour selection). The vector has a f1 phage origin of replication, a colE1 origin and contains the β lactamase ampicillin resistance gene for bacterial selection. (figure 3.3.3.4.e)

f: pMAMneo Tgase.

The expression vector contains a functional 3.3 kb human cytosolic tissue transglutaminase insert that was blunt end cloned into the *EcoR1* site in the multiple cloning site. The SV40 constitutive promoter and enhancer controls the neomycin resistance sequence that allows post transfection selection while the cDNA insert is controlled by the mouse mammary tumour virus (MMTV) promoter. The MMTV promoter is inducible by either the addition of glucocorticoids or heavy metals. The vector contains the β lactamase gene to

code for ampicillin resistance and the pBR322 site of origin. (figure 3.3.3.4.f)

g: pMAMneo CAT

The expression vector contains the chloramphenicol acetyl transferase gene cloned into the multiple cloning site. The SV40 constitutive promoter and enhancer controls the neomycin resistance sequence that allows post transfection selection while the cDNA insert is controlled by the mouse mammary tumour virus (MMTV) promoter. The MMTV promoter is inducible by either the addition of glucocorticoids or heavy metals. The vector contains the β lactamase gene to code for ampicillin resistance and the pBR322 site of origin. (figure 3.3.3.4.g)

h: pSV CAT

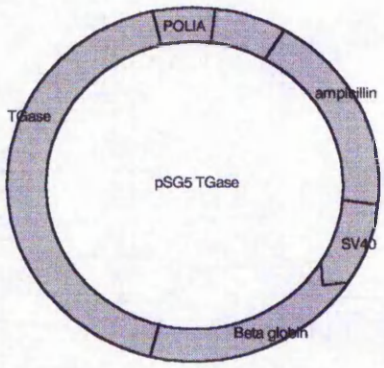
The vector contains the SV40 early promoter and enhancer unit that drives transcription of the chloramphenicol acetyl transferase gene and the β lactamase sequence conferring ampicillin resistance. (figure 3.3.3.4.h)

i: pSV_2neo

The vector contains the SV40 early promoter and enhancer unit that drives transcription of the neomycin resistance gene and the β lactamase sequence conferring ampicillin resistance. (figure 3.3.3.4.i)

Figure 3.3.3.4.

a:



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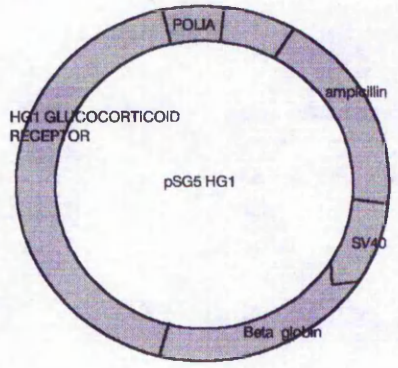

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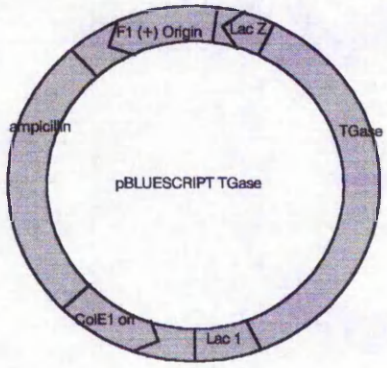

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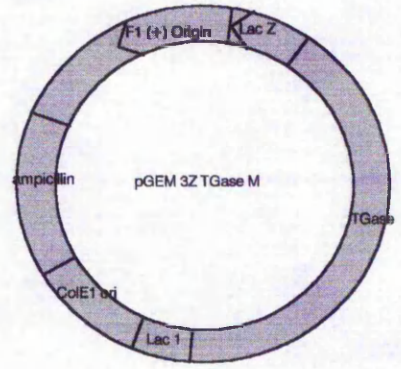

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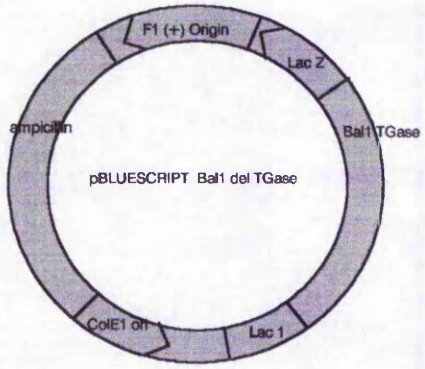

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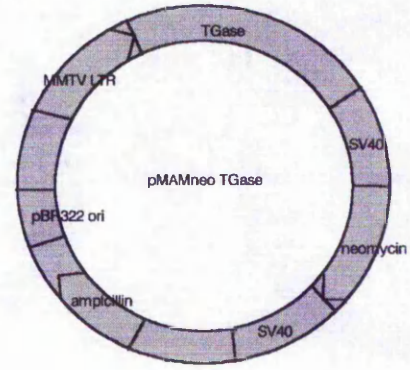

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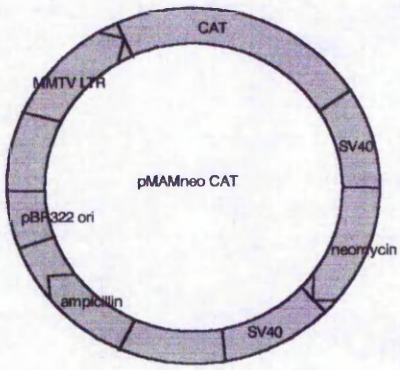
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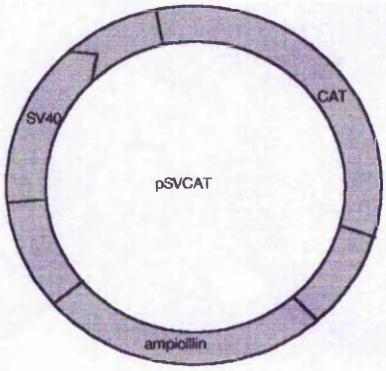
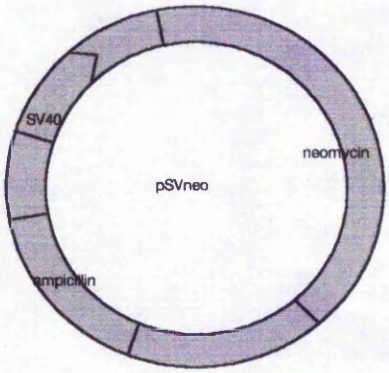
MMTV LTR
SV40
pMAMneo CAT
pBR322 ori
neomycin
ampicillin
SV40

h:


SV40
CAT
pSVCAT
ampicillin

i:


SV40
neomycin
pSVneo
ampicillin

3.4.: Biochemical techniques.

3.4.1.: Chromatography.

3.4.1.1: FPLC separation of tissue transglutaminase into its three forms.

If cells from tissue culture were to be analysed approximately 2 x 10^7 cells were harvested using either 5 mM Tris, 1 mM EDTA, with cell scraping or by trypsinisation (0.25% (w/v)), washed twice in PBS and then resuspended in 500 μl of homogenising buffer (5 mM Tris, 1 mM EDTA, 0.32 M sucrose, 5 mM benzamidine, 0.5 mg ml^{-1} leupeptin, 1 mM PMSF (phenylmethylsulphonyl fluoride), 1% (w/v) Lubrol Px.

If tissue was to be studied, fresh tissue was quickly fine chopped on ice and resuspend in homogenising buffer to give a 20% (w/v) homogenate in homogenising buffer.

Homogenisation and separation

a: The sample was homogenised with several passes of a hand driven glass / teflon homogeniser on ice. The particle free supernatant was separated by centrifugation at 4°C for 45 minutes at 70 000 x g. The particle free supernatant was filtered through a 0.22 μm filter and loaded onto a 5 mM Tris / 2 mM EDTA equilibrated Mono Q column (2 ml) (Pharmacia). The entire volume of the homogenate from tissue culture source (approximately 2 mg protein) or 500 μl of the 20% (w/v) tissue homogenate (approximately 50 mg protein) was loaded.

b: The protein was eluted from the column using a 0-0.5 M sodium chloride gradient applied over a 30 minute period. Flow rate was set at 0.5 ml per minute and 500 μl fractions collected every 1 minute into tubes kept on ice. Gradient rate and flow rate were altered on occasion to give a flow rate of 350 μl per minute and a gradient run over 20 minutes to provide a more

concentrated fraction and allow a higher number of samples to be run in one day.

c: Each fraction was assayed for transglutaminase activity and antigen as outlined in methods 3.4.3.1. and 3.4.2.3. respectively.

3.4.1.2:: Polyacrylamide gel electrophoresis of proteins.

The polyacrylamide gel was prepared using a Biorad Mini Protean 2 gel apparatus and the 5 x 1 mm well comb. A 10 % (w/v) polyacrylamide gel was prepared as described. Into a Buchner flask, 10 ml of a 30% (w/v) acrylamide, 0.8%(w/v) bis-acrylamide solution was pipetted followed by 15 ml of 0.75M Tris pH 8.8. 1 ml of freshly prepared 1% (w/v) ammonium persulphate solution was then added and the solution made up to 30 ml with distilled water. This was degassed under vacuum for 5 minutes and then 35 μl of TEMED (N,N,N',N'-tetramethylenediamine) added. This was mixed gently and poured immediately between the gel plates and overlaid with butan-2-ol to exclude oxygen and allowed to polymerise for 1 hour at room temperature. A stacking gel was poured on to the resolving gel into which the comb was set to provide loading wells. This was done by removing the butan-2-ol and washing the surface of the resolving gel with distilled water twice. The top of the resolving gel was dried with filter paper and the comb placed into position. The remaining area between the plates was filled to the top of the plates with a 4% stacking gel which was prepared as described. 10 ml of 0.75 M Tris pH 6.8 was added to 3 ml of a 30%(w/v) acrylamide, 0.8% (w/v) bisacrylamide solution followed by 1 ml of a freshly prepared 1% (w/v) ammonium persulphate solution. This was made up to 21 ml with distilled water. 35 μl TEMED was added and the stacking gel poured immediately. This was allowed to set for 30 minutes before use.

The comb was removed from the apparatus and the wells dried with filter paper. The wells were filled with running buffer (0.025M Tris pH 8.5, 0.2M glycine, 0.1% (w/v) SDS) and the samples loaded in to the wells carefully using a Hamilton syringe. The gel was then clipped into the electrode housing and

placed into the tank. Both chambers were filled with running buffer and the gel run at constant voltage set between 70 and 100 volts until the bromophenol blue front reached approximately 1 cm short of the bottom of the gel. This took approximately 2 hours. The gel was then removed from the gel plates and was ready to be stained or Western blotted.

Sample preparation

Preparation of the homogenate was designed to optimise electrophoresis for a denaturing gel (10 cm x 10 cm x 1 mm) to be run on a Biorad Mini Protean 2 system using a 5 x 1 mm well.

i: Cultured cells. Cells were harvested from the plate using either trypsin or a cell scraper if the cell line grew as a monolayer or by centrifugation if the cell line grew in suspension. Cells were washed twice in PBS and counted on a haemocytometer. A volume containing $5x10^5$ cells was aliquoted into a microfuge tube and the cells pelleted down with a 1 minute spin in a microfuge at maximum g force. The cells were resuspended in 20 μl of loading buffer (10 mM Tris pH 6.8, 2.5% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 0.02% (w/v) bromophenol blue.) and completely homogenised by 3 x 5 second bursts of sonication on ice interspersed by 30 second cooling periods. Samples were boiled for 5 minutes to allow complete reduction of the proteins. The homogenate was then loaded directly on to the polyacrylamide gel. The cell number used in the homogenate was on occasion used at 1 x 10^6 cells with only a small loss in resolution. If the gel was to be Western blotted and probed for a protein of low abundance then this was further raised to 1 x 10^7 cells, but resolution was impaired with this high protein loading.

ii: Tissue (tumours). The tissue was removed from animal and the homogenisation commenced immediately or snap frozen in liquid nitrogen until ready. A 20% (w/v) homogenate of the tissue was prepared in 5 mM Tris, 2 mM EDTA, 0.32 M sucrose homogenising buffer (STE) using 3 x 10

second bursts of an Ultra Turrax on the maximum setting interspersed with 30 second cooling periods. This was done in a class 1 safety cabinet to contain aerosols. Complete homogenisation was ensured using 3 x 5 second bursts of sonication on ice again interspersed with 30 second cooling periods. 1 μl of the homogenate was diluted in 5 μl STE and then 20 μl of loading buffer added. This was boiled for 5 minutes to allow reduction of the proteins and then loaded on to the gel.

3.4.2.: Immunological.

3.4.2.1.: Western blotting.

This method was designed for use with the L.K.B. multiphor 2 dry blot system.

a: The anode and cathode graphite plates were saturated with distilled water, allowed to soak for 5 minutes and excess water removed with a tissue.

b: 18 pieces of 3 MM filter paper were cut to the same size as the gel and soaked in blocks of 9 in continuous buffer (39 mM glycine, 48 mM Tris pH 8.8, 0.0375% (w/v) SDS 20% (v/v) methanol) by capillary action to avoid trapping air bubbles in between the papers. The first block of 9 papers was placed on to the anode and smoothed out to remove any air bubbles.

c: A piece of nylon membrane (Amersham's Hybond C super) was cut to the gel size, soaked in continuous buffer and placed on to the filter papers on the anode, again removing air bubbles.

d: The gel was removed from the gel plates and soaked in continuous buffer for 5 minutes. The gel was positioned onto the nylon and any air bubbles eased out. The second block of 9 filter papers was soaked in continuous buffer and placed on top of the gel again using precautions to remove air bubbles.

e: The cathode plate was placed on top of the gel, nylon, filter paper sandwich and the electrode pins connected. The transfer was under taken by applying

0.6 mA / cm^2 gel surface area for 45 minutes. For a gel run in a miniprotan 2 system this was 45 mA constant voltage for 45 minutes for a single gel.

f: On completion of the blot, transfer of proteins from the gel to nylon was checked by staining the nylon membrane with 0.1% (w/v) Ponceau S red in 4% (w/v) trichloroacetic acid and destaining with distilled water. A good transfer showed strong banding the complete length of each lane.

3.4.2.2.: Immunoprobing of Western blots for tissue transglutaminase.

All processes were carried out at room temperature on a rotary shaker.

a: Any Ponceau red stain was removed from the blot by washing in immunoblot B (50 mM Tris pH 7.4, 200 mM sodium chloride, 0.1% (w/v) Tween 80) until all red colouration was removed. Any remaining protein binding sites on the nylon membrane were blocked by incubating in immunoblot A (100 mM Tris pH 9.0, 150 mM sodium chloride, 0.05% (v/v) Tween 80, 2% (w/v) marvel). Sixty minutes at room temperature was sufficient if time did not allow an overnight incubation.

b: The Western blot was then probed with Goat 202 anti guinea pig liver transglutaminase antibody (Gift from Dr. P.J.A. Davies, University of Texas) diluted 1:1000 in immunoblot A for 2 hours. The blot was then washed twice with immunoblot B for 10 minutes to remove any non specifically bound antibody.

c: To commence the antibody revealing procedure an affinity purified anti goat biotin conjugate (Dako or Sigma) diluted in immunoblot B (dilution depended on the supplier and batch number) was placed onto the Western blot. In general a 1:600 dilution for the Dako supplied antibody and a 1:200 dilution for the Sigma chemicals supplied antibody were adequate. A 1 hour incubation was sufficient for equilibrium to be reached. The membrane was then washed twice for 10 minutes each in immunoblot B to remove the non specifically bound antibody.

d: The blot was then incubated in an extra-avidin - horse radish peroxidase conjugate (Sigma Chemicals) diluted 1:1000 in immunoblot B for 30 minutes. It was particularly important to thoroughly wash the blot twice in immunoblot B, once in distilled water and twice in PBS pH 7.4 at this stage. Each wash lasted 10 minutes.

e: To visualise the site of primary antibody binding the HRP substrate reagent was added and the blot left until bands became visible. This generally took about 5 to 10 minutes but depended on the level of the transglutaminase present. The substrate reagent was prepared immediately before use. To 20 mg 4-chloro-napthol, 2.5 ml of dimethylsulphoxide was added and the solution shaken gently until the chloronapthol fully dissolved. To this 47.5 ml of PBS was slowly added while stirring. 15 μl of 30% (v/v) hydrogen peroxide was then added and the substrate used immediately.

3.4.2.3.: Transglutaminase antigen determination by ELISA.

Enzyme linked immunosorbent assay is one of the most accurate and quickest methods of determining levels of a specific protein within a mixture of proteins. The ELISA used for tissue transglutaminase was only used to assess transglutaminase protein levels within fractions from chromatography separations of homogenates as in the complete homogenate high background made the assay unreliable and inaccurate.

a: The wells of an ELISA plate were coated with 100 μl of a 1:20000 dilution of sheep anti guinea pig liver transglutaminase antibody (produced by Dr. C.R.L. Knight, Nottingham Trent University) diluted in 0.05 M sodium hydrogen carbonate and incubated overnight at 4^{o}C. The plate was washed twice in PBS pH 7.4 to remove any unbound antibody.

b: Any remaining protein binding sites were blocked with 150 μl per well of 3% (w/v) BSA in PBS using a 1 hour incubation at 37^{o}C.

c: The plate was washed once in PBS pH 7.4 before the addition of 100 μl of the transglutaminase standards (100 ng, 50 ng, 25 ng, 12.5 ng, 6.25 ng, 3.125 ng, 1.56 ng.) and 100 μl of the samples to be measured. This was then incubated at 37°C for 2 hours and the plate washed 3 times in PBS, 0.1% (v/v) Tween 20 (PBS-T) to remove unbound protein.

d: To each well 100 μl of a 1:5000 dilution of rabbit - anti guinea pig liver transglutaminase (Produced by Dr C.R.L. Knight, Nottingham Trent University) diluted in PBS containing 3% (w/v) BSA was added and incubated at 37°C for 2 hours. The plate was washed 3 times with PBS-T to remove unbound antibody.

e: To each well 100 μl of anti - rabbit biotin conjugate (Sigma Chemicals) diluted 1:2500 in PBS containing 3%(w/v) BSA was added and incubated for 1 hour at room temperature. Again the plate was washed 3 times in PBS-T.

f: 100 μl of extravidin, horse radish peroxidase conjugate (Sigma Chemicals) diluted 1:5000 in PBS-T was added to each well and incubated for 30 minutes at 37°C. The plate was then washed twice with PBS-T and then twice with distilled water.

g: 100 μl of the horse radish peroxidase substrate was then added to each well. This was prepared immediately before use as described below. To 20 ml of 0.1 M sodium citrate pH 6 (with acetic acid), 150 μl of 10 mg ml^{-1} tetramethyl benzene diluted in dimethyl sulphoxide was added. To this 25 μl of 3% (v/v) hydrogen peroxide was added. Once the substrate was added, the plate was left at room temperature in the dark for 5 minutes for colour to develop. The reaction was then stopped with the addition of 50 μl of 2.5 N sulphuric acid.

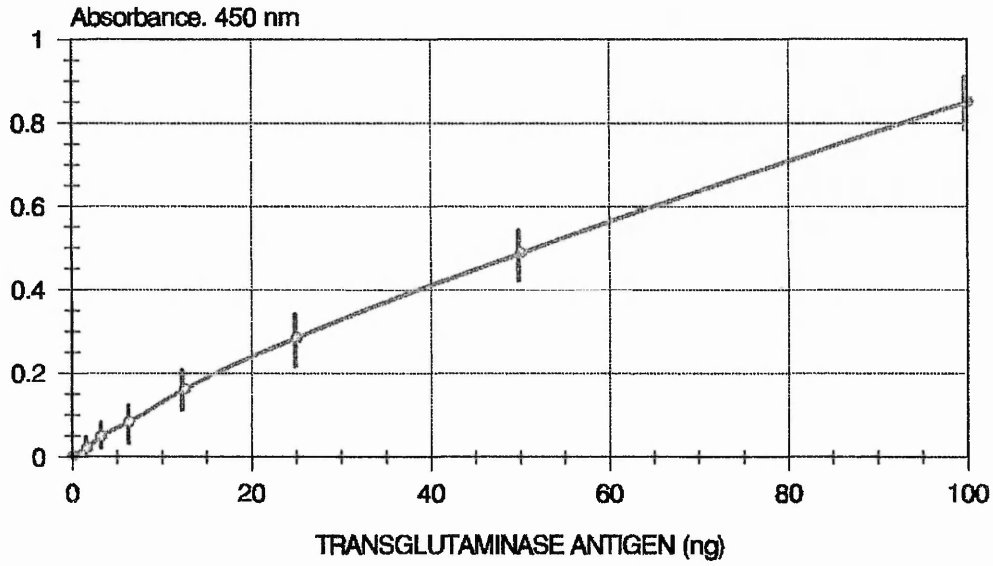
h: The absorbance of each well was measured by reading the plate at 450 nm in an ELISA plate spectrophotometer.

The transglutaminase antigen level in the samples can then be determined by reading from the standard curve. Figure 3.4.6. shows a typical standard curve for the ELISA with sensitivity down to 1 ng of antigen

Figure 3.4.6.: A typical standard curve from a transglutaminase ELISA.

Transglutaminase standards of 100μg, 50μg, 25μg, 12.5μg, 6.25μg, 3.125μg and 1.56μg were subjected to the transglutaminase ELISA procedure as outlined in section 3.4.6. Data represents mean absorbance at 450 nm $\pm$ S.E.M.

Figure 3.4.6.



3.4.3.: Enzyme assays

3.4.3.1.: Transglutaminase activity assays.

This assay was based upon the ability of tissue transglutaminase to incorporate putrescine in to N,N'-dimethylcasein, and thus the activity was measured by determining the amount of radiolabelled putrescine that was incorporated in to TCA precipitable protein in a specific time. Two types of radioisotope labelled putrescine were used. A ^{14}C labelled putrescine (1,4 ^{14}C-putrescine) was used in the main. This was used for accurate determination of activity as ^{14}C has a high scintillation counting efficiency but low specific activity. In addition a ^{3}H labelled putrescine was used. This was supplied at a much higher specific activity than the ^{14}C version and therefore provided a much more sensitive assay being able to detect much lower enzyme activity. Therefore the ^{3}H labelled putrescine was used only were relative activity needed to be determined where activity was low, that was in fractions from FPLC separations of homogenates. The ^{14}C labelled putrescine was used for the determination of activity in straight homogenates from cultured cells and tissues where definitive values were required.

Sample preparation.

i: **From cultured cells.** Cells were harvested from a plate using either trypsin or a cell scraper if the cell line grew in a monolayer or by centrifugation if it grew in suspension. Cells were washed twice in PBS and counted on a haemocytometer. A volume containing $1x10^6$ cells was aliquoted into a microfuge tube and the cells pelleted down with a 1 minute spin in a microfuge on high. The cells were resuspended in 90 μl of homogenising buffer (5 mM Tris pH 7.4, 2 mM EDTA, 0.32 M sucrose) that contained the protease inhibitors benzamidine (5 mM), PMSF (1 mM) and leupeptin (20 μg ml^{-1}) and the cells homogenised using 3 x 5 second bursts of sonication interspersed with 30 second cooling periods. All procedures were performed at 4^oC .

ii: **From tissue (tumours).** Tissue was removed from the animal and homogenised immediately or snap frozen in liquid nitrogen until assayed. A 20% (w/v) homogenate of the tissue was prepared in homogenising buffer (5 mM Tris, 2 mM EDTA, 0.32 M sucrose (STE)) that contained the protease inhibitors benzamidine (5 mM), PMSF (1 mM) and leupeptin (20 μg ml^{-1}). The tissue was homogenised using 3 x 10 second bursts of an Ultra Turrax on the maximum setting interspersed with 30 second cooling periods. This was done in a class 1 safety cabinet to contain aerosols that occurred. Complete homogenisation was ensured by 3 x 5 second periods of sonication again interspersed by 30 second cooling periods. The homogenate was assayed immediately.

^{14}C labelled putrescine method.

a: A working stock of ^{14}C putrescine was prepared by adding 50 μl of a 243 mM putrescine solution made up in 1.05M Tris at pH 7.4, to 1 ml of the ^{14}C putrescine (Amersham) which was supplied at approximately 50 μCi ml^{-1} or 109 mCi $mmol^{-1}$. This gave a working stock solution of 12.2 mM putrescine with a specific activity of 3.97 mCi $mmol^{-1}$.

b: The reaction mixture was prepared in an Eppendorf tube by adding 5 μl of 25 mM calcium chloride in 50 mM Tris pH 7.4, 5 μl dithiothreitol in 50 mM Tris, 10 μl of 25 mg ml^{-1} N,N'-dimethylcasein in 50 mM Tris pH 7.4 and 5 μl of the putrescine working stock solution. If several assays were to be performed then the reaction mixture was prepared in bulk and aliquoted out in to individual tubes. In addition to each tube a duplicate tube was produced in which the calcium chloride was replaced with 5 μl of 100 mM EDTA in 50 mM Tris pH 7.4. This acted as a control for non enzymatic incorporation of putrescine.

c: The reaction was commenced by the addition of 25 μl of the sample to be measured. This was gently mixed and placed at 37^{o}C for the reaction to proceed. The time the reaction was left was critical due to enzyme kinetics

as reaction rate decreases with time and substrate level. Reaction times were optimised so that incubation times were chosen when the reaction rate was found to be linear, i.e. 30 minutes. An example of determining reaction rate is shown in Figure 3.4.5. This shows a rate of reaction graph from clone Met B pSG5 Tgase 36.

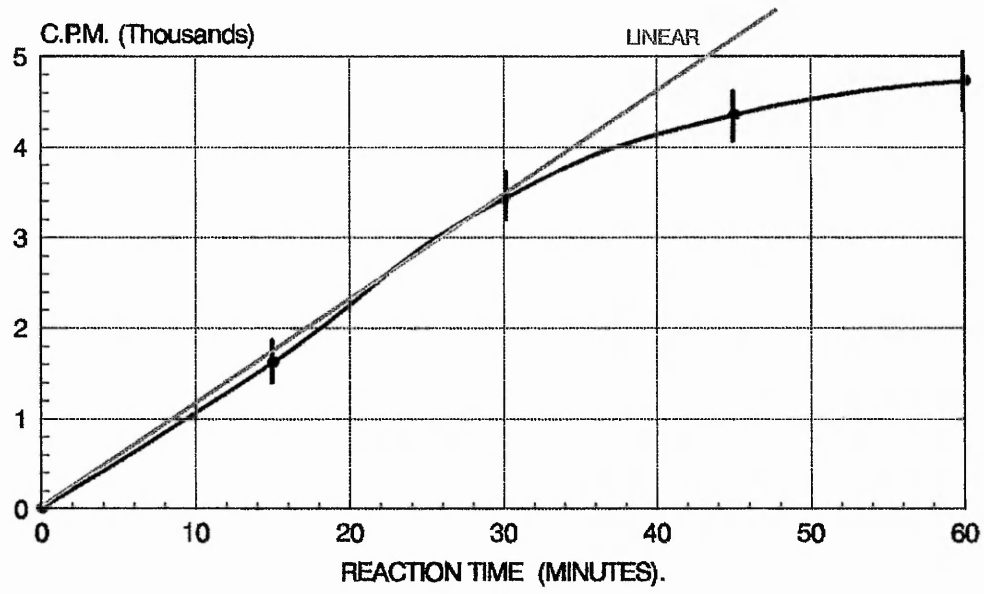
d: After the desired incubation period it was necessary to determine the level of incorporation of the radio labelled putrescine. This was done by pipetting 10 μl of the reaction mixture on to a 1 cm^2 piece of 3 MM filter paper (pre-soaked in 50 mM EDTA, 5 mM methylamine and then dried prior to use) in triplicate. At the same time the procedure was repeated with the EDTA duplicate tube. The filter papers were immediately dropped into a beaker containing ice cold 10% (w/v) trichloroacetic acid. The TCA immediately precipitated the protein in the reaction mixture on to the filter paper and subsequently trapped it there. Unincorporated putrescine did not precipitate.

e: Unincorporated ^{14}C putrescine was removed via a series of washes. Filter papers were left washing in the cold 10% (w/v) TCA for at least 10 minutes. This wash was repeated for a further 10 minutes. Filters were then washed 3 times for 5 minutes for each wash in ice cold 5% (w/v) TCA, followed by a 5 minute washes in 50%(w/v) ethanol, 50% (w/v) acetone and finally in 100 % (v/v) acetone for 10 minutes.

Figure 3.4.5.: Transglutaminase rate of reaction graph in Clone Met B pSG5 Tgase 36.

A tube was set up containing 6 times the normal level of transglutaminase assay reaction mixture and the reaction was initiated by addition of 6 times the normal level of sample homogenate. 10 μl aliquots of reaction mixture were removed at time 0, 15, 30, 45 and 60 minutes and the level of ^{14}C putrescine incorporation into dimethylcasein determined as described in section d. Data represents Mean C.P.M. of ^{14}C incorporated putrescine $\pm$ S.E.M. n=5

Figure 3.4.5.



f: The filter papers were allowed to dry for at least 1 hour at room temperature although overnight was preferable. They were then scintillation counted. A quench correction curve could not be used here to correct for disintegrations per minute due to the physical quenching caused by various orientation of the filter papers in the scintillation fluid. Therefore efficiency was determined by counting a known level of the isotope and working out the percentage counted compared with actual d.p.m.. This was done by taking an aliquot out of the reaction tube, but not putting it through the wash procedure and counting it directly. This also accounted for pipetting discrepancies.

g: Once the counts had been measured, they were corrected for non enzymatic incorporation by taking the background EDTA value away from the calcium value. This was then corrected for the amount of the reaction mixture counted and the percentage of the sample added to the reaction mixture. Further corrections were made for counting efficiency and then knowing the specific activity of the ^{14}C putrescine the counts were converted to nmol of putrescine incorporated. A unit of transglutaminase activity is nmols of putrescine incorporated per hour.

^{3}H putrescine incorporation assay

This was essentially the same assay as the ^{14}C putrescine assay with a few experimental changes outlined below.

a: The ^{3}H putrescine supplied by Amersham at a concentration of 28 Ci $mmol^{-1}$ (1 mCi ml^{-1}) was used neat in the reaction mixture to increase sensitivity. This gave a final putrescine concentration of 90 pM in the reaction solution.

b: The reaction volume and sample volume was halved to reduce cost. Relative concentrations of components were maintained. Smaller volumes meant that the sample could only be spotted onto filter paper in duplicate rather than triplicate.

c: Washing times were increased to 20 minutes for the 10% (w/v) TCA washes and to 15 minutes for the 5% wash. The washes with ethanol / acetone and acetone were replaced by a single 10 minute wash in 100% (v/v) ethanol due to the high quenching properties of acetone residues on tritium emissions. This was left overnight at room temperature to dry.

3.4.3.2: Chloramphenicol acetyltransferase assay (CAT assay).

Chloramphenicol acetyltranferase (CAT) is a bacterial enzyme that catalyses the transfer of the acyl group from acetyl CoA or acyl CoA cofactors to chloramphenicol. The enzyme evolved as a bacterial resistance factor to the antibiotic chloramphenicol. It is not found in the mammalian cell and is subsequently commonly used as a reporter gene to either measure the incorporation of a transfected gene or as a measure of a promoter sequences effectiveness in a particular cell. Measurement of CAT activity is quick simple and accurate which facilitates its use as a reporter gene. The most common CAT assay relies on the acylation of ^{14}C labelled chloramphenicol which is then separated from the non acetylated form using thin layer chromatography. The acetylated band can then be accurately measured using scintillation counting.

a: Cells were harvested from tissue culture dishes using trypsin and washed three times in PBS and the cell number determined by counting on a haemocytometer. A volume was aliquoted into a Eppendorf tube equivalent to 2 $x10^6$ cells and the cells pelleted by a 1 minute spin in a microfuge on high. The pellet was then resuspended in 100 μl of ice cold 0.25 M Tris-HCl pH 7.5.

b: Cells were lysed using a freeze thaw procedure. The cells were frozen in liquid nitrogen and then incubated at 37°C for 5 minutes, this was repeated twice more for an effective lysis. The lysate was cooled on ice and then micro centrifuged for 5 minutes. The supernatant was transferred to a fresh tube on ice. The cytosol extract contained the CAT enzyme. The sample was frozen immediately at -20°C at this stage and the assay carried out a later date or the assay was performed immediately.

c: 20 μl of extract was assayed by adding it to the following reaction cocktail:

2 μl 200 μCi ml^{-1} ^{14}C chloramphenicol (35 - 55 mCi / ml).
20 μl 4 mM acetyl CoA. (store at -20^{o}C for 2 weeks only)
32.5 μl 1 M Tris-HCl pH 7.5.
75.5 μl of distilled water.

If ^{14}C chloramphenicol was only available at 20 μCi ml^{-1} then this was increased to 20μl volume and the water reduced to 68.5 μl to maintain the 150 μl reaction volume to maintain the Tris at 0.25 M.

d: 20 μl of extract was added to the 130 μl of reaction cocktail in a microfuge tube and incubated for 1 hour at 37^{o}C. If the CAT activity was very low then this was extended, but the 4 mM acetyl CoA was replaced with 40 mM as the acetyl CoA was unstable under reaction conditions. It was also essential that the reaction be performed in a linear time range. This was verified by setting up a 600 μl volume reaction and taking 145 μl aliquots at various time points and analysing the conversion to the acetylated form as described below. Detection of the diacetylated product indicated a fall from the linear reaction.

e: 1 ml of ethyl acetate was added to the reaction tube and vortexed immediately. The phases were separated by micro centrifugation for 1 minute at maximum g force. The top ethyl acetate layer was transferred into a clean tube. The chloramphenicol and acetylated chloramphenicol were extracted into the ethyl acetate. The ethyl acetate was dried down in a rotary evaporator for 45 minutes or left to evaporate overnight in a fume hood. Leaving overnight to dry down could result in breakdown products being present.

f: The sample was resuspended in 30 μl of ethyl acetate. This was spotted 5 μl at a time onto a precoated plastic backed silica gel TLC sheet (type 60F_{254} 0.2 mm thick layer, Merck) approximately 2 cm from the bottom of the sheet. The TLC was developed in a chromatography tank containing 200 ml

of 19:1 chloroform to methanol solvent mixture. The tank was equilibrated for 2 hours previous to adding the thin layer sheet using a piece of filter paper in place of the TLC plate. The chromatograph was allowed to run for 2 hours or until the solvent front was close to the top of the sheet. The TLC plate was removed and air dried, wrapped in cling film and exposed to X ray film such as Kodak X-OMAT AR. An overnight exposure was usually sufficient. The resulting autoradiograph had up to five spots as shown in figure 3.2.4.1. The presence of a diacetylated spot showed the reaction was out of the linear range and the sample should be diluted or the incubation time reduced.

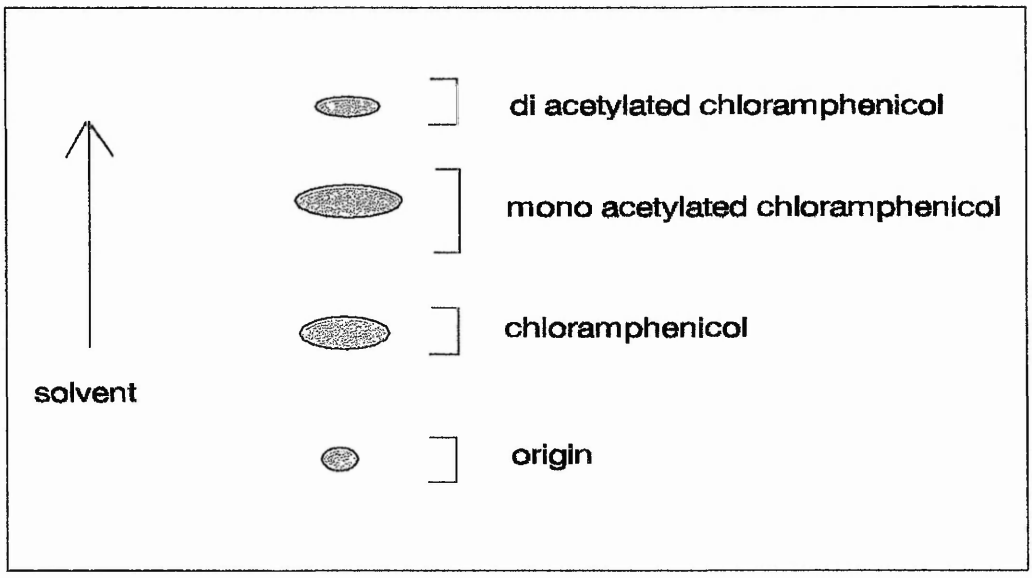
g: The activity of the extract was calculated by first determining the percentage of counts in the mono acetylated chloramphenicol species. To determine the amount of label in each spot, the TLC and autoradiograph were aligned using a light box using the origins to align the two. The position of each spot on the TLC was outlined using a pencil and the piece of TLC cut out and counted in a scintillation counter. CAT activity from the assay was calculated as below.

$$\text{percentage acetylated} = \frac{\text{CPM acetylated species}}{\text{CPM acetylated species + CPM non acetylated}} \text{ X 100}$$

Figure 3.4.7. Schematic diagram of a CAT assay autoradiograph

A diagrammatic representation of an autoradiograph that would result from a typical CAT assay as performed using the method outlined in section 3.4.7. The diacetylated product only becomes detectable when the assay exceeds the linear range

figure 3.4.7.



3.4.4.: Miscellaneous

3.4.4.1.: Determination of transglutaminase mediated apoptotic index.

The number of cells in culture undergoing spontaneous apoptosis involving transglutaminase can be determined by isolating and counting apoptotic envelopes using the method of Knight et al 1991. The apoptotic envelopes are the protein shells that are formed at the end of the apoptotic program by the crosslinking of the membrane proteins via ε (γ-glutamyl) lysine crosslinks. These strong covalent links result in a detergent insoluble structure which is the bases of the apoptotic envelope. It is important to note that when using this method to determine the apoptotic index that only 5% of the cells entering apoptosis may result in bodies that are isolated and counted as calculated from Fesus et al (1989).

a: Cells were grown in a 150 cm^2 flask to approximately 50% confluency, the medium removed and the cells washed with fresh medium. New medium was then added and the flask returned to the incubator for 24 hours.

b: The medium was poured of and saved in a 50 ml centrifuge tube. The medium contained a lot of apoptotic bodies that had formed over the 24 hours since the medium change. The monolayer of cells was harvested using 0.25%(w/v) trypsin and combined with the medium component. All cells / apoptotic bodies were pelleted with a 400 x g spin for 5 minutes and resuspended in 10 ml of PBS.

c: Cell number was counted using a haemocytometer and the cells then pellet as before. The cells were then resuspend in PBS to give a cell concentration of 2×10^7 cells ml^{-1}. This was divided into 500 μl aliquots in Eppendorf tubes. To each aliquot SDS was added to a final concentration of 2% (w/v) (50 μl of 20% (w/v) stock) and dithiothreitol (DTT) to a final concentration of 0.1% (w/v) (10 μl of a 5% (w/v) stock).

d: These tubes were boiled for 5 minutes in a water bath to allow the complete solubilisation of any detergent soluble material and then cooled. DNase 1 was added to a concentration of 20 units ml^{-1} and incubated at 37°C for 1 hour. Digestion of all the DNA in the sample prevented the bodies forming into large clumps due to the 'sticky' nature of DNA which made counting impossible.

e: All the detergent insoluble bodies were pelleted with a 5 minute spin in a microfuge set to maximum g force and subsequently washed 3 times in 2% (w/v) SDS, 0.01% (w/v) DTT, 10 units ml^{-1} DNase 1.

f: The apoptotic bodies were pelleted using a microfuge and as much liquid as possible removed. Any remaining liquid was removed with a 10 minute spin in a rotary evaporator on the highest heat setting with pulse vent. When removing the liquid with a pipette and during the washes 10-15 μl of liquid was left in the bottom of the tube to minimise the number of bodies that were disturbed and lost. The pellet was not completely dried as this caused clumping of the apoptotic bodies.

g: The apoptotic bodies were resuspended in a small volume of 2% (w/v) SDS, 0.01% (w/v) DTT, 10 units ml^{-1} DNase 1. The apoptotic bodies were then counted using a haemocytometer or by pipetting a volume (e.g. 10 μl) on to a haemocytometer without the coverslip and counting all the bodies in the volume using the lines on the haemocytometer as a guide. Resuspending the bodies in about 20 μl gave a suitable dilution for use with the haemocytometer and about 60 μl for the spot method when using the cell lines in this study.

3.4.4.2.: Total protein determination.

Protein concentration in cell and tumour homogenates was determined using the Bio-rad protein assay kit (Bio-rad Laboratories). This kit is based on the method first described by Bradford 1976 which relies on the principle that the maximum absorbance for an acidic solution of Coomassie brilliant blue G-250

shifts from 465 nm to 595 nm when binding to protein occurs. This method was chosen to determine protein concentration because it was quick and simple requiring the addition of just one reagent to the sample which was then read in 5 minutes and secondly because it was relatively inexpensive. The method also has the advantage of being accurate and reliable with little chemical interference although it did show a significant protein to protein variation if measuring purified proteins. The micro-assay procedure was used.

a: Protein standards ranging from 1 to 40 μg ml^{-1} and a blank containing just sample buffer were prepared. Standards were prepared using purified Ovalbumin as this protein gave consistent results if measured by either the Biorad, Lowry or Biuret methods. 800 μl of the standards were placed into a clean tube.

b: When using tissue culture cells a pellet of 1 x 10^6 cells was taken and homogenised by sonication in 100 μl of homogenising buffer (5 mM Tris, 2 mM EDTA, 0.32 M sucrose (STE)). 20 μl of this was placed in to a clean tube and made up to 800 μl with homogenising buffer. When using tissue, a 20% (w/v) homogenate was prepared in homogenising buffer. 2 μl of this was placed into a clean tube and made up to 800 μl with homogenising buffer.

c: 200 μl of the Bio-rad dye reagent concentrate was added to the samples and standards. The tubes were then vortexed avoiding foaming and left for 5 minutes to an hour for colour to develop. The optical density was measured at 595 nm against a blank tube using a 1 cm light path cuvette.

d: The absorbance at 595 nm versus the concentration of the standards was plotted and the unknowns determined from this calibration curve. Values were corrected for sample dilution.

3.4.4.3.: Separation of particulate and cytosolic tissue transglutaminase by ultracentrifugation.

a: Approximately 2 x 10^7 cells were harvested using either 5 mM Tris, 1 mM EDTA, cell scraping or trypsinisation, washed twice in PBS and then resuspended in 500 μl of homogenising buffer (5 mM Tris, 1 mM EDTA, 0.32 M sucrose, 5 mM benzamidine, 0.5 mg ml^{-1} leupeptin, 1 mM PMSF (phenylmethylsulphonyl fluoride). The sample was homogenised with several passes of either a hand driven glass Teflon homogeniser on ice. The particle free supernatant was separated by centrifugation at 4°C for 45 minutes at 90000 x g in a Beckman 2870 ultracentrifuge. The supernatant contained the cytosolic tissue transglutaminase.

b: The pellet was then resuspended in a second 500μl of homogenising buffer containing 1% (v/v) Lubrol Px and further homogenised to release membrane bound components. This was again clarified by centrifugation at 4°C for 45 minutes at 90000 x g. The supernatant contained the particulate (membrane bound) tissue transglutaminase.

3.4.4.4.: Statistical analysis.

Statistical comparison of data groups was performed using the non paired students t test with significance tested at both $p \geq 0.05$ and $p \geq 0.01$. Statistical analysis of line plots was via linear regression and correlation analysis. All statistical manipulations were performed on Microsoft Excel version 4.0a (Microsoft Ltd)

3.5: Materials

3.5.1.: Chemicals and reagents

All chemicals and reagents were purchased from Sigma Chemicals Ltd unless specified in the text. Specified materials are listed below:

[α-^{32}P] dCTP	ICN/Amersham International
^{14}C Chloramphenicol	Amersham International
Anti Goat Biotin Conjugate	Dako
Digoxigenin DNA Labelling / Detection System	Boehringer Mannheim
Dulbecos Modified Eagles Medium (DMEM)	Imperial Laboratories
Fast Track mRNA Kit	British Biotechnology
Foetal Calf Serum	Imperial Laboratories
Gene Clean	Distributed by Biogenesis
Hybond C Super Nitrocellulose Membrane	Amersham International
Hybond N Nylon Membrane	Amersham International
Lipofectin	Gibco BRL Life Technologies
Multiprime System	Amersham International
Penicillin/Streptomycin Solution	Imperial Laboratories
Pharmacia mRNA Extraction System	Pharmacia Ltd

Prep-A-Gene	Biorad
Prime-A-Gene Kit	Promega
Restriction Enzymes	Gibco BRL Life Technologies
RNAsol B	Distributed by Biogenesis
Thin Liquid Chromatography Plates	British Drug Houses

3.5.2 Equipment

β Scintillation Counter	Hewlett Packard
15 x 15 cm Flat Bed Electrophoresis Tank	Genetic Research Instrumentation
3000V Power Pack	Biorad
8 x 10 cm Flat Bed Electrophoresis Tank (mini gel)	Biorad
Bench Top Centrifuge	MSE
BH2 Phase Contrast Microscope	Olympus
C35 AD4 Camera Attachment System	Olympus
Carbon Dioxide Incubators	Flow Laboratories
CK2 Inverted Microscope	Olympus
Fast Protein Liquid Chromatograph System	Pharmacia
Laminar Flow Cabinets	Flow Laboratories
LKB Dry Blot Electroblotter	Hoefer Scientific Instruments

LKB Power Pack	Hoefer Scientific Instruments
Microfuge	MSE
Microsoft Excel computer software	Microsoft Corporation
Mini Protean II Vertical Electrophoresis Tank	Biorad
Mono Q IOW Exchange Column	Pharmacia
MSE 24 Super Speed Centrifuge	MSE
Rotary Evaporator	Jouan
Sonication Unit and Probe	British Drug Houses
Ultra Turrax Homogeniser	British Drug Houses
Ultracentrifuge (Beckman model 2870)	Beckman
UV Stratalinker	Stratagene
UV Transilluminator	Genetic Research Instrumentation

3.5.3 Manufacturers and distributors.

Amersham International Plc	Amersham Place Little Chalfont Buckinghamshire HP7 9NA
Beckman Instruments UK Ltd	Progress Road Sands Industrial Estate High Wycombe Buckinghamshire HP12 4JL

Gibco BRL	Life Technologies Ltd PO Box 35 Trident House Renfrew Road Paisley Renfrewshire PA3 4EF
Biogenesis Ltd	12 Yeomans Park Yeomans Way Bournemouth BH8 0BJ
Biorad Laboratories Ltd	Biorad House Maylands Avenue Hemel Hempstead Hertfordshire HP2 7TD
Boehringer Mannheim	Bell Lane Lewes East Sussex BN7 1LK
British Biotechnology	R&D Systems 4-10 The Quadrant Barton Lane Abington Oxon OX14 3YS
British Drug Houses	Merck House Poole Dorset BH15 1TD

DAKO	16 Manor Courtyard Hukhenden Avenue High Wycombe Buckinghamshire HP13 5RE
Flow Laboratories	Woodlock Hill Harefield Road Rickmansworth Hertfordshire WD3 1PQ
GRI (Genetic Research Instrumentation)	Gene House Dunmow Road Felsted Dunmow Essex CM6 3LD
Hewlett Packard	Camberra Packard Ltd Brook House 14 Station Road Pangbourne Berkshire RG8 7DT
Hoefer Scientific instruments Ltd	Unit 12 Croft Road workshops Hempstlle Lane Newcastle under Lyme Staffordshire ST5 OTW
ICN Biomedicals Ltd	Eagle House Penekrine Business Park Gomm Road High Wycombe Buckinghamshire HP13 5BR

Imperial Laboratories	West Portway Andover Hampshire SP10 3LF
Jouan UK Ltd	130 Western Road Trink Hertfordshire HP23 4BU
Microsoft corporation	Phoenix Technologies, Phoenix, USA.
Millipore UK Ltd	Winster House Heronsway Chester Business Park Wrexham Road Chester CH4 9QR
MSE	Sanyo Gallengamp Plc Park House Meridian Business Park Leicester LE3 2UZ
Olympus	Olympus Optical Co Ltd 2-8 Honduras Street London EC1Y 0TX
Pharmacia Biosystems Ltd	Davy Avenue Knowlhill Milton Keynes MK5 8PH

Promega	Enterprise Road Chilworth Research Centre Southampton SD1 7NS
Sigma Chemical Company Ltd	Fancy Road Poole Dorset BH17 7NH
Stratagene Ltd	140 Cambridge Innovation Centre Cambridge Science Park Milton Road Cambridge CB4 4GF

RESULTS.

CHAPTER 4.

THE INACTIVE TRANSGLUTAMINASE. CONFIRMATION OF ITS EXISTENCE AND SEARCH FOR A mRNA.

4.1: Introduction

In 1990 Knight et al (1990a,d) described the existence of an inactive transglutaminase that was found to be present in a range of malignant cells. This work also demonstrated an inverse relationship between the amount of inactive enzyme and the amount of active cytosolic tissue transglutaminase enzyme present in these malignant cells. Furthermore, it suggested that the amount of inactive enzyme protein within cells could be directly related to the malignant tendency of these cells. The relationship appeared to be independent of the amount of particulate tissue transglutaminase that was present.

The outstanding question was whether this larger (120 kDa) inactive form of the enzyme was a result of inappropriate gene expression which could occur at the transcriptional, translational or post-translational level. Therefore this investigation was undertaken to search for the presence of a transglutaminase mRNA that could encode for a protein of the size of the inactive transglutaminase. Such a mRNA might be expected to have a size of approximately 4 to 5 kb. The sequence of the native enzyme proposed by Gentile et al (1991) indicated a 3.3 kb (human) or 3.5 kb (mouse) mRNA consisting of a 2.3 kb coding region plus a 1 kb (human) or 1.2 kb (mouse) non coding sequence. This mRNA has enough bases downstream from the coding sequence to make a protein that is equivalent to the size of the inactive tissue transglutaminase. It is therefore possible that if read through occurred and the non coding region was translated the mRNA could remain unaltered. This is unlikely since computer analysis to insert frame shift mutations indicates that other translational stop sequences appear downstream (V. Gentile, personal communication). Work (Knight, unpublished) involving exposure of Met cells to *all-trans* retinoic acid demonstrated that active cytosolic transglutaminase expression could be induced independently of inactive transglutaminase. This indicates independent control rather than "read through" of the cytosolic transglutaminase mRNA and correlates with the computer analysis results.

Initially it was necessary to confirm the existence of the inactive tissue transglutaminase using FPLC (Pharmacia Ltd) separation of a cell homogenate using a Mono Q column (Pharmacia Ltd) and a sodium chloride elution gradient.

4.2.: Confirmation of the presence of inactive tissue transglutaminase in the hamster fibrosarcoma Met B.

In Figure 4.2.1. antigen and activity traces for the separation of tissue transglutaminase are shown from the normal cell line BHK-21. Two major peaks of transglutaminase antigen are identified using the transglutaminase sandwich ELISA. The peak eluting at 0.2 M NaCl (20 minutes) corresponds to particulate tissue transglutaminase, while the peak eluting at 0.4 M NaCl (34 minutes) corresponds to cytosolic tissue transglutaminase (Knight et al 1990c). Both peaks are mirrored by the trace of enzyme activity when measured by the incorporation of ^{3}H putrescine into casein.

In figure 4.2.2. where the same separation procedure is applied to the malignant hamster fibrosarcoma cell line Met B, as seen with extracts from BHK cells, two eluting peaks are present corresponding to both activity and antigen of the particulate and cytosolic tissue transglutaminase. In addition a third antigen peak can be identified that has no corresponding peak on the activity trace in figure 4.2.2.. This is the inactive tissue transglutaminase as described by Knight et al (1990a,d). Having verified the existence of the inactive transglutaminase in the cells to be studied attentions were then turned towards investigating the presence of a possible second transglutaminase mRNA.

Figure 4.2.1.: The separation of tissue transglutaminase from the 'normal' cell line BHK-21 cell homogenates using FPLC separation on a Pharmacia Mono Q column.

Cells from a 90% confluent culture of BHK-21 cells were harvested, counted using a haemocytometer and $2x10^7$ cells homogenised in 1 ml of homogenising buffer. All the homogenate was loaded onto a 2 ml Mono Q column and separated using a FPLC system (Pharmacia Ltd) and a 0-0.5M sodium chloride gradient at a flow rate of 0.5 ml min^{-1} as described in methods section 3.4.1.1. 0.5 ml fractions were collected and analysed for transglutaminase activity as described in methods section 3.4.3.1 (red trace) and transglutaminase antigen as described in methods section 3.4.2.3. (blue trace). Representative trace from 2 experiments.

Figure 4.2.2.: The separation of tissue transglutaminase from the highly metastatic Met B cell homogenates by FPLC separation using a Pharmacia Mono Q column.

Cells from a 90% confluent culture of Met B cells were harvested, counted using a haemocytometer and $2x10^7$ cells homogenised in 1 ml of homogenising buffer as described in methods section 3.4.1.1.. All the homogenate was loaded onto a 2 ml Mono Q column and separated using a FPLC system (Pharmacia Ltd) and a 0-0.5M sodium chloride gradient at a flow rate of 0.5 ml min^{-1} as described in figure 4.2.1.. 0.5 ml fractions were collected and analysed for transglutaminase activity (red trace) and transglutaminase antigen (blue trace) as described in figure 4.2.1.. Representative trace from 2 experiments.

Figure 4.2.1.

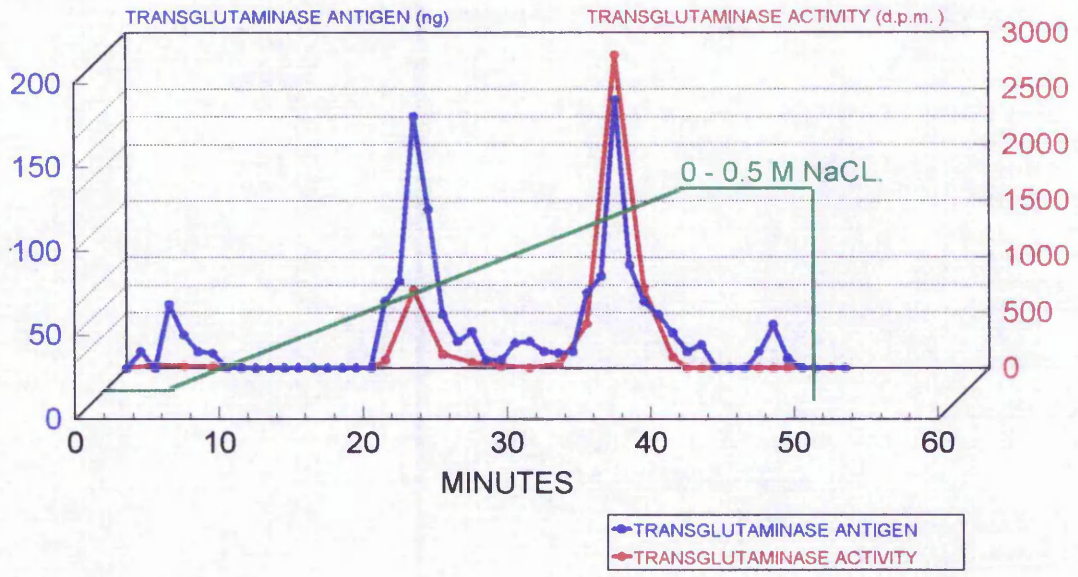
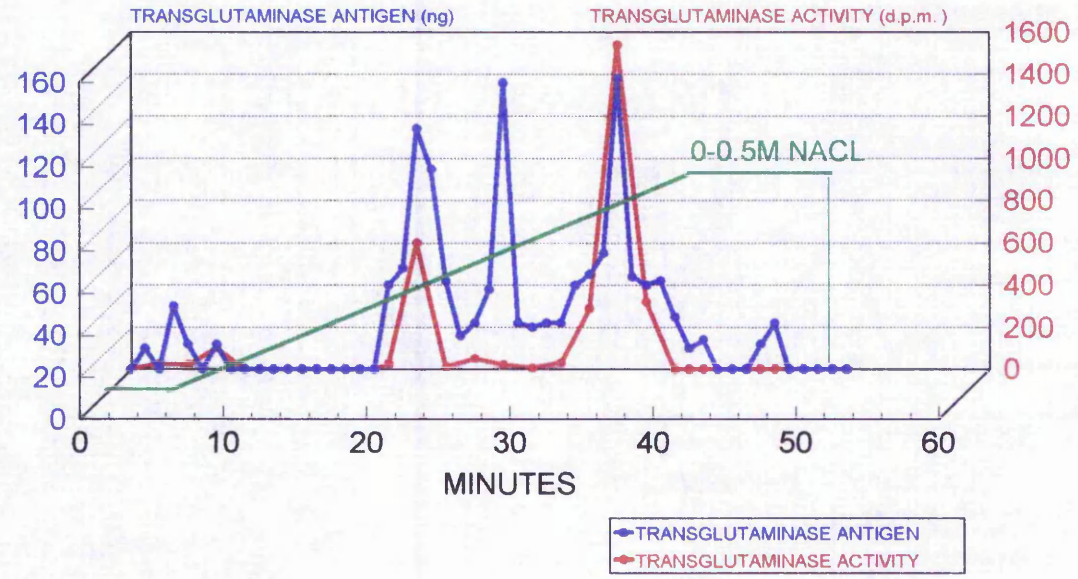


Figure 4.2.2.



4.3.: Search for an inactive transglutaminase mRNA

In order to undertake these experiments a suitable cDNA probe was required that was capable of hybridising with the mRNA from hamster. The ideal probe would have been a sequence from the hamster tissue transglutaminase since investigations were being undertaken with a hamster fibrosarcoma. Unfortunately the transglutaminase gene from hamster had not been isolated and no sequence data was available, a compromise had to be reached. The next best candidate was the use of a cDNA from a closely related species. For this reason the probe finally chosen was the complete cDNA of the mouse tissue transglutaminase. In choosing this as the probe it was necessary to make two assumptions. Firstly it was necessary to assume that mouse transglutaminase cDNA is of sufficient homology to that of the hamster to bind to the hamster mRNA. This would seem reasonable since the homology between the coding regions of transglutaminase from all mammalian species isolated so far is over 80% and since mouse and hamster are related species then the homology is likely to be greater. Secondly that the cytosolic tissue transglutaminase cDNA has sufficient regions of homology to the inactive transglutaminase DNA, to allow hybridisation to the mRNA of the inactive enzyme. This is a reasonable assumption since both proteins are immunoreactive with antibodies raised to the cytosolic transglutaminase and further partial proteolysis of the inactive leads to an active transglutaminase (Knight et al 1990a,d) indicating that the inactive contains a section of 'normal' transglutaminase protein. The decision to use the entire sequence was taken to maximise the chance of homology between the target mRNA and sequences of a random primed probe.

Total RNA was first extracted using a caesium chloride gradient and then the semi purified RNA was separated using agarose borate gel electrophoresis. After Northern blotting the nylon blots were probed with a mouse transglutaminase cDNA probe and after drying exposed to Kodak X OMAT RP film for 12 days. Examination of the autoradiograph (Figure 4.3.1.) shows that no bands are visible

in the total RNA extracted from Met B including bands around 3.5 kb that should code for normal cytosolic transglutaminase. Since the probe recognised the human mRNA transglutaminase standard obtained from a cell line (Met B) that had been transiently transfected with the cDNA of human tissue cytosolic transglutaminase (methods section 3.1.3.) this validated the technique. Furthermore as the agarose gel showed no signs of degradation, it was therefore concluded that the transglutaminase mRNA was present in such small amounts to be beyond the sensitivity of this method. It was evident that there were two problems. In order to increase the transglutaminase message more RNA needed to be loaded on to the gel, however with loadings of over 30 μg separation of the RNA was affected during electrophoresis. Loading more RNA would also accentuate the problem, since the phenomenon of 'blinding' would be increased where the signal of interest is masked from the probe by other sequences. Experiments were therefore undertaken to isolate mRNA rather than total RNA. By doing this the signal could be amplified at least 10 fold by removing ribosomal RNA which accounts for approximately 90% of the total RNA.

Using the Invitrogen mRNA fast track kit (methods section 3.3.2.3.) mRNA was isolated and separated by agarose borate gel electrophoresis, capillary blotted and hybridised to a mouse cDNA probe in the same manner as previously outlined. Using this methodology it was found that the mouse transglutaminase cDNA probe bound to a single band on the extracted mRNA which corresponds to a message of around 3.5 kb., that was comparable to the size of cytosolic tissue transglutaminase mRNA standard (figure 4.3.2.). No bands were visible that might represent a mRNA of a greater size that may be representative of the inactive transglutaminase. Since the cytosolic transglutaminase mRNA band is visible, then if a second inactive transglutaminase mRNA existed it is likely that it would have been detected given the comparable antigen levels in the cell.

Figure 4.3.1.: Northern blot of total RNA from Met B cells, separated on a 1%(w/v) Agarose gel and probed with a mouse transglutaminase cDNA.

Total RNA was extracted from two, 90% confluent Petri dishes of Met B cells using a guanidium isothiocyanate / caesium chloride gradient method as described in methods section 3.3.2.1.. The extracted RNA was electrophoresed on a agarose / borate / formaldehyde gel, stained with ethidium bromide and viewed under UV to verify loading and sample degradation and subsequently capillary blotted on to hybond N (Amersham Ltd) as detailed in methods sections 3.3.2.4. and 3.3.2.5.. The Northern blot was then probed using a mouse cytosolic transglutaminase cDNA labelled with ^{32}P using the Amersham multi prime system (methods sections 3.3.2.6 and 3.3.2.7.). The hybridised blot was dried and exposed to Kodak X OMAT RP film for 12 days using intensifying screens. Lane 1 = cytosolic transglutaminase mRNA standard (1 μg), Lane 2 = total RNA extract from Met B cells (30μg).

Figure 4.3.1.

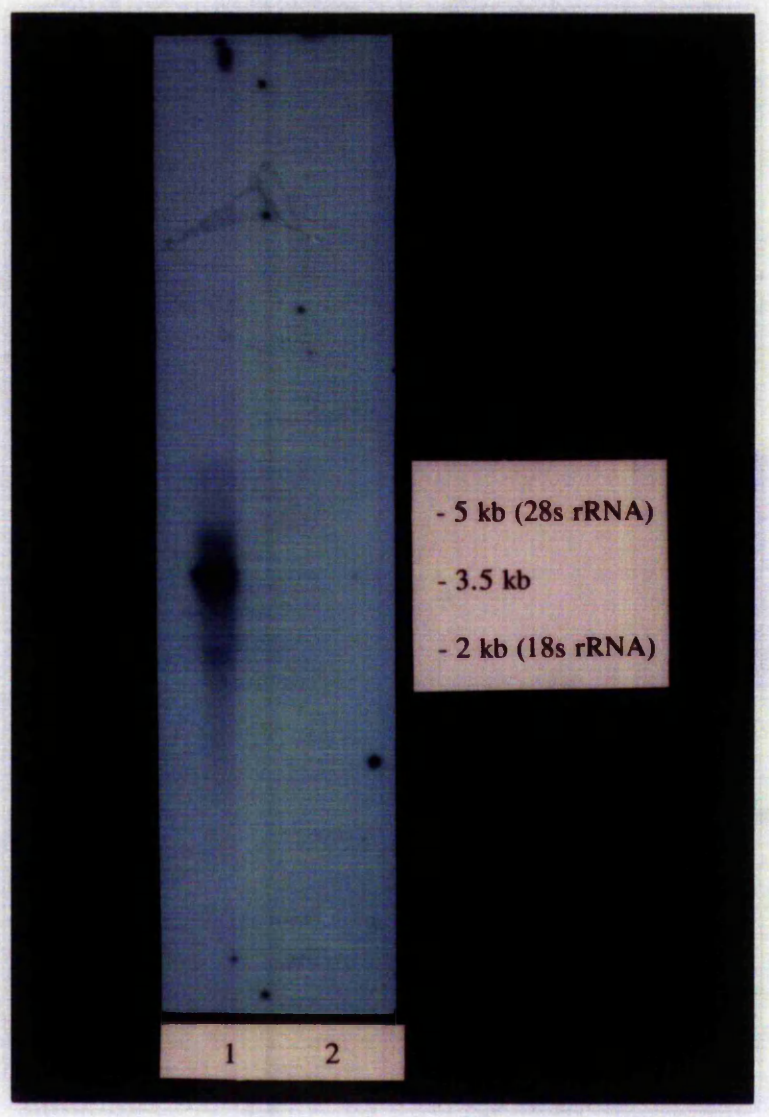
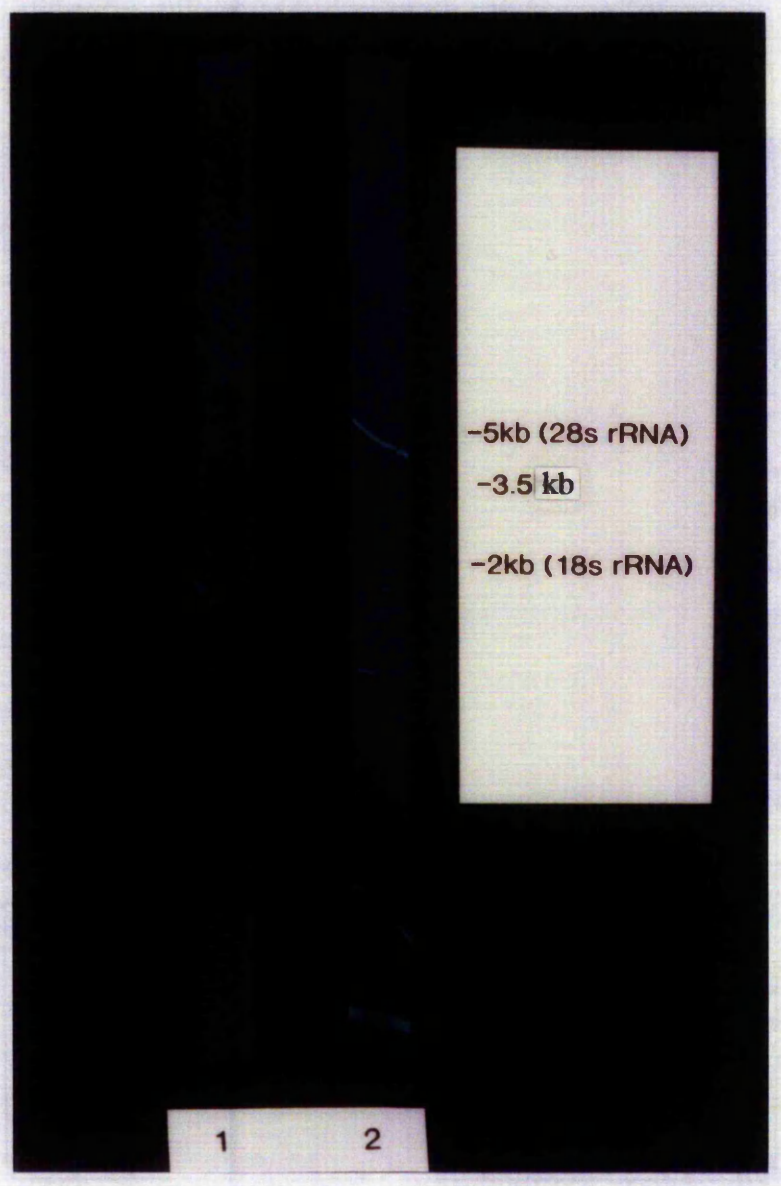


Figure 4.3.2: Northern blot of mRNA separated on a 1%(w/v) Agarose gel and probed with mouse transglutaminase cDNA.

Messenger RNA was extracted from two, 90% confluent Petri dishes of Met B cells using the Invitrogen mRNA micro fast track kit as described in methods section 3.3.2.3.. The extracted mRNA was electrophoresed on a agarose / borate / formaldehyde gel, stained with ethidium bromide and viewed under UV to verify loading and sample degradation and subsequently capillary blotted on to Hybond N (Amersham Ltd) as detailed in methods sections 3.3.2.4. and 3.3.2.5.. The Northern blot was then probed using a mouse cytosolic transglutaminase cDNA labelled with ^{32}P using the Amersham multi prime system (Methods sections 3.3.2.6. and 3.3.2.7.). The hybridised blot was dried and exposed to Kodak X OMAT RP film for 12 days using intensifying screens. Lane 1 = cytosolic transglutaminase mRNA standard (1μg), Lane 2 = mRNA extract from Met B cells (4 μg).

Figure 4.3.2.



4.4. Discussion

The mouse transglutaminase cDNA probe was found to hybridise to a single band on the extracted mRNA which corresponds to a message of approximately 3.5 kb, which is the approximate size of cytosolic tissue transglutaminase mRNA (Gentile et al 1991) (figure 4.3.2.). This band also corresponds to that seen with the tissue transglutaminase mRNA standard obtained from transiently transfected cells. No bands were detected that were indicative of a larger mRNA that might be representative of the inactive transglutaminase which has a molecular weight of 120 kDa. It is therefore likely that the inactive tissue transglutaminase results from an event occurring at or after translation.

CHAPTER 5

THE EFFECTS OF PHARMACOLOGICAL AGENTS ON TRANSGLUTAMINASE AND APOPTOSIS IN THE HIGHLY MALIGNANT HAMSTER FIBROSARCOMA MET CELL LINES AND THE 'NORMAL' FIBROBLAST CELL LINE BHK-21.

5.1: Introduction

Earlier studies by Knight et al (1990a,d) indicated an inverse relationship between transglutaminase activity and detergent insoluble apoptotic body formation. In a series of Met hamster fibrosarcoma cell lines, it was suggested by these authors that the production of the inactive transglutaminase protein in host cells may be one of the reasons for the reduced incidence of apoptotic body formation in the malignant cell.

Having shown the existence of an inactive transglutaminase that could account for the reduction in transglutaminase activity seen in the hamster fibrosarcoma Met B, further investigations were directed towards increasing the level of transglutaminase activity in these cells. If transglutaminase is a key player in the apoptotic program then increasing its activity should affect the cells ability to enter apoptosis. This may in turn affect the malignant state of a tumour cell. The easiest way to modulate transglutaminase activity is pharmacologicaly.

5.2.: The effects of all-*trans* retinoic acid on the expression of transglutaminase and transglutaminase mediated apoptotic body formation.

5.2.1: Transglutaminase expression

All-*trans* retinoic acid has been reported as an inducer of transglutaminase activity by a number of groups on a variety of cell lines (Gentile et al 1989, Davies et al 1990). It has been also reported as a treatment for promyelocytic leukaemia (Castaigne et al 1990). On this bases it was decided to see if this would modulate the activity of transglutaminase in the MET cell lines where active transglutaminase is in part thought to be lost due to the expression of the inactive form.

Transglutaminase activity

When transglutaminase activity is measured in cell homogenates of Met B by the incorporation of radiolabelled putrescine into N,N'-dimethylcasein (methods section 3.4.3.1), after 15 hours exposure of cells to all-*trans* retinoic acid (methods section 3.1.2) this drug causes a 6 fold increase in transglutaminase activity, which restores it to a level comparable to that seen in the 'normal' cell line BHK (figure 5.2.1.). All-*trans* retinoic acid treatment of BHK causes a smaller increase in transglutaminase activity when expressed as a percentage, leading to an increase of approximately 40%.

Transglutaminase antigen

When a Western blot of cell homogenates from cultures of Met B grown with and without the presence of all-*trans* retinoic acid was immunoprobed with a goat polyclonal antibody raised to the guinea pig liver transglutaminase then it can be seen that this increase in activity was due to a raised level of antigen which corresponded to the internal standard of the guinea pig liver enzyme (figure 5.2.2.).

Transglutaminase mRNA

Having ascertained that the increase in transglutaminase activity from all-*trans* retinoic acid treatment is a result of an increase in transglutaminase antigen, the question remained as to whether all-*trans* retinoic acid causes an increase in activity and antigen by post translational modification (possibly by activation of the inactive protein) or by *de novo* synthesis of a new protein. Work by Knight et al (1990a,d) suggested that the increase may be from synthesis of new protein as the separation of cell homogenates on the Mono Q column from cells treated with all-*trans* retinoic acid showed no alteration to levels of the inactive transglutaminase which could account for an increase in the levels of the cytosolic tissue

transglutaminase. To answer this question completely, it was necessary to investigate if there was an increase in the production of transglutaminase mRNA.

An autoradiograph of a Northern blot of total RNA extracted from Met B cells grown with and without all-*trans* retinoic acid is shown in figure 5.2.3. After probing with a mouse cytosolic transglutaminase cDNA, the presence of a mRNA band corresponding to around 3.5 kb mRNA indicates that the increase in transglutaminase activity and protein was dependent on the synthesis of new mRNA. Unfortunately, the non all-*trans* retinoic acid treated sample did not have sufficient transglutaminase mRNA to form a band. The experiment was repeated using a mRNA extraction (Micro Fast Track, Invitrogen, methods section 3.3.2.3.). The resulting autoradiograph of a Northern blot (figure 5.2.4.) shows a weak band in the non all-*trans* retinoic acid treated sample with a much stronger band in the retinoic treated sample. The presence of a band in the non treated sample allowed densitometry studies to be performed which indicated a 3-4 fold increase in transglutaminase mRNA production following all-*trans* retinoic acid treatment.

To see if this was a phenomenon unique to the Met lines the extraction of mRNA was repeated in a completely different cell line using the same methods as for Met B. The cell line K562 was selected for this experiment. In this cell line Northern blotting indicated the presence of a weak band for the non all-*trans* retinoic acid treated K562 cells, with a much stronger band present around 3.5 kb in the all-*trans* retinoic acid treated cell extracts (figure 5.2.5). Densitometry studies of the autoradiographs indicate a 3 fold increase in transglutaminase mRNA. It can be concluded therefore that all-*trans* retinoic acid results in a mRNA synthesis dependent increase in transglutaminase activity that is not unique to hamster fibroblast.

Figure 5.2.1: The effect of 1 μM all-*trans* retinoic acid (RA) for 15 hours on transglutaminase activity in Met B and the normal cell line BHK.

A semi confluent plate of either Met B or BHK-21 cells was exposed to 1μM all-*trans* retinoic acid as outlined in methods, section 3.1.2.. Cells were harvested using trypsin and cell number determined using a haemocytometer. Transglutaminase activity was measured in cell homogenates by the incorporation of ^{14}C putrescine into N,N' dimethylcasein (methods, section 3.4.3.1.). Data represents mean transglutaminase activity $\pm$ S.E.M. from 5 or more experiments.

Figure 5.2.1

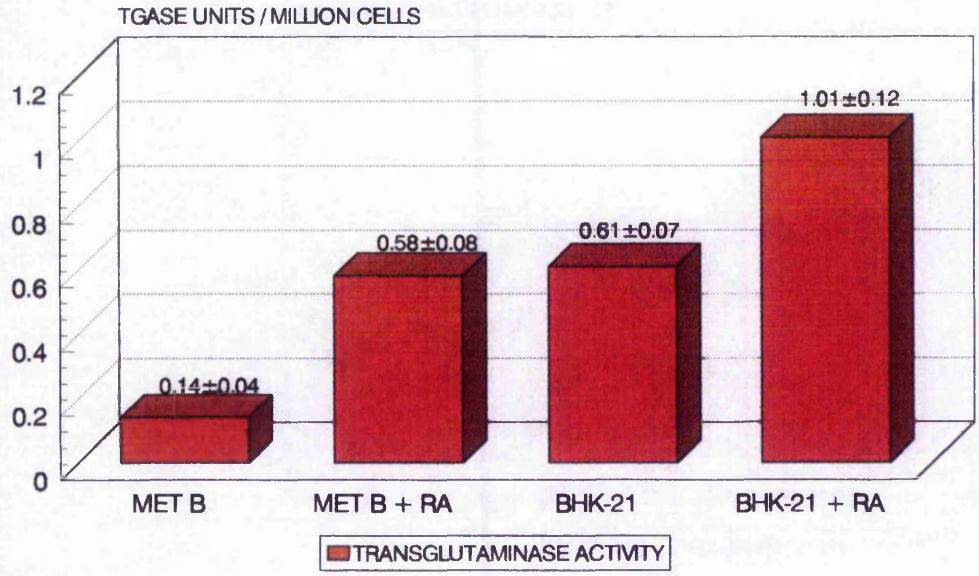

TGASE UNITS / MILLION CELLS
1.2
1
0.8
0.6
0.4
0.2
0
0.14±0.04
0.58±0.08
0.61±0.07
1.01±0.12
MET B
MET B + RA
BHK-21
BHK-21 + RA
TRANSGLUTAMINASE ACTIVITY

Figure 5.2.2.: Western blot of cell homogenate from Met B cells treated with 1 μM all-*trans* retinoic acid for 15 hours and probed for cytosolic transglutaminase.

A semi confluent plate of Met B cells was exposed to 1μM all-*trans* retinoic acid as outlined in methods, section 3.2.1.. Cells were harvested using trypsin and cell number determined using a haemocytometer before being homogenised. A volume equivalent to 1 million cells (20 μl) was loaded onto a 10% (w/v) polyacrylamide gel, electrophoresed and electroblotted on to Hybond C super nylon as described in the methods sections 3.4.1..1 and 3.4.2.1.. The Western blot was immunoprobed with goat 202 anti guinea pig transglutaminase antibody (methods, section 3.4.2.2.). Lane 1 = Guinea pig liver cytosolic transglutaminase standard, Lane 2 = Met B, Lane 3 = Met B exposed to 1 μM all-*trans* retinoic acid for 15 hours.

Figure 5.2.2:

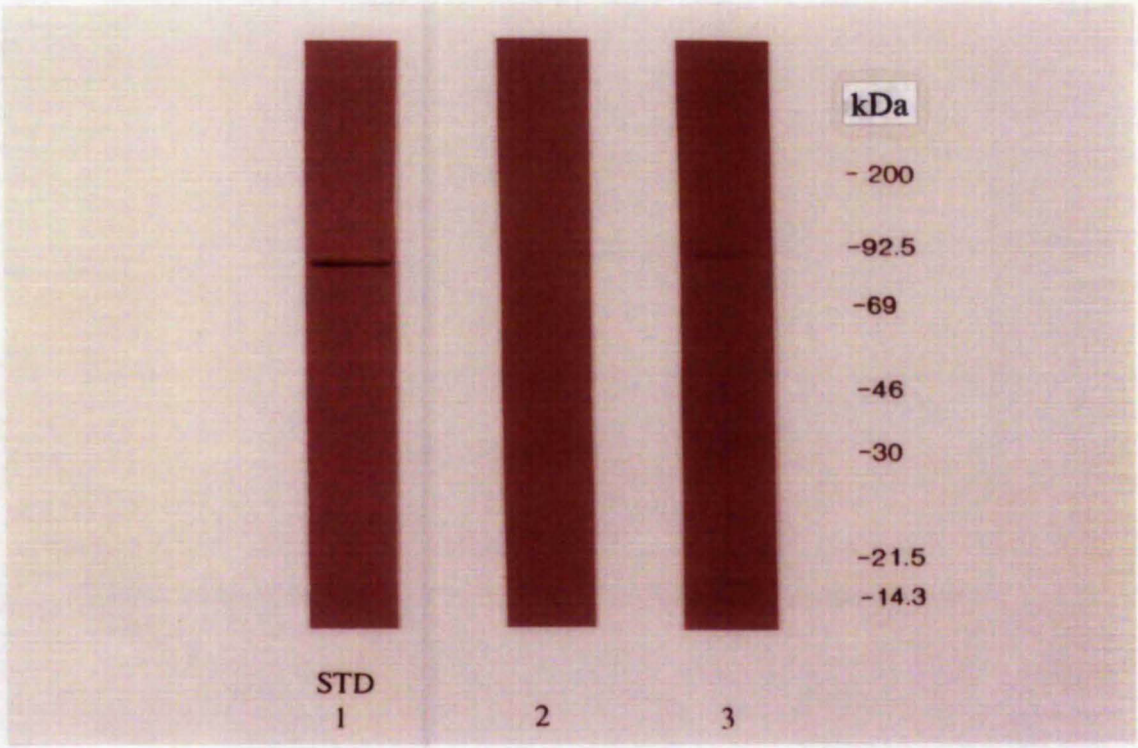


Figure 5.2.3: Northern blot of total RNA extracted from Met B cells treated with 1μM all-*trans* retinoic acid separated on a 1% (w/v) agarose borate gel and probed with mouse transglutaminase cDNA labelled with ^{32}P.

Total RNA was extracted from a 90% confluent 75 cm^2 flask of Met B cells using a guanidium isothiocyanate / caesium chloride gradient method as described in methods section 3.3.2.1.. The extracted RNA was electrophoresed on a agarose / borate / formaldehyde gel, stained with ethidium bromide and viewed under UV to verify loading and sample degradation and subsequently capillary blotted on to hybond N (Amersham Ltd) as detailed in methods sections 3.3.2.4. and 3.3.2.5.. The Northern blot was then probed using a mouse cytosolic transglutaminase cDNA labelled with ^{32}P using the Amersham 'multi-prime' system (methods sections 3.3.2.6. and 3.3.2.7.). The hybridised blot was dried and exposed to Kodak X OMAT RP film for 12 days using intensifying screens. Lane 1 = cytosolic transglutaminase mRNA standard (1μg), Lane 2 = total RNA extract from Met B cells (5 μg), Lane 3 = total RNA extracted from Met B cells treated with 1 μM all-*trans* retinoic acid (5 μg).

Figure 5.2.3.

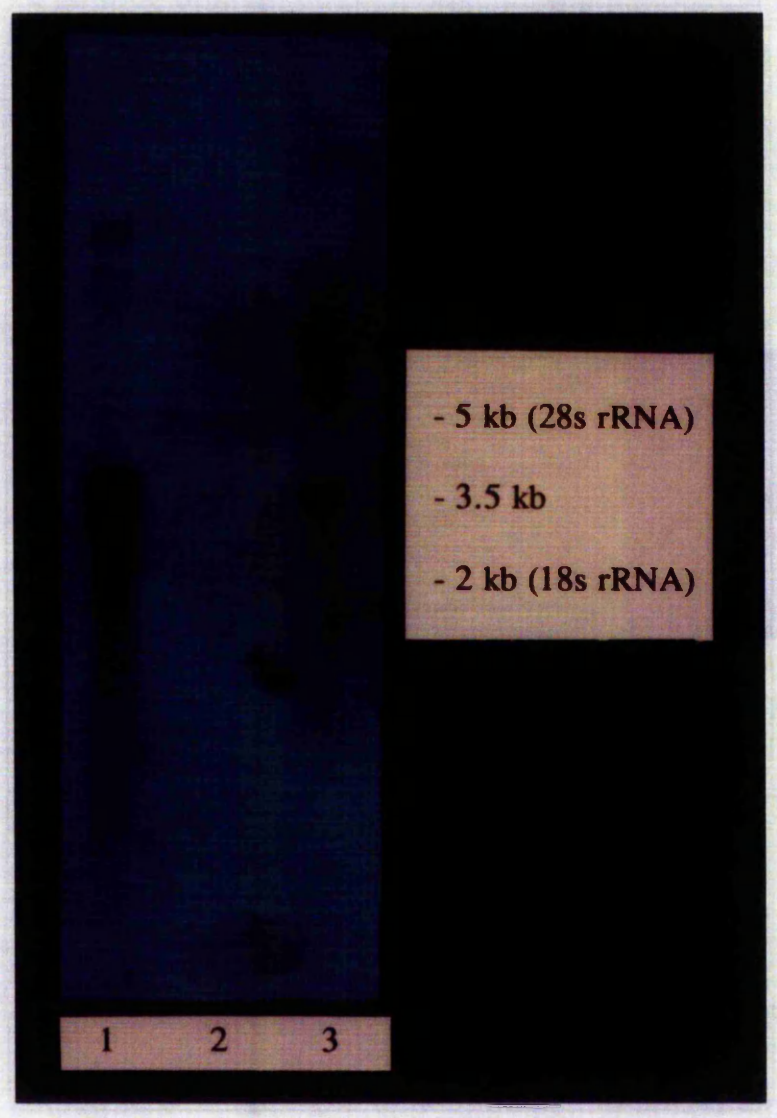


Figure 5.2.4: Northern blot of mRNA extracted from Met B cells treated with 1μM all-*trans* retinoic acid separated on a 1% (w/v) agarose borate gel and probed with mouse transglutaminase cDNA labelled with ^{32}P.

Messenger RNA was extracted from a 90% confluent 75 cm^2 flask of Met B cells using the Invitrogen mRNA micro fast track kit as described in methods sections 3.3.2.3.. The extracted mRNA was electrophoresed on a agarose / borate / formaldehyde gel, stained with ethidium bromide and viewed under UV to verify loading and sample degradation and subsequently capillary blotted on to Hybond N (Amersham Ltd) as detailed in methods sections 3.3.2.4. and 3.3.2.5.. The Northern blot was then probed using a mouse cytosolic transglutaminase cDNA labelled with ^{32}P using the Amersham 'multi-prime' system (methods sections 3.3.2.6. and 3.3.2.7.. The hybridised blot was dried and exposed to Kodak X OMAT RP film for 12 days using intensifying screens. Lane 1 = cytosolic transglutaminase mRNA standard (1 μg), Lane 2 = mRNA extract from Met B cells (4 μg), lane 3 = mRNA extract from Met B cells treated with 1 μM all-*trans* retinoic acid (4 μg).

Figure 5.2.4.

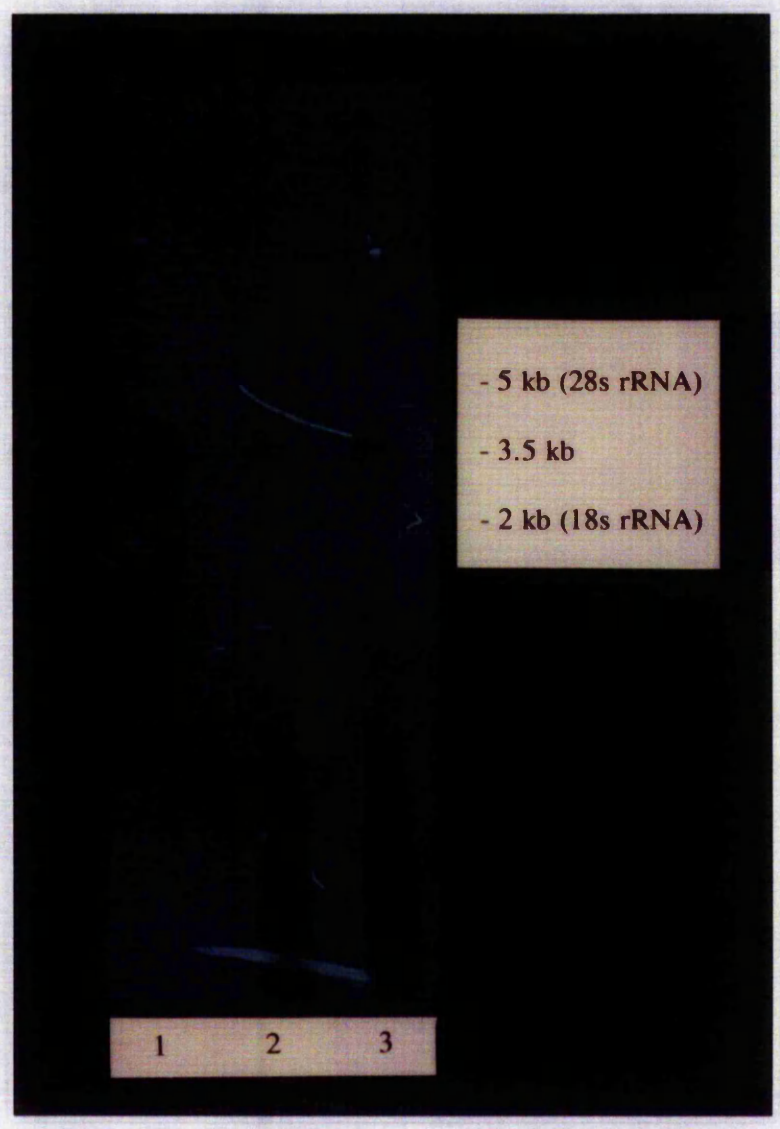
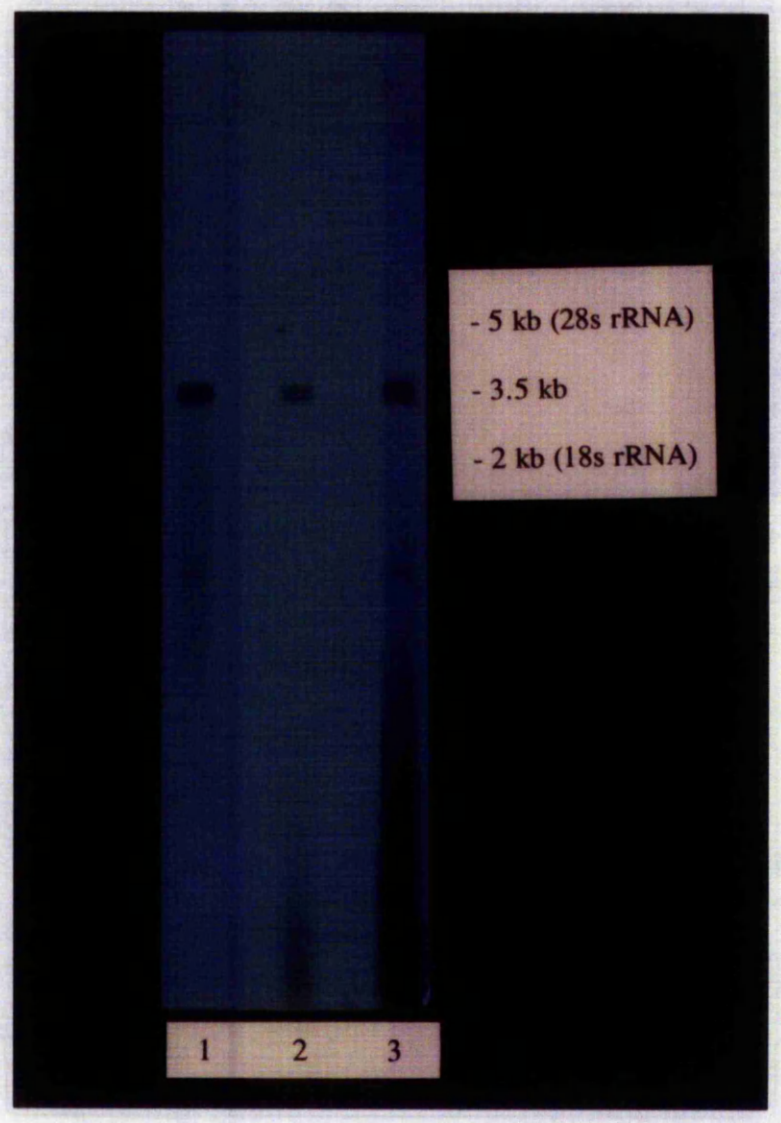


Figure 5.2.5.: Northern blot of mRNA extracted from K562 cells treated with 1μM all-*trans* retinoic acid separated on a 1% (w/v) agarose borate gel and probed with mouse transglutaminase cDNA labelled with ^{32}P.

Messenger RNA was extracted from a 50 ml volume of densely growing K562 cells using the Invitrogen mRNA micro fast track kit as described in methods sections 3.3.2.3. and analysed for transglutaminase mRNA as in figure 5.2.4.. Lane 1 = cytosolic transglutaminase mRNA standard (1 μg), lane 2= mRNA extracted from K562 cells (4 μg), lane 3 = mRNA extracted from K562 cells treated with all-*trans* retinoic acid (4μg).

Figure 5.2.5.



5.2.2: Apoptosis (apoptotic body formation)

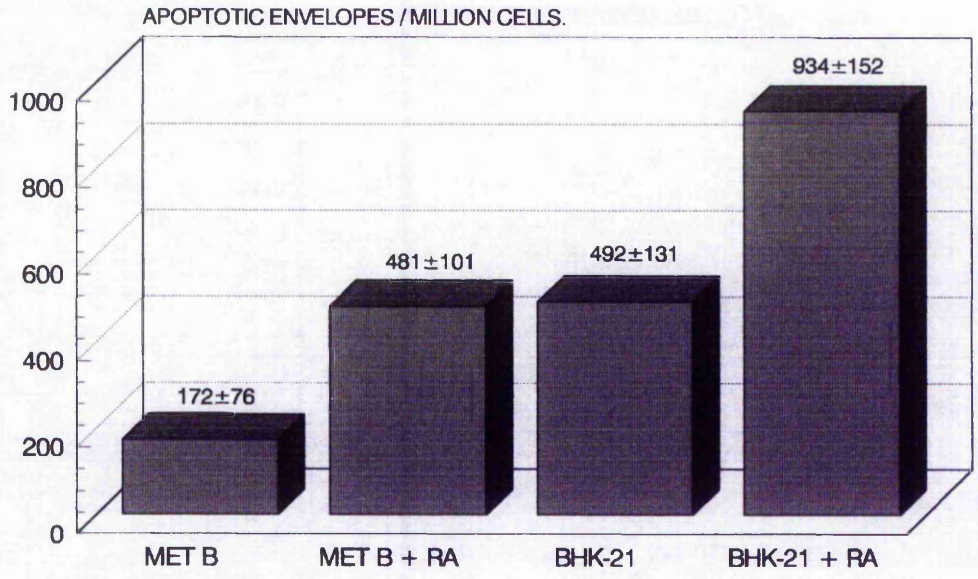
Having shown that transglutaminase increases and how this increase occurs in the presence of retinoids, it was necessary to see if this increase in transglutaminase was accompanied by any cytosolic transglutaminase mediated variation in apoptotic body formation. Figure 5.2.6. shows the apoptotic index in Met B and BHK-21 as determined by the presence of detergent insoluble apoptotic envelopes in populations treated with and without 1μM all-*trans* retinoic acid for 24 hours. In Met B a 2.8 fold rise from 172 to 481 envelopes per million cells was observed. In the 'normal' BHK-21 cell line a smaller increase in percentage terms of 1.9 fold was observed (469 to 891 envelopes per million cells). Both groups showed a statistically significant increase ($p \leq 0.05$). The rise in transglutaminase is similar to the rise in apoptotic index when calculated in percentage terms. This suggests that there may be a relationship between increases in transglutaminase activity and apoptosis when measured by detergent insoluble body formation.

Although all-*trans* retinoic acid treatment shows an increase in transglutaminase activity and shows a concomitant increase in apoptosis, it is still not established whether this increase in transglutaminase activity is a symptom rather than the cause of the apoptotic event. These two events may not even be related. To demonstrate a direct effect of transglutaminase on the amount of apoptosis present in a cell line it is necessary to cause a specific increase the expression of enzyme. This is unlikely with the use of pharmacological agents which are likely to cause increased expression of other proteins in addition to that of transglutaminase. One way to do this is by inserting the full cDNA of tissue transglutaminase into a cell with its own independent promoter.

Figure 5.2.6.: The effect of 1μM all-*trans* retinoic acid for 15 hours on the apoptotic index of the hamster fibrosarcoma Met B and the normal hamster fibroblast cell line BHK-21.

Met B cells were treated for 15 hours with 1 μM all-*trans* retinoic acid as described in methods section 3.1.2.. Cells were recovered from both the medium and attached to the plate using trypsin and pooled. Total cell number was determined using a haemocytometer and detergent insoluble bodies isolated by boiling the cells in the presence of SDS as detailed in methods section 3.4.4.1.. Detergent insoluble bodies were counted using both a haemocytometer and number of bodies per μl of solution and the results averaged. Counting was performed by at least 3 workers who were unaware of the experimental groups. Data represents number of apoptotic bodies per 1 x 10^6 cells $\pm$ S.E.M. from 5 or more experiments.

Figure 5.2.6.


APOPTOTIC ENVELOPES / MILLION CELLS.
1000
800
600
400
200
0
172±76
481±101
492±131
934±152
MET B
MET B + RA
BHK-21
BHK-21 + RA

5.3.: The effect of dexamethasone on the expression of transglutaminase and transglutaminase mediated apoptotic body formation.

Initial studies to try and specifically increase transglutaminase activity by insertion of the cDNA centred on the use of the inducible vector pMAMneo carrying the transglutaminase cDNA in the multiple cloning site of this vector (methods section 3.3.3.4.). The MMTV promoter sequence controlling the expression of the inserted cDNA can be induced by heavy metals or glucocorticoids such as dexamethasone. Dexamethasone is a man made glucocorticoid that shows high activity and is more resistant to breakdown in tissue culture due to the reorganisation of its hydroxyl groups and addition of a fluoride atom. When dexamethasone was incubated with non transfected Met B control cells it was noted that there was an increase in transglutaminase activity.

Dexamethasone has been reported as an inducer of apoptosis in lymphoid cells, in particular the immature lymphocytes of the thymus gland (thymocytes) by a number of authors (Wyllie 1980, McConkey et al 1989, Compton and Cidlowski 1992), but non of these make a reference to concomitant changes in transglutaminase activity and there are no reported cases of dexamethasone inducing apoptosis outside lymphoid tissue. An extensive literature search revealed a small amount of work by Goldman et al (1987) where transglutaminase activity changes had occurred in bone marrow derived mononuclear phagocytes, macrophage like cell lines and human myeloid leukaemia cell lines as a result of dexamethasone treatment. This new observation of transglutaminase induction by glucocorticoid in fibroblasts was therefore studied in greater depth particularly as it provided a second pharmacological stimulator of transglutaminase in addition to retinoids.

5.3.1.: Transglutaminase expression

Transglutaminase activity

Dexamethasone treatment (1 μM) of cell lines for 24 hours causes increases in transglutaminase activity of 8.5 fold in Met B, 4.4 fold in Met D, 2.2 fold in Met E, 3.56 fold in BHK-21 and 1.65 fold in B16 (figure 5.3.1.). In all these cases the increase in transglutaminase activity was highly significant at $p \leq 0.01$ except for the B16 cells where $p \leq 0.05$ In the cell lines Met B and BHK-21 dose response experiments were performed indicating a dose response relationship (figure 5.3.2). To determine if there was an increase in the level of transglutaminase antigen with activity, immunoprobing of Western blots was under taken using antiserum to the tissue transglutaminase from guinea pig liver

Transglutaminase antigen

The immunoprobed Western blot shown in figure 5.3.3. demonstrates an increase in transglutaminase antigen following dexamethasone treatment in all cell lines tested with the exception of B16. The lack of a transglutaminase band in B16 may be due to the increase being so small the transglutaminase level is not detectable by this method (technique sensitive to approximately 5 to 10 ng). This would be supported by the activity results in B16 which show a very low basal transglutaminase level and is only increased moderately with dexamethasone. The Western blot suggests that dexamethasone is causing an increase in protein synthesis, however it is still possible that there is activation of an existing protein that the antibody is unable to recognise.

Transglutaminase mRNA

As with the all-*trans* retinoic acid study (section 5.2) it is important to determine if there is an increase in transglutaminase mRNA levels. Initial studies for detection of transglutaminase mRNA were carried out using a total RNA extraction by caesium chloride gradient and separation of mRNA on an agarose borate and then probing with a mouse transglutaminase cDNA. Using this method (figure 5.3.4.), there was no detectable transglutaminase mRNA in either the untreated or dexamethasone treated samples. The inability to detect transglutaminase mRNA in a total RNA extraction from untreated samples was not surprising, but in the inability to detect transglutaminase mRNA in dexamethasone treated sample was a surprise since in the all-*trans* retinoic acid treated sample (section 5.2) where the increase in activity and antigen were lower than seen with dexamethasone, an increase in mRNA was easily detectable in a total RNA extraction. This indicated that dexamethasone could be acting in one of 3 ways. Dexamethasone could be increasing transglutaminase antigen and activity directly by activating existing transglutaminase protein, which seemed unlikely due to the method of action of glucocorticoids (Cohn and Duke 1984). Alternatively, dexamethasone could be increasing mRNA synthesis of a protein that may activate existing inactive or active transglutaminase antigen or that dexamethasone was causing a very small increase in transglutaminase mRNA which is not detected by the methods used and at the same time facilitating increased translation of this mRNA.

To determine one or other possibility, rather than total RNA, mRNA was extracted from treated cells using the Pharmacia mRNA extraction kit, separated by agarose gel electrophoresis and probed with a human cDNA probe. The autoradiograph resulting from this can be seen in figure 5.3.5.. This confirms that there is no increase in transglutaminase mRNA following dexamethasone treatment and thus dexamethasone dose not act directly on transglutaminase transcription.

Figure 5.3.1.: The effects of 1μM dexamethasone treatment on transglutaminase activity in the Met cell lines, BHK-21 and the malignant melanoma cell line B16.

A 80-90% confluent cell culture in a 75 cm^2 flask were treated for 24 hours with 1μM dexamethasone as outlined in methods section 3.1.2.. The monolayer of cells was then harvested using trypsin and counted on a haemocytometer. $1x10^6$ aliquots of cells were homogenised and the transglutaminase activity determined by the incorporation of ^{14}C labelled putrescine into N,N'-dimethylcasein (methods section 3.4.3.1.). Blue bars indicate basal transglutaminase level, red bars indicate cells treated with dexamethasone. Data represents transglutaminase activity (expressed in units (nmol putrescine incorporated per hour) / million cells) $\pm$ S.E.M. from 8 or more experiments.

Figure 5.3.2.: Dexamethasone dose response in Met B and BHK-21. 48 hours exposure to dexamethasone.

60-70% confluent cell cultures of either Met B or BHK-21 in 75 cm^2 flasks were treated for 48 hours with either 1 nM, 10 nM, 100 nM, 1μM or 10μM of dexamethasone as outlined in methods section 3.1.2.. The monolayer of cells was then harvested using trypsin and counted on a haemocytometer. $1x10^6$ aliquots of cells were homogenised and the transglutaminase activity determined by the incorporation of ^{14}C labelled putrescine into dimethyl casein (methods section 3.4.3.1.). Blue bars indicate transglutaminase level in BHK-21, red bars indicate transglutaminase activity in Met B. Data represents transglutaminase activity (expressed in units (nmol putrescine incorporated per hour / million cells) $\pm$ S.E.M. from 4 experiments.

Figure 5.3.1.

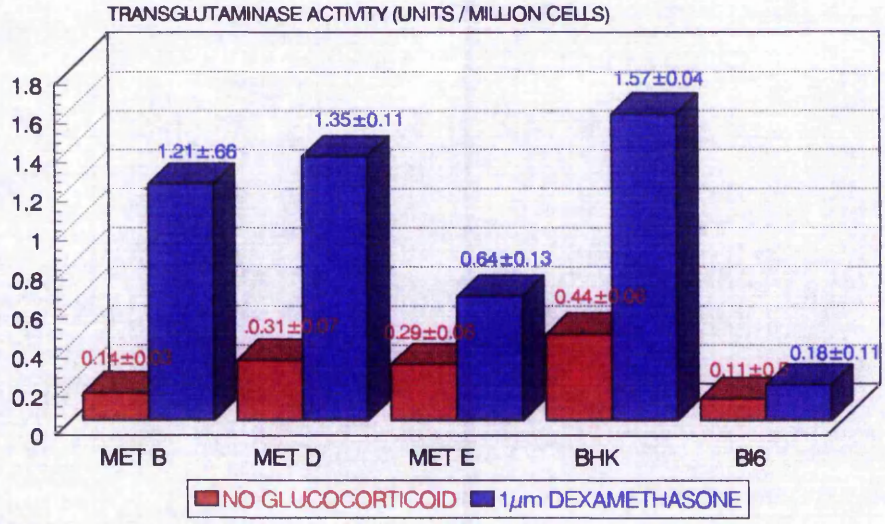


Figure 5.3.2.

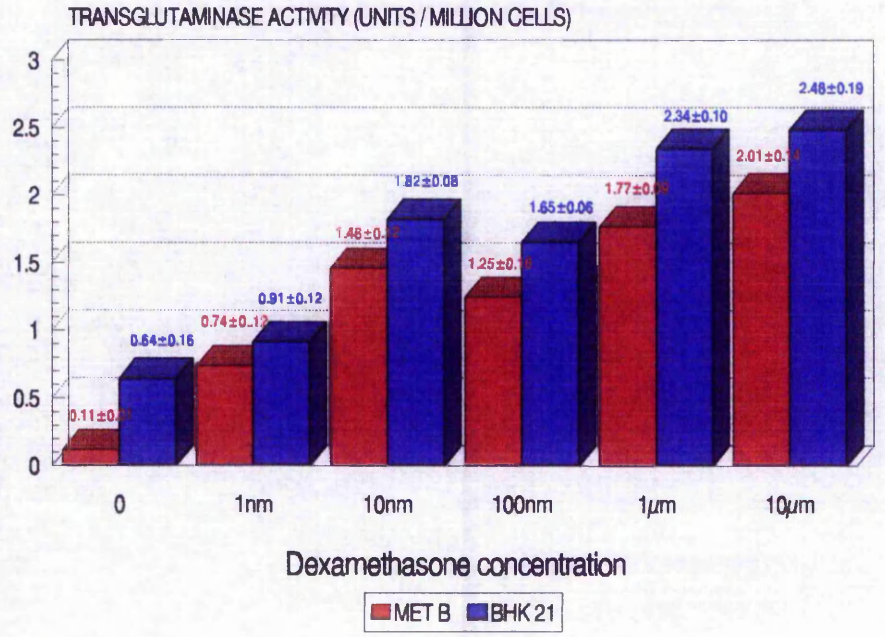


Figure 5.3.3.: Immunoblot blot of cell homogenate from the MET cell lines, BHK and B16 previously treated for 48 hours with 1μM dexamethasone.

A 60-70% confluent 75 cm^2 flask of cells was exposed to 1μM dexamethasone for 48 hours as outlined in methods, section 3.1.2.. Cells were harvested using trypsin and cell number determined using a haemocytometer before being homogenised. A volume equivalent to 1 million cells was loaded onto a 10% (w/v) polyacrylamide gel. Following electrophoresis this was electroblotted on to Hybond C super nylon (Amersham Ltd) as described in methods sections 3.4.1.2. and 3.4.2.1.. The Western blot was immunoprobed with goat 202 anti guinea pig transglutaminase antibody (methods section 3.4.2.2). Lane 1 = Transglutaminase standard (100 ng), lane 2 = Met B, lane 3 = Met D, lane 4 = Met E, lane 5 = BHK-21, lane 6 = B16.

Figure 5.3.3

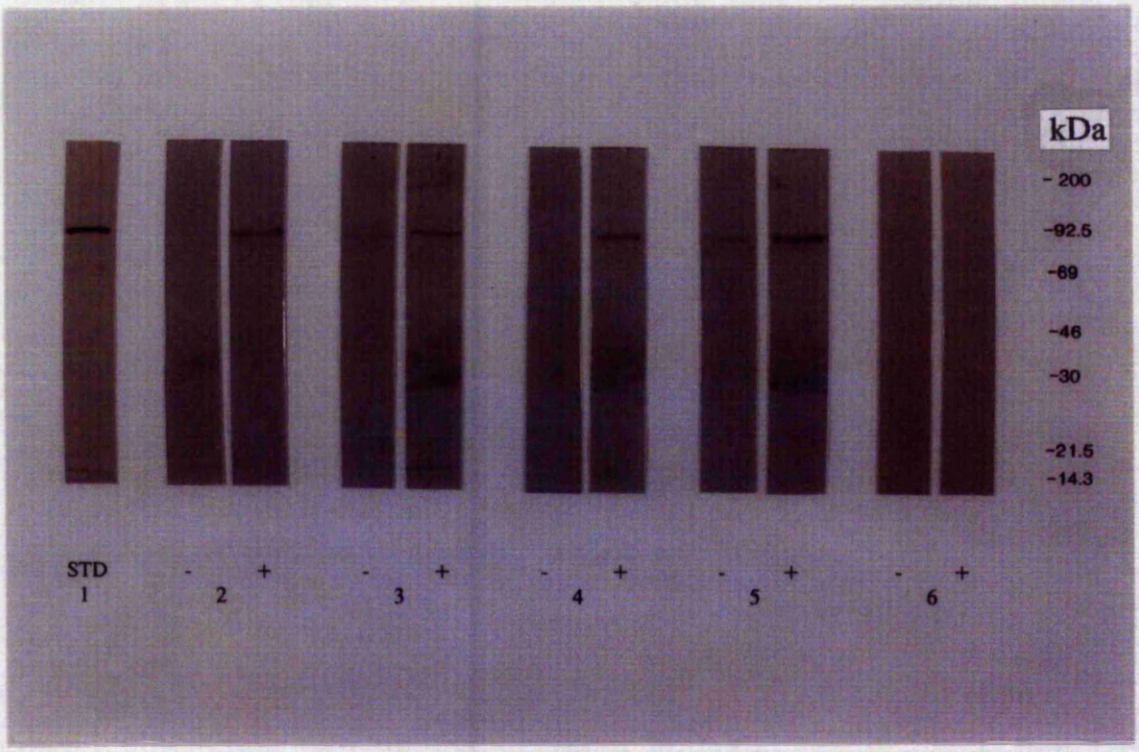


Figure 5.3.4.: Northern blot analysis of total RNA from dexamethasone treated Met B cells

Total RNA was extracted from a 90% confluent 75 cm^2 flask of Met B cells using a guanidium isothiocyanate / caesium chloride gradient method as described in methods section 3.3.2.1.. The extracted RNA was electrophoresed on a agarose / borate / formaldehyde gel, stained with ethidium bromide and viewed under UV to verify loading and sample degradation and subsequently capillary blotted on to Hybond N (Amersham Ltd) as detailed in methods sections 3.3.2.4. and 3.3.2.5.. The Northern blot was then probed using a mouse cytosolic transglutaminase cDNA labelled with ^{32}P using the Amersham multi prime system (methods sections 3.3.2.6. and 3.3.2.7.). The hybridised blot was dried and exposed to Kodak X OMAT RP film for 12 days using intensifying screens. Lane 1 = cytosolic transglutaminase mRNA standard (1 μg), Lane 2 = total RNA extract from Met B cells (30μg), Lane 3 = total RNA extracted from Met B cells treated with 1 μM dexamethasone (30 μg).

Figure 5.3.4.

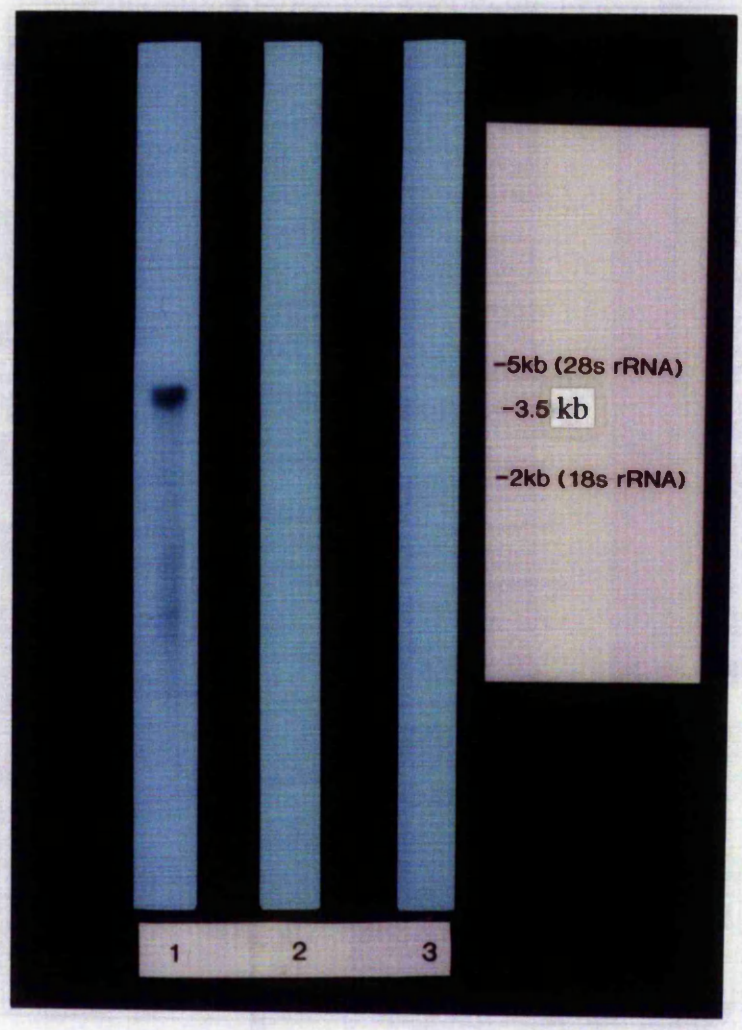
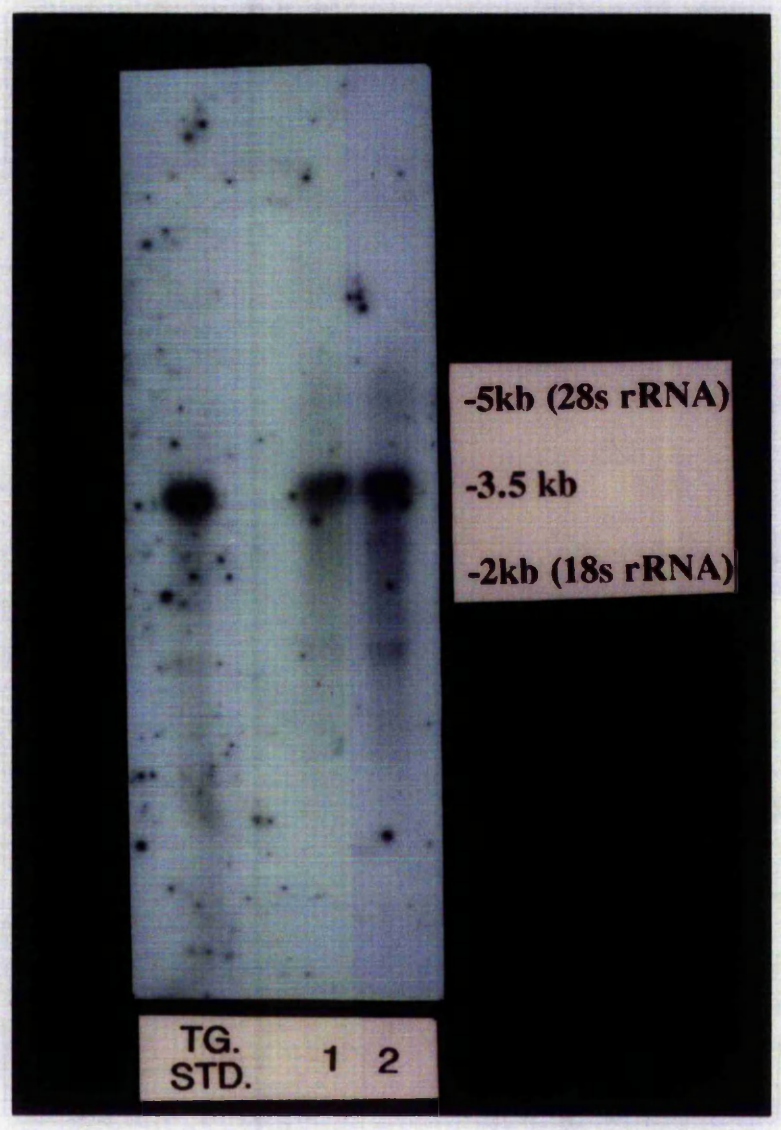


Figure 5.3.5. Northern blot analysis of mRNA from dexamethasone treated Met B cells separated on a MOPS / formaldehyde / agarose gel.

Messenger RNA was extracted from a 90% confluent 75 cm^2 flask of Met B cells using the Pharmacia mRNA kit as described in methods sections 3.3.2.3.. The extracted mRNA was electrophoresed on a agarose / MOPS / formaldehyde gel, stained with ethidium bromide and viewed under UV to verify loading and sample degradation and subsequently capillary blotted on to N^+ Nylon (Boehringer Ltd) as detailed in methods sections 3.3.2.4. and 3.3.2.5.. The Northern blot was then probed using a human cytosolic transglutaminase cDNA (complete sequence) labelled with ^{32}P using the Promega 'random prime' system (methods sections 3.3.2.6. and 3.3.2.7.). The hybridised blot was dried and exposed to Kodak X OMAT RP film for 8 days using intensifying screens. Lane 1 = cytosolic transglutaminase mRNA standard (1 μg), Lane 2 = mRNA extract from Met B cells (5 μg), lane 3 = mRNA extract from Met B cells treated with 1 μM Dexamethasone (5 μg).

Figure 5.3.5.



5.3.2.: Apoptosis (Apoptotic body formation).

In view of the different mechanisms used to increase transglutaminase activity by all-*trans* retinoic acid and dexamethasone, it was essential to see if dexamethasone would have any affect on apoptosis as already observed with all-*trans* retinoic acid (section 5.2). The apoptotic index was determined by counting the number of detergent insoluble apoptotic bodies present in cell cultures and the results of which are shown in figure 5.3.6.. This shows that in all cell lines tested the application of dexamethasone gave rise to a statistically significant ($p \leq 0.05$) rise in apoptosis when determined by apoptotic envelope counts. To see if there was any relationship between the increase in apoptosis and transglutaminase activity the percentage increase in transglutaminase activity was compared to that for apoptosis (figure 5.3.7.).

This shows that there is no direct correlation between the increase in transglutaminase and apoptosis. What it does demonstrate is that the cells with the highest percentage increase in transglutaminase also have the highest percentage increase in apoptosis, but that the increase in apoptosis appears to plateau when a 3 fold increase is reached. This is a similar to that seen with retinoid treatment of Met B (section 5.2).

Figure 5.3.6.: The apoptotic index as determined by detergent insoluble apoptotic envelopes in the Met cell lines. BHK-21 and B16 with and without the presence of 1μM dexamethasone for 24 hours.

Cells were treated for 24 hours with 1 μM dexamethasone as described in methods section 3.1.2.. Cells were recovered from both the medium and attached to the plate using trypsin harvest and pooled. Total cell number was determined using a haemocytometer and detergent insoluble bodies isolated by boiling the cells in the presence of SDS as detailed in methods section 3.4.4.1.. Detergent insoluble bodies were counted using both a haemocytometer and number of bodies per μl of solution and the results averaged. Counting was performed by at least 3 workers who were unaware of the experimental groups. Data represents detergent insoluble bodies / 1 x 10^6 cells $\pm$ S.E.M. from 5 experiments.

Figure 5.3.7.: The percentage increase in transglutaminase activity compared with the percentage increase in apoptotic envelopes following 1μM dexamethasone treatment for 24 hours.

The increases in transglutaminase activity and apoptotic bodies following exposure to dexamethasone were calculated for each cell line from the data in figures 5.3.1. and 5.3.6. respectively

Figure 5.3.6.

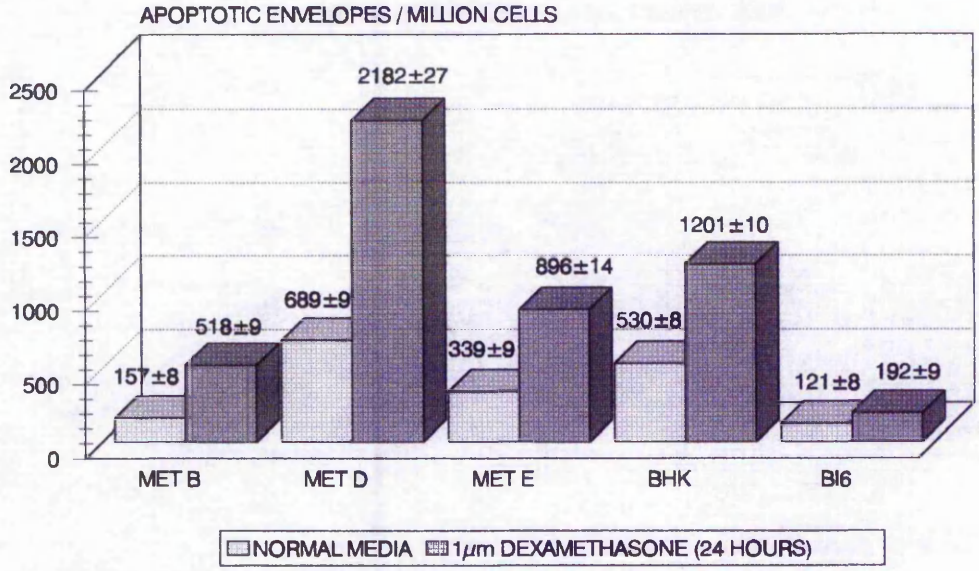
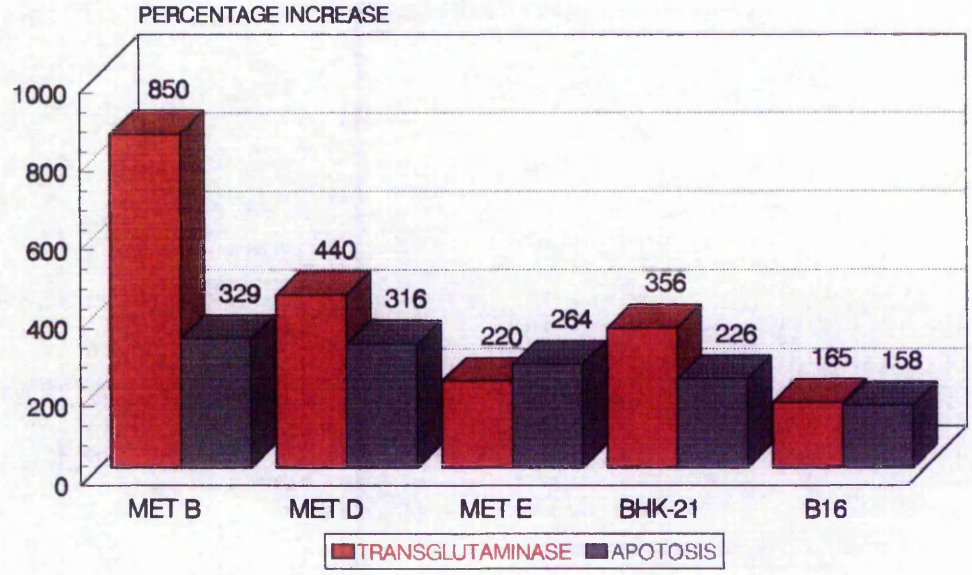


Figure 5.3.7.



5.4.: The relationship between dexamethasone responsive receptors, transglutaminase activity and apoptosis in the Met cell lines, BHK-21 and B16.

Further experiments were designed to ascertain whether the increase in transglutaminase activity was dependent on the presence of glucocorticoid receptors and whether the responses observed were proportional to the number of glucocorticoid receptors a cell contains. To determine this, a reporter construct carrying the glucocorticoid response element MMTV (Mouse Mammary Tumour Virus) positioned up stream to a reporter gene (chloramphenicol acetyl transferase (CAT)) was used. This is shown in methods section 3.3.3.4..

Chloramphenicol acetyl transferase is not found in mammalian cells, but is the product of the chloramphenicol resistance gene found in bacteria where its function is to convert the harmful antibiotic chloramphenicol to harmless mono acetylated and diacetylated derivatives. If the CAT cDNA is attached to the MMTV promoter it can be activated by either heavy metals or the binding of a number of glucocorticoid receptor proteins, hence if this vector is transfected into a mammalian cell then it provides a method of determining the level of glucocorticoid receptor proteins in the cell. When the MMTV promoter is activated by glucocorticoid receptor binding, determining the amount of conversion of chloramphenicol to its acetylated products by a cell homogenate gives a measure of receptor protein levels at a set dexamethasone concentration.

Since five cell lines express differing levels of transglutaminase response to dexamethasone, further experiments were designed to ascertain any relationship between a pMMTV CAT response and the transglutaminase response. A correlation between these two parameters would confirm the link between the expression of transglutaminase activity and the level of glucocorticoid receptors expressed by a cell.

It was also important to assess each cell lines ability to accept DNA by the lipofection transfection method used in these studies (methods section 3.1.3.). This was carried out using the CAT reporter gene, but attached to a constitutive promoter sequence rather than inducible promoter so that any CAT activity would be a direct measurement of the DNA inserted. The CAT was used with the SV40 constitutive promoter sequence inserted into the pSV plasmid (methods section 3.3.3.4.). The results of this experiment are shown in figure 5.4.1.. The results indicate a very low transfection level for B16, while BHK has a much higher percentage acetylation for the same amount of pSV CAT in the transfection mixture, and thus has a higher transfection efficiency. Correspondingly all results obtained from the use of the inducible vector must be corrected for these differences of transfection.

The CAT activity (percent acetylation) occurring after transfection with 10 μ g of pMMTV CAT is shown in figure 5.4.2. with the amount of acetylation corrected for transfection efficiency in each cell line. Although this data cannot be used to give a definitive value for the level of dexamethasone receptor level, what it does indicate is the relative responsive levels of the receptor protein between the different cell lines. This indicates that Met B > Met D > Met E > BHK > B16 level of dexamethasone responsive receptors.

The level of receptor in relation to the increase seen in transglutaminase activity with dexamethasone treatment is compared in figure 5.4.3.. This showed a relationship between the dexamethasone receptor relative level and the percentage increase in transglutaminase activity in each cell line with the cell lines expressing higher levels of dexamethasone receptor having a larger transglutaminase response than those with lower receptor levels. The increase in apoptosis with dexamethasone receptor level is compared in figure 5.4.4. which demonstrates a similar relationship to that found for transglutaminase between the dexamethasone receptor levels and apoptosis.

Figure 5.4.1.: An assessment of transfection efficiency for Met cell lines, BHK-21 and B16 using the transfection vector pSV CAT.

Cell lines were transfected with 10 μg of the expression vector pSVCAT using lipotransfection (methods section 3.1.3.). The constitutive expression of the CAT gene by the SV40 promoter in pSVCAT results in CAT activity being an indirect determination of the level of incorporation of the vector and hence transfection. CAT activity was measured by incubating cell homogenates (from $2x10^6$ cells) with 0.4 μCi of ^{14}C labelled chloramphenicol and acetyl CoA for 1 hour. The acetylated and non acetylated chloramphenicols were then separated by TLC and visualised using autoradiography. Bands were cut from the TLC plates and accurately measured by scintillation counting. CAT activity is expressed as the percentage acetylation occurring in 1 hour per $2x10^6$ cells, calculated by dividing the CPM of the monoacetylated product by the total CPM (acetylated + non acetylated). Methods section 3.4.4.2.. Data represents mean values $\pm$ S.E.M. from 3 experiments.

Figure 5.4.1.

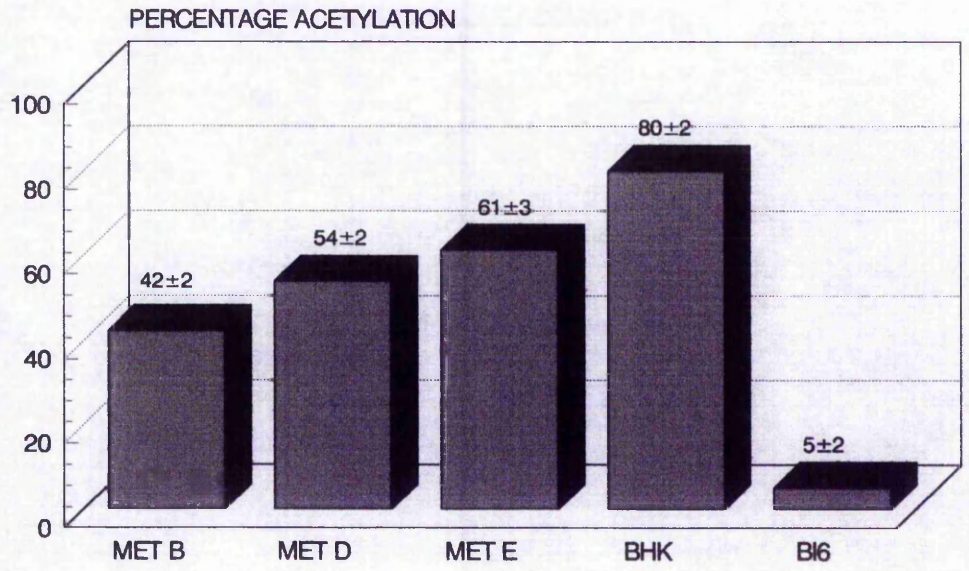

PERCENTAGE ACETYLATION
100
80
60
40
20
0
42±2
54±2
61±3
80±2
5±2
MET B
MET D
MET E
BHK
Bl6

Figure 5.4.2.: The relative levels of Dexamethasone responsive receptor protein in the cell lines Met B. Met D. Met E. BHK. and B16.

Cell lines were transfected with 10 μg of the dexamethasone inducible vector pMAMneo CAT using lipotransfection (methods section 3.1.3.). The induced expression of the CAT gene by the action of the dexamethasone activated glucocorticoid receptor protein on the MMTV promoter in pMAMneo CAT results in CAT activity being an indirect determination of the level of receptor protein available in the cell at a set concentration of dexamethasone. All CAT activities were corrected for transfection efficiency. CAT activity was measured by incubating cell homogenates (from $2x10^6$ cells) with 0.4 μCi of ^{14}C labelled chloramphenicol and acetyl CoA for 1 hour. The acetylated and non acetylated chloramphenicols were then separated by TLC and visualised using autoradiography. Bands were cut from the TLC and accurately measured by scintillation counting. CAT activity is expressed as the percentage acetylation occurring in 1 hour per $2x10^6$ cells, calculated by dividing the CPM of the monoacetylated product by the total CPM (acetylated + non acetylated). Methods section 3.4.4.2.. Cells were exposed to 1 μM dexamethasone for 48 hours on completion of the transfection procedure (methods section 3.1.3.) Data represents mean values $\pm$ S.E.M. corrected for transfection efficiency of each cell line from 3 experiments.

Figure 5.4.2.

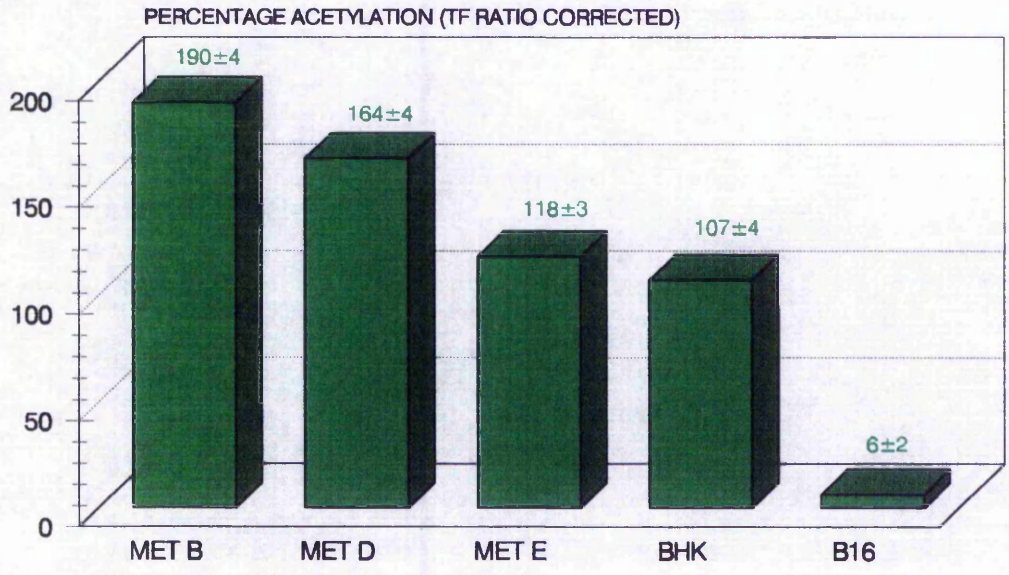

PERCENTAGE ACETYLATION (TF RATIO CORRECTED)
190±4
164±4
118±3
107±4
6±2
200
150
100
50
0
MET B
MET D
MET E
BHK
B16

Figure 5.4.3.: The relative levels of dexamethasone responsive receptor protein in the cell lines Met B, Met D, Met E, BHK, and B16 compared to percentage increase in transglutaminase levels with 1 μM Dexamethasone.

The relative levels of dexamethasone responsive receptor protein as determined in figure 5.4.2. were compared to the increase in transglutaminase activity seen with dexamethasone treatment as calculated in figure 5.3.1.

Figure 5.4.4.: The relative levels of dexamethasone responsive receptor protein in the cell lines Met B, Met D, Met E, BHK, and B16 compared to the percentage increase in apoptosis following 1 μM dexamethasone treatment.

The relative levels of dexamethasone responsive receptor protein as determined in figure 5.4.2. were compared to the increase in apoptosis seen following dexamethasone treatment as calculated in figure 5.3.7.

Figure 5.4.3.

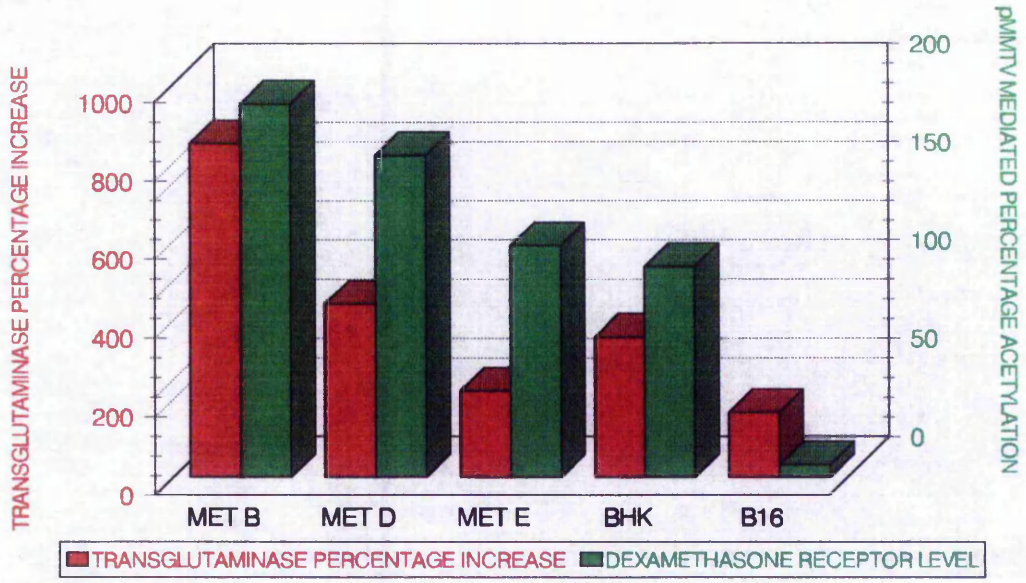
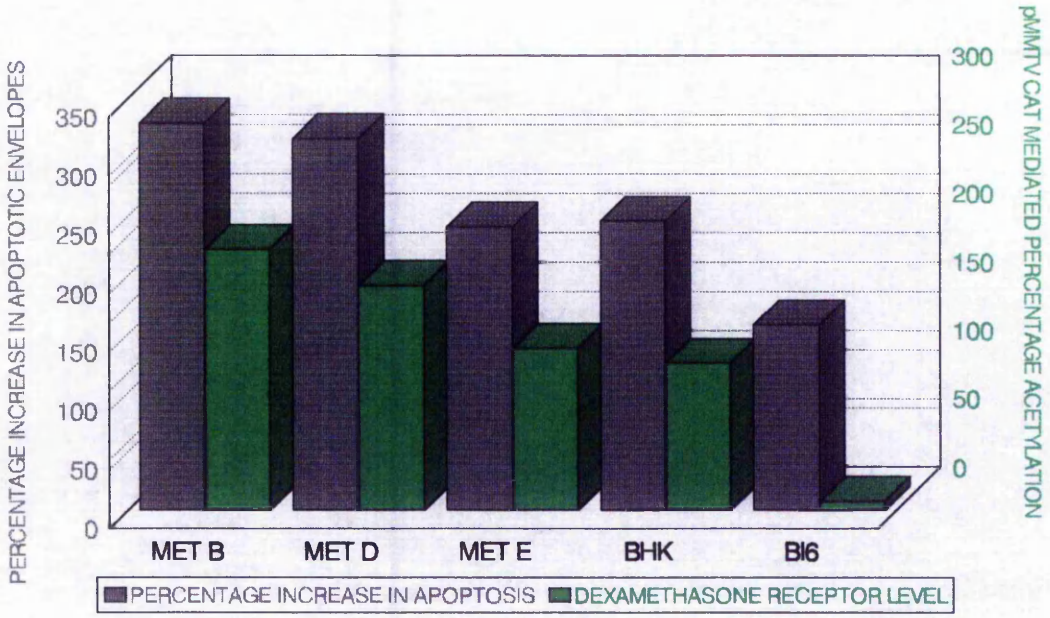


Figure 5.4.4.



5.5.: The effect on transglutaminase activity and apoptosis of increasing the number of the dexamethasone responsive receptor proteins in a cell line.

Previous studies in section 5.4 demonstrate a potential link between the level of dexamethasone responsive receptor protein and dexamethasone induced transglutaminase expression and apoptosis. To substantiate this correlation and to see if it was possible to increase the level of apoptosis still further it was decided to artificially increase the level of dexamethasone receptors by transfecting Met B with the glucocorticoid receptor HG1 carried in the vector pSG5 (methods section 3.3.3.4.). Seven experimental groups were set up as described in table 5.5.1..

Met B - pMMTV was used as a transfection standard, just receiving the vector pSVneo. Met B + pMMTV received the inducible CAT reporter gene and was exposed to dexamethasone. This was to be used as a normal response for CAT activity and transglutaminase activity in response to dexamethasone in the cell line. The HG labelled groups received increasing levels of the HG1 receptor protein, 5 μ g of the CAT reporter gene and the total DNA in the transfection mixture made up to 10 μg using pSVneo. A final group was included transfected with the maximum level of HG1 and the CAT reporter gene but not exposed to dexamethasone. This was to be used as a control to assess the affect of HG1 on the basal levels of CAT.

Table 5.5.1.: Experimental groups and vectors transfected for the introduction of the HG1 glucocorticoid receptor protein into Met B.

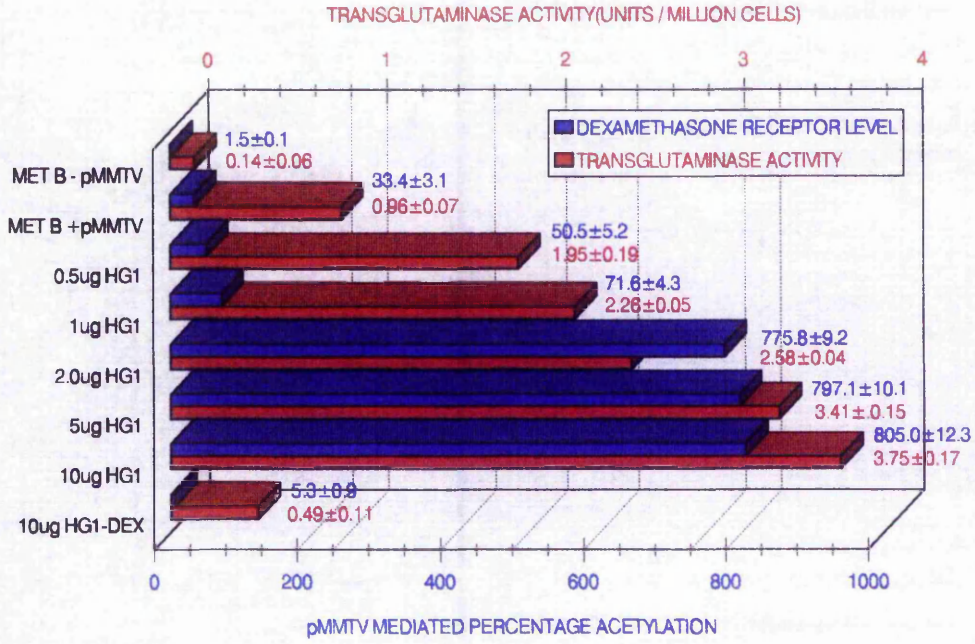
DESCRIPTION	pSG5 HG1 μg	pMMTV CAT μg	pSVneo μg	Dexamethasone μM
Met B - pMMTV	0	0.0	10.0	0
Met B + pMMTV	0	5.0	5.0	1
0.5 HG1	0.5	5.0	4.5	1
1 HG1	1.0	5.0	4.0	1
2 HG1	2.0	5.0	3.0	1
5 HG1	5.0	5.0	0.0	1
5 HG1 - DEX	5.0	5.0	0	0

This table contains the amounts of DNA transfected of each vector for each experimental group of Met B. The pMAMneo CAT vector used to monitor the level of the receptor protein for varying levels of HG1 transfected remains constant. The expression vector pSVneo is used to maintain a constant concentration of DNA being transfected to maintain transfection efficiency and also as a control for non specific protein expression. A control with maximum level of HG1 transfected, but not exposed to dexamethasone is used to ensure changes are as a result of the action of the steroid.

Figure 5.5.1.: The effect on transglutaminase activity of increasing the glucocorticoid receptor protein in Met B.

Various amounts of the glucocorticoid receptor protein gene HG1 carried in the constitutive expression vector pSG5 (SV40 promoter) were co transfected using lipofectin into Met B with equal amounts of the dexamethasone inducible expression vector pMAMneo CAT as described in methods section 3.1.3.. Details of the transfection DNA are given in table 5.5.1.. Transfection of increasing levels of HG1 gene results in an increasing expression of the glucocorticoid receptor protein which was monitored by measuring the CAT induction resulting from activation of the pMAMneo CAT vector when exposed to 1μM dexamethasone for 48 hours in the same manor as in figure 5.4.2.. The relative levels of dexamethasone responsive receptor protein are shown as blue bars. Transglutaminase activity was measured (methods section 3.4.3.1.) in the cells carrying increased glucocorticoid receptor protein when exposed to 1μM dexamethasone for 48 hours. Transglutaminase activity is shown as the red bars and expressed as units per $1x10^6$ cells. Values represent mean $\pm$ S.E.M. from 3 experiments.

Figure 5.5.1.


TRANSGLUTAMINASE ACTIVITY(UNITS / MILLION CELLS)
0
1
2
3
4
DEXAMETHASONE RECEPTOR LEVEL
TRANSGLUTAMINASE ACTIVITY
MET B - pMMTV
MET B +pMMTV
0.5ug HG1
1ug HG1
2.0ug HG1
5ug HG1
10ug HG1
10ug HG1-DEX
1.5±0.1
0.14±0.06
33.4±3.1
0.96±0.07
50.5±5.2
1.95±0.19
71.6±4.3
2.26±0.05
775.8±9.2
2.58±0.04
797.1±10.1
3.41±0.15
805.0±12.3
3.75±0.17
5.3±0.9
0.49±0.11
0
200
400
600
800
1000
pMMTV MEDIATED PERCENTAGE ACETYLATION

The transfection standard showed no alteration in levels of transglutaminase activity and CAT activity as previously recorded. The normal dexamethasone response group again behaved as expected with a transglutaminase increase within the statistical range normally seen (1 to 1.4 units per $1x10^6$ cells), although the CAT activity was lower than normal as the pMMTV CAT was added at 5 μg rather than 10 μg used in the previous experiments (figure 5.4.2.). In the HG1 transfected groups transglutaminase activity increased from 0.96 units per million cells in the normal response group to 3.4 units in the 5 μg HG1 group. This was a steady, non linear increase as the level of HG1 increased in the transfection mixture thus confirming the link of dexamethasone receptor level to transglutaminase response. Each HG1 transfected group was assessed for the level of HG1 receptor protein actually incorporated into the cell population by cotransfection of the inducible reporter gene pMMTV CAT. This showed that with 0.5 μg HG1 transfected gave a 1.3 fold increase in CAT activity ($p \geq 0.05$), 1 μg, a 1.8 fold increase ($p \geq 0.05$), 2 μg, a 20 fold increase ($p \geq 0.01$) and 5 μg a 21 fold increase ($p \geq 0.01$). This result was surprising in the fact that there was such a large increase between 1 and 2 μg transfected in the CAT activity and that the increase was not mirrored by the rise in transglutaminase activity. Thus it appears that the relation between transglutaminase activity increase and receptor number has reached a saturation level at approximately 1 μg of HG1 receptor protein transfected. This is confirmed by the sample receiving 0.5 μg of receptor where a 30% increase in receptor level doubles transglutaminase activity and at 1 μg where a 80% increase from the pMMTV transfection control only results in a 2.5 fold increase in transglutaminase.

To determine if the increase in transglutaminase activity was unique to the MET lines, the experiment was repeated in a less complex form on the human melanoma cell line B16 which had shown the lowest level of transglutaminase activity and glucocorticoid receptor level in the previous experiments (figure 5.5.2.). Three experimental groups were used. B16 that was not exposed to dexamethasone but had been transfected with 5 μg pMMTV CAT and 5 μg pSVneo,

a second group identical to the first but exposed to dexamethasone and a third group transfected with 5 μg pMMTV CAT and 5 μg pSG5 HG1. Figure 5.5.2. shows that the response to dexamethasone seen in B16 can be increased more than 3.5 fold by transfection of the vector pSG5 HG1 (5 μg) thus confirming the results seen in Met B.

Having assessed the effects of increasing the glucocorticoid receptor levels on transglutaminase activity in Met B, its corresponding effects on apoptosis were determined. Cultures of Met B were transfected with 5 μg HG1 and 5 μg pSVneo, treated with dexamethasone and the apoptotic index determined by the counting of detergent insoluble apoptotic envelopes. The results in figure 5.5.3. indicate a statistically significant ($p \leq 0.05$) increase of 35% in apoptosis in the cultures transfected with HG1. Hence a 1.35 fold increase in apoptosis compared with a 3.5 fold increase in transglutaminase activity when transfected with 5 μg of pSG5 HG1 in the presence of dexamethasone.

Figure 5.5.2.: The effect on transglutaminase activity of transfecting the dexamethasone receptor protein HG1 into the B16 mouse melanoma cell line.

5 μg of the glucocorticoid receptor protein gene HG1, carried in the constitutive expression vector pSG5 (SV40 promoter), was co transfected using lipofectin into B16 melanoma cells with an equal amount of the dexamethasone inducible expression vector pMMTV CAT as described in methods section 3.1.3.. Transfection of the HG1 gene (5μg) results in an increasing expression of the glucocorticoid receptor protein which was monitored by measuring the CAT induction resulting from activation of the pMMTV CAT vector when exposed to 1μM dexamethasone for 48 hours in the same manner as in figure 5.5.2.. pMMTV CAT was also transfected into two groups of B16 that were either untreated or exposed to dexamethasone and acted as controls. The relative levels of dexamethasone responsive receptor protein are shown as blue bars. Transglutaminase activity was measured (methods section 3.4.4.1.) in the cells carrying increased glucocorticoid receptor protein when exposed to 1μM dexamethasone for 48 hours. Transglutaminase activity is shown as the red bars and expressed as units per $1x10^6$ cells. Data represents mean values $\pm$ S.E.M. from 3 experiments.

Figure 5.5.2.

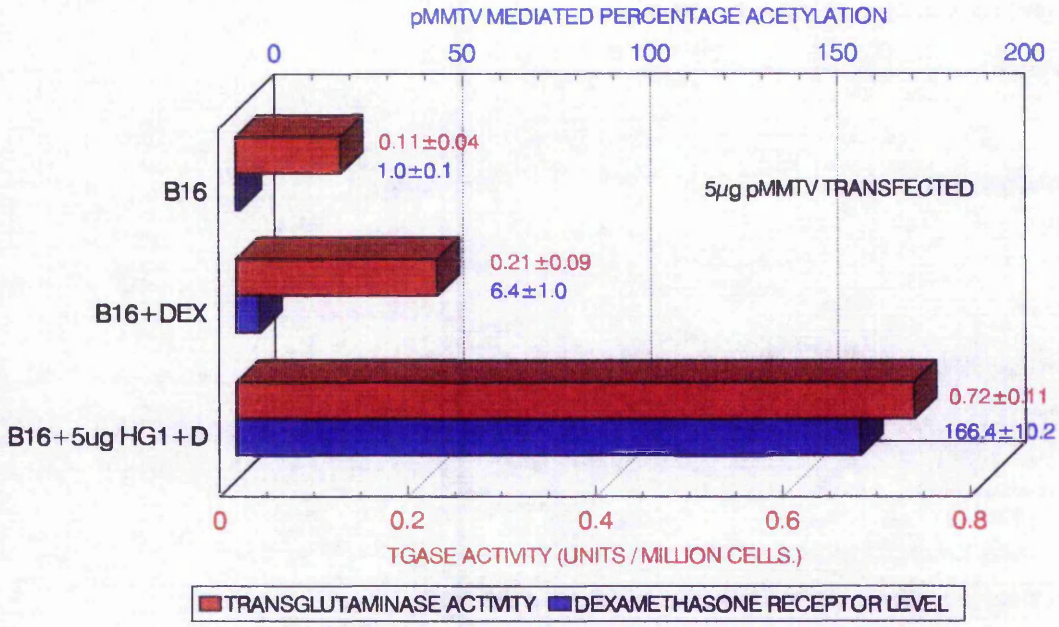
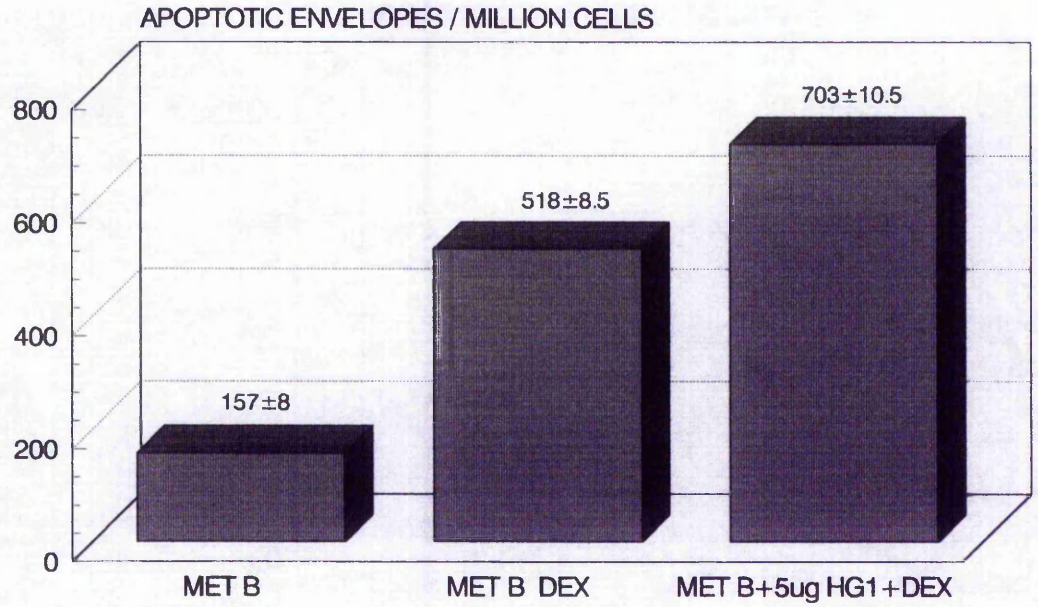

pMMTV MEDIATED PERCENTAGE ACETYLATION
0
50
100
150
200
B16
B16+DEX
B16+5ug HG1+D
0.11±0.04
1.0±0.1
0.21±0.09
6.4±1.0
0.72±0.11
166.4±10.2
5μg pMMTV TRANSFECTED
0
0.2
0.4
0.6
0.8
TGASE ACTIVITY (UNITS / MILLION CELLS.)
TRANSGLUTAMINASE ACTIVITY
DEXAMETHASONE RECEPTOR LEVEL

Figure 5.5.3.: The dexamethasone induced increase in apoptosis in Met B cells receiving the HG1 glucocorticoid receptor.

Met B cells were transfected using lipofectin with 5 μg of the glucocorticoid receptor expression vector pSG5 HG1 as described in methods section 3.1.3. and then incubated overnight under normal tissue culture conditions (methods section 3.1.1.) to allow full expression of the transfected protein. The transfected cell line was then exposed to 1 μM dexamethasone for 24 hours and the apoptotic index determined by counting detergent insoluble bodies in the same manner as in figure 5.3.6.. Data represents mean numbers of detergent insoluble bodies $\pm$ S.E.M. from 5 experiments.

Figure 5.5.3.


APOPTOTIC ENVELOPES / MILLION CELLS
800
600
400
200
0
157±8
518±8.5
703±10.5
MET B
MET B DEX
MET B+5ug HG1+DEX

5.6. Discussion

These studies have indicated that both glucocorticoids and retinoids can cause increases in transglutaminase activity and apoptosis in the cell lines used. Interestingly, these agents appear to cause induction of transglutaminase by differing mechanisms. Retinoids act directly causing a mRNA synthesis dependent increase in transglutaminase where dexamethasone results in a greater increase in activity that appears not to be dependant on mRNA synthesis. Dexamethasone may therefore act indirectly via a regulatory protein which activates existing transglutaminase antigen. One possible explanation could be the activation of the inactive tissue transglutaminase present in the Met cells as described by Knight et al (1990a,d). As an increase in antigen is detected by Western blotting then this activation would have to result in a conformational change in the proteins structure to reveal further epitopes to the anti-cytosolic transglutaminase antibody that were not accessible in the inactive state, especially as the antibody used to probe the Western blot (goat 202) has poor cross reactivity with the inactive transglutaminase. The failure to identify inactive transglutaminase in a 'normal' cell line such as BHK-21 which also shows a transglutaminase response to dexamethasone would seem to contradict this hypothesis, but it could be further hypothesised that all cells contain a certain level of a cryptic transglutaminase and that it is only in the tumour cell lines studied, which also show a reduced apoptotic rate, that the inactive transglutaminase is in a high enough concentration to be detected by the methods used by Knight et al (1990a,d). An alternative is simply that a regulatory protein increases the stability and / or translation of existing transglutaminase mRNA. These results therefore show that the regulation of one of the most ubiquitous enzymes found in mammalian tissues is likely to be under the control of a complex regulatory mechanism, particularly as current work has identified a glucocorticoid response element in the human transglutaminase promoter region (personal communication P.J.A. Davies) which would suggest that an increase in transglutaminase mRNA should occur in response to glucocorticoids.

The finding that differing levels of glucocorticoid receptors cause differing transglutaminase responses and differing levels of apoptosis within cells shows that the ability to alter these parameters may be dependent on these receptors, i.e. glucocorticoid regulation of transglutaminase and apoptosis would have a large effect on tissues rich in the receptor with almost no effect on tissues low in the receptor number such as B16. If this was then coupled with a response to a second agent such as retinoids, it could be hypothesised that the balance between the different receptors that a cell contains can dictate its transglutaminase response to the bodies messenger signals. The same hypothesis could then be applied to apoptosis and may help to explain why different tissues show differing apoptotic rates in different environmental conditions. In addition it may also explain why induced apoptosis in response to dexamethasone treatment has only been previously recorded in lymphoid tissues where a much greater induction of apoptosis is observed (Schwartzman and Cidlowski 1993).

An interesting observation is that the levels of transglutaminase activity increase (3.6 fold) much more than the increase in apoptotic body formation (1.4 fold) following transfection of the HG1 receptor cDNA when compared to increases caused by dexamethasone in non transfected cells. This would indicate that transglutaminase is not the limiting factor for spontaneous apoptosis to take place in Met B, and that other factors are required for apoptosis to proceed in this cell line that are not activated by dexamethasone or the resulting increase in transglutaminase. This limitation to the increase in apoptotic index is also seen in the non transfected cells treated with dexamethasone where increases in apoptosis would appear to plateau at approximately a 3 fold increase compared to the basal level although the increase in transglutaminase reaches almost 9 fold in some cell lines. This observation reinforces the proposal that apoptosis is under a complex regulatory process.

Although the relationship between transglutaminase and apoptosis is reinforced by these results using glucocorticoids and retinoids, it still does not answer the question as to whether the stimulating agent induces apoptosis which is accompanied by an increase in transglutaminase to allow the program to proceed, or does the inducer cause an increase in transglutaminase which pushes the cell into apoptosis. In fact the increase in apoptosis may simply be an event triggered that is totally independent of transglutaminase activity. To determine which is the case it was necessary to specifically increase transglutaminase activity in the Met B cells using the cDNA of the tissue transglutaminase attached to an independent promoter. Transfection of cells with such a promoter would cause a specific increase in transglutaminase activity. Having already tried the use of an inducible vector to carry the gene, the other alternative was to use a constitutive vector that would need no pharmacological inducer.

CHAPTER 6

THE TRANSFECTION AND CLONING OF THE HAMSTER FIBROSARCOMA MET B WITH THE CONSTITUTIVE EXPRESSION VECTOR pSG5 TGASE.

6.1. Introduction

The vector pSG5 contains a SV40 viral promoter sequence adjacent to a multiple cloning site for the insertion of exogenous DNA. The pSG5 Tgase plasmid contains the human endothelial transglutaminase cDNA (Gentile et al 1991) within the multiple cloning site (methods section 3.3.3.4.). The SV40 promoter is classically termed a constitutive promoter meaning that it will continually cause a stable level of transcription of the DNA it controls, although the efficiency of this promoter has been shown to be increased by sodium butyrate (Lee et al 1987) and it is less effective in cells not expressing the T antigen. Unlike the MMTV promoter in pMAMneo, the SV40 promoter requires no inducer. Therefore once pSG5 Tgase is transfected into the cell it requires no further addition of physiological or pharmacological agents for activation, thus minimising any non specific effects. The Met B cell line to be used in this study is derived from an HSV transformed cell, and contains the large T antigen (Teale and Rees 1987). It therefore provides a good cell line to use this vector in.

Since the pSG5 vector carries no antibiotic resistance gene to allow selection of cells that have been transfected, it was necessary to co-transfect cells with a second vector that carries a gene coding for antibiotic resistance to allow selection of transfected clones. In the transfections used in the following studies the antibiotic resistance vector pSV_2neo was used which provides resistance to neomycin (G418 sulphate) which is toxic to mammalian cells in high concentrations. As a transfection control the pSV_2neo vector was used alone.

The DNA ratios chosen for each of the vectors were 1 μg of pSV_2neo to 9 μg of pSG5 Tgase transfected into 60% confluent cells contained in 9 cm diameter Petri dishs. This ratio of cDNA was chosen to ensure that the majority of cells acquiring resistance to G418 sulphate would also contain the transglutaminase cDNA insert.

6.2.: Expression of transglutaminase in pSG5 Tgase transfected clones

Fifty G418 sulphate (neomycin) resistant clones were isolated and screened for raised expression of transglutaminase activity by the ^{14}C putrescine incorporation into dimethylcaesin assay. Sixteen clones contained a raised level of transglutaminase activity, (figure 6.2.1.). Of these, 3 clones were chosen which were then subjected to a more thorough characterisation. Clone 1 and clone 36 showed the highest levels of transglutaminase activity and were selected for this reason. Clone 32 was chosen for characterisation since it contained approximately 50% the activity of clones 1 and 36 and would therefore give a range of clones with different transglutaminase activity levels.

More accurate determination of transglutaminase activity in selected clones (figure 6.2.2.). shows that the transfection control pSV_2neo (Met B transfected with the G418 Sulphate resistance vector pSV_2neo) has a similar transglutaminase activity to non transfected Met B at 0.18 and 0.14 units per million cells respectively. The clone with the least activity, clone 32 (C32) has a value of 13.69 units per million cells which is a 74 fold increase in activity over the transfection control, where as the higher activity clones, clone 1 (C1) and clone 36 (C36) have values of 28.72 and 20.01 units per million cells with give increases of around 156 and 108 fold respectively.

Further investigations were under taken in order to show that the increase in activity is a result of increased protein synthesis. This was done by immunoprobing Western blots of proteins from cell homogenates (separated by SDS PAGE) using the methods outlined in figure 6.2.3..

The immunoblot in figure 6.2.3. confirms that the increase in transglutaminase activity in all clones is a result of increased expression of the transglutaminase

protein. The intensity of staining of the slower moving band (transfected human transglutaminase, not visible in the controls) was found to reflect the relative activities in figure, 6.2.2.. Densitometry of immunoblots blots indicate the increase in transglutaminase antigen resulting from the transfection is approximately 22 fold greater in C1, 10 fold in C32 and 15 fold greater in clone 36 when compared to the transfected pSV_2neo control. The presence of the fainter, faster moving transglutaminase band was also present in the protein extracts obtained from the control cells indicating this to be the endogenous transglutaminase present in these cells.

To verify that the increase in transglutaminase is via increased transcription as a result of the exogenous cDNA insert it was necessary to demonstrate the presence of a mRNA increase in the transfected clone. Northern blot analysis (figure 6.2.4.) shows transcripts of approximately 3.5 kb in all transglutaminase transfected clones indicating that the increase in transglutaminase activity and antigen in all clones is a mRNA dependent increase indicating the successful incorporation of the transfected DNA into the genome. Densitometry studies indicate an approximate 14, 4 and 8 fold increase in transglutaminase mRNA in clones C1, C32 and C36 respectively compared to RNA isolated from cells transfected with pSV_2neo. In clone 1 another major transcript in the region of 5 to 6 kb was found. Interestingly multiple transcripts of 2.8, 4.2, 5.0 and 6.2 kb were found in balb-c 3T3 fibroblasts stably transfected with the pSG5 Tgase vector (Gentile et al 1992). It was suggested that these multiple transcripts result from random multiple integrations of plasmid DNA into the recipient cell genome and when transcribed these cells lead to diverse transcripts accumulating in the transfected cells (Eghbali et al 1990, Jiang and Jordan 1992).

Having determined that the clones 1 ,32 and 36 had a raised transglutaminase activity, antigen and mRNA it was important to confirm that the increase in transglutaminase was cytosolic tissue transglutaminase alone and not particulate.

Clone 36 was used for this purpose. Cell homogenates extracted in either the presence or absence of Lubrol PX from C36 was separated into particulate and cytosolic fractions by high speed ultracentrifugation as outlined in the methods section 3.4.4.3.. Figure 6.2.5. shows that the increase in transglutaminase activity following transfection with the vector pSG5 Tgase is, in the main, due to the cytosolic form of tissue transglutaminase with the particulate activity remaining at a comparable level to that of the control.

Figure 6.2.1.: Initial screen of transglutaminase activity in all transfected clones resistant to G418 sulphate.

Isolated clones were allowed to grow to approximately 90 % confluency in a 25 cm^3 flask. These were then harvested using 0.25 % (v/v) trypsin resuspended in PBS and counted using a haemocytometer. Aliquots of cells containing $1x10^6$ cells were then assayed for transglutaminase activity by the incorporation of ^{14}C putrescine into N,N-dimethylcasein as described in methods section 3.4.3.1.. Transglutaminase activity is represented by the level of incorporated ^{14}C putrescine over 30 minutes per 1 $x10^6$ cells.

Figure 6.2.2: Repeated determination of transglutaminase activity of clones pSG5 Tgase 1, 32 and 36.

Cells were allowed to grow to approximately 90% confluency in a 75 cm^2 flask before being harvested using trypsin. The cells were resuspended in PBS and counted using a haemocytometer. A volume equivalent to $1x10^6$ cells was placed into an Eppendorf tube and the cells pelleted prior to being assayed for transglutaminase activity as in methods section 3.4.3.1.. An equal volume of the cell suspension was used to determine protein levels using the Biorad protein assay reagent (method section 3.4.4.2). Data represents transglutaminase activity expressed as units per $1x10^6$ cells or per mg of total cellular protein $\pm$ S.E.M. from 15 or more experiments.

Figure 6.2.1.

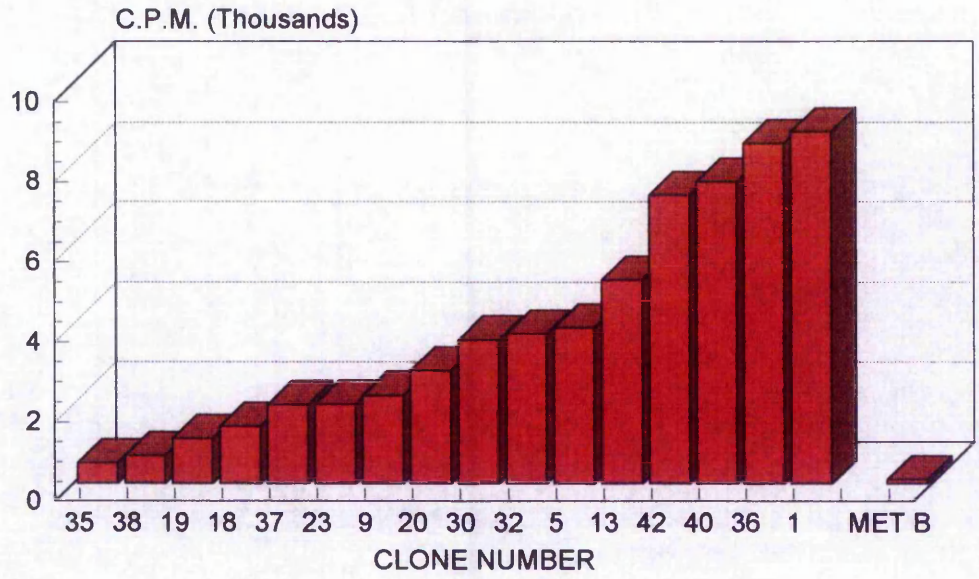


Figure 6.2.2.

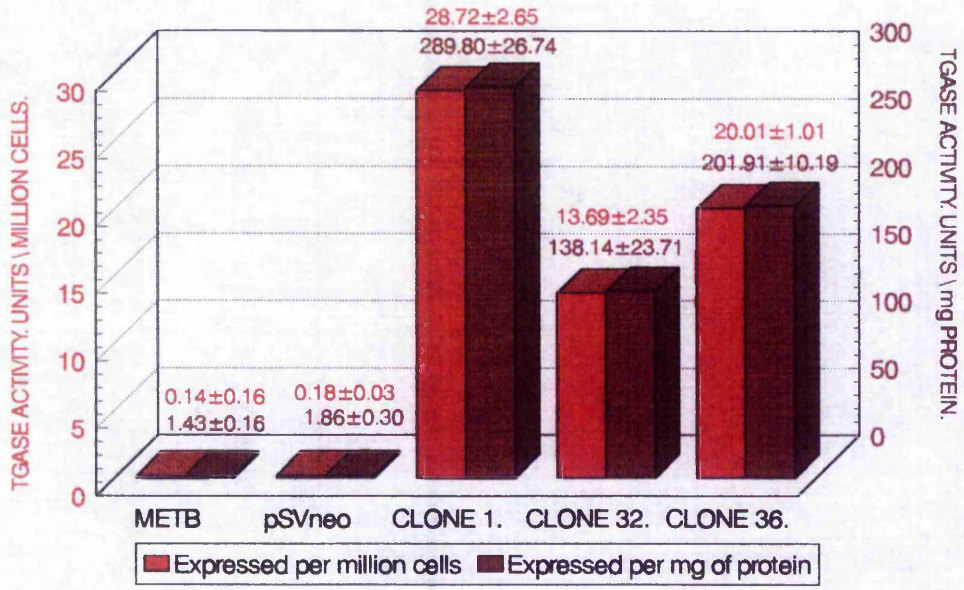


Figure 6.2.3: Immunoblot of protein from cell homogenates of clones pSG5 Tgase 1.32 and 36.

Ninety percent confluent plates of either Met B, Met B cells transfected with pSV_2neo, or pSG5 Tgase clones 1, 32 and 36 cells were prepared in 75 cm^2 flasks. Cells were harvested using trypsin and cell number determined using a haemocytometer before being homogenised. A volume equivalent to 1 million cells was loaded onto a 10% (w/v) polyacrylamide gel, electrophoresed and electroblotted on to Hybond C super nylon (Amersham) as described in methods sections 3.4.1.2. and 3.4.2.1.. The Western blot was immunoprobed with goat 202 anti guinea pig transglutaminase antibody (methods, section 3.4.2.2.). Lane 1 = Guinea pig liver cytosolic transglutaminase standard, Lane 2 = Met B, Lane 3 = Met B pSV_2neo, Lane 4 = C1, Lane 5 = C32, Lane 6 = C36.

Figure 6.2.3.

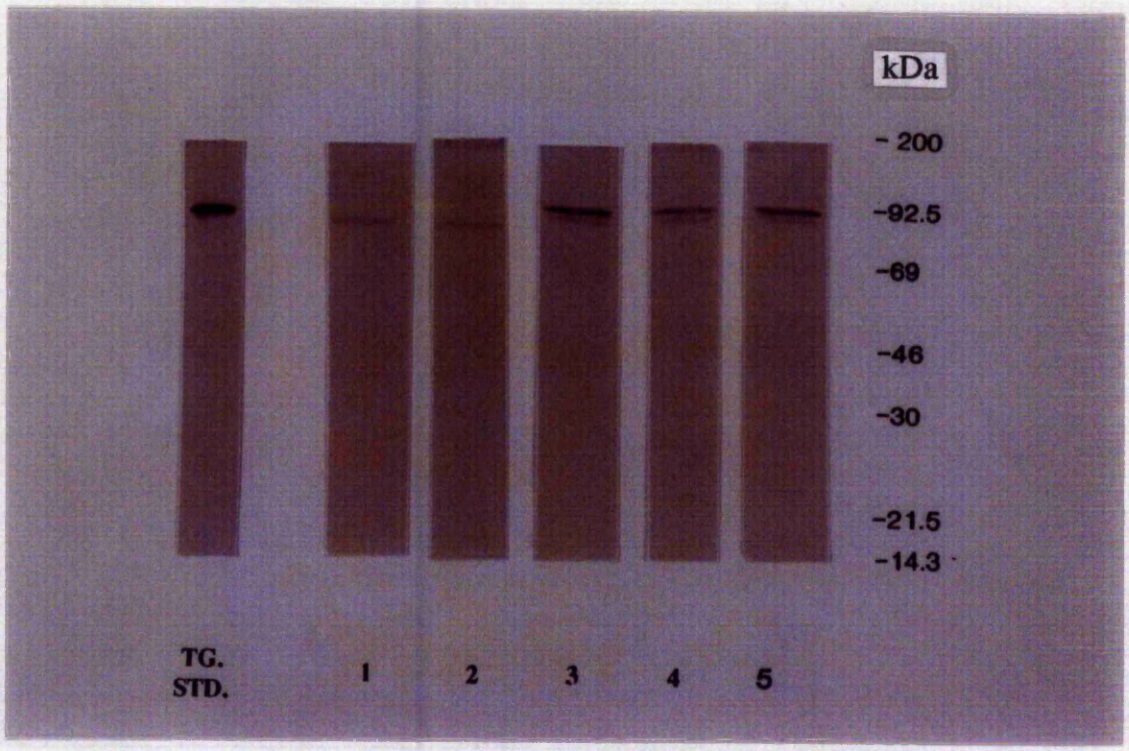


Figure 6.2.4: Northern blot of total RNA from clones pSG5 Tgase 1, 32 and 36 probed with Human transglutaminase cDNA (coding sequence) labelled with ^{32}P.

Total RNA was extracted from a 90% confluent 75 cm^2 flasks of either Met B, Met B cells transfected with pSV_2neo, or pSG5 Tgase clones 1, 32 and 36 cells using the RNAsol B method as described in methods section 3.3.2.1.. The extracted RNA was electrophoresed on a agarose / MOPS / formaldehyde gel, and viewed under UV to verify loading and sample degradation and subsequently capillary blotted on to N^+ nylon (Boehringer) as detailed in methods sections 3.3.2.4. and 3.3.2.5.. The Northern blot was then probed using a human coding sequence cytosolic transglutaminase cDNA labelled with ^{32}P using the Promega random prime system (methods sections 3.3.2.6. and 3.3.2.7.). The hybridised blot was dried and exposed to Kodak X OMAT RP film for 12 days using intensifying screens. Lane 1 = cytosolic transglutaminase mRNA standard (1 μg), Lane 2 = total RNA extract from Met B cells (10 μg), Lane 3 = total RNA extracted from Met B pSV_2neo cells (10 μg), Lane 4 = total RNA extract from C1 (10 μg), Lane 5 = total RNA extract from C32 (10 μg), Lane 6 = total RNA extract from C36 (10 μg).

Figure 6.2.4.

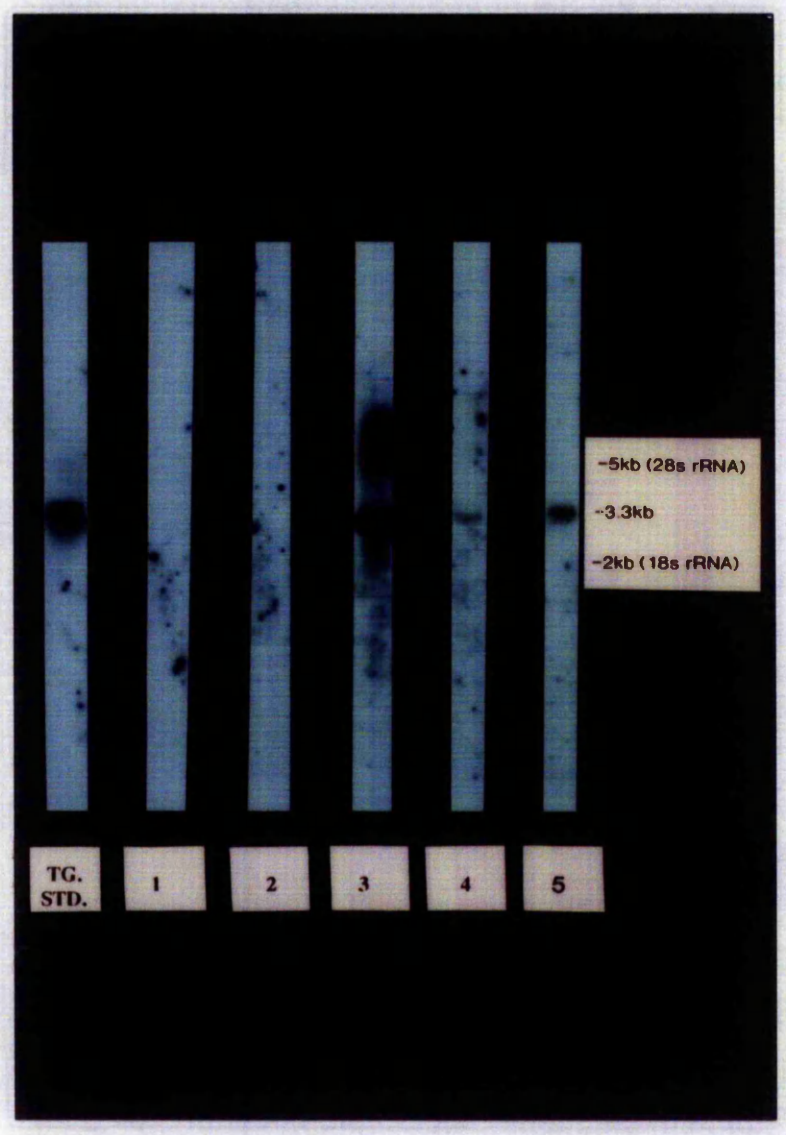
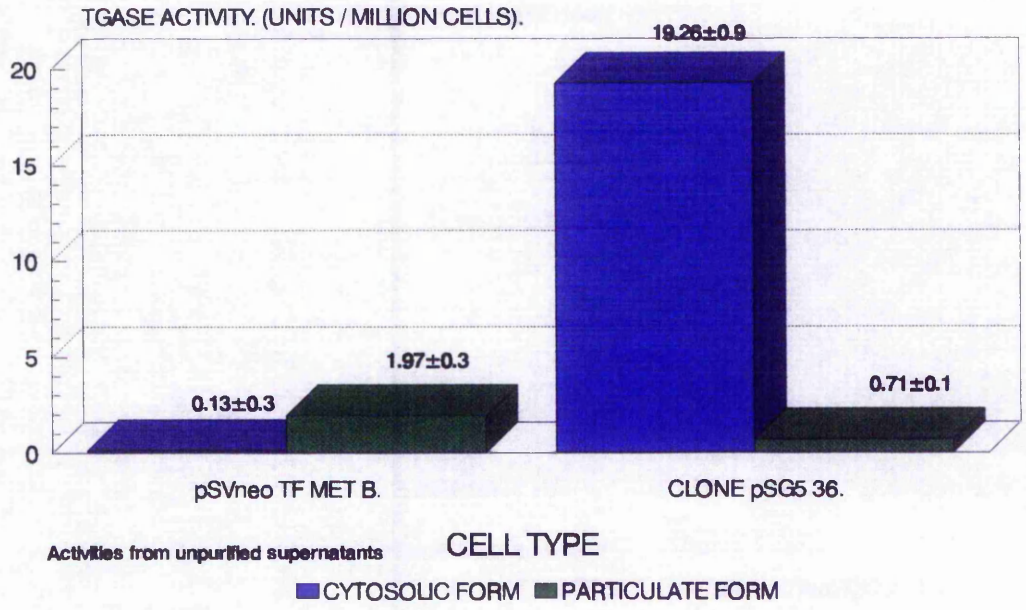


Figure 6.2.5.: The distribution of tissue transglutaminase activity in the pSG5 Tgase transfected clone 36.

Cells were grown to 90% confluency in a 150 cm^2 flask and harvested using trypsin. $1x10^7$ cells were homogenised in the absence of Lubrol Px to release the cytosolic component which was clarified by centrifugation at 90000 x g for 1 hour (methods section 3.4.4.3.). The supernatant was collected and the pellet re extracted in the presence of 1% (v/v) Lubrol Px to release membrane bound components. This was again clarified by centrifugation with the supernatant representing the particulate fraction. Each fraction was then was then assayed for transglutaminase activity as in methods section 3.4.3.1.. Data represents mean transglutaminase activity expressed as units per 1 x 10^6 cells from 3 experiments.

Figure 6.2.5.


TGASE ACTIVITY. (UNITS / MILLION CELLS).
20
15
10
5
0
19.26±0.9
1.97±0.3
0.13±0.3
0.71±0.1
pSVneo TF MET B.
CLONE pSG5 36.
Activities from unpurified supernatants
CELL TYPE
CYTOSOLIC FORM
PARTICULATE FORM

6.3.: The effect of increased expression of transglutaminase activity on cell morphology

The next parameter to characterise was cell morphology. Gentile et al (1992) following transfection of the transglutaminase gene into normal mouse 3T3 fibroblasts had reported a flattening of many of the cells and it was therefore important to see if this was also occurring in the fibrosarcoma lines being used in this investigation. Figures 6.3.1. and 6.3.2. show Clones 1, 13, 32, 36 and the control cell lines Met B and pSV_2neo at medium (100 X) and high magnification 200 X) on inspection using light microscopy. Both figures show very healthy looking cells with no apparent differences in morphology between the clones and control cell lines. All clones retain a classical fibroblast shape with no apparent flattening but maintaining a bipolar form. Unlike Gentile et al (1992) there was not a significantly higher level of cells that were either blebbing or rounding which are classical morphological signs of cells undergoing apoptosis. Hence initially by these methods there was no discernible changes in the clones endogenous rate of apoptosis.

Figure 6.3.1. Morphological appearance of Met B pSG5 Tgase transfected clones 1. 13 .32 36 and the control cell lines Met B and pSV_2neo transfected Met B as observed in the inverted light microscope (100 X magnification.)

Cells were grown in 75 cm^2 flasks to between 60 and 80% confluency. Cells were photographed on the Olympus CK2 Opitare inverted light microscope using the 10 X objective lens (100 X overall magnification) with a dedicated Olympus camera. Exposure and aperture were set to automatic using Kodak Gold film, 100 ISO

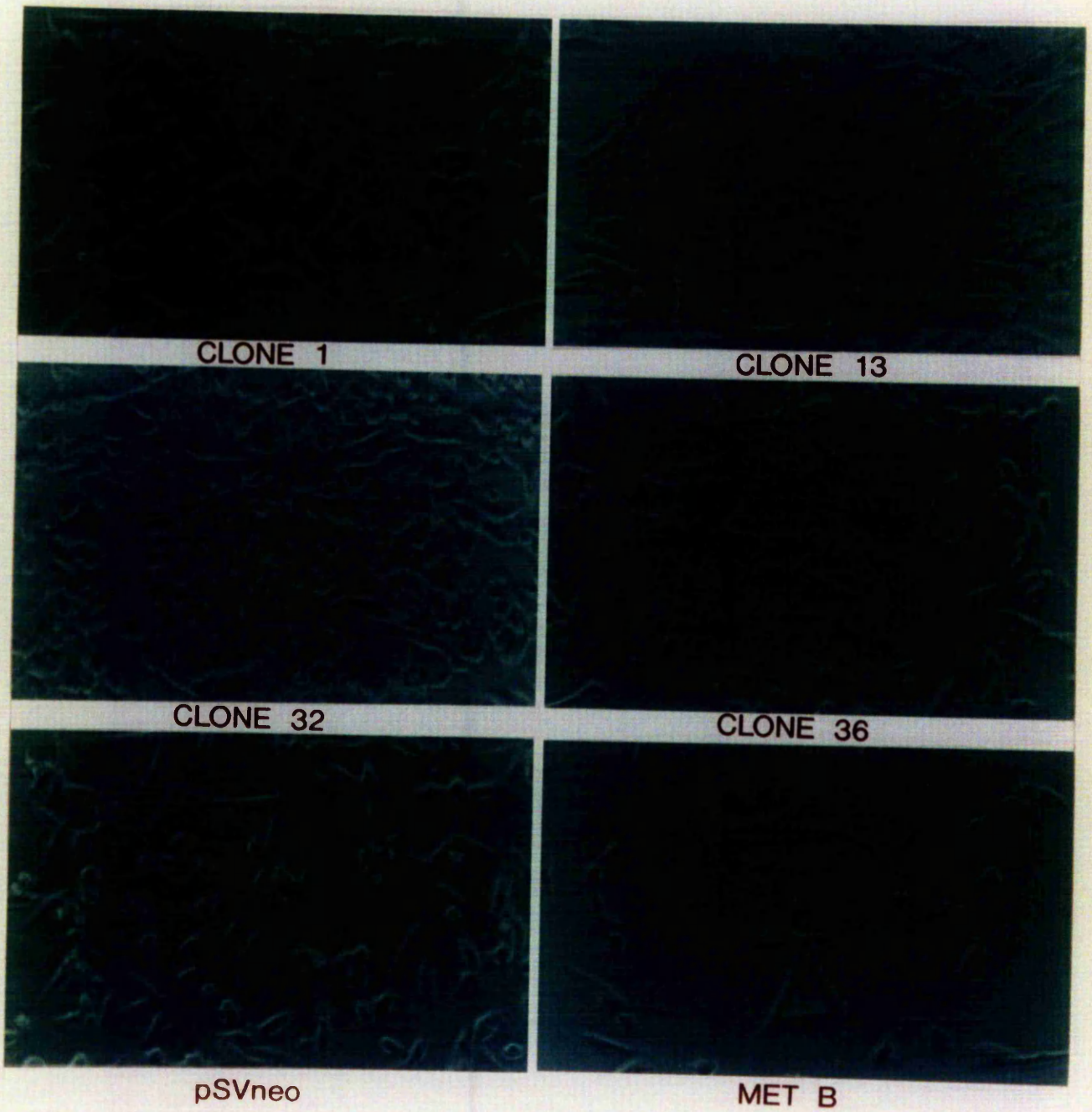
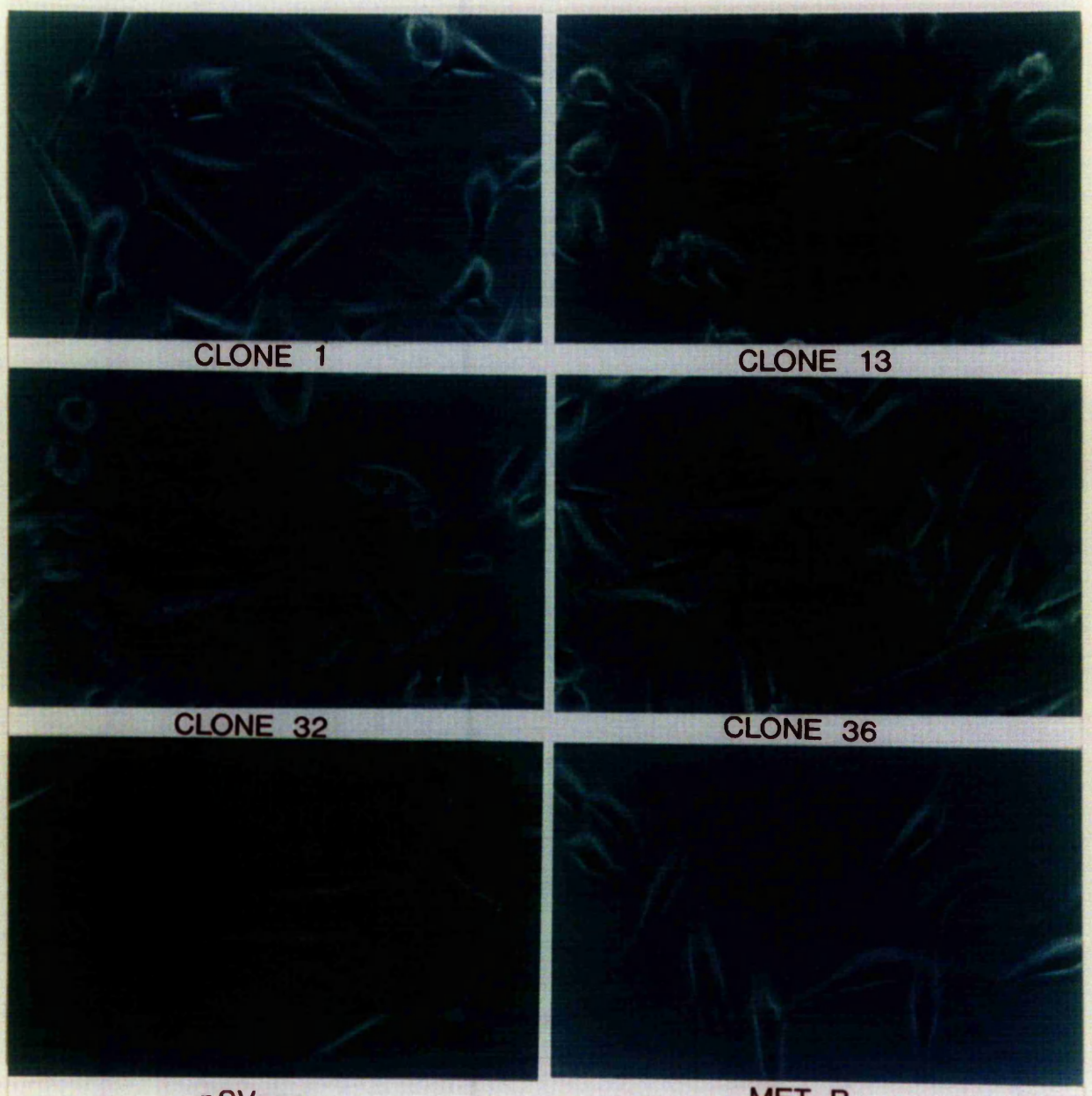

CLONE 1
CLONE 13
CLONE 32
CLONE 36
pSVneo
MET B

Figure 6.3.2.: Morphological appearance of Met B pSG5 Tgase transfected clones 1, 13 ,32 36 and the control cell lines Met B and pSV_2neo transfected Met B as observed in the inverted light microscope (200 X magnification).

Cells were grown in 75 cm^2 flasks to between 60 and 80% confluency. Cells were photographed on the Olympus CK2 Opitare inverted light microscope using the 20 X objective lens (200 X overall magnification) with a dedicated Olympus camera. Exposure and aperture were set to automatic using Kodak Gold film, 100 ISO.


CLONE 1
CLONE 13
CLONE 32
CLONE 36
pSVneo
MET B

6.4.: The effect of increased transglutaminase activity on cell adhesion and attachment.

A number of workers (Cai et al 1991, Gentile et al 1992) have reported changes in a cells adhesive properties when transglutaminase activity is raised in these cells. There has also been a number of reports indicating that transglutaminase, which is essentially an intracellular enzyme may work at the cell surface (Upchurch et al 1991, Aeschlimann and Paulsson 1991, Barsigian et al 1991, Jensen et al 1993, Beninati et al 1994, Martinez et al 1994, Dolynchuk et al 1994). Changes in cell adhesion and cell attachment were therefore measured in the transfected fibrosarcomas. It has been suggested that changes in a cells adhesive abilities may be one of the factors that may influence a cells invasiveness and metastatic ability and hence progression of a cancer. It may be expected that a cell would require a low adhesion level to be released from the primary tumour, but then require a rapid attachment ability to lodge and develop a secondary growth. Experiments were designed to examine the cells relationship to its substratum in two ways, the ability of the cells to attach to a substratum and then the cells ability to remain attached to a substrate once adhered.

Attachment

Attachment studies were performed as described in methods section 3.1.5.. Cells were harvested using trypsin, washed in PBS containing a trypsin inhibitor and then the cells placed at a known concentration on to differing surfaces for 90 minutes and the percentage of cells attached determined. Five different surfaces were examined: uncoated plastic; plastic coated with bovine serum albumin (BSA), collagen type IV or with fibronectin; commercially supplied tissue culture coated plastic. After 90 minutes incubation there was no attachment of any cell lines to uncoated plastic or plastic coated with BSA. The level of attachment seen with the other surfaces can be seen in figure 6.4.1.. In all cells tested there was no surface

tested that was favoured by all cell lines. Clone 1 and clone 32 showed lower levels of attachment to all surfaces than the control cell lines Met B and pSV_2neo, although clone 36 showed comparable attachment to the controls. Thus transfection of cells with transglutaminase had no conclusive affect on the attachment of these cells to other substratum.

Adhesion

Adherence of cells to a surface was measured using the cells ability to resist proteolytic digestion by trypsin after overnight attachment. Cells were plated out to a known cell density and then left overnight to attach to the substrate. They were then washed in DMEM (no serum) and then incubated with trypsin at either 0.025%, 0.0025% or 0.00025% (w/v) for 30 minutes. The percentage of cells remaining attached was then determined. Following overnight incubation, no attachment of any cells was observed with the plastic control, or plastic coated with BSA. In addition, although all cell lines attached to collagen, following overnight incubation the strength of the attaching bonds became so weak that the physical stresses encountered during the washing procedure removed the majority of the cells from the substratum. Thus cells would attach to type IV collagen but were unable to survive long term culture on this surface. As a result adherence was only determined on the commercially coated tissue culture plastic and fibronectin coated plastic. When 0.025% (w/v) trypsin was applied to the cells on both substratum (fibronectin and commercial plates) then apart from the normal cell line BHK where over 90% of cells on both substrates remained attached, all cell lines had less than 5% of cells still attached after 30 minutes. When 0.00025 % trypsin was applied to cells grown on fibronectin and commercial plates, then in all cases adherence was 90% or greater. It was only at 0.0025% trypsin that any differences between cell lines could be seen.

In figure 6.4.2. it can be seen that pSV_2neo and Met B cells have approximately 30% of the cells still attached after 30 minutes incubation with trypsin on both fibronectin and commercial plates. All three clones show a significant increase in the level of adherence to both surfaces ($p \geq 0.05$) when compared to the control cells. Clone 36 shows little preference for either substrate, with the percentage of cells attached doubling for both substrata compared to the control cell lines. Clones 1 and 32 express a preference for fibronectin ($p \geq 0.05$) as a substratum where adhesion reaches 70% for fibronectin compared to approximately 50% for commercial tissue culture plates. The normal fibroblast cell line BHK shows in excess of 90 % cells still attached following trypsin treatment. This result indicates that although transglutaminase is apparently influential on a cells ability to adhere to both fibronectin and commercially coated plates, since BHK has a lower transglutaminase activity than the clones it would suggest that transglutaminase is not the sole factor in determining a cells ability to adhere to these substrates. These results also show that a cells adherence is effected by the extracellular matrix since both collagen and BSA failed to act as a surface for attachment or adherence for cells. The differences seen between the cloned cell lines regarding adhesion to fibronectin would suggest that transglutaminase was not the most influential factor in causing adherence to this surface.

In terms of cancer progression these attachment / adhesion results indicate that a reduced transglutaminase level may well facilitate the release of a cell from a primary tumour when transglutaminase activity is low. When the attachment studies are considered, then 2 of the 3 clones with raised transglutaminase show a lower level of attachment than the wild type fibrosarcoma. Although not conclusive, it is tempting to speculate that low adherence and raised attachment in the low transglutaminase activity containing malignant fibroblasts may be a parameter in metastasis. It is important to note that as not all the transglutaminase transfected clones tested showed a reduced attachment and the results shown could simply be a clonal effect and thus further clones need to be studied to show a significant change.

Figure 6.4.1.: The attachment of Met B, pSV_2neo transfected Met B and Met B pSG5 Tgase clones 1, 32, 36, to fibronectin, collagen IV and commercially coated tissue culture plastic following a 90 minute incubation.

Plastic 96 well plates were coated with either fibronectin, BSA, or collagen type IV as described in section 3.1.5.. A healthily growing population of cells was harvested with trypsin, washed in PBS containing trypsin inhibitor and counted on a haemocytometer. Aliquots containing $2x10^4$ cells in complete medium were then placed into each well of the previously treated plates. These were incubated at 37°C for 90 minutes in a 5% (v/v) CO_2 environment. Each well was then carefully washed with PBS to release any unattached cells. Cells remaining attached to the surface were then fixed and stained using 70% (v/v) ethanol, 0.5% (w/v) crystal violet. The plate was washed with PBS until no more stain could be released, before the cells were solubilised using 30% (v/v) acetic acid and the resulting crystal violet stain released measured by spectrophotometry on a 96 well plate reader. Determination of cell number remaining attached was by reference to a a cell number standard curve, methods section 3.1.5.. There was no attachment of cells to the controls of uncoated plastic and BSA coated plastic. Data represents mean percentage values for attached cells $\pm$ S.E.M. from 5 experiments.

Figure 6.4.1.

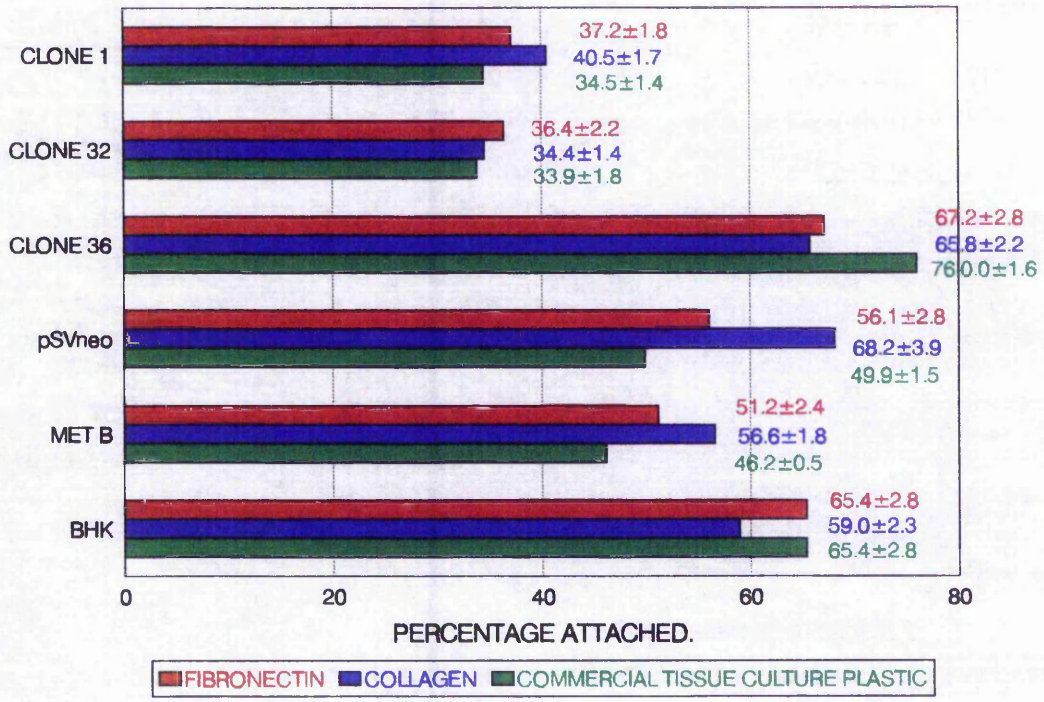
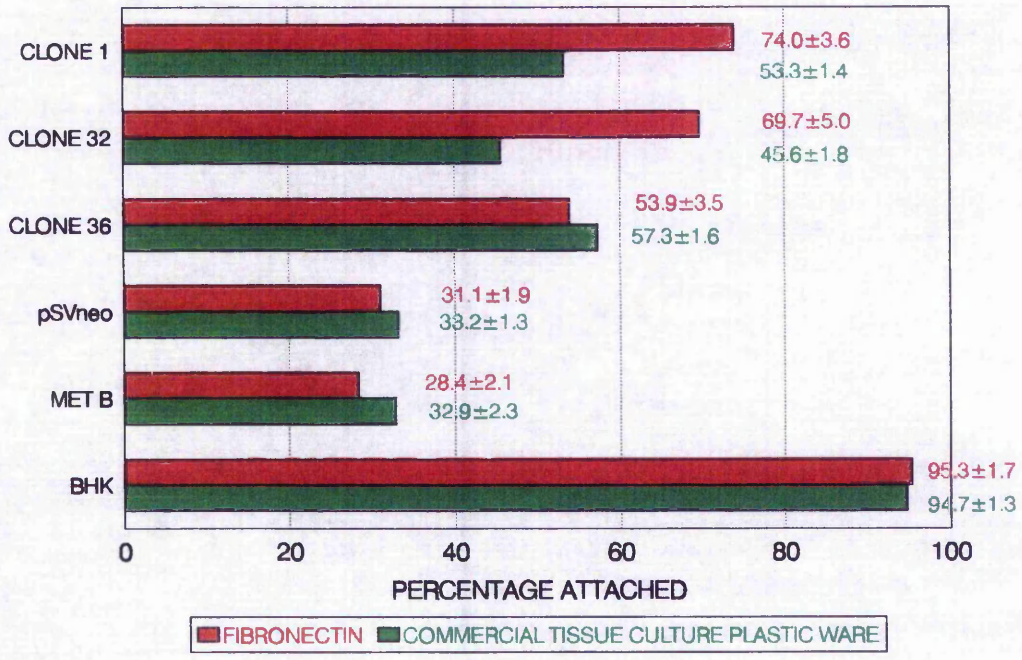

CLONE 1
37.2±1.8
40.5±1.7
34.5±1.4
CLONE 32
36.4±2.2
34.4±1.4
33.9±1.8
CLONE 36
67.2±2.8
65.8±2.2
76.0±1.6
pSVneo
56.1±2.8
68.2±3.9
49.9±1.5
MET B
51.2±2.4
56.6±1.8
46.2±0.5
BHK
65.4±2.8
59.0±2.3
65.4±2.8
0
20
40
60
80
PERCENTAGE ATTACHED.
FIBRONECTIN
COLLAGEN
COMMERCIAL TISSUE CULTURE PLASTIC

Figure 6.4.2.: The adhesion of Met B, pSV$_2$neo transfected Met B and Met B pSG5 Tgase clones 1, 32, 36, to fibronectin and commercially coated tissue culture plastic following a 30 minute incubation with 0.0025% trypsin.

Plastic 96 well plates were coated with either fibronectin, BSA, or collagen IV as described in section 3.5.1.. A healthily growing population of cells was harvested with trypsin, washed in trypsin inhibitor and counted on a haemocytometer. Aliquots containing $2x10^4$ cells in complete medium were then placed into each well of the previously treated plates. These were incubated at 37°C overnight in a 5% (v/v) CO_2 environment. Each well was then carefully washed with PBS and the cells incubated for 30 minutes in 0.0025% (w/v) trypsin before a second PBS wash to remove released cells. Cells remaining attached to the surface after the proteolytic treatment were then fixed and stained using 70% (v/v) ethanol, 0.5% (w/v) crystal violet. The plate was washed with PBS until no more stain could be released, before the cells were solubilised using 30% (v/v) acetic acid and the resulting crystal violet stain released measured by spectrophotometry at 410 nm on a 96 well plate reader. Determination of cell number remaining attached was by reference to a standard curve of cells not exposed to trypsin as detailed in section 3.1.5.. There was no attachment of cells to the controls of uncoated plastic and BSA coated plastic. Attachment to collagen type IV following the overnight incubation was so weak that all cells were removed in the PBS wash. Data represents mean percentage values of cells attached to the plate following trypsin digestion ± S.E.M. from 5 experiments.

Figure 6.4.2.


CLONE 1
74.0±3.6
53.3±1.4
CLONE 32
69.7±5.0
45.6±1.8
CLONE 36
53.9±3.5
57.3±1.6
pSVneo
31.1±1.9
33.2±1.3
MET B
28.4±2.1
32.9±2.3
BHK
95.3±1.7
94.7±1.3
0
20
40
60
80
100
PERCENTAGE ATTACHED
FIBRONECTIN
COMMERCIAL TISSUE CULTURE PLASTIC WARE

6.5.: The effect of increased transglutaminase expression on apoptosis

Since all the previous pharmacological studies using dexamethasone and retinoids had suggested that a rise in transglutaminase had been accompanied by a rise in apoptosis, studies were under taken to determine if similar observations were found with the clones carrying increased transglutaminase activity. This was undertaken by counting the number of detergent insoluble apoptotic envelopes present in cell cultures as shown in figure 6.5.1..

Figure 6.5.2. indicates no significant difference ($p \leq 0.05$) in the apoptotic index measured in the transfected control pSV_2neo and Met B. The clone 36 also shows no change in apoptosis when compared to Met B or pSV_2neo. However, this is contradicted by the other 2 clones.

Clone 1 shows a significantly ($p \geq 0.05$) lower apoptotic index than pSV_2neo control whereas clone 32 shows a raised apoptotic index. It is important to note that the differences between the populations are very small, and as one clone is the same as the control, with one clone higher and one clone lower than the changes observed in apoptotic index are more likely to result from cloning rather than from raised transglutaminase activity. These results suggest that the increased levels of tissue transglutaminase in a cell do not induce the apoptotic program and that the raised levels of transglutaminase activity seen with the induction of apoptosis by pharmacological agents is likely to involve the induction of other factors, in addition to that of transglutaminase, that are important in triggering the apoptotic event.

Figure 6.5.1.: A detergent insoluble apoptotic envelope

An example of a detergent insoluble apoptotic envelopes isolated from a Met B culture. Cells were collected from both medium and plate surface. These were then pelleted and resuspended in PBS containing 2% (w/v) SDS and 0.1% (w/v) dithiothreitol (DTT) before being boiled for 5 minutes. Following cooling DNase 1 was added to a concentration of 20 units ml^{-1} and the solution incubated at 37 ^{o}C for 1 hour. Apoptotic envelopes were then pelleted and subsequently washed 3 times in 2% (w/v) SDS, 0.01% (w/v) DTT, 10 units ml^{-1} DNase 1. Bodies were again pelleted and resuspended in 10 μl of the wash solution. (section 3.4.4.1.). The apoptotic envelopes were photographed under a inverted light microscope at 100X magnification.

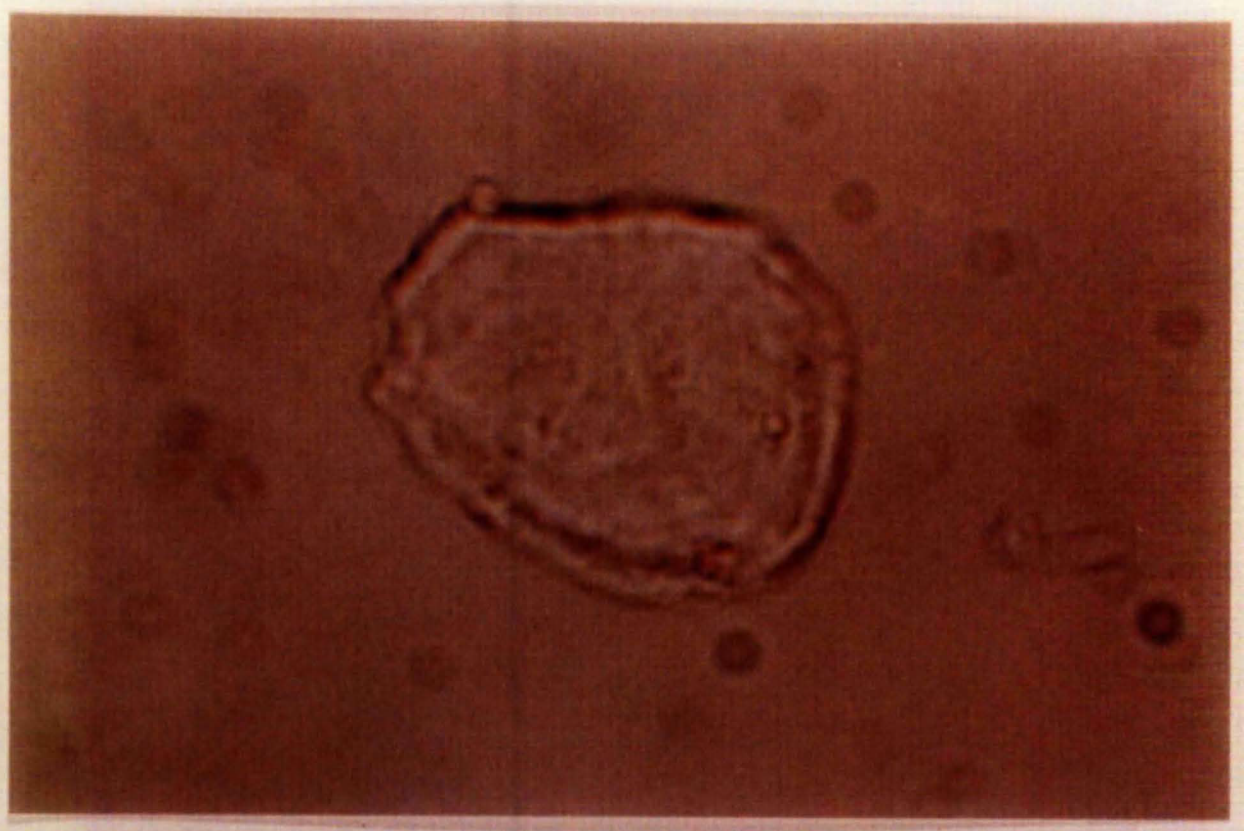
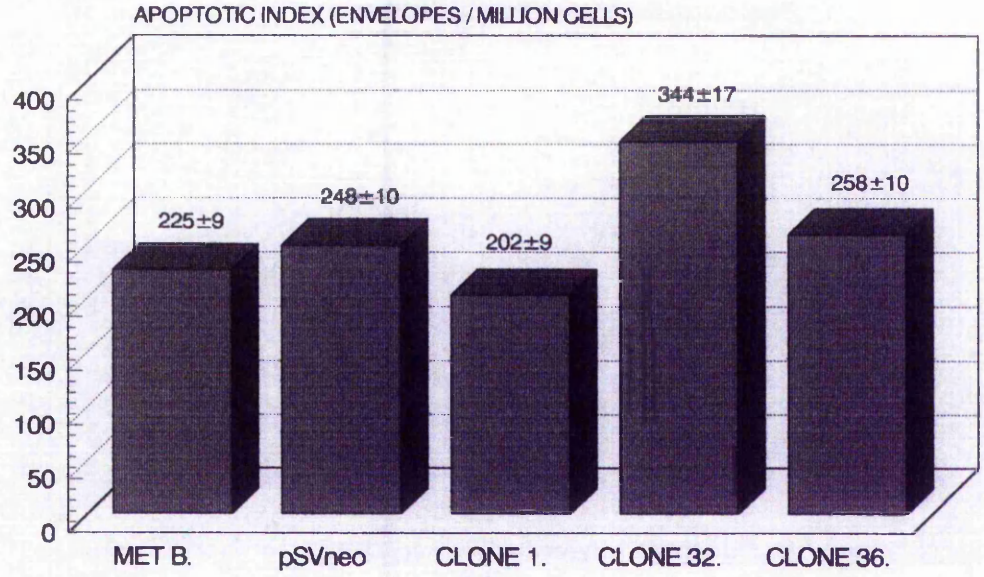


Figure 6.5.2.: Apoptotic index of clones pSG5 Tgase C1, C32 C36, pSV_2neo and Met B determined by counting the number of SDS insoluble apoptotic envelopes.

Cells were grown to 90% confluency in a 150 cm^2 flask before the medium was removed and the cells washed several times in sterile PBS and fresh complete medium added. The flask was then returned to the incubator for 24 hours at which stage cells were collected from both medium and plate surface. These were then counted on a haemocytometer and analysed for detergent insoluble bodies as described in fig. 6.5.1.. Bodies were then counted under an inverted light microscope by at least three workers that were unaware of the experimental groups and the figures averaged. Apoptotic envelopes were counted using either a haemocytometer or the number of bodies per μl volume depending on the workers preferred method of counting. Data represents mean numbers of detergent insoluble bodies / 1 $x10^6$ cells $\pm$ S.E.M. from 8 or more experiments.

Figure 6.5.2.


APOPTOTIC INDEX (ENVELOPES / MILLION CELLS)
400
350
300
250
200
150
100
50
0
225±9
248±10
202±9
344±17
258±10
MET B.
pSVneo
CLONE 1.
CLONE 32.
CLONE 36.

6.6.: Discussion

Characterisation of the transglutaminase transfected clones indicated some changes in cell biochemistry as a result of the specific increase in transglutaminase concentration. Analysis of cell adherence showed that increasing transglutaminase expression increased the ability of the cell to stay fixed to a substrate, with some suggestion that this may be associated with fibronectin as adhesion to fibronectin was significantly increased over adhesion to commercial tissue culture plates in 2 of the 3 clones characterised in detail. In addition 2 of the 3 clones showed a reduced ability to attach to a range of substrates. It is important to note here that the clone that showed neither reduced attachment nor increased adhesion to fibronectin was clone 36. This may suggest that clone 36 is biochemically different to the other clones isolated owing to clonal variation; before conclusions can be made it is therefore necessary to characterise a larger number of the raised transglutaminase clones. If clones 1 and 32 are truly representative of cells with a phenotype for raised transglutaminase expression then reduced attachment and increased adhesion would suggest a decreased tendency for the metastatic process (Willis et al 1974).

As clone 1 has a higher transglutaminase activity than clone 36 and clone 32 then it might be expected that this difference should also be reflected in the attachment and adhesion studies if transglutaminase was a key player. This criticism should be qualified using an important argument raised by Gentile et al (1992) who undertook similar experiments on transglutaminase transfected mouse balb c 3T3 fibroblasts. Although transglutaminase activity is high when measured using the putrescine incorporation assay, activity is being measured in a cell homogenate with factors present to both maximally activate transglutaminase and to provide a substrate for its action. In the intact cell the transglutaminase may be controlled at the protein level (as results in chapter 3 would suggest) either by tertiary structure, calcium dependency (Hand et al 1985) or the binding of GTP (Achyuthan and Greenberg 1987). Alternatively the availability of the substrates for

transglutaminase could control its cellular function. When considering at the primary extracellular mechanisms of cell adhesion and cell attachment then the passage of transglutaminase through the cell membrane may also regulate its action. Therefore in the studies undertaken here which show equal changes in adhesion and attachment in clone 1 and clone 32 where clone 1 has more than double the transglutaminase activity of clone 32 then a saturated level of transglutaminase activity may have been reached in the cells. Transglutaminase protein above this level may therefore be redundant.

Studies on transglutaminase mediated apoptosis showed that there was no conclusive change in apoptotic index of the clones containing raised transglutaminase activity compared to the controls when measured by detergent insoluble apoptotic body formation. This suggests that the pharmacological induction of apoptosis seen in chapter 3 using dexamethasone and all-*trans* retinoic acid was not mediated by transglutaminase alone or at least needs the expression of other factors in addition to transglutaminase to induce apoptosis. Transglutaminase is therefore likely to be involved in the apoptotic process, but as a effector protein rather than as a regulatory protein. This further indicates that in the Met cell lines the low apoptotic index (Knight et al 1990a,d) is not solely a result of low transglutaminase expression

The next logical step was to see if the transglutaminase mediated changes in the cloned cells would effect the growth of the fibrosarcoma both *in vitro* and in tumour development and progression.

CHAPTER 7

THE EFFECT OF RAISED TRANSGLUTAMINASE EXPRESSION ON *IN VITRO* CELL GROWTH AND *IN VIVO* TUMOUR GROWTH OF THE HAMSTER FIBROSARCOMA MET B.

7.1. Introduction.

The effects of raising transglutaminase expression in the hamster fibrosarcoma Met B are characterised *in vitro* in chapter 6. The true effects of raising transglutaminase in the fibrosarcoma can not be determined until the clones are returned to the *in vivo* situation where they are exposed to all factors and pressures that act on a cell in its natural environment. *In vivo*, a number of host / tumour cell interactions may influence its growth and development which can not be reproduced *in vitro* during cell culture. These are described in chapter 1.3.. Hence if tumour development, progression and metastasis are to be studied in this transfected cell line it is necessary to introduce transfected clones into the animal.

7.2: Raised transglutaminase expression on the rate of *in vitro* cell growth rate in BHK-21, Met B, transglutaminase transfected clones 1, 32 and 36, and the transfection control pSV$_2$neo transfected Met B.

Measurement of growth rates of clones and control cell lines were undertaken by seeding a known cell number and counting the cells attached to the plate at 24 hour periods using a haemocytometer. The assumption made is that cells still attached to the plate after washing are viable. For each individual cell line 5 independent growth studies were performed in triplicate. For each study a growth curve of the natural log plot was determined and the line linear regressed and the equation of the line calculated. An average value for the equation of the line was then calculated for each cell line from the 5 independent experiments. The equation of each line (natural log plot as growth was exponential) can be seen in table 7.2.1. and the resulting growth curves produced from the equation shown in figure 7.2.1..

Figure 7.2.1. indicates there is no significant difference between the growth curves of clones 1, 32, 36, pSV$_2$neo, Met B, but all show a significant difference (P

$\leq$ 0.05) to the normal cell line BHK-21 which grows at a slower rate. The coefficient of correlation of a natural log plot (ln) of cell number against time for all cell lines is greater than 0.97, indicating exponential growth, hence a regressed linear plot can be used to calculate doubling times which correlate to growth. The doubling times for each cell line are shown in figure 7.2.2..

Table 7.2.1.: The *in vitro* growth curve linear regressed line equation for a natural log plot of cell number against time.

Cells from a healthily growing population of cells were harvested using trypsin, washed twice in complete medium to deactivate the trypsin and then counted on a haemocytometer. Aliquots of cells containing $1x10^5$ cells were placed into twenty one, 10 cm Petri dishes and incubated under normal tissue culture conditions. Every 24 hours all plates received fresh medium and 3 of the dishes were washed in PBS and the cells harvested using trypsin. Each harvested dish had the total cell number determined by counting on a haemocytometer, with 3 counts per dish. The mean from each dish was then averaged with the other dishes at that time point. This process was repeated for 6 days until all dishes were harvested. The average from the 3 dishes at each point constituted 1 growth curve. The natural log (exponential growth) was taken of cell number and plotted against time to give a linear plot. This line was then regressed to give the equation of the line. In all experiments Correlation Coefficient values exceeded 0.97 confirming linearity. For each cell line this was repeated a total of 5 times. The mean of the 5 line equations was then calculated and the resulting equation placed in table 7.2.1..

Table 7.2.1.

Cell line	Line Equation
Met B	Y=10.4 + 0.04444 X
pSV_2neo	Y=10.216 + 0.04548 X
CLONE 1	Y=9.67 + 0.0456 X
CLONE 32	Y = 11.0 + 0.0440 X
CLONE 36	Y =10.29 + 0.0468 X
BHK-21	Y=9.94 + 0.0186 X

Y=bo + b1 X

Y=Y axis (cell number)

X=X axis (time)

bo=Y intercept

b1=gradient

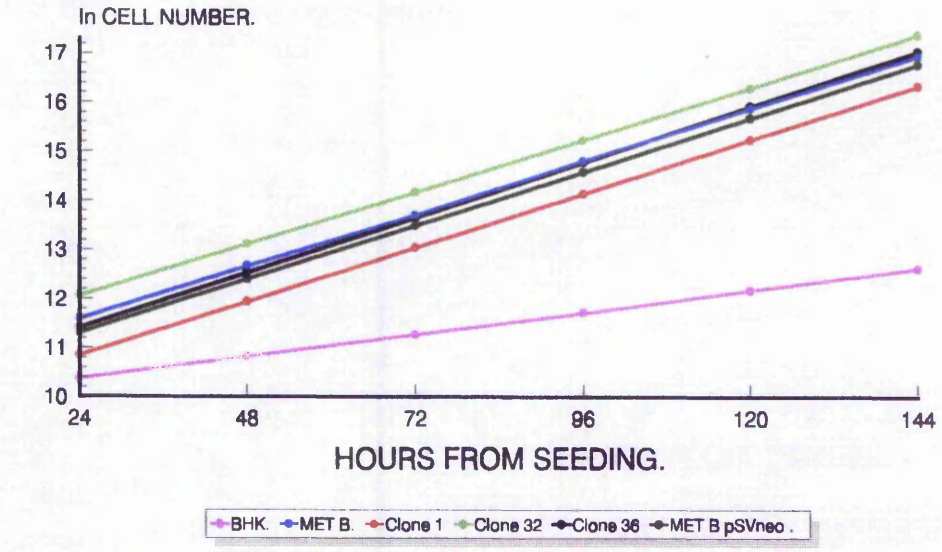
Figure 7.2.1.: *In vitro* growth curves of Met B, pSV2neo transfected Met B, the 'normal' cell line BHK-21 and clones 1, 32 and 36.

Growth curve experiments were performed exactly as specified in table 7.2.1. The line equations in this table were used to calculate the points for the growth curve.

Figure 7.2.2.: *In vitro* doubling times for growth of Met B, pSV2neo transfected Met B, the 'normal' cell line BHK-21 and clones 1, 32 and 36.

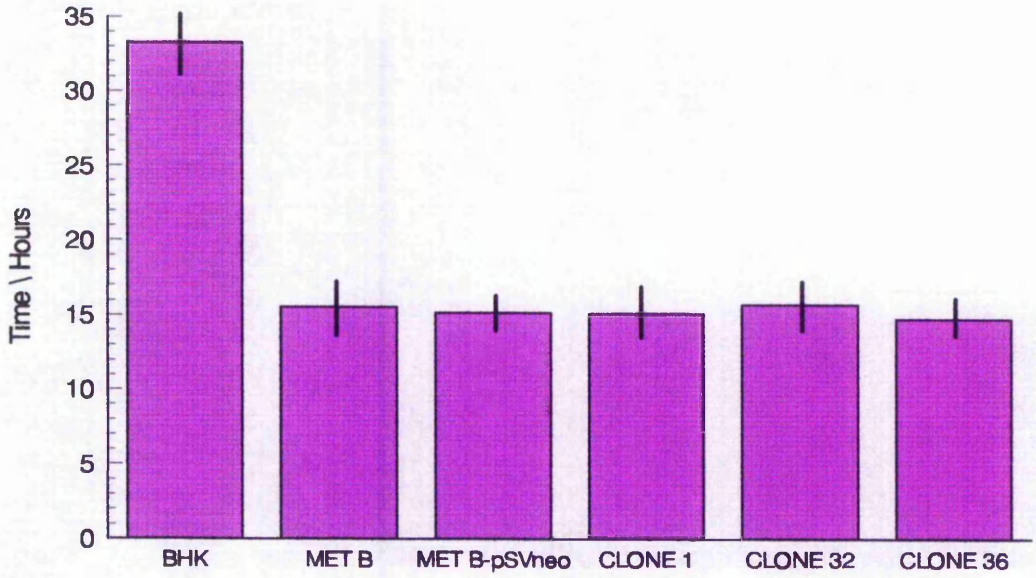
Doubling times were calculated from the line equations in table 7.2.1.

Figure 7.2.1.



AVERAGE OF 5 CURVES. 100000 CELLS SEEDED

Figure 7.2.2.



7.3.: Tumour growth of the transglutaminase transfected clones 1, 32 and 36 and control cell lines Met B and pSV_2neo transfected Met B

In vitro growth cannot be correlated with *in vivo* growth because cell growth in a plastic dish fails to simulate the host / tumour cell interactions in the animal. Therefore to observe the true effects of raising transglutaminase expression in a metastatic fibrosarcoma it is essential to return the cells to the animal. The easiest way to implant cultured cells and subsequently observe the growth is by subcutaneous injection of cells into the flank of the animal as in methods section 3.2.1.. This was done using injections containing 3 different cell numbers, i.e. 10^5, 10^4, and 10^3 of the cell lines Met B, pSV_2neo Met B and three transglutaminase transfected clones 1, 32 and 36. The total number of animals for each experiment are shown in table 7.3.1., while the growth curves calculated for tumours that did grow are shown in figure 7.3.1..

Growth rates for the different tumours indicate that once a tumour is detected then the growth rate remains constant between all cell lines injected and regardless of the cell number injected. Thus the growth rate of tumours *in vivo* mirrors what occurs for cells *in vitro*. What it is important to note however is that this is the growth once the tumour has started to develop and can be detected by touch. If the time for the tumour to actually be detected is looked at then immediate differences become evident between the transglutaminase transfected clones and the control cell lines. In figure 7.3.2. the average time for tumours to develop is shown for each cell line at the 3 injected cell concentrations used in the growth experiment. A summary of the total results is displayed in table 7.3.2..

Table 7.3.1.: Summary of total animal numbers used in growth curve experiments

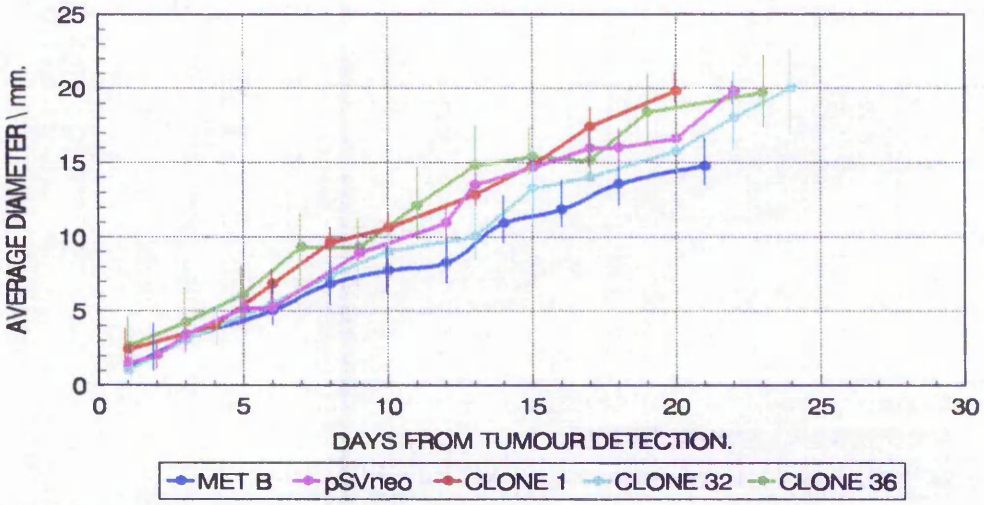
Cell line	Number of cells injected subcutaneously		
	10^3	10^4	10^5
Met B	4	9	20
pSV_2neo	5	13	29
clone 1	5	5	5
clone 32	5	5	3
clone 36	4	12	24

The total number of Syrian hamsters used in tumour growth curve experiments. These are divided into the number of animals injected at 10^3, 10^4 and 10^5 cells injected subcutaneously.

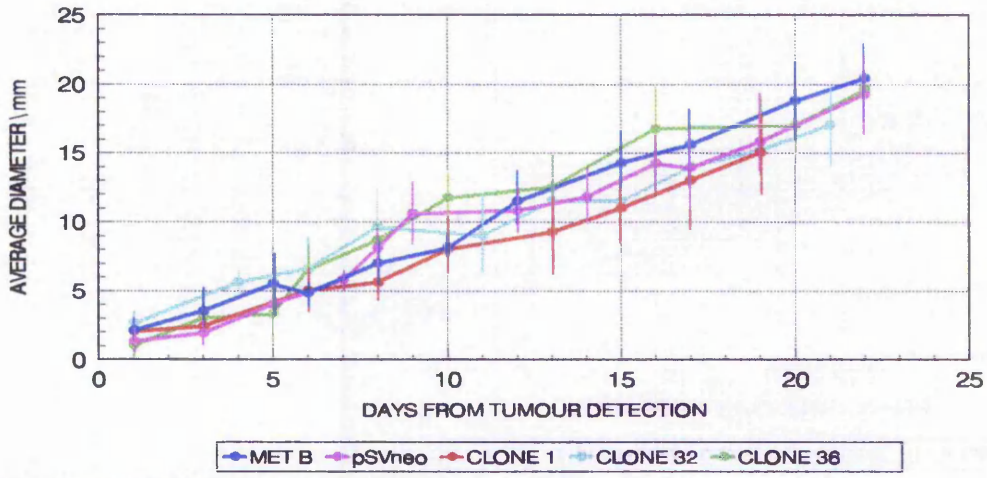
Figure 7.3.1.: Tumour growth curves in hamster following implantation by subcutaneous injection of Met B. pSV$_2$neo and clones 1, 32 and 36.

Healthily growing populations of cells at approximately 90% confluency were harvested using trypsin and then washed in complete medium to deactivate the trypsin, washed twice in sterile PBS and then counted on a haemocytometer. Cells were then resuspended in sterile PBS such that 100 μl volume would contain either 10^3, 10^4 or 10^5 cells depending on the growth curve under study. Cells were maintained at 4^{o}C. 100 μl volumes of cells were then injected subcutaneously on to the right rear flank of hamsters of approximately 4 months of age under ether anaesthesia. Following injection animals were checked daily for tumour development. On detection of a tumour the growing tumour was measured every 2 days using fat callipers and the tumour size determined as in section 3.2.1.. Data represents the average tumour diameter $\pm$ S.E.M. from the day the tumour was detected (not implantation). Sample numbers are listed in table 7.3.2..

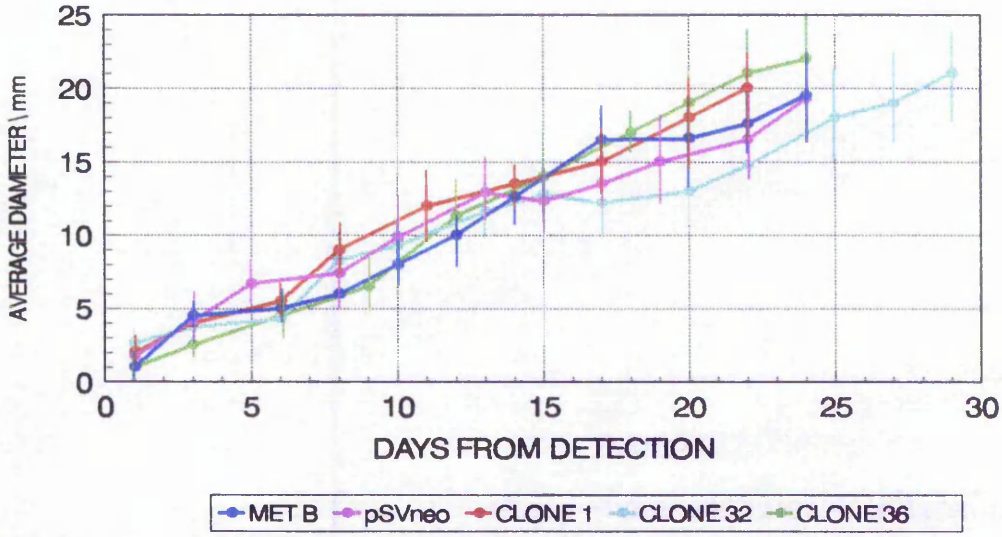
Figure 7.2.2.1.



100 thousand cells injected subcutaneously



10 thousand cells injected subcutaneously



1 thousand cells injected subcutaneously

Table 7.3.2.: Number of hamsters injected with transglutaminase transfected clones of control cells. the numbers of animals developing tumours and the day of detection of tumour.

Cell line 10^5 injected	N° animals injected	Percentage animals developing tumours	Mean day of tumour detection ± S.E.M.
Met B	20	100	13.8 ± 1.7
pSV_2neo	29	100	16.8 ± 2.6
Clone 1	5	100	26.4 ± 2.8
Clone 32	3	100	23.5 ± 1.5
Clone 36	24	100	26.4 ± 2.7

Cell line 10^4 injected	N° animals injected	Percentage animals developing tumours	Mean day of tumour detection ± S.E.M.
Met B	9	100	24.6 ± 2.0
pSV_2neo	15	87	24.9 ± 2.2
Clone 1	5	100	40.2 ± 2.3
Clone 32	5	60	26.3 ± 1.5
Clone 36	12	33	31.2 ± 2.4

Cell line 10^3 injected	N° animals injected	Percentage animals developing tumours	Mean day of tumour detection ± S.E.M.
Met B	4	100	34.0 ± 2.4
pSV_2neo	5	100	33.5 ± 2.1
Clone 1	5	40	59.5 ± 2.5
Clone 32	5	60	34.0 ± 3.5
Clone 36	4	50	49.5 ± 2.2

Table containing data on sample numbers, percentage of injected animals developing tumours and day of tumour detection for tumour growth experiments as outlined in figure 7.3.2..

Figure 7.3.3.: Tumour development time in hamster following subcutaneous injection of Met B. pSV_2neo and clones 1, 32 and 36.

Healthily growing populations of cells at approximately 90% confluency were harvested using trypsin and then washed in complete medium to deactivate the trypsin, washed twice in sterile PBS and then counted on a haemocytometer. Cells were then resuspended in sterile PBS such that 100 μl volume would contain either 10^3, 10^4 or 10^5 cells depending on the growth curve under study. Cells were maintained at 4°C. 100 μl volumes of cells were then injected subcutaneously on to the right rear flank of hamsters of approximately 4 months of age under ether anaesthesia. Following injection animals were checked daily for tumour formation and tumour development time recorded on the first day of detection. Detailed methodology is given in section 3.2.1.. Data represents the mean day of tumour detection for each cell line. Sample numbers and $\pm$ S.E.M. for the first day of tumour detection are listed in table 7.3.2.

Figure 7.3.4.: The percentage of animals developing tumours 75 days from subcutaneous injection of Met B. pSV_2neo, clones 1, 32, and 36.

The experiment was performed exactly as detailed for figure 7.3.3. and the sample numbers given in table 7.3.2.. Data represents the percentage of animals developing tumours following 10^5, 10^4 and 10^3 cells injected subcutaneously.

Figure 7.3.3.

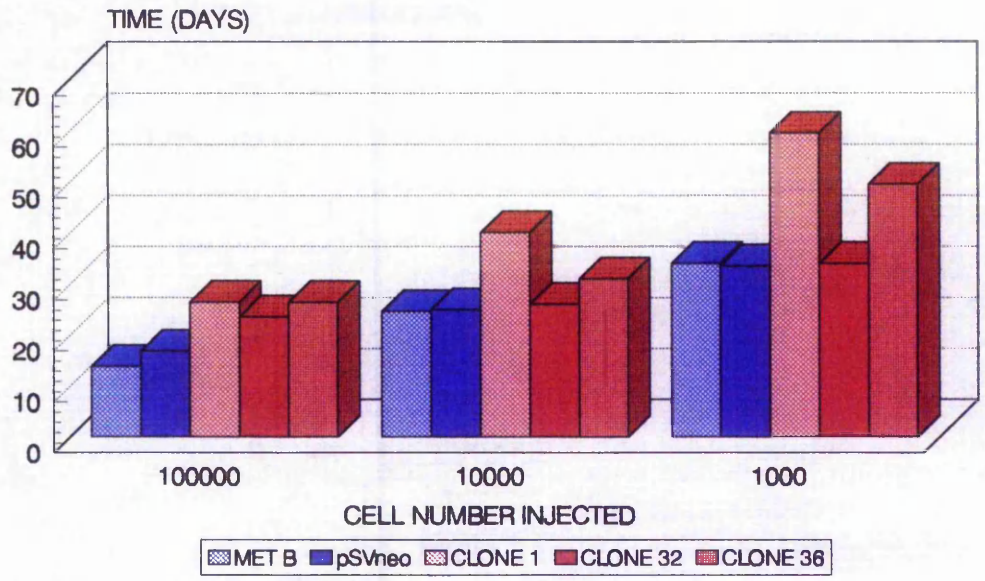
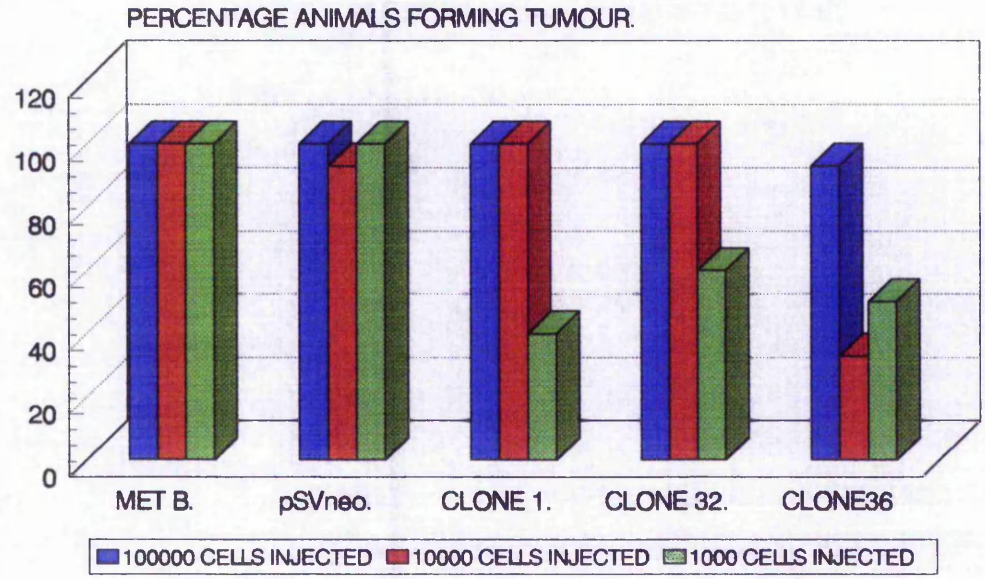


Figure 7.3.4.



In figure 7.3.3. the time for tumours to become detectable from the day of implantation is shown. If the Met B and pSV_2neo cell lines are compared then there is no significant difference in the time for tumours to develop at any concentration of cells injected, and thus the transfection procedure does not effect the cells ability to form a tumour. If the transglutaminase transfected clones are then compared to the control cell lines then it is clear that there is an increase in the time taken for the tumours to become detectable. When 10^5 cells are injected then in clones 1 and 36 which express higher levels of transglutaminase, the average time for tumour development increases by 10 days, and in clone 32 by 9 days. If 10^4 cells are injected, then this lag period in tumour development increases to 15 days in the highest transglutaminase containing cell clone 1, decreases to 6 days in clone 36. Clone 32 fails to show a significant lag time. If 10^3 cells are introduced in to the animal then in clone 1 the tumour development lag period is extended to 25 days, clone 36 to 16 days with clone 32 still failing to show a significantly different lag period to that of control cells. Thus there is a clear significant difference in time taken for tumours to develop with the transglutaminase transfected clones. It is interesting to note that in the high transglutaminase activity containing clones the lag period increases in duration as the number of cells injected subcutaneously decreases. This is particularly prominent in clones 1 than 36. The lowest transglutaminase activity clone (clone 32) fails to show a statistically significant lag period as cell number injected decreases. When 10^5 and 10^3 cells are injected there is a good correlation between the transglutaminase activity of the clone and the lag period in tumour development. This further emphasises that transglutaminase is having a specific effect on tumour development.

In figure 7.3.4. the percentage of injected animals which form a tumour following implantation are shown. When 10^5 cells are injected then there is no difference between the control cell lines and the clones in the numbers developing tumours with all animals injected forming a tumour, except in clone 36 where only about 90% of the animals develop a tumour. If 10^4 cells are injected then the

results are comparable to those obtained with 10^5 cells injected with clone 36 being the exception where only 30% of animals show tumour development. It is not until 10^3 cells are injected that a major difference between all the clones and the control cell lines becomes clear.

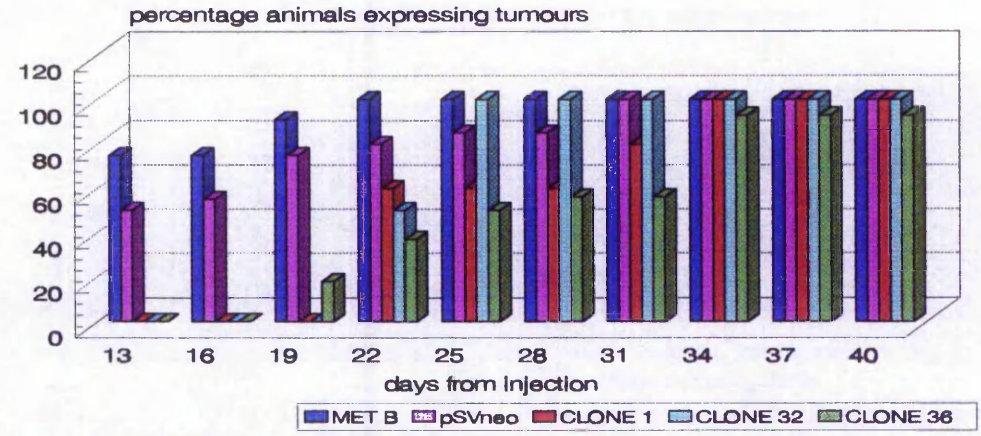
When 10^3 are cells injected then Met B and pSV_2neo still form tumours in all animals injected. The transglutaminase transfected clones show a large reduction in the percentage of animals developing a tumour. The clone with the highest transglutaminase activity, clone 1 only forms a tumour in 40% of animals injected, clone 36, the clone with the next highest transglutaminase only forms a tumour in 50% of the animals while the lowest activity clone, clone 32 results in tumour formation in 60% of animals. Thus a clear correlation between transglutaminase level and tumour development results.

In figure 7.3.5. results for the percentage of animals forming a tumour and time taken for tumour development are combined and provides a better if more complex overall view. This figure demonstrates that the time span for tumour development at each injected cell concentration is relatively tight with at maximum a 10 day span between the emergence of the first and last tumours in a particular group. In addition it shows that the time distribution in tumour development within each group is uniform for the transfected clones and control lines.

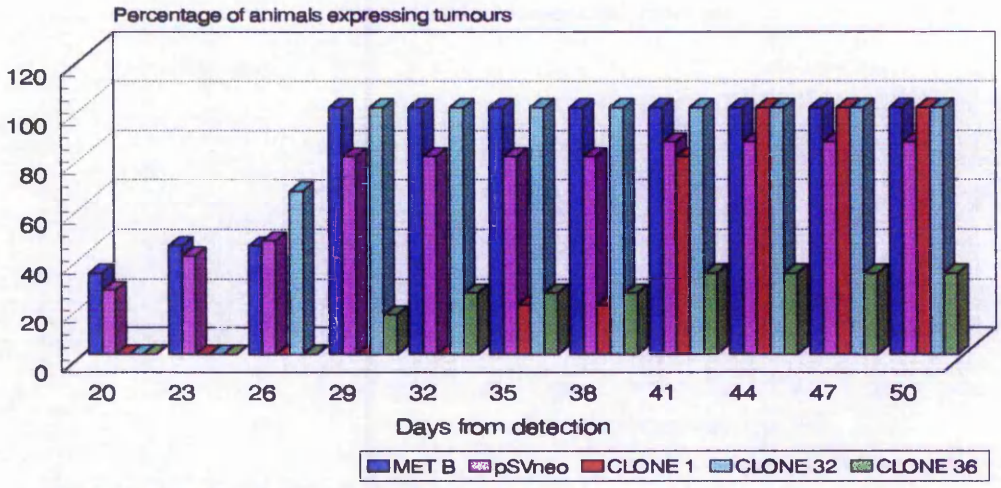
Figure 7.3.5.: Percentage of animals developing tumours at various time points following injection of Met B, pSV_2neo and clones 1, 32 and 36 into hamster

The experiment was performed exactly as detailed for figure 7.3.4. and the sample numbers given in table 7.3.2. Data represents the percentage of animals developing tumours following 10^5, 10^4 and 10^3 cells being injected subcutaneously at various times from injection.

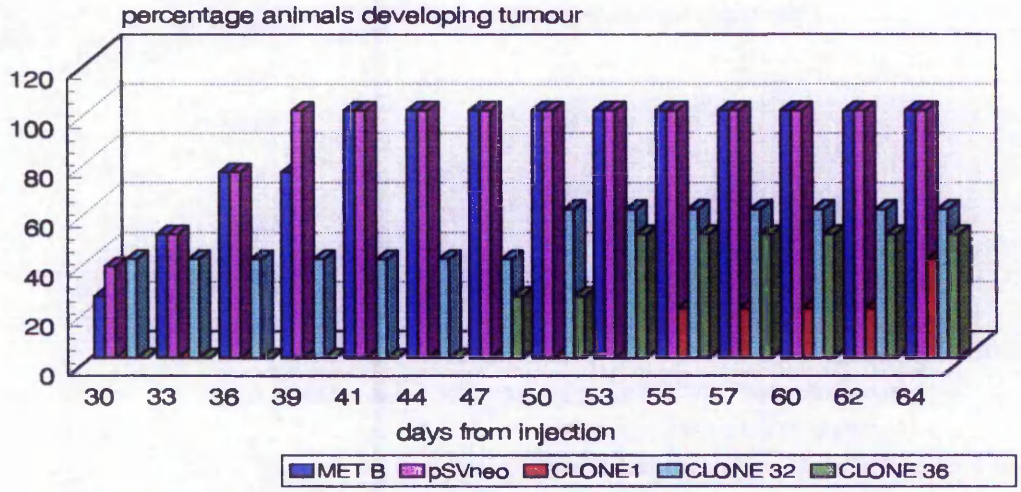
Figure 7.2.2.5.



100 thousand cells injected subcutaneously



10 thousand cells injected subcutaneously



1 thousand cells injected subcutaneously

7.4.: Transglutaminase activity measured in tumours obtained from transglutaminase transfected clones and control cell lines.

Prior to injection into the animal the activity of the clones was 28.72, 20.01 and 13.69 units per million cells for clones 1, 32 and 36 respectively. This correlated to increases of 156, 108 and 74 fold over pSV_2neo. When the tumours had developed to approximately 2 to 3 cm in diameter the tumours were removed and assayed for transglutaminase activity by the ^{14}C-putrescine incorporation into N,N'-dimethyl casein (methods section 3.4.3.1.), the results of which can be seen in figure 7.4.1.. The data clearly indicates that the huge increases in transglutaminase activity seen in the clones when compared to the controls had been reduced in the growing tumours with no or a very small increase over the control cell lines. Clone 1 has a transglutaminase activity level lower than pSV_2neo and tumours from clones 32 and 36 only showed a transglutaminase activity approximately 1.5 times greater than pSV_2neo. Liver and lung tissue were assayed as 'normal' tissue controls and gave values as expected (Barnes et al 1985, Hand et al 1990a). At first sight these results were surprising. The need for reduced transglutaminase activity has been reported for tumour development (Barnes et al 1985, Delcros et al 1986, Hand et al 1987a, Griffin et al 1989), but the loss of transglutaminase activity in a stably transfected cell with the gene attached to an independent promoter sequence was unexpected. This phenomena could be explained in a number of ways. Initially it was thought that cells expressing transglutaminase at higher levels may grow at a slower rate and therefore eventually through natural host selection the clone expressing a reduced transglutaminase would become dominant. However, this theory was unlikely since as *in vitro* growth (figure 7.2.1.) studies showed no differences in growth rate between the transglutaminase transfected clones, Met B and pSV_2neo transfected Met B, furthermore the transglutaminase activity in clone 36 had remained constant through several months of *in vitro* passage (data not

shown). Additionally it would be unlikely that the increase in transglutaminase activity observed in cell culture would be lost completely.

To clarify if the assumption that cells expressing a reduced transglutaminase level in the whole population would become dominant and lead to a reduction in the overall transglutaminase level in the tumour, transglutaminase activity was assessed at various times during tumour growth. Tumours were removed as soon as they were detected and large enough to be removed. This corresponded to approximately 0.5 cm diameter. In addition tumours were removed at approximately 1.5 cm diameter and then at about 3 cm, the maximum size without causing mobility problems or distress to the animal. It was found that the transglutaminase activity in Met B and pSV_2neo decreased by approximately 80 units per gram of wet tissue between days 20 and 55 from injection (figure 7.4.2.) which correlated with previous work by Knight et al (1990a,d), but clone 36 had already fallen to the same level as the control cell lines by the time the tumour was large enough to measure the activity, and then fluctuated around this level rather than showing a steady decrease. Since transglutaminase activity in clone 36 had already decreased to the control level at the first detection of tumour development then this reinforced the belief that growth selection of low transglutaminase containing cells was not responsible for the loss of activity. It would have been expected that so early in tumour growth that the transglutaminase transfected clone would still show a significantly raised transglutaminase activity that would then continue to decrease. It seemed unlikely that in a cloned cell line that was stable *in vitro* that such a high proportion of the cells would have lost their raised transglutaminase activity prior to injection to account for the rapid reduction in activity and that a few low transglutaminase containing cells able to grow could form a detectable tumour only 13 days behind the control.

Figure 7.4.1.: Transglutaminase activity in tumour arising from Met B pSV_2neo Met B and transglutaminase transfected clones 1, 32 and 36.

Animals were terminated by lethal exposure to ether and tumours quickly removed. Tumours were sliced open, non necrotic tissue extracted and homogenised using an 'Ultra Turrax' in homogenising buffer to give a 20% (w/v) homogenate. The homogenate was then assayed for transglutaminase activity by the ^{14}C putrescine incorporation assay (methods section 3.4.3.1). Data represents units of transglutaminase activity $\pm$ S.E.M (expressed as either per mg of protein or per gram of wet weight tissue). Sample numbers are between 13 and 49 (table 7.3.2.).

Figure 7.4.2.: Variations in transglutaminase activity with tumour development in Met B, pSV_2neo Met B and clone 36.

Animals were injected with 10^5 cells of either Met B, pSV_2neo transfected Met B or the transglutaminase transfected clone 36 as previously detailed in figure 7.3.4.. Tumours were allowed to develop and at various times into tumour growth, tumours were removed and assayed for transglutaminase activity as in figure 7.4.1. except that tumours were homogenised using a glass / teflon homogeniser (section 3.4.3.1.). Data represents transglutaminase activity $\pm$ S.E.M. per gram wet tissue weight from 4 or more experiments.

figure 7.4.1.

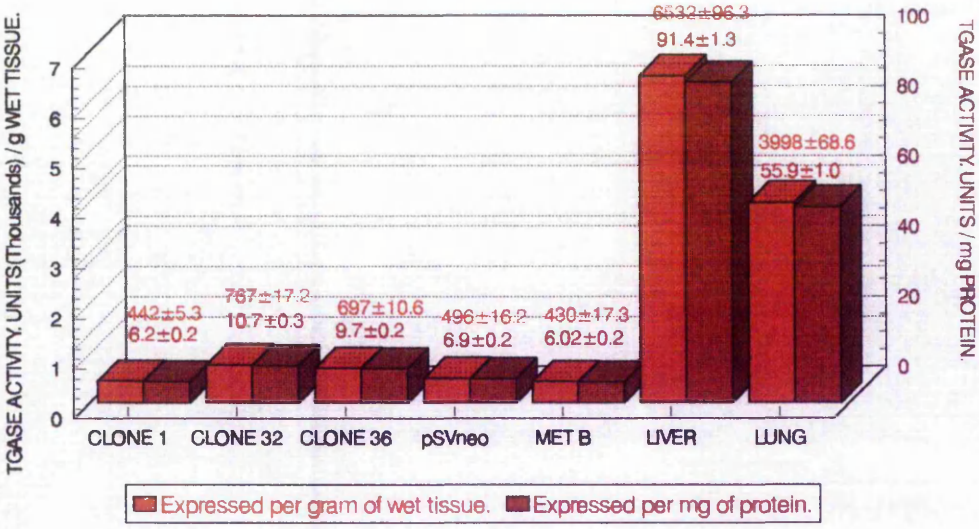
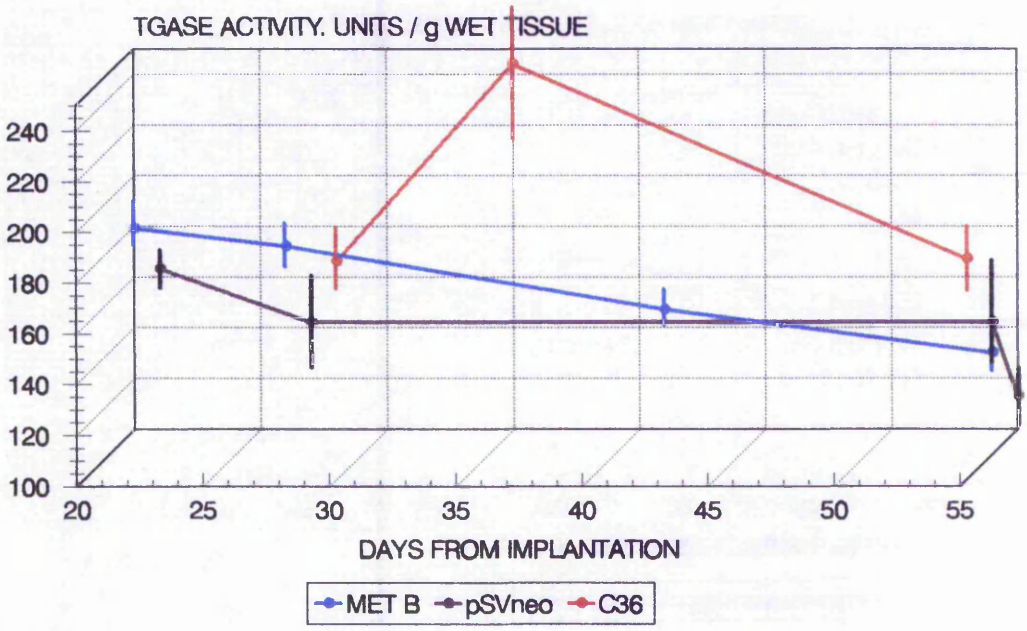


Figure 7.4.2.



If the cells within the population were not being selected for by virtue of their low transglutaminase activity and these cells within a population becoming dominant due to increased growth rate, then it is possible that cells were losing there transglutaminase activity by a regulatory process resulting from host/tumour cell interactions. As the transglutaminase was being controlled by an independent promoter sequence it seemed likely that the loss in activity was due to a post translational modification of the transglutaminase. One obvious product would be the previously reported inactive transglutaminase protein (Knight et al 1990a,d). Tumours from Met B, pSV_2neo and clone 36 were therefore analysed for the presence of the inactive transglutaminase as described in section 3.4.1.1. whereby a Pharmacia Mono Q column was used to separate transglutaminase in tumour homogenates into its distinct forms prior to enzyme assay. In figure 7.4.3. traces of activity and antigen for each cell line (Met B, pSV_2neo Met B, C36) forming tumour investigated are shown. The figure shows the average values and retention times from eight separations per cell line. Each tumour separation shows a successful separation of tissue transglutaminase into its 3 forms (eluted at approximately 0.2M, 0.3M and 0.4M NaCl respectively), although the level of particulate transglutaminase is lower than expected as no Lubrol Px extraction was performed.

Figure 7.4.4. compares the values of antigen and activity directly for each tumour type and indicates there is little activity difference between all types of tumour tissue analysed, with the control cell lines actually expressing a little more cytosolic tissue transglutaminase activity than clone 36. The inactive fraction (eluting at salt concentrations of around 0.3 M) shows no activity in any tumour. When antigen is compared then no difference exists between all tumour types if particulate and cytosolic forms are considered, if the inactive form is studied then as expected no difference exists between Met B and pSV_2neo Met B, but in clone 36 the inactive antigen peak is increased in quantity by approximately 100%. Although there is an increase in the inactive transglutaminase, this increase in the inactive

alone can not account for the total loss of transglutaminase activity by post translational modification as the inactive level only doubles were Western blot analysis of the clone prior to injection shows at least a 10 - 15 fold greater level of cytosolic tissue transglutaminase than the controls. As these traces also show that there is no differences in the tumours between the transglutaminase transfected clone and control cells level of cytosolic tissue transglutaminase this suggests that the cell is failing to produce transglutaminase at the transcriptional level.

This could result from any of three reasons. The clone may not actually be transcribing the DNA inserted in to its genome. Selective isolation of a non cellular controlled independent promoter is unlikely, but there may be sequences on the non coding regions of the inserted transglutaminase cDNA were regulatory proteins may bind to inhibit transcription. Alternatively the cell is not translating the mRNA into protein, thus regulation of the mRNA may be occurring at the translational level by inhibiting translation. The third possibility is that at some stage in tumour development the insert has been selectively removed from the genome. Whichever the reason, the transglutaminase level has remained raised long enough to cause a increase in the time for tumour development and to increase the number of cells required to induce tumour development.

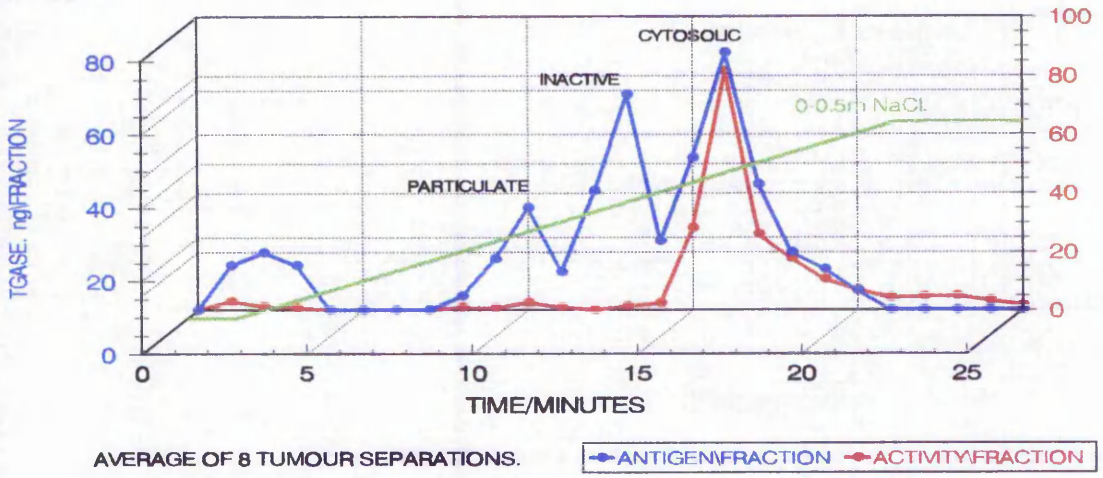
To determine which of these three proposals was the most probable a range of experiments were undertaken. These experiments were initially performed on solid tumours and then repeated on tumour cells that had been reintroduced into cell culture. This would facilitate an *in vitro* environment where it is possible to control and remove any systemic factors present in the animal that may be acting as regulators of transglutaminase, further this would help to determine if it was regulation or deletion of the inserted gene.

Figure 7.4.3.: Mono Q separation of tissue transglutaminase from tumour homogenates of Met B. pSV_2neo and clone 36.

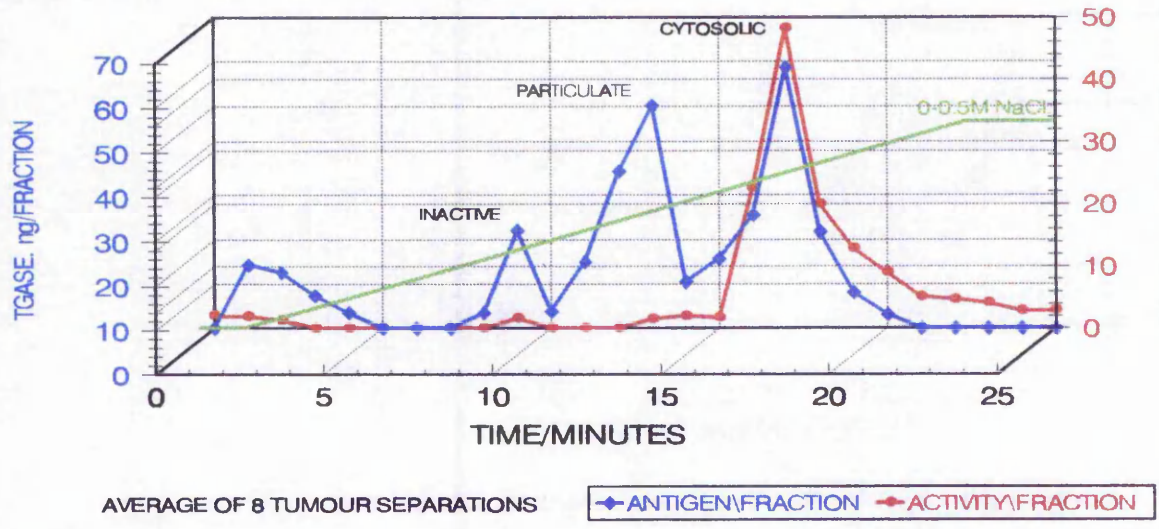
Cells were prepared and injected into hamsters as described in figure 7.3.4.. Following injection animals were checked daily for tumour development. Tumours were allowed to develop. When tumours were between 1 and 3 cm in diameter animals were terminated by lethal exposure to ether and tumours quickly removed. Tumours were longitudinally sectioned and non necrotic tissue excised and homogenised using a glass teflon homogeniser in homogenising buffer to give a 20% (w/v) homogenate. 500 μl of the homogenate was then loaded onto a Mono Q column and separated using a FPLC system (Pharmacia Ltd) using a 0-0.5 M sodium chloride gradient over 20 minutes at a flow rate of 0.35 ml min^{-1} as described in methods section 3.4.1.1.. Each fraction was then analysed for transglutaminase activity by the ^{14}H putrescine incorporation assay (section 3.4.3.1.) and transglutaminase antigen by ELISA as described in methods section 3.4.3.3.. Data represents mean transglutaminase antigen and activity per 1 minute fraction. For Met B and pSV_2neo n=8 and for clone 36 n=4. S.E.M are not shown for figure clarity.

Figure 7.2.3.3.

i: Met B



ii: pSV_2neo



iii: Clone 36

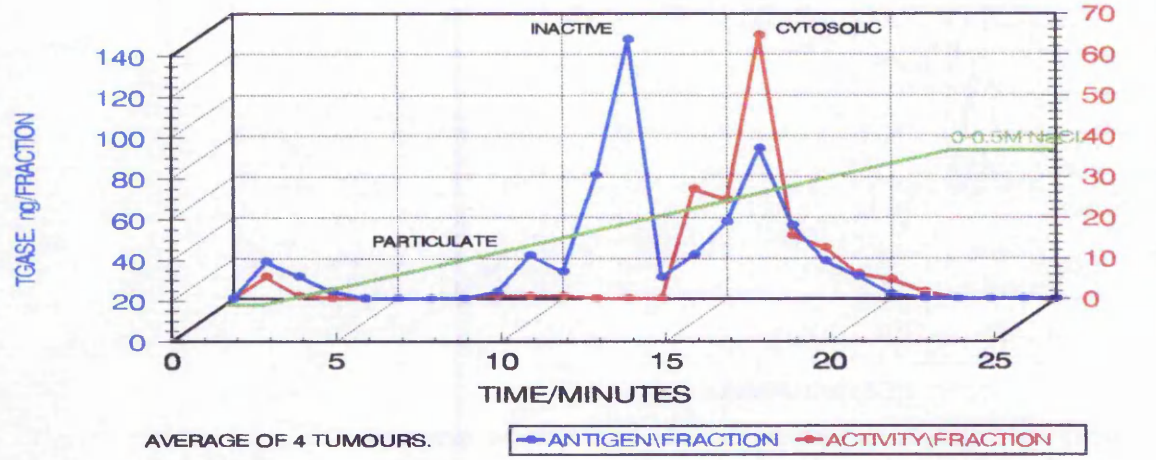


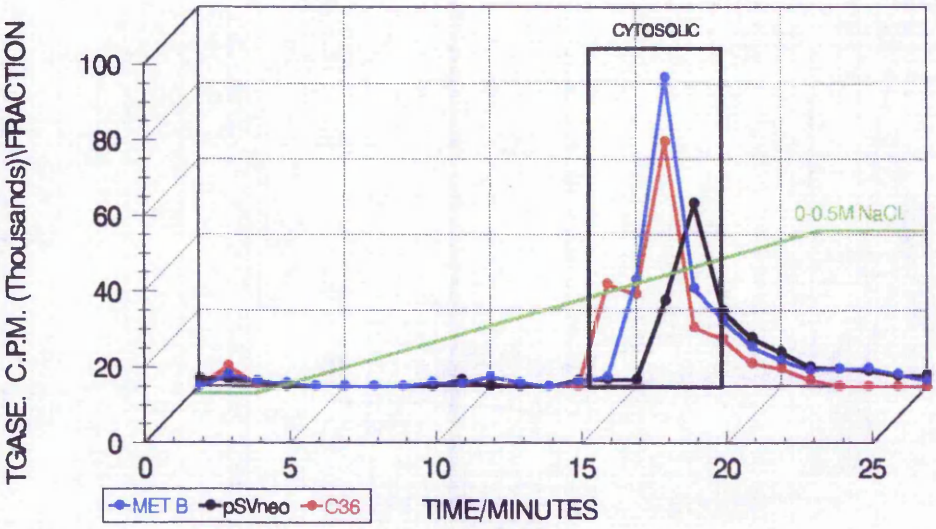
Figure 7.4.4.. Direct comparison of activity and antigen traces for tumours of Met B. pSV_2neo and clone 36 following separation on a Mono Q column.

Transglutaminase activity and antigen traces from C36, Met B and pSV_2neo tumours in figure 7.4.3. have been taken and summarised into activity and antigen charts to show variations between tumour types.

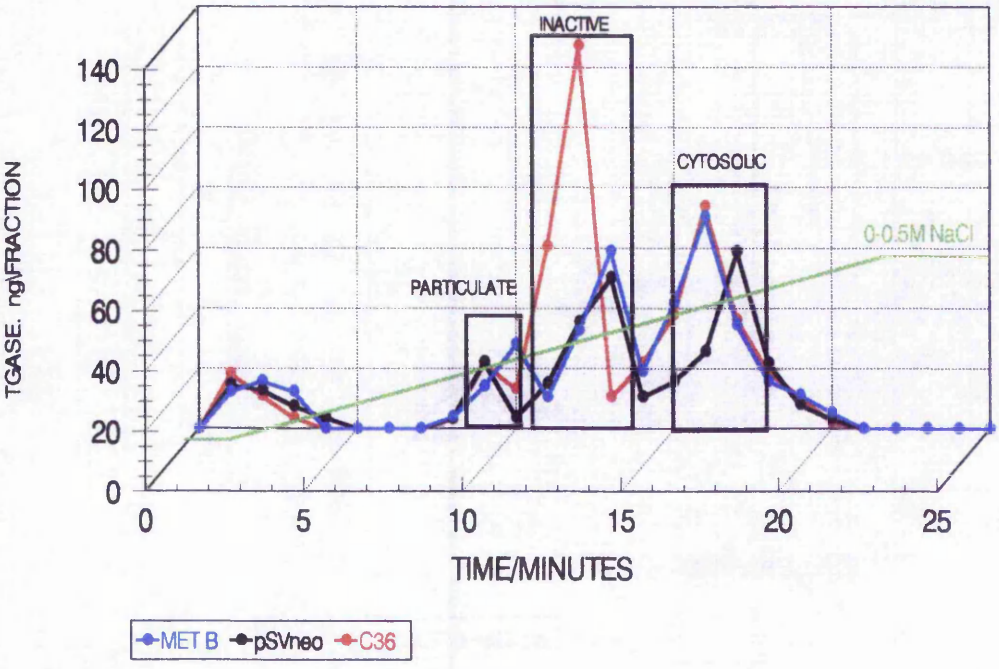
It is important to note that not all peaks occur in exactly the same fraction in this diagram. This results from the fact that these experiments were performed over several weeks and resulted in the Mono Q column being heavily used. This resulted in slight alterations to the retention and flow characteristics of the column. Data represents mean transglutaminase activity and antigen. For Met B and pSV_2neo $n=8$ and for clone 36 $n=4$. S.E.M. are not shown for figure clarity.

Figure 7.4.4.

i: Activity



ii: Antigen.



7.5.: Molecular analysis of Met B, pSV$_2$neo and Clone 36 tumours.

To verify the reduction of the cytosolic tissue transglutaminase antigen in transfected clones, a Western blot procedure was undertaken on tumour material previously fractionated by SDS-gel electrophoresis as described in section 3.4.3.3.. Figure 7.5.1. shows the resulting immunoprobed Western blot and clearly demonstrates that the antigen level of transglutaminase in clones 1, 32, and 36 has fallen to the same level as seen in the control cell lines. This confirms that a loss of the cytosolic form of the antigen has occurred.

Secondly total RNA was isolated from the tumours and a Northern blot analysis performed. The Northern blot in figure 7.5.2. which was probed with the complete human transglutaminase cDNA probe (section 3.3.2.6.) shows that there is such a low level of transglutaminase now present in the tumours that it is not detectable in a total RNA extraction. This is in contrast to the strong transglutaminase mRNA bands observed in the clones when grown in culture as shown in figure 6.2.4. in chapter 6. The validity of the extraction and probing technique is assured from the banding observed in an extraction from hamster liver performed at the same time. It must be therefore concluded that there is a failure of the cells to produce transglutaminase mRNA from the inserted cDNA. The final and definitive experiment was to verify that the cDNA insert was still present in the tumour material and the cells carrying the insert had not been excluded from the population. DNA was extracted from Met B, pSV$_2$neo Met B, C1, C32 and C36 tumour cells using proteinase K digestion and phenol extraction (methods section 3.3.1.1.). DNA was then digested with EcoR1 to fractionate the genomic DNA. EcoR1 was used since the transglutaminase insert had been inserted into the pSG5 plasmid using the EcoR1 restriction site in the multiple cloning site, hence in theory the EcoR1 digestion should release a 3.3 kb cDNA fragment. This digest was then run on a agarose gel and Southern blotted before being probed with the ^{32}P labelled

human transglutaminase cDNA. The pSG5 Tgase EcoR1 digest was used as a control. The autoradiograph resulting from this experiment is shown in figure 7.5.3.. It clearly demonstrates that the tumour tissue arising from Clones 1, 32 and 36 have a strong band at approximately 3.3 kb which correlates to the transglutaminase cDNA standard. Tumours from Met B and pSV_2neo do not have this band, hence the transglutaminase cDNA insert is still in the cloned cells once a tumour has developed.

These results indicate that suppression of tissue transglutaminase resulting from the exogenous cDNA insert must be occurring at the DNA level as no raised mRNA for transglutaminase can be detected, but the cDNA insert is still in the cells. This is a surprising result as control at the DNA level is usually via regulation of a promoter sequence and in this case the insert is controlled by an independent viral promoter. This suggests that there may be a sequence on the transglutaminase cDNA non coding region that may be controlled by a regulatory protein. Alternatively, but unlikely is that the mRNA from this cDNA is much less stable in the tumour environment leading to its degradation.

Figure 7.5.1.: Western blot of tumour homogenates from Met B, pSV_2neo and clones 1, 32 and 36 probed with goat 202 anti guinea pig liver transglutaminase.

Cells were prepared and injected into hamsters as described in figure 7.3.4.. Tumours were allowed to develop. When tumours were between 1 and 3 cm in diameter animals were terminated by ether asphyxiation and tumours quickly removed. Tumours were sliced open and non necrotic tissue excised and homogenised using an Ultra Turrax homogeniser in homogenising buffer to give a 20% (w/v) homogenate. 1 μl of the homogenate was run on a reducing 10%(w/v) polyacrylamide gel (section 3.4.1.2), and then Western blotted (section 3.4.2.1.) onto Hybond C Super (Amersham Ltd). The resulting blot was then probed with the goat anti guinea pig liver transglutaminase antibody (202) as described in methods section 3.4.2.2.. Lane 1 = Met B, lane 2 = pSV_2neo transfected Met B, lane 3 = clone 1, lane 4 = clone 32, lane 5 = clone 36.

Figure 7.5.1.

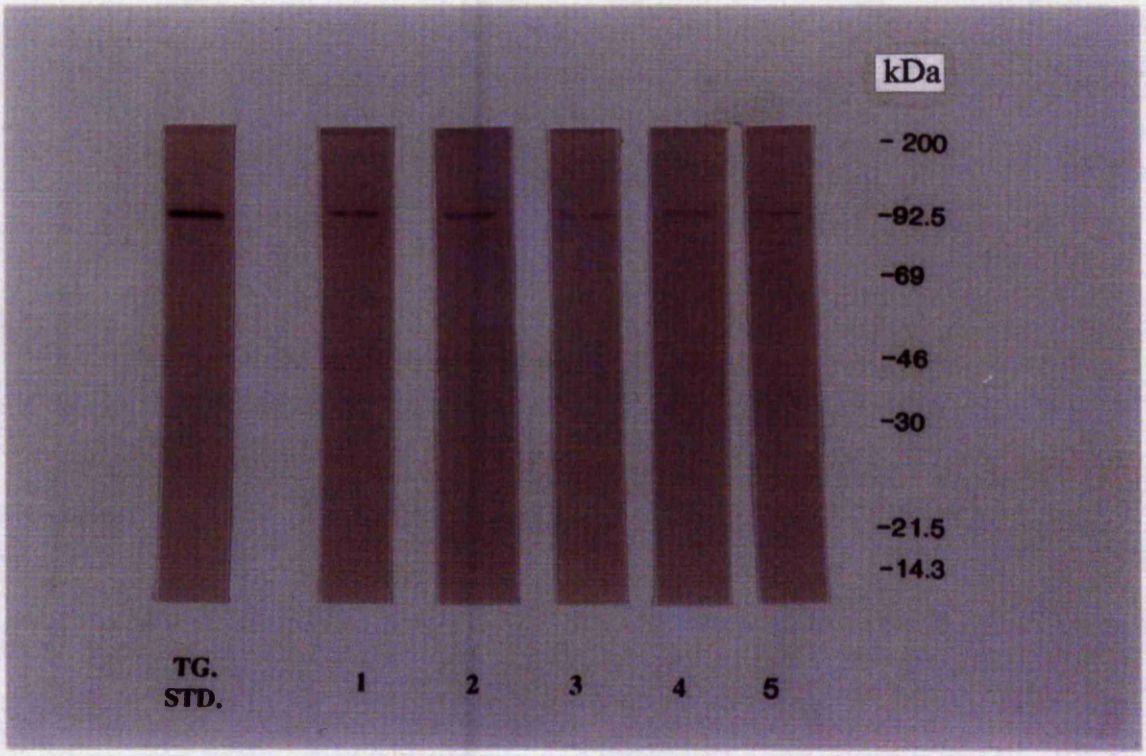


Figure 7.5.2.: Northern blot analysis for transglutaminase using total RNA extracted from tumours of Met B. pSV$_2$neo and clones 1, 32 and 36.

Cells were prepared and injected into hamsters as described in figure 7.3.4.. Tumours were allowed to develop. When tumours were between 1 and 3 cm in diameter animals were terminated by lethal ether exposure and tumours quickly removed. Tumours were sliced open and non necrotic tissue excised. Total RNA was extracted using the RNAsol B method as described in methods section 3.3.2.2.. The extracted RNA was electrophoresed on a agarose / MOPS / formaldehyde gel, and viewed under UV to verify loading and sample degradation and subsequently capillary blotted on to N^+ nylon (Boehringer) as detailed in methods sections 3.3.2.4. and 3.3.2.5.. The Northern blot was then probed using a complete human Tgase cDNA labelled with ^{32}P using the Promega random prime system (methods section 3.3.2.6.). The hybridised blot was dried and exposed to Kodak X OMAT RP film for 12 days using intensifying screens. Lane 1 = total RNA extract from Met B tumour (10 μg), Lane 2 = total RNA extracted from Met B pSV2neo tumour (10 μg), Lane 3 = total RNA extract from C1 tumour (10 μg), Lane 4 = total RNA extract from C32 tumour (10 μg), Lane 5 = total RNA extract from C36 tumour (10 μg), Lane 6 = total RNA extract from hamster liver (10 μg), Lane 7 = cytosolic transglutaminase mRNA standard (RNA extracted from transglutaminase transiently transfected cells) (1 μg).

Figure 7.5.2

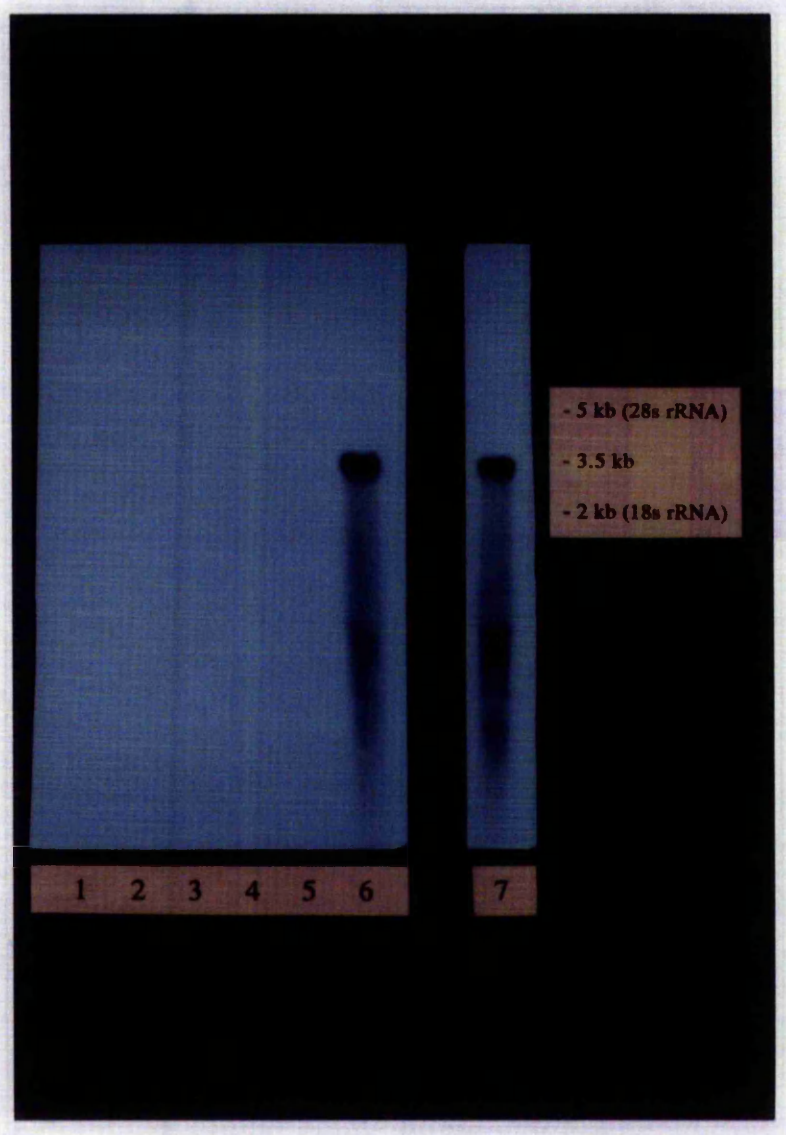
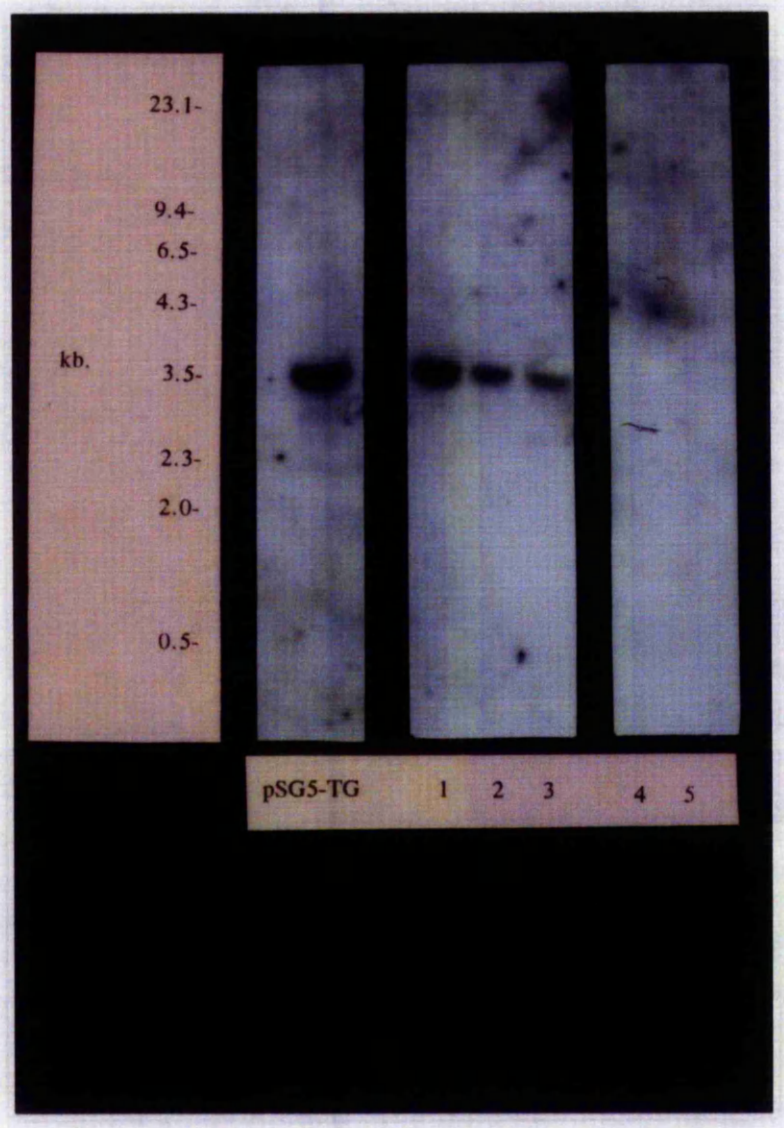


Figure 7.5.3.: Southern blot analysis of DNA extracted from Met B, pSV_2neo and transglutaminase transfected clones 1, 32 and 36 tumours for the 3.5 kb transglutaminase cDNA

Cells were prepared and injected into hamsters as described in figure 7.3.4.. Tumours were allowed to develop. When tumours were between 1 and 3 cm in diameter animals were terminated by lethal ether exposure and tumours quickly removed. Tumours were sliced open and non necrotic tissue excised. Genomic DNA was extracted using a proteinase K digestion and phenol extraction method as described in methods section 3.3.1.1.. Extracted DNA (5 μg) was subjected to a EcoR1 digestion and electrophoresed on a 1% (w/v) agarose / TAE gel, and viewed under UV light to verify loading and sample condition and subsequently capillary blotted on to N^+ nylon (Boehringer) as detailed in methods section 3.3.1.3.. The Southern blot was then probed using a complete human transglutaminase cDNA labelled with ^{32}P using the Promega random prime system (methods sections 3.3.2.6. and 3.3.2.7.). The hybridised blot was dried and exposed to Kodak X OMAT RP film for 6 days using intensifying screens. pSG5 Tgase (EcoR1 digest) standard, Lane 1 = EcoR1 digested genomic DNA extracted from C1, Lane 2 = EcoR1 digested genomic DNA extracted from C32, Lane 3 = EcoR1 digested genomic DNA extracted from C36, Lane 4 = EcoR1 digested genomic DNA extracted from Met B cells, Lane 5 = EcoR1 digested genomic DNA extracted from Met B pSV_2neo cells.

Figure 7.5.3.



7.6: Analysis of Clone 36, pSV_2neo and Met B tumours following return to tissue culture.

To verify that the loss of raised transglutaminase expression in tumours arising from transglutaminase transfected cells was a regulatory phenomenon and not an error in the insert itself it was necessary to show whether reactivation of the inserted tissue transglutaminase cDNA could be achieved following return of tumour cells to culture. Since there was no information on what was the controlling factor in this suspected regulation the easiest way to remove all systemic influences which may act on the cell was to return the tumours to an *in vitro* environment. Thus four clone 36, four pSV_2neo transfected Met B cells and two Met B tumours were returned to culture as described in section 3.1.6.. Each recultured tumour was grown both in a selective medium containing the selection agent G418 (800 units ml^{-1} for 14 days, then reduced to 200 units ml^{-1}) and in normal complete medium without G418. This was intended to show if a percentage of the tumour cell population had lost the insert in which case cells with no insert would die in the G418 groups. Following reintroduction to cell culture, when the cells had been in culture for approximately 3 days (passage 1) all the cells were tested for changes in transglutaminase activity, antigen and mRNA. At this stage no change in any of these factors was apparent except for a small increase in activity observed in the transglutaminase transfected clones compared with the control cell lines. At this stage, with all systemic factors and physiological pressures removed and no increase in transglutaminase activity it seemed probable that loss of transglutaminase expression in the tumours may have resulted from permanent deletion or damage to the transglutaminase cDNA. These recultured tumours were then left growing and periodically the transglutaminase activity measured. As the time and number of passages increased a further decrease in transglutaminase activity was observed followed by a increase in the transglutaminase activity measured in the clone 36 recultured cells (figure 7.6.1.). Initially the activity in clone 36 decreases, but this is then reversed and there is a steady increase in the activity which occurs over 22

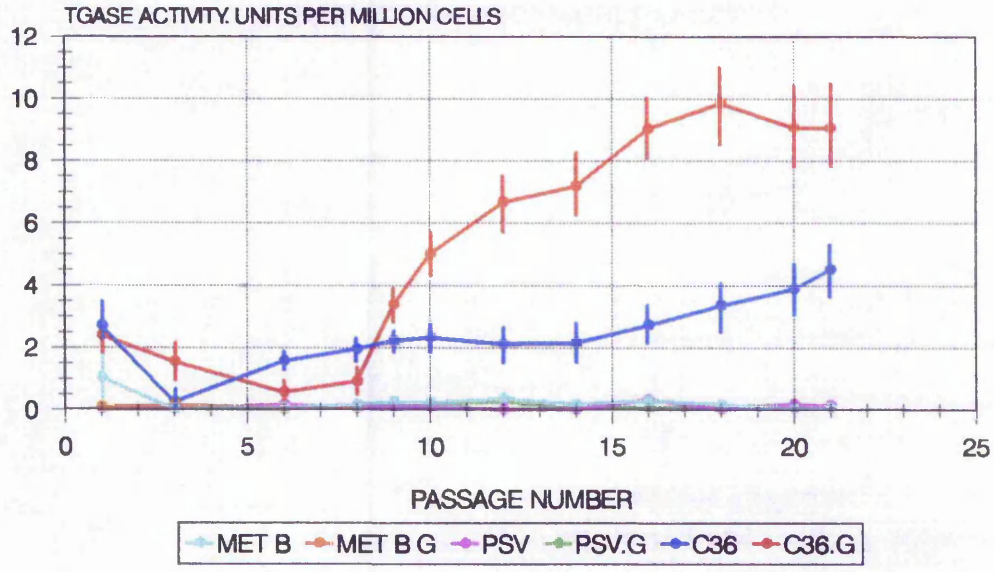
passages or 65 days. This increase in activity increases to approximately 10 units in the cells grown in a G418 environment and 5 units per million cells in a G418 free medium. Thus transglutaminase activity can be returned to approximately 50% of its initial value on removal from the animal. This figure which is only half the value of the original cells introduced into the animal suggests that some of the cells have lost the insert for transglutaminase during tumour formation and confirms that tumour development is favouring cells not expressing higher levels of transglutaminase. The value of only 5 units seen in the clone 36 cells grown in a G418 free environment is again half that seen in the cells grown in G418 again indicating approximately half of the cells which have lost the transglutaminase insert have also lost the pSV_2neo insert.

Having observed an increase in transglutaminase activity in the recultured clone 36 tumour cells it was necessary to check further parameters concerned with transglutaminase expression including antigen, mRNA and the presence of the cDNA insert. Western blots, Northern blots and Southern blots for recultured cells at passage numbers of approximately 20 can be seen in figures 7.6.2., 7.6.3. and 7.6.4. respectively. These figures demonstrate the presence of transglutaminase antigen and mRNA which in each case is higher in the G418 grown cells, and is approximately half that seen in the pre implant clone 36 cells when analysed by densitometric studies. The Southern blot analysis shows the cDNA insert ($\approx$ 3.5 kb) released by *EcoR1* digestion is still present thus paralleling the result found in the tumour samples and is not changed in size or band pattern.

Figure 7.6.1.: The variation of tissue transglutaminase activity with time and passage number following reintroduction to tissue culture of clone 36. Met B and pSV_2neo tumours.

Cells were prepared and injected into hamsters as described in figure 7.3.4.. Tumours were allowed to develop. When tumours were approximately 2.5 cm in diameter (40 - 50 days from injection) animals were terminated by lethal ether exposure. The animal was placed in a laminar flow cabinet, the skin sterilised with ethanol and the tumour quickly removed aseptically, cells aseptically released from the tumour and returned to tissue culture as described in section 3.1.6.. Cells were passaged when confluency reached 95%. Every 2 to 3 passages cells were harvested using trypsin and assayed for transglutaminase activity. The cells were resuspended in PBS and counted using a haemocytometer. A volume equivalent to $1x10^6$ cells was placed into an Eppendorf tube and the cells pelleted prior to being assayed for transglutaminase activity as in methods section 3.4.3.1.. Data represents mean transglutaminase activity (units) $\pm$ S.E.M. per $1x10^6$ cells. For pSV_2neo and C36 groups n=4 and for Met B groups n=2. G subscript indicates grown in G418 containing medium.

i: By passage number.



ii: By day

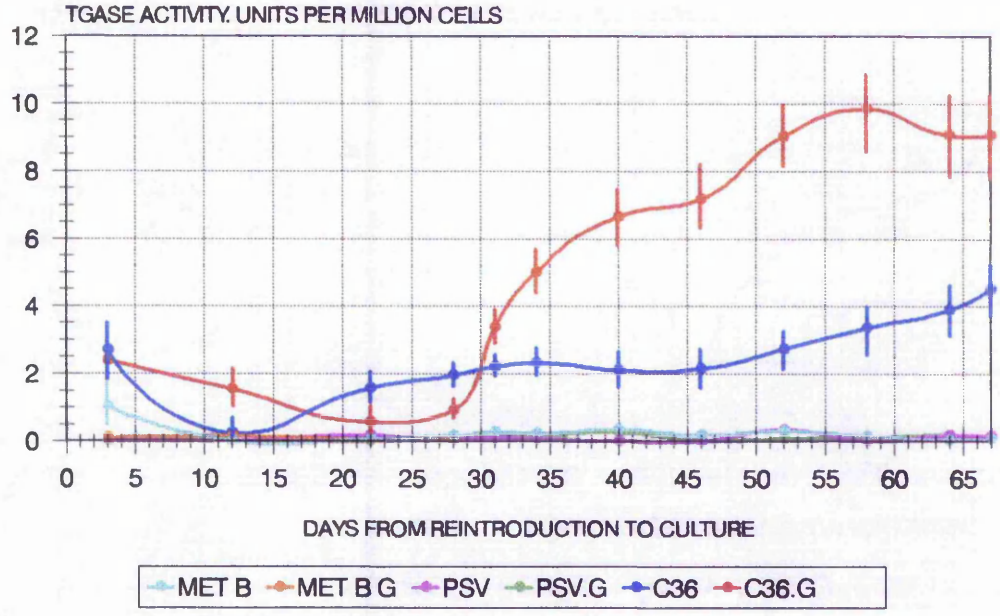


Figure 7.6.2.: Western blot of homogenates from cultured tumours of Met B, pSV_2neo transfected Met B and clones 1, 32 and 36 (passage number 19) probed with goat 202 anti guinea pig liver transglutaminase.

95 % confluent plates of cells recultured from tumours of Met B, Met B cells transfected with pSV_2neo, or clone 36 cells were grown in 75 cm^2 flasks in the presence of G418. Cells were harvested using trypsin and cell number determined using a haemocytometer before being homogenised. A volume equivalent to 1 million cells was loaded onto a 10% (w/v) polyacrylamide gel, electrophoresed and electroblotted on to Hybond C Super nylon (Amersham) as described in methods sections 3.4.1.2 and 3.4.2.1.. The Western blot was immunoprobed with goat 202 anti guinea pig transglutaminase antibody (methods, section 3.4.2.2.). Lane 1 = Guinea pig liver cytosolic transglutaminase standard, Lane 2 = Met B, Lane 3 = Met B pSV_2neo, Lane 4 = C36.

Figure 7.6.2.

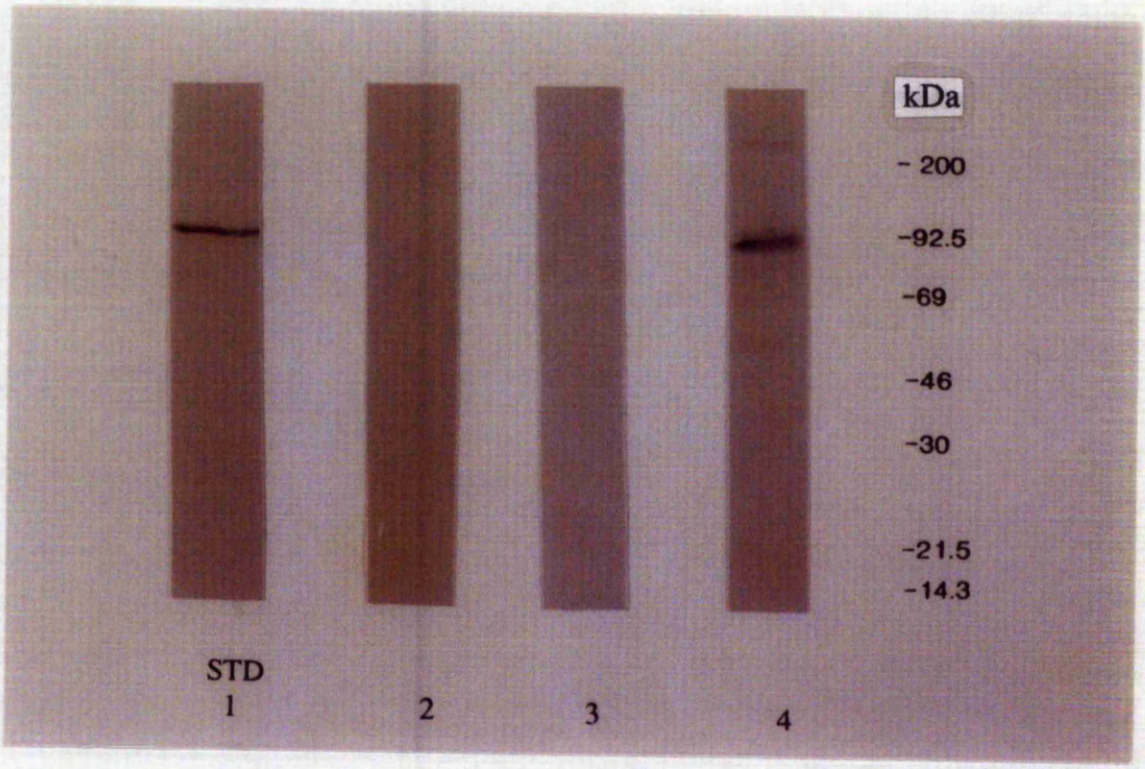


Figure 7.6.3.: Northern blot analysis of total RNA extracted from cultured tumours of Met B, pSV_2neo and transglutaminase transfected clone 36 (passage 20)

Total RNA was extracted from a 95% confluent 75 cm^2 flasks of either recultured Met B, recultured Met B cells transfected with pSV_2neo, or recultured transglutaminase transfected clone 36 cells grown in the presence of G418 using the RNAsol B method as described in methods section 3.3.2.1.. The extracted RNA was electrophoresed on a agarose / MOPS / formaldehyde gel, and viewed under UV to verify loading and sample degradation and subsequently capillary blotted on to N^+ nylon (Boehringer) as detailed in methods sections 3.3.2.4. and 3.3.2.5.. The Northern blot was then probed using a complete human cytosolic transglutaminase cDNA labelled with ^{32}P using the Promega random prime system (methods sections 3.3.2.6. and 3.3.2.7.). The hybridised blot was dried and exposed to Kodak X OMAT RP film for 12 days using intensifying screens. Lane 1 = total RNA extracted from recultured Met B tumour cells (10 μg), Lane 2 = total RNA extract from recultured Met B pSV_2neo tumour cells (10 μg), Lane 3 = total RNA extract from recultured C36 tumour cells (10 μg), Lane 4 = cytosolic transglutaminase mRNA standard (from transiently transfected cells) (1 μg).

Figure 7.6.3.

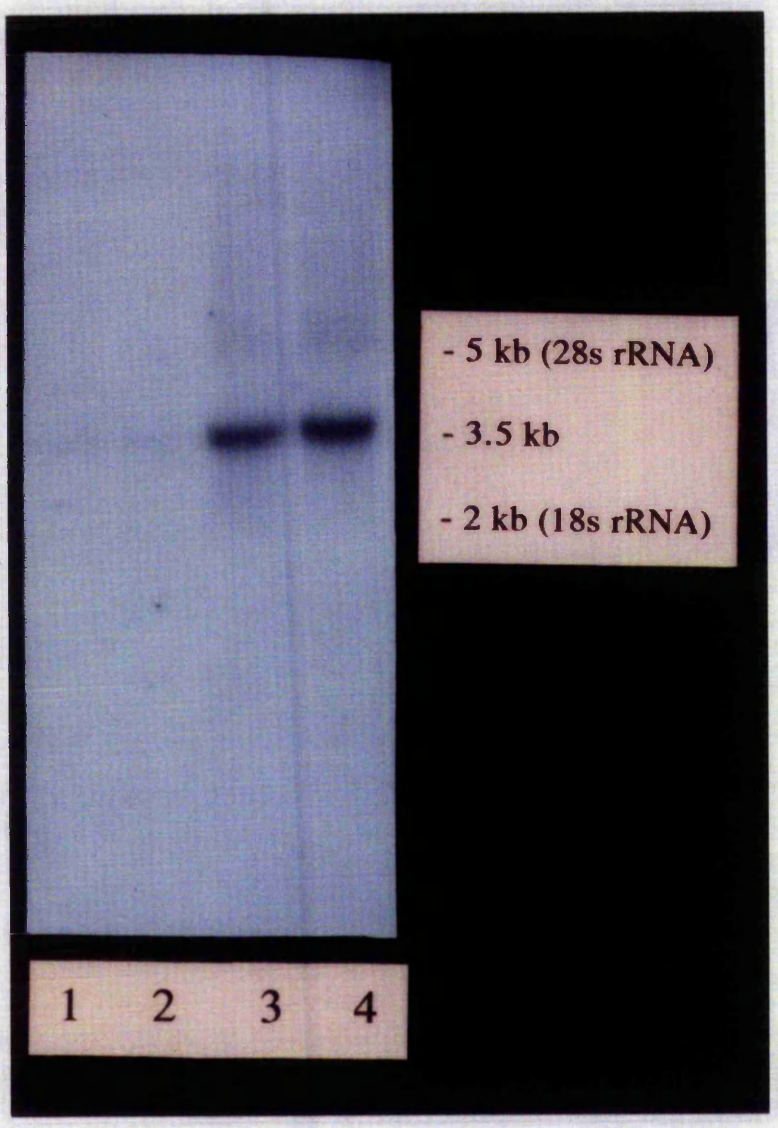
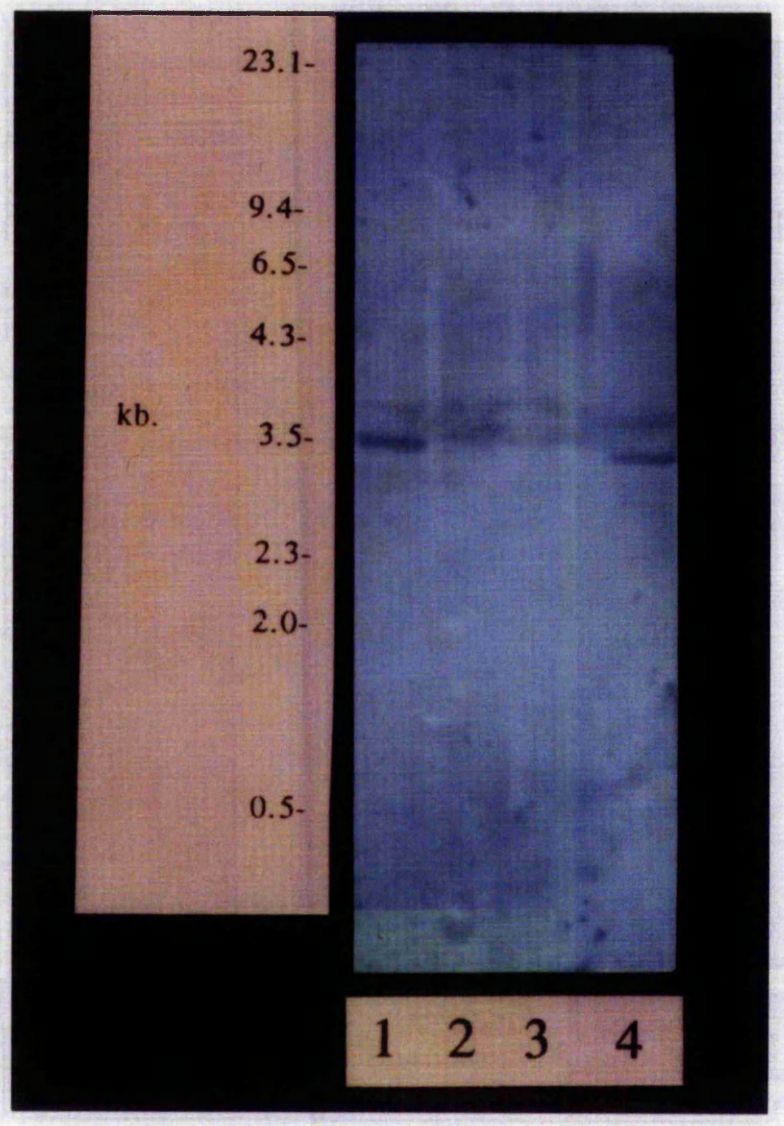


Figure 7.6.4.: Southern blot analysis for cytosolic transglutaminase of DNA extracted from recultured tumours of Met B, pSV_2neo and clone 36 (passage number 21).

Genomic DNA was extracted from a 95% confluent 75 cm^2 flasks of either recultured Met B, recultured Met B cells transfected with pSV_2neo, or recultured pSG5 Tgase clone 36 cells grown in the presence of G418 using a proteinase K digestion and phenol extraction method as described in methods section 3.3.1.1.. Extracted DNA (5 μg) was subjected to a *EcoR1* digestion and electrophoresed on a 1% (w/v) agarose / TAE gel, and viewed under UV to verify loading and sample degradation and subsequently capillary blotted on to N^+ nylon (Boehringer) as detailed in methods section 3.3.1.3.. The Southern blot was then probed using a complete human transglutaminase cDNA labelled with digoxigenin using the Boehringer digoxigenin nucleic acid detection system (methods sections 3.3.1.4., 3.3.1.5., 3.3.1.6. and 3.3.1.7.). The hybridised blot was exposed to Kodak X OMAT RP film for 1 day. Lane 1 = pSG5 Tgase (*EcoR1* digest) standard (250 ng), Lane 2 = *EcoR1* digested genomic DNA extracted from recultured Met B tumour cells, Lane 3 = *EcoR1* digested genomic DNA extracted from recultured Met B pSV_2neo tumour cells, Lane 4 = *EcoR1* digested genomic DNA extracted from recultured C36 tumour cells.

Figure 7.6.4.



To briefly summarise, on reintroducing the clones into an animal by subcutaneous injection, it was found that the cells containing raised transglutaminase activity took longer to form a tumour, but once tumour development was detected, tumour growth occurred at the same rate in all clones and the control cell lines. This suggested that either a large number of the injected population were killed, or the growth of the cells severely arrested for a number of days. The death of a large majority of the population appeared to be the most feasible idea. This was reinforced by the finding from dose response studies, which indicated that the numbers required to form a primary tumour in the transglutaminase transfected clones required a factor of 10 more cells Previous studies in chapter 6 had not indicated a raised apoptotic index for the cloned cells *in vitro*, but it could be feasible that on injection the raised transglutaminase cells may be induced to enter the apoptotic program to a greater extent when exposed to the physiological stimuli and pressures present *in vivo*. Additionally it may be a reduced ability of the clones containing raised transglutaminase activity to attach to the tissue they are injected into that results in the suspected cell death since earlier results (chapter 6) on cell attachment indicated a reduced level of attachment to substrates in 2 of the 3 clones tested. Alternatively the increased adhesion of the raised transglutaminase clones (chapter 6) may make these cells less invasive. The loss of transglutaminase activity in the cloned cell tumours apparently by a further unknown regulatory process may explain why not all the cells die following subcutaneous injection.

In summary, the data suggests that there are strong physiological pressure (e.g. immune, hormonal, physical, biochemical) acting on the clones carrying raised transglutaminase activity when they are placed into the *in vivo* situation.

7.7.: Tumour growth in immuno compromised animals.

It had previously been reported by Knight et al (1993) that both Natural Killer cells and lymphocytes showing LAK activity following treatment with IL-2 can trigger entry of target cells into the apoptotic program. Other factors related to the immune system may also have an effect on this process. Initially as a means of investigating if the immune system plays a part in selecting against cells containing increased expression of transglutaminase, tumour growth experiments were repeated in nude mice (balb c, (nu^+/nu^+)), which have a severely compromised immune system. These mice have a small, involuted, rudimentary thymus gland and are subsequently deficient in immune functions involving this gland. Functionally this means that nude mice are congenitally deficient in T cell function, but have a full complement of B lymphocytes and high levels of Natural Killer cells. Therefore in nude mice the effects on tumour development due to the action T lymphocytes would not occur. Reintroduction of transfected and non transfected cells was repeated in nude mice in order to undertake tumour growth studies where the immune system was compromised.

Figures 7.7.1. and 7.7.2. show that in the nude mice, tumour development and tumour growth rate is identical for clone 36 and the control cell lines pSV_2neo transfected Met B and Met B. This is different to tumour growth studies observed in hamsters since there is no lag period in tumour development in the transglutaminase transfected clone 36. This result suggests that the hamster immune system may be a factor in the lag period occurring in hamster and in the *in vivo* situation may cause the cells containing raised transglutaminase to enter the apoptotic program and thus result in the reduced ability of these cells to form tumours.

If transglutaminase was making cells more susceptible to the action of the immune system thus leading to their death, then in nude mice where this action was

reduced it might be expected that the resulting tumour should still have a raised transglutaminase activity as down regulation would not be essential to tumour cell survival. Figure 7.7.3. shows the transglutaminase activity in tumour resected from nude mice. In nude mice, transglutaminase activity in cells used to induce tumour growth (pre implant) was over 100 fold higher in clone 36 than in the control cell lines, however, on resection of the tumour at approximately 20 days post implant there was no difference in the transglutaminase activity found in tumours arising from clone 36, pSV_2neo and Met B cells. Comparison of these results with those found in hamster would suggest that the loss of transglutaminase activity during tumour growth is independent of the immune system. Hence the nude mice are likely to exert a regulatory system for those cells containing high levels of transglutaminase activity. Alternatively those cells containing low levels of transglutaminase when placed in the *in vivo* situation are those which can proliferate more rapidly

These studies undertaken on nude mice suggest that the immune system may be responsible for the lag period seen when cells containing raised transglutaminase activity are reintroduced into hamster for primary tumour development probably by inducing the cells with higher transglutaminase to die. To substantiate this finding further studies were designed to test whether hamsters could be immunised against clones carrying raised transglutaminase.

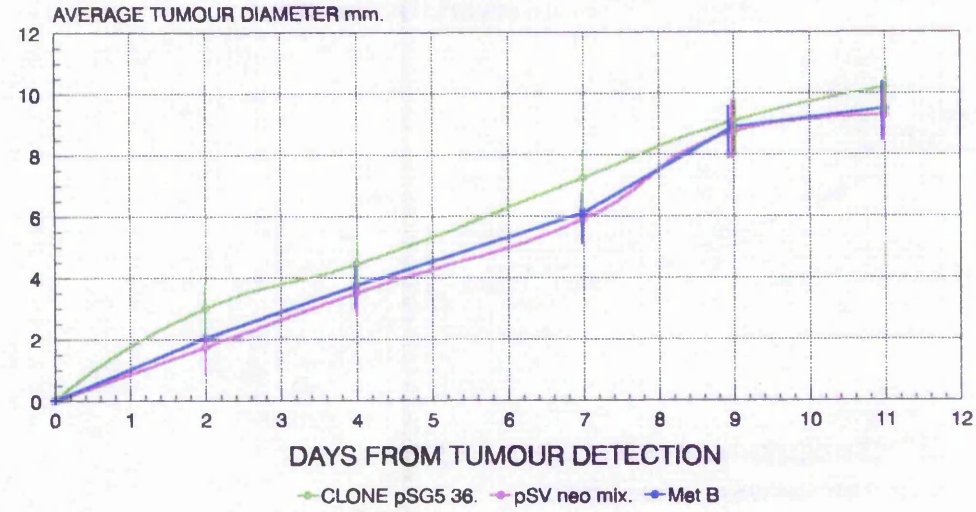
Figure 7.7.1.: Tumour growth curve in nude mice (balb c) when $1x10^5$ cells of Met B. pSV_2neo and clone 36 are implanted by subcutaneous injection.

Cells were prepared for injection as described in figure 7.3.4.. 100 μl volumes of cells (1 $x10^5$ cells) were injected subcutaneously on to the right rear flank of nude mice of approximately 3 months of age. Following injection animals were checked daily for tumour development. On detection of a tumour the growing tumour was measured every 2 days using fat callipers and the tumour size determined as in section 3.2.1.. Data represents the average tumour diameter $\pm$ S.E.M. for each cell line from the day the tumour was detected (not implantation) from 8 determinations.

Figure 7.7.2: Tumour development time in nude mice (balb c) following subcutaneous injection of 10^5 cells of Met B. pSV_2neo and clone 36.

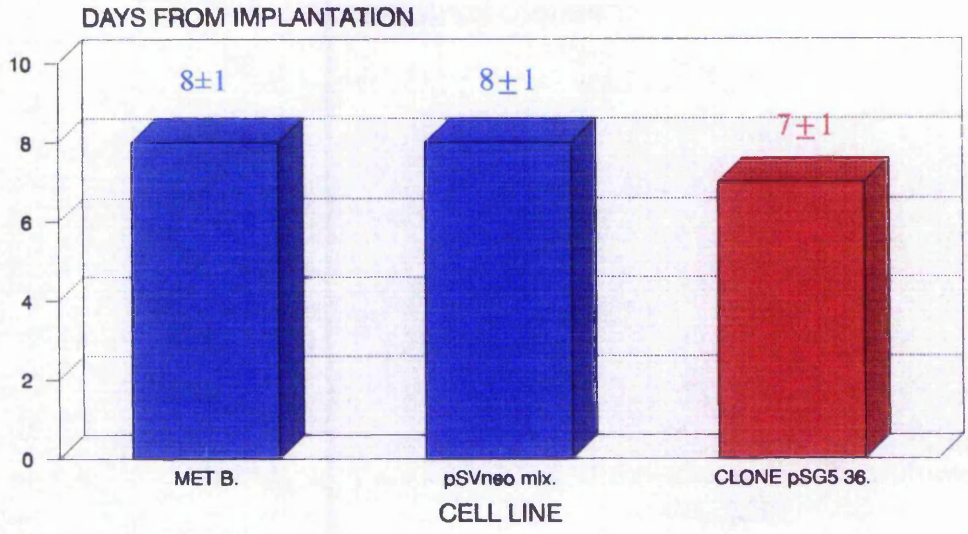
1 x 10^5 cells of either Met B, pSV_2neo transfected Met B or transglutaminase transfected clone 36 were subcutaneously injected into nude mice and tumours allowed to develop as described in figure 7.7.1.. Data represents the Mean day of tumour detection $\pm$ S.E.M. for each cell line from 8 determinations.

Figure 7.7.1.



100 000 CELLS INJECTED SC.

Figure 7.7.2.

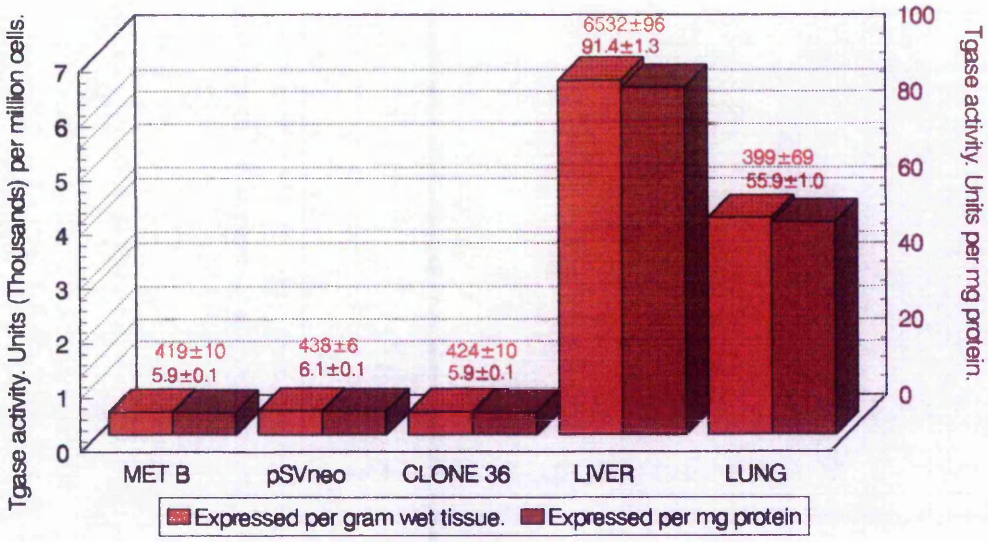


100 000 CELLS INJECTED SC.

Figure 7.7.3.: Transglutaminase activity of Met B. Met B transfected with the vector pSV_2neo and the transglutaminase transfected clone 36 post implant into nude mice

Animals from the experiment in figure 7.7.1. were terminated by exposure to ether and tumours quickly removed. Tumours were longitudinally sliced and non necrotic tissue excised and homogenised using an Ultra Turrax in homogenising buffer to give a 20% (w/v) homogenate. The homogenate was then assayed for transglutaminase activity by the ^{14}C putrescine incorporation assay (section 3.4.3.1.). Data represents units of transglutaminase activity $\pm$ S.E.M expressed either per mg of protein or per gram of wet tissue from 8 determinations.

Figure 7.7.3.


Tgase activity. Units (Thousands) per million cells.
Tgase activity. Units per mg protein.
0
1
2
3
4
5
6
7
0
20
40
60
80
100
419±10
5.9±0.1
438±6
6.1±0.1
424±10
5.9±0.1
6532±96
91.4±1.3
399±69
55.9±1.0
MET B
pSVneo
CLONE 36
LIVER
LUNG
Expressed per gram wet tissue.
Expressed per mg protein

7.8.: Tumour growth in pre immunised hamsters

Hamsters were pre-immunised with γ-irradiated cells that may or may not trigger an immune response, which would limit the growth of the tumour cells. Six groups of eight hamsters all of similar size and age were used. These groups were then pre immunised under the regime described in table 7.8.1.. It was expected that this immunisation regime would cause activation of T lymphocytes, particularly cytotoxic T cells and potentially T helper cells. NK, LAK and B cells would be unlikely to be activated (Personal communication Dr. R.C. Rees), although the exact effects of the immunisation on immune system activation were not fully investigated.

The results of tumour growth in pre-immunised animals can be seen in figure 7.8.1.. The X axis in this figure represents days from detection of the first tumour in either the irradiated or PBS inoculated populations. The Y axis is a percentage scale calculated by working out the number of animals developing tumours in the irradiated cell inoculated populations as a percentage of the PBS inoculated group. For example on day one if 3 animals from an irradiated population developed a tumour and 3 from the PBS inoculated group also showed tumour growth then the value would be 100 %, indicating identical development times. What these results show is that in animals pre immunised with Met B and pSV_2neo cells there is no difference in the tumour development times in the animals that have been pre immunised with the irradiated cells or PBS. In contrast when the healthy cells are introduced into the animal, in the populations that have been immunised with clone 36 irradiated cells, tumours begin to develop at different times in the PBS and irradiated clone 36 populations. There is an increase in tumour development time in the animals pre immunised with irradiated cells of between 8 and 19 days. Therefore challenging the animals immune system with known cells only alters the tumour development time if the animals are pre immunised with cells which contain raised transglutaminase activity. This suggests

that the immune system may be an agent in limiting tumour growth in the transglutaminase transfected clone resulting in the lag in tumour development time.

Table 7.8.1.: Experimental vaccination regime details for the pre immunisation of hamsters with irradiated cells.

6 groups of 8 hamsters were selected of mixed sex and of approximately 4 months of age. 2 groups were then assigned to either Met B, pSV$_2$neo transfected Met B or transglutaminase transfected clone 36. One of these groups acted as a control receiving a PBS inoculation while the second received irradiated cells. The first three inoculations were 1 week apart and then the fourth 14 days later. The animals were then left 10 days for the immune response to complete at which stage the animals were injected subcutaneously with $1x10^5$ non irradiated cells. These were harvested, injected and tumour growth monitored as previously described in figure 7.3.4..

Cells to be irradiated were harvested using 5 mM Tris, 1 mM EDTA, washed twice in sterile PBS and finally resuspended in PBS and subsequently irradiated as described in methods section 3.2.2. by exposure to 15 000 rads of ionising radiation. This required approximately 30 minutes exposure to the γ source.

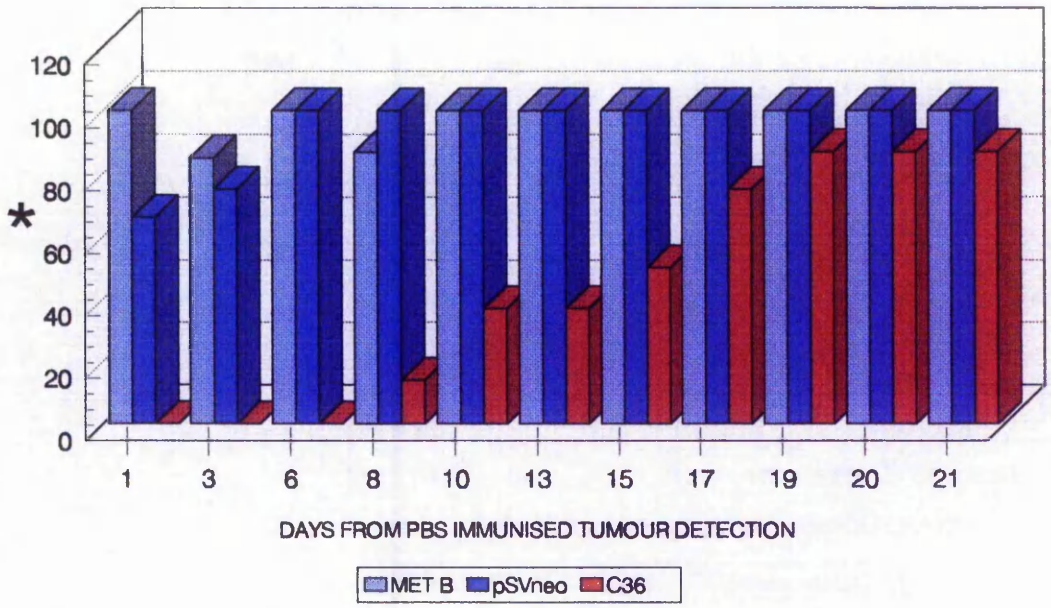
Table 7.8.1.

group	Day and injection contents				
	1	7	21	35	45
1	$1x10^6$ irradiated Met B	$2x10^5$ irradiated Met B	$2x10^6$ irradiated Met B	$1.5x10^6$ irradiated Met B	$1x10^5$ Met B
2	100 μl PBS	100 μl PBS	100 μl PBS	100 μl PBS	$1x10^5$ Met B
3	$1x10^6$ irradiated pSV$_2$neo	$2x10^5$ irradiated pSV$_2$neo	$2x10^6$ irradiated pSV$_2$neo	$1.5x10^5$ irradiated pSV$_2$neo	$1x10^5$ pSV$_2$neo
4	100 μl PBS	100 μl PBS	100 μl PBS	100 μl PBS	$1x10^5$ pSV$_2$neo
5	$1x10^6$ irradiated clone 36	$2x10^5$ irradiated clone 36	$2x10^6$ irradiated clone 36	$1.5x10^6$ irradiated clone 36	$1x10^5$ clone 36
6	100 μl PBS	100 μl PBS	100 μl PBS	100 μl PBS	$1x10^5$ clone 36

Figure 7.8.1.: The effects on tumour detection time of pre immunising hamsters with irradiated Met B. pSV_2neo and clone 36 cells followed by injection of a healthy population of the same cell line.

The experiment was performed exactly as outlined in the legend of table 7.2.6.1.. Data represents the percentage of pre-immunised animals expressing tumours compared to non immunised animals following tumour detection in non immunised animals. Actual animal numbers are given in the accompanying table.

Figure 7.8.2.



* = Y axis = $\frac{\text{Number of animals inoculated with irradiated cells expressing tumours.}}{\text{Number of animals inoculated with PBS expressing tumours}} \times 100$

Cell line		Number of animals expressing tumours on days following Detection of first tumour in PBS inoculated population										
		1	3	6	8	10	13	15	17	19	20	21
Met B	irradiated	4	6	7	7	8	8	8	8	8	8	8
	PBS	4	7	7	8	8	8	8	8	8	8	8
pSV$_3$neo	irradiated	2	3	8	8	8	8	8	8	8	8	8
	PBS	3	4	8	8	8	8	8	8	8	8	8
C36	irradiated	0	0	0	1	3	3	4	6	7	7	7
	PBS	1	4	7	7	7	8	8	8	8	8	8

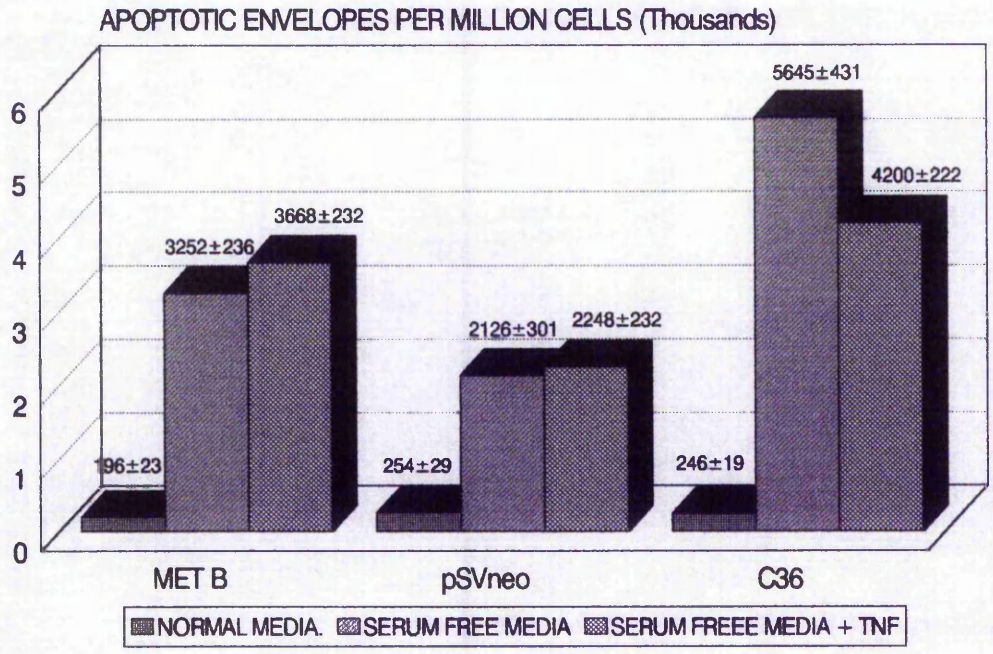
7.9.: The effect of Tumour necrosis factor alpha (TNFα) on apoptotic index *in vitro.*

Having suggested a possible role for the hamster immune system in the suspected death of Met B clones carrying raised transglutaminase activity, it was important to investigate further which components of the immune response involved were important. As tumour development in nude mice showed no differences in development times between control and transglutaminase transfected cells and pre immunisation had an effect in attenuating the delay in tumour development time in transglutaminase transfected fibrosarcoma cells, T lymphocytes (cytotoxic killer cells, T helper cells and T cells) were prime candidates particularly as these have already been implicated in recognition of tumour antigens by many authors (Reviews; Pardoll 1994, Tsomides and Elsen 1994). One particular agent specifically synthesised in quantity by T cells (Famularo et al 1994, Parra et al 1994) and frequently related to tumour regression (potentially via c-myc) is tumour necrosis factor alpha (TNF α) (Gridly et al 1994, Blum et al 1994, Janike et al 1994). Furthermore, TNF_{α} has been shown to be a potent inducer of spontaneous apoptosis *in vitro* in a number of cell lines (Fernandez and Ananthaswamy 1994, Dao et al 1994, Leist et al 1994, Polunovsky et al 1994). Therefore it was important to investigate if this cytokine was able to induce apoptosis in the raised transglutaminase clones to a greater extent than the control cell lines and could thus be a player in causing the suspected cell death in the injected clones containing raised transglutaminase activity. The results for TNF α with respect to the induction of apoptosis can be seen in figure 7.9.1.. This figure demonstrates that TNF_{α} has no effect *in vitro* on the level of apoptosis as measured by detergent insoluble body formation, although the removal of serum essential for the action of TNF α causes a large increase in cells developing apoptosis in all cell lines.

Figure 7.9.1.: The effect of TNFα on the induction of apoptosis in Met B, pSV_2neo transfected Met B and clone 36.

Cells were allowed to grow to approximately 80% confluency in duplicate 150 cm^2 flasks. Medium was removed and the cells washed in serum free medium, incubated in serum free medium for 30 minutes and then washed in serum free medium again. Serum free medium was then added to the flasks. One was used as a serum free control, while the second had TNFα added to a concentration of 500 units ml^{-1}. Both flasks were incubated for 24 hours. Cells were collected from both medium and plate surface. These were then counted on a haemocytometer, pelleted and resuspended in PBS containing 2% (w/v) SDS and 0.1% (w/v) dithiothreitol (DTT) before being boiled for 5 minutes. Following cooling DNase 1 was added to a concentration of 20 units ml^{-1} and the solution incubated at 37 ^oC for 1 hour. Apoptotic envelopes were then pelleted and subsequently washed 3 times in 2% (w/v) SDS, 0.01% (w/v) DTT, 10 units ml^{-1} DNase 1. Bodies were again pelleted and resuspended in 10 μl of the wash solution. (section 3.4.4.1.). Bodies were then counted under a inverted light microscope by at least three workers who were unaware of the experimental groups and the figures averaged. Apoptotic envelopes were counted using either a haemocytometer or the number of bodies per μl volume depending on the workers preferred method of counting. Data represents mean number of detergent insoluble bodies ± S.E.M. from 5 experiments.

Figure 7.9.1.


APOPTOTIC ENVELOPES PER MILLION CELLS (Thousands)
6
5
4
3
2
1
0
196±23
3252±236
3668±232
254±29
2126±301
2248±232
246±19
5645±431
4200±222
MET B
pSVneo
C36
NORMAL MEDIA.
SERUM FREE MEDIA
SERUM FREEE MEDIA + TNF

7.10.: The effects of raised transglutaminase expression on experimental metastasis

Since the first links between transglutaminase and tumour progression were reported (Laki et al 1966), the relationship between transglutaminase and tumour metastasis has been studied. The relationship between transglutaminase and metastatic potential was characterised comprehensively in the Met cell line (A range of cells of differing metastatic potential originating from a single parent) by Knight et al in 1990a,d where an inverse relationship was shown between the cells metastatic potential and transglutaminase activity. Therefore it was important to investigate this relationship with the clones containing raised transglutaminase activity. This was not be possible by allowing a tumour to develop in hamster and then resecting the primary tumour since by the time the tumour had developed to a detectable level, all increases in transglutaminase activity had been lost. Therefore it was only possible to do this by experimental metastasis, a technique usually carried out in nude mice to allow characterisation of the changed phenotype without immune response complications. Experimental metastasis is a technique where cells are removed from culture and injected directly into the venous blood supply so the cells ability to adhere and colonise to a new environment can be assessed without changes occurring to the cells in primary tumour development.

Early experiments done in hamster allowing primary tumour development which were then resected to allow secondary tumours time to become detectable showed no reduction in metastasis in animals with clone 36 tumours compared Met B tumours (data not shown). This was not surprising due to the total loss of raised transglutaminase activity. Experimental metastasis experiments in nude mice on first site appeared to show a reduction in the number of metastasis present in the lungs and a more comprehensive pathological examination performed by the Royal Hallamshire Hospital pathology department showed that a greater number of metastatic foci were present in lungs from animals injected with Met B and pSV_2neo

transfected Met B than in clone 36. Unfortunately when this work was repeated, the results were inconclusive in 2 further experiments where no detectable difference between cell lines could be detected. Further study needs to be undertaken to draw definitive conclusions on the link between transglutaminase and metastatic potential.

7.11: Discussion.

When the growth rate of the 3 cloned cell lines is measured *in vitro* it is identical to that seen in the pSV_2neo transfected control and the parent fibrosarcoma cell line Met B. It can therefore be concluded that the transfection process, plasmid integration and raising transglutaminase activity within the cell are not affecting growth rate. When the growth rate is measured *in vivo* in hamsters following subcutaneous injection of the cells then again growth rate is the same in transfected clones and controls once the tumour is detected. However, in animals injected with the transglutaminase transfected clones then the time for a tumour to develop to detectable size is increased, which is approximately proportional to the transglutaminase activity of the cell line at all 3 levels of cell injection. This indicates that the lag must be a result of the higher transglutaminase activity since it is the only difference between the control and transglutaminase transfected clones. In addition to this, cell dose response experiments indicate that the transglutaminase transfected clones need a greater number of cells injected to facilitate tumour development, again showing a correlation between transglutaminase activity levels and tumour development. This is particularly true when lower numbers of cells are injected (i.e. 10^3).

The identical growth rate of tumours once they are detected indicates that the reason for the lag time in tumour development is not a result of the clones having a slower growth rate, but suggests that the population of cells that are able to form a colony on injection is smaller, probably due to a greater proportion of cells being

killed on injection of the transglutaminase transfected clone populations. This is supported by the cell dose response experiments which also suggest a lower survival rate of the transglutaminase transfected clones on injection, since more cells need to be injected to cause tumour formation.

When these experiments involving hamsters are repeated in nude mice, where the immune system is severely compromised by a T cell deficiency, then again growth in controls and clones is identical, but here the lag time of several days found in hamsters does not occur in nude mice. This suggests a role for the immune system (particularly T cells) in the establishment of this lag period, which initially may indicate a change in cell surface antigenicity due to the transfection procedure. However as the transfection control shows no lag period then the transfection procedure can not be responsible and thus it can only be attributed to the presence of increased transglutaminase activity. The pre-immunisation experiments on hamster substantiate this conclusion.

If transglutaminase was responsible for the failure of the transglutaminase transfected cloned cell lines to survive , the question remains as to why all the cells were not killed on injection and hence why did a tumour develop. This was partly answered by the transglutaminase activity studies undertaken on tumours which showed that by the time a tumour could be detected then transglutaminase had fallen to that of the control cells. Studies to ascertain the loss of activity showed that the this was in the main not a result of cells losing the cDNA insert as Southern blotting demonstrated an intact insert in the tumours. Furthermore, when cells were recultured from tumours they showed more than 50% of the activity returned once the physiological pressures present in the *in vivo* environment were removed. Western blot analysis showed a loss of transglutaminase antigen in the tumours arising from transglutaminase transfected clones, while the FPLC / Mono Q analysis demonstrated that in clone 36 the level of the inactive transglutaminase had doubled. This demonstrates that some of the loss in activity may be due to post

translational modification, but the levels of inactive enzyme protein could not explain the huge loss of transglutaminase activity and antigen. Northern blot analysis revealed that transglutaminase mRNA had fallen to the level of the control cells. This was extremely surprising as the insert remained in the tumour and the cDNA is controlled by an independent constitutive SV40 promoter that is unlikely to fall under cellular control. Therefore the only conclusion available was that the regulation was occurring at the transcriptional level, possibly by the binding of a regulatory protein directly onto the insert, which could be associating with a sequence on the non coding 5' region of the cDNA insert. A possible system could be where coarse regulation was occurring at the gene level with an apparent fine tuning by post translational modification.

To suggest a possible explanation for the events occurring during tumour development of clones carrying increased transglutaminase, it is necessary to link all this information together with the characterisation studies undertaken in chapter 6.

On injection of the cloned cells into the hamster the clones contain high transglutaminase activity. Immediately the cells are injected they are exposed to physiological factors present in the *in vivo* situation. The cloned cells appear to come under two selective pressures, the first to die, possibly by entering the apoptotic pathway and the second to proliferate and survive which appears to be accompanied by a down regulation of transglutaminase activity.

The most appealing theory to account for the apparent death of the injected cells containing high transglutaminase activity is activation of the apoptotic program by the immune system, possibly by cytotoxic T cells. Although there is evidence from the nude mice and hamster pre immunisation experiments to suggest a role for the immune system in these events, we have no evidence to suggest that the raised transglutaminase clones are more susceptible to apoptosis. As an alternative to apoptotic induced death, it may be that changes in cell adhesion and attachment that

make the cells less viable. Classically the increases in attachment and decreases in adhesion would not favour the metastatic phenotype, and by injecting cells subcutaneously one may be (at least in part) be placing cells in an environment similar to that seen by a cell released from a primary tumour.

A reduced cell attachment as noted in the raised transglutaminase clones in chapter 6 could be important in increasing the numbers of cells that die on injection. High transglutaminase may result in more extracellular proteins (collagens, fibronectins, laminins, proteoglycans) being crosslinked (section 1.1.2.4.), thus resulting in the bonds available for crosslink being reduced and hence the cells ability to attach to a surface reduced. Thus, a higher percentage of the cells being injected may simply die via a reduced ability to attach to the host tissue, but to account for decreased transglutaminase activity in the tumours then transglutaminase must still come under a regulatory process. Although this hypothesis explains *in vitro* characterisation results it is interesting to note that clone 36 on which the majority of tumour work was performed actually failed to show a reduction in attachment unlike clones 1 and 32, and must cast doubt on this conclusion indicating that changes in attachment may be clonal.

Initially a more likely candidate to explain the lag times and dose response effects in tumours formed from transglutaminase transfected clones is the role of cellular adhesion as this was increased in all 3 clones tested, although it was not proportional to the transglutaminase activity in the different clones. As with attachment, adhesion has classically been associated with metastasis with reduced adhesion allowing detachment from the primary tumour. Here, where adhesion is increased, it is difficult to see how this factor could influence primary tumour formation from injected cells. It can only be postulated that if cellular adhesion is greater, then this may restrict the invasive properties of the tumour cell by increasing tumour cell matrix attachment (reducing the effectiveness of any protease activity in the tumour cell) which would not only reduce the tumours ability to enter

surrounding tissues but reduce vascularisation. Thus it may be that while transglutaminase activity remains raised these factors lead to slow initial tumour development and result in the lag period seen in tumour growth with transglutaminase transfected clones. It is however difficult to postulate how increased adhesion could explain the dose response results.

CHAPTER 8

DISCUSSION

The major aim of this study was to increase the expression of cytosolic tissue transglutaminase in neoplastic cell lines that demonstrate low transglutaminase activity (Knight et al 1990a,d) and to characterise changes in both the biochemistry and malignancy of these cells with a view to gaining a greater understanding of the involvement of transglutaminase in apoptosis and in the development of the malignant phenotype. In 1990a,d Knight et al suggested that the low transglutaminase activity in the Met (hamster fibrosarcoma) cell lines (Teale and Rees 1987) was a result of expression of an inactive tissue transglutaminase characteristic of the malignant phenotype. The initial work in this study was therefore undertaken to verify the existence of the inactive transglutaminase and then to ascertain if the inactive transglutaminase resulted from errors in the genome itself, transcription or translation. When the cell extracts from Met B cells were separated using ion exchange chromatography (Mono Q column, Pharmacia) 3 protein peaks were isolated that were immunoreactive with a polyclonal anti transglutaminase antibody. Only 2 of these 3 peaks showed transglutaminase activity which corresponded to the particulate and cytosolic peaks (Knight et al 1990a,d). Following verification of an inactive, immunological detectable transglutaminase, mRNA extracts from the same cell line were probed with a mouse transglutaminase cDNA (Gentile et al 1991) with the aim of determining if this inactive transglutaminase was translated from a separate mRNA transcript. Development of the autoradiographs revealed that the probe bound to a single band corresponding to a mRNA of approximately 3.5 kb, the approximate size of the mouse cytosolic tissue transglutaminase mRNA. Since there was no further band evident that could code for the larger 120 kDa inactive transglutaminase (Knight et al 1990a,d) it is likely that the inactive transglutaminase results from an event occurring at or after translation as a genomic or transcriptional perturbation would result in a second, larger mRNA. As the ion exchange analysis of the Met B cell extracts revealed approximately equal levels of inactive and cytosolic transglutaminase protein, detection of an inactive transglutaminase mRNA may be

expected to occur at the same sensitivity as that for the active cytosolic transglutaminase.

The idea of post-translational modification involvement in the inactive transglutaminase is further enhanced by treatment of the inactive transglutaminase with proteases such as thrombin or trypsin. Knight et al (1990a,d) demonstrated that proteolysis of the inactive transglutaminase resulted in an active transglutaminase with biophysical properties similar to cytosolic tissue transglutaminase. Similar results were also shown by Zirvi et al (1991) in human colonic tumour cell lines.

Knight et al (1990a,d) suggested that the inactive transglutaminase was a characteristic of the malignant phenotype, but protease induced activation of tissue transglutaminase has been described in 'normal' tissue (Chung et al 1988b) that is independent of protein synthesis and ATP levels. This has been recorded in both lung and liver tissue and is associated with a reduction in the molecular weight of the cytosolic tissue transglutaminase in a similar manner to that recorded in the malignant cell line (Knight et al 1990a,d). Chung et al (1988b) also suggested the protease induced activation in 'normal' tissues indicated the existence of an inactive transglutaminase. Thus it is not inconceivable to suggest that regulation of transglutaminase activity may occur at the post translational level, and in the malignant cell line it is errors at this level that lead to perturbations in transglutaminase activity and the greater levels of inactive enzyme present. If regulation of other transglutaminases is observed such as Factor XIII (Lorand 1986, Lewis et al 1987, Ichinose and Davie 1988), keratinocyte transglutaminase (Rice et al 1990) and epidermal transglutaminase (Negi et al 1985) then proteolytic activation is observed in all of these. Therefore it would seem feasible that the family trait of proteolytic activation should be extended to the most abundant member of the transglutaminase enzyme family.

Studies on the physiological functions of transglutaminase in the malignant phenotype initially used pharmacological agents to raise transglutaminase activity. In the first instance 1 μM all-*trans* retinoic acid was used as an inducer. This was first reported to increase transglutaminase activity following experiments where serum was devoid of lipids and 'add back' techniques used to determine what constituents of the serum activated transglutaminase in macrophages (Davies et al 1985). All-*trans* retinoic acid was demonstrated to activate transglutaminase at 1-10 nmol in macrophages. Following trial experiments with the mouse mammary tumour virus promoter in the inducible transfection vector pMAMneo Tgase, it was noted that dexamethasone, a potent inducer of apoptosis in thymocytes (Wyllie et al 1980) led to increases in transglutaminase activity in the Met cell lines. Thus 2 pharmacological inducers of cytosolic tissue transglutaminase were used in this study.

Both dexamethasone and all-*trans* retinoic acid caused increases in transglutaminase activity and apoptotic index when determined by detergent insoluble bodies in all cell lines tested. Retinoids act through the nuclear retinoic acid receptors (RAR) and retinoid X receptors (RXR) that are ligand activated transcription factors belonging to the superfamily of steroid receptors (Mangelsdorf and Evans 1992). Similarly, dexamethasone, an artificial glucocorticoid also regulates gene transcription via nuclear receptors (Cohn and Duke 1984). From the results of these preliminary studies on the Met cell line it would appear that induction of transglutaminase by these agents occurs by different mechanisms. Retinoids act directly via increased mRNA synthesis of the tissue transglutaminase gene as was previously described in macrophages (Davies et al 1988a). It has been indicated that both RXR and RAR receptors are responsible for the induction of tissue transglutaminase (Davis et al 1994). In contrast, dexamethasone, which in the cell lines studied causes a greater induction of cytosolic tissue transglutaminase (approximately double that of all-*trans* retinoic acid) but does not appear to cause an increase in transglutaminase mRNA synthesis. This finding was surprising,

particularly as recent work by P.J.A. Davies (personal communication) has identified a glucocorticoid response element on the human transglutaminase promoter which should result in increased transglutaminase mRNA in the presence of glucocorticoids. Therefore, assuming that in the cell lines used in these experiments that dexamethasone does not result in an increase in transglutaminase mRNA, by the very nature of steroid action via transcriptional regulation it must be that dexamethasone is activating transglutaminase either via a further regulatory protein or stabilises mRNA and / or increases translation of existing transglutaminase mRNA. Stabilisation / increased translation is observed within other proteins thought to be involved in apoptosis. For example, c-myc has its mRNA stabilised / de stabilised by regulatory proteins as a means of controlling the c-myc levels involved in apoptosis regulation (Jones and Cole 1987, Bonnieu et al 1988).

An interesting proposal with regard to dexamethasone activation of transglutaminase activity is a situation whereby activation may be via a proteolytic enzyme that acts on existing antigen in a similar manner to thrombin on factor XIII (Lorand 1986, Ichinose and Davie 1988), an unidentified protease in keratinocyte transglutaminase (Rice et al 1990) and epidermal transglutaminase (Negi et al 1985). Potentially this may be via activation of the inactive enzyme (Knight et al 1990a,d, Chung et al 1988b). Although no increase in transglutaminase mRNA is detected in cells following dexamethasone treatment, an increase in antigen corresponding to the active enzyme antigen is detected by Western blotting. This would indicate that increased stabilisation and / or translation of transglutaminase mRNA is the cause of the increased transglutaminase activity, alternatively the increased transglutaminase antigen could result from a conformational change in a the protein (such as that caused by proteolysis) to reveal further epitopes to the antibody not available in the inactive conformation. This is likely as the antibody used (Goat 202 anti guinea pig liver transglutaminase) was raised using purified active cytosolic transglutaminase which involved separation by ion exchange. To answer this question, further work

is required to separate cell extracts from dexamethasone treated cells in the same manner as performed in chapter 3 using anti transglutaminase antibodies that recognise the inactive transglutaminase. The goat 202 antibody used to perform the Western blot analysis from dexamethasone treated cells failed to recognise the inactive form of transglutaminase on Western blots using cell homogenates.

Studies using Met B, Met D, Met E, BHK-21, and B16 show that cells expressing the greater number of dexamethasone responsive receptors also show the greatest induction of transglutaminase in response to the glucocorticoid. Furthermore cell lineages with a greater number of dexamethasone receptors also show a greater induction of apoptosis as determined by numbers of detergent insoluble bodies within the scope of this study. This relationship is further enhanced by transfection of the glucocorticoid receptor protein HG1 into Met B and B16 cells where the greater the level of HG1 transfected the greater the increases in transglutaminase activity and apoptosis. These findings would indicate that regulation of transglutaminase and apoptosis by glucocorticoids would have a large effect on tissues rich in the receptors, with almost no effect on cells low in receptors to glucocorticoid such as B16. As found with the regulation of apoptosis (Review Martin et al 1994) these results suggest that transglutaminase is also controlled by multiple factors, this being the case then it could be suggested that the balance between different receptor proteins a cell contains would dictate its response to transglutaminase expression, for example retinoids and glucocorticoids. This may explain why apoptosis in response to glucocorticoids has only previously been reported in lymphoid tissue where a much greater induction of apoptosis is observed (Schwartzman and Cidlowski 1993) and it would thus be interesting to compare relative numbers of glucocorticoid receptors between thymocytes and the cells used in this study, especially as it has been reported that glucocorticoid induction of apoptosis in thymocytes is accompanied by increases in transglutaminase activity (Fesus et al 1987).

Conclusions on the physiological function of tissue transglutaminase have been hampered by the lack of techniques capable of selectively modifying the activity of the enzyme in intact cells. Transglutaminase inhibitors have been used to deduce information on the function of transglutaminase (Davies et al 1985), but most of the inhibitors are not entirely specific and can inhibit other enzymes and physiological processes (Cornwell et al 1983, Lee et al 1985, Bungay et al 1986). An alternative approach is to induce expression of the enzyme and correlate changes in enzymatic activity with a biological response as performed in this study with both all-*trans* retinoic acid and dexamethasone. In keeping with similar studies using transglutaminase inducers` such as all-*trans* retinoic acid and sodium butyrate (Moore et al 1984, Davies et al 1985, Lee et al 1987, Chliocca et al 1989) these agents produce diverse effects on cellular function and it is difficult to identify alterations in responses that are specifically attributable to the induction of transglutaminase. Thus, the relationship seen between transglutaminase in response to all-*trans* retinoic acid and dexamethasone in this study can add weight to the role of transglutaminase, but it does not answer the question as to whether the stimulating agent induces apoptosis, by increasing transglutaminase activity , or whether the inducer causes an increase in transglutaminase which is a symptom rather than the cause of apoptosis. One cannot rule out that increases in apoptosis may be independent of the increases in transglutaminase activity. Therefore to specifically increase expression of transglutaminase an alternative approach was used which involved transfecting the cells to be studied with the full length cDNA of the tissue transglutaminase under the control of an independent promoter (Gentile et al 1991).

One of the major discussions concerning the transfection of the transglutaminase cDNA was the vector used to carry the cDNA, or more specifically the promoter used to control expression. Two options were available, one using an inducible vector such as pMAMneo (Clontech) which contains the MMTV inducible promoter or a constitutive vector such as pSG5 (Stratagene) where

the enzyme is under the control of a SV40 constitutive promoter. Initial studies concentrated on the inducible pMAMneo vector containing the transglutaminase cDNA. This inducible vector gave the advantage of being able to transfect, select and clone the transfected cell line without the cell expressing elevated transglutaminase which could itself affect cell viability and cloning if the transglutaminase mediated induction of apoptosis and other cell changes (e.g. differentiation / proliferation, extracellular matrix stabilisation, secretion coupling (section 1.1.2.4)) proved to be correct. Unfortunately, dexamethasone, which is the most effective activator of the MMTV promoter affected both apoptosis and the expression of transglutaminase in controls (and potentially other biochemical and physiological factors that were not measured) . It was also found that the promoter was not effective in inducing the transglutaminase insert significantly above that seen in the control cells. Consideration was given to other inducible promoters (e.g. The 'Lac switch' inducible mammalian expression vectors pOPRSV1 and pOP13 (Stratagene) that use the RTL-LTR promoter that is induced by IPTG (Isopropyl-β-D-thio-galactopyranoside)), but the pharmacological agents used to activate the promoter always had the potential to effect cell biochemistry and physiology and the level of induction attainable by inducible promoters is low compared to their constitutive counterparts. For these reasons the decision was made to use the constitutive expression vector pSG5 Tgase.

The use of the constitutive expression vector carrying the transglutaminase cDNA raised similar concerns to those of Gentile et al (1992) where similar transfection protocols were used on 3T3 fibroblast. Questions as to the feasibility of developing cell lines capable of tolerating high levels intracellular transglutaminase were raised. Transglutaminase induces irreversible protein crosslinking and it may be anticipated that the expression of high levels of cytosolic tissue transglutaminase inside a cell might be associated with a profound inhibition of proliferative activity. Several cell types such as endothelial cells (Greenberg et al 1987, Korner et al 1989, Nova et al 1989) and activated or retinoid stimulated macrophages (Murtaugh

et al 1983, Moore et al 1984) have been shown to contain high levels of intracellular transglutaminase (as much as 1 to 2% of total cellular protein), but these cells are usually terminally differentiated and show little proliferative activity *in vitro*. Lee et al (1987) have shown that transglutaminase activity tends to be low in undifferentiated or rapidly proliferating cell types and to rise when the cells are induced to differentiate or to undergo growth arrest. In spite of these observations the data in chapter 6 demonstrates that it is possible to isolate viable cell lines expressing high transglutaminase with high proliferative activity comparable to control and control transfected cells. The results obtained with Met B are similar to those seen in 3T3 cells by Gentile et al (1992) suggesting that high transglutaminase by itself does not affect proliferation or differentiation. There is no effect over a large transglutaminase activity range as demonstrated by the isolation of 16 clones with raised transglutaminase activity ranging from 1 unit to 30 units per $1x10^6$ cells. In the clones characterised, there is no reduction in proliferation (doubling times) of the transfected cell lines and no obvious change in cell morphology.

The viability of these cells may be attributed to a number of causes. Tissue transglutaminase is a Ca^{2+} dependent enzyme and it is likely that the levels of Ca^{2+} (5 μM) sufficient to activate the enzyme are not normally achieved in the intact, viable cell (Hand et al 1985) although these are achieved in the transglutaminase activity assay used. Furthermore guanine nucleotides have been reported to inhibit tissue transglutaminase activity (Achyuthan and Greenberg 1987) and some cells have been reported to contain specific intracellular transglutaminase inhibitors (Korner et al 1989). Determination of these parameters were outside the scope of this study, and therefore we can only postulate that the effects of Ca^{2+}, GTP and inhibitors in the transglutaminase transfected cell lines and must therefore be an area where additional work is required particularly as these could be involved in the regulation of transglutaminase via pharmacological agents such as dexamethasone. What can be said with certainty is that although transglutaminase is raised, for instance in clone 36 by some 110 fold, the product of the enzyme, the ε-(γ-

glutamyl)-lysine crosslink increased by less than 2 fold (24.76 - 54.5 pmol / 10^6 cells or approximately 240 - 540 pmol / mg protein (Johnson et al 1994)) indicating that the transglutaminase was not highly activated / functional in clone 36. These figures are similar to those reported in non malignant tissues (260-440 pM / mg protein) (Fesus et al 1989, Hand et al 1990a,b), they show similar percentage increases to that seen in 'Normal' transfected cells (Gentile et al 1992).

Characterisation of transglutaminase transfected clones 1, 32 and 36 *in vitro* revealed no statistically significant change in apoptosis as determined by detergent insoluble bodies, this was not surprising considering the requirement for raised intracellular calcium in the majority of cell types for apoptosis to proceed (Bellamo et al 1992) . Characterisation of the clones did show that changes in cell adhesion increased with increasing levels of transglutaminase, with some suggestion that this may be linked to fibronectin since adhesion to fibronectin was considerably increased over adhesion to commercial tissue culture plates in 2 of the 3 clones characterised. In addition 2 of the raised transglutaminase clones showed increased attachment to a range of substrates. There must be some concern over the attachment and adhesion results as not all transglutaminase clones showed increases and therefore it may be simply a clonal effect. Characterisation of a greater number of clones is required to answer this question. It is important to note that the trypsin used in the adhesion studies was highly diluted (0.0025% (w/v)) compared to 0.125% (w/v) used in similar experiments on 3T3 cells (Gentile et al 1992). Thus the affects on cell adhesion, although significant, must be considered to be small compared to the studies on 'normal' cells. There is considerable evidence available to suggest that the link between transglutaminase and adhesion involves enzymatic reactions occurring outside the cell (Barsigian et al 1991, Upchurch et al 1991, Aeschlimann and Paulsson 1991, Jensen et al 1993, Beninati et al 1994, Martinez et al 1994). In this environment the Ca^{2+} concentration is high enough to support transglutaminase activity and the enzyme may be freed from inhibitory factors such as GTP or inhibitory proteins that restrict its activity within the cell. An

involvement of transglutaminase in the processing of extracellular matrix proteins may explain the accumulation in basement membranes wound healing and tissue repair (Dolynchuk et al 1994). There are reports of transglutaminase being involved in crosslinking fibronectin to collagens I, II and III (Mosher 1984), transglutaminase involvement in binding low density lipoprotein to collagen III (Bowness et al 1989) and crosslinking of fibronectin to osteopontin. Substrate sites have been identified on fibronectin (Fesus et al 1986) and collagen III (Bowness et al 1987) for transglutaminase. An unanswered question is how the enzyme gains access to the extracellular space. Like Factor XIII and several other extracellular proteins, transglutaminase does not contain a leader sequence or obvious secretory signal and it remains to be determined how the enzyme becomes deposited in the extracellular compartment.

There is consistent evidence to indicate that reduced transglutaminase activity accompanies development of the malignant phenotype (Birckbichler et al 1977b, Barnes et al 1985, Hand et al 1987b, 1988, Zirvi et al 1991, Beninati et al 1994). In 1990a,d Knight et al demonstrated that the highly malignant hamster Met cell lines (Teale and Rees) showed an inverse relationship between transglutaminase and tumour malignancy. Therefore the injection of the transglutaminase transfected Met B clones *in vivo* would indicate if loss of transglutaminase activity alone was a prerequisite or consequence of the malignant phenotype in these cells.

Subcutaneous injection of the transglutaminase transfected clones and control transfected cells into hamsters demonstrated differences between the groups. Determination of growth rate once the tumour had reached a detectable size was identical between transglutaminase transfected clones 1, 32 and 36, the transfected controls and the non transfected Met B, confirming the *in vitro* growth rate studies. However, in animals injected with transglutaminase transfected clones, the time for development of a detectable tumour was increased, with the increase in time being generally proportional to the transglutaminase activity of the cloned cell line. This

'lag' time in tumour development in the transglutaminase transfected clones must be a result of the higher transglutaminase expression as this is the only controlled altered parameter. In addition, cell dose response experiments indicated the transglutaminase transfected clones required greater numbers of cells to be injected to facilitate tumour development, once again showing a correlation between transglutaminase activity levels and tumour development. These findings indicate that reduction in transglutaminase activity may be a prerequisite to tumour growth and progression and confirm the importance of the enzyme in the maintenance of the normal differentiated state.

The identical growth rate of tumours originating from the control cells and transglutaminase transfected cells once tumours are detected, coupled with the *in vitro* growth study indicates that the lag time in tumour development in the transglutaminase transfected clones is unlikely to be a result of a reduced growth rate, but suggests that the population of cells that are able to form a tumour colony on injection is reduced. This implies that a greater proportion of the cells from the transglutaminase transfected cell lines are killed on injection. This conclusion is supported by the *in vivo* dose response experiments which also suggest a lower survival rate of the clones containing the highest transglutaminase activity since more cells needed to be injected to cause tumour formation.

The reasons why transglutaminase can suppress tumour growth when such a large number of cells are injected into the host animal was not evident from our studies. However, the diverse roles which have been ascribed to tissue transglutaminase and observations seen in this study allow a number of explanations to be put forward. The inability of increased transglutaminase expression in the fibrosarcoma to affect growth rates or the spontaneous level of apoptosis *in vitro* means that the *in vivo* effects can not be ascribed directly to these factors. However the possibility that cells carrying increased tissue transglutaminase are more susceptible to the induction of apoptosis either by the hosts cytotoxic killer cells or

cytokines produced by other invading cells in the tumours cannot be ruled out, given recent evidence to suggest that cytosolic tissue transglutaminase may be a key player in cell death mediated by NK and LAK cells (Knight et al 1993).

Biochemical assessment of the tumours once detectable revealed interesting and surprising findings. Tumours arising from transglutaminase transfected and cloned cells had lost their raised transglutaminase activity and antigen as compared against tumours from both the pSV_2neo transfected control cells and Met B cells. This loss was accompanied by a loss of transglutaminase mRNA which indicated that the cells forming the tumour had lost the transfected insert, possibly by selection of cells within the injected population that contained lower transglutaminase expression due to epigenetic effects (i.e. higher transglutaminase containing cells may grow at a reduced rate). This conclusion of selection by reduced growth rates was questionable particularly as all the experimental results on growth rates contradicted this theory. *In vitro* growth rates in control and transglutaminase transfected cells were comparable and maintenance of transglutaminase clones cultured continuously for in excess of 6 months had showed no decrease in activity. Furthermore, *in vivo* growth rates of growing tumours were again comparable. In addition the complete loss of the raised transglutaminase expression in such an early stage in tumour development was surprising as one would have expected a slow, continual decline for selection with the cells of a population containing raised transglutaminase gradually forming a minority of the tumour population.

Further investigation via Southern blot analysis revealed that cells from the tumours originating from the transglutaminase transfected clones still contained the transglutaminase cDNA insert, and the return of approximately 50% of the initial transglutaminase activity when the tumours were returned to the *in vitro* environment suggested a more complex explanation. Furthermore ion exchange

separation of the total transglutaminase from the tumours showed an approximate doubling of the inactive form of transglutaminase.

Therefore it can be concluded from these results that the major reason for the reduction in transglutaminase in the transglutaminase transfected clones is not due to the loss of the insert. It is more likely to be due to blocking of transcription of the transglutaminase cDNA insert or a reduction in the half life of the transglutaminase mRNA. In addition it would appear that there is also a small amount of post translational modification of the transglutaminase protein to the inactive form (or alternatively a failure of the inactive transglutaminase to be activated). The increased transglutaminase activity found in the transglutaminase transfected tumour cells when returned to *in vitro* culture compared to those of the transfection control shows that the insert and the promoter are not damaged and that the viral T antigen required for the enhanced activation of the SV40 promoter (Green et al 1988) has not been lost from the tumour cells. Furthermore the transfected clones were stable in culture prior to tumour formation and the finding that transglutaminase activity can be returned to the tumour cells once removed from the *in vivo* environment suggests that the down regulation of transglutaminase in tumours arising in the transglutaminase transfected clones is likely to be a result of the action of factors present only in the *in vivo* environment. Furthermore, the transfected construct (Gentile et al 1991) is believed not to contain any known regulatory region in either the 120 bases up stream or 1 kb down stream of the coding region identified so far. Future work should concentrate on repeating this work with an alternative promoter to verify promoter function and on the identification of a regulatory element on the transglutaminase 3' non coding region.

Therefore two major questions arise from this work. Primarily what agent(s) and by what mechanism does the stably transfected cDNA insert become down regulated in the *in vivo* environment and secondly by what mechanism do the cells containing raised transglutaminase die. On both of these points we can only

postulate, but it is significant that the transglutaminase activity in the injected cells is lost prior to tumour development and detection thus demonstrating the requirement for reduced transglutaminase expression in neoplasia.

Transglutaminase activity can be induced by a number of naturally occurring compounds including retinoids and glucocorticoids as demonstrated in this study and cytokines such as TGFβ1 (Jetten et al 1986). Therefore it is possible that other 'messengers' found *in vivo* have a negative regulator effect on the enzyme particularly as negative intra cellular inhibitors have already been identified (Korner et al 1993) which must themselves fall into a regulatory pathway. Identification of these negative regulators would be best pursued using add back techniques similar to those used by Davis et al (1985) in the identification of transglutaminase activators. It has recently been shown that tissue transglutaminase can be down regulated by P53 (P.J.A. Davies, personal communication).

A more posing question is by what mechanism the cells containing raised transglutaminase die on subcutaneous injection. As previously mentioned transglutaminase has been ascribed to a number of physiological processes such as growth regulation (Birckbichler and Patterson 1978), endocytosis (Davies and Murtaugh 1984), antigen presentation (Teshigawara et al 1985), exocytosis / secretion (Sener et al 1985, Bungay et al 1986), cellular morphology (Byrd and Litchi 1987, Nara et al 1989), cellular adhesion (Cai et al 1991), stabilisation of the extracellular matrix (Bowness et al 1987, Aeschlimann and Paulsson 1991) and most recently apoptotic cell death (Knight et al 1991, Fesus et al 1992).

The most obvious physiological process to account for the suspected death of the injected cells would be apoptosis, but the *in vitro* characterisation of the clones suggests this is not the case, although this cannot be completely ruled out. As previously mentioned, it is possible that the cells carrying the increased transglutaminase activity are more susceptible to the induction of apoptosis by

cytotoxic killer cells or cytokines secreted by these or other cells given the recent evidence to suggest that transglutaminase may be a key player in cell death mediated by NK and LAK cells. (Knight et al 1993).

The only changes seen in the characterisation of the transglutaminase transfected clones was in reduced attachment and increased adhesion. Alterations in these two parameters would typically be expected to decrease the metastatic potential of the cells by reducing the cells ability to be released from the primary tumour (increased adhesion) and decreasing the released cells ability to arrest in secondary areas (reduced attachment) (Willis et al 1974). Preliminary studies on metastatic potential of the transglutaminase transfected clones (data not shown) using resection of primary tumours to allow secondary development in hamsters and experimental metastasis experiments in nude mice were both inconclusive. It is conceivable that the reduced attachment could affect the cells viability following subcutaneous injection, but this was only seen in 2 of the 3 clones characterised. In addition, it is difficult to see how the data obtained from the nude mice experiments and pre immunisation experiments can be explained by attachment changes alone.

With the suggestion that the hosts immune system may play a part in the death of transglutaminase transfected clones from the experiments undertaken in nude mice (balb c, Nu^+/Nu^+) mice and pre immunised hamsters, it is important to try and determine which parts of the immune system may be important. Nude mice have a small, involuted, rudimentary thymus gland and are subsequently deficient in immune functions involving this gland. Functionally this means that nude mice are congenitally deficient in T cell function (T Lymphocytes, T helper cells and cytotoxic T cells) but have a full complement of B lymphocytes and high levels of Natural Killer cells. LAK cells (approximately 95% NK, 5% T cells) are unlikely to play a role in nude mice due to there requirement for activation by the cytokine IL-2 that is synthesised by T cells. The failure to observe a retardation in nude mice with raised transglutaminase clones suggests a T cell involvement.

With regard to the pre-immunisation experiments in hamster, it is difficult to say which particular cells of the immune system are activated by the immunisation procedure without a full characterisation. Similar immunisation experiments were performed by Teale and Rees (1987). In a personal communication with R.C. Rees, it was suggested that following immunisation it could be expected that B lymphocytes were probably not activated, LAK cells have the potential to be activated, but not necessarily with the immunisation regime used and that NK cells exist already and could mediate anti tumour killing but would not be likely to be effected by the immunisation. It is highly probable that cytotoxic T cells are placed into an activated state and direct killing is likely to result from this activation of cytotoxic T cells, although the mechanism of kill could be indirect. Thus both the experiments in nude mice and immunisation of hamsters suggest a strong role for T cells which further suggests that there is alteration to the tumour antigens of Met B.

Previous studies by Teale and Rees (1987) using cross immunisation techniques indicated that Met B has a weak tumour antigen expression. The results of which are contained in table 8.1..

Table 8.1.: Met B cross immunisation experiment results (Teale and Rees 1987)

Immunised	Challenged	Result
Parent	Parent	Immune
Met B	Met B	Non immune
Met B	Parent	Non immune
Parent	Met B	Immune
Met B + Adjuvant (BCG)	Met B	Immune

In these experiments Teale and Rees immunised hamsters with either the parent cell line of Met B, Met B or Met B plus an adjuvant. When the animals were challenged then it can be seen that Met B parent cells must be expressing a tumour antigen since when immunised animals are challenged an immune response is seen. Opposingly, animals immunised with Met B are unable to initiate an immune

response when challenged. It can be concluded that Met B is expressing low levels of tumour antigen as following immunisation with Met B plus an adjuvant or parent cells then a Met B challenge does infact initiate an immune action.

In the pre immunisation experiments performed in this study with the clone carrying raised transglutaminase (clone 36), it would appear that the increased expression of the enzyme is enhancing expression of the tumour antigen as an immune response is now seen when clone 36 immunised animals are challenged with clone 36 cells. No change in immune response is seen with either the Met B or Met B pSVneo transfected control. To verify this result it is imperative to perform cross immunisation experiments similar to those performed by Teale and Rees (1987). Basically animals would need to be immunised with either a raised transfected clone, Met B. Met B transfection control or PBS. All groups would then be challenged with Met B. If the group immunised with the raised transglutaminase clone then showed an immune response while the other 3 groups failed to demonstrate any change in their immune recognition, then this would clearly demonstrate transglutaminase is acting to increase expression of the Met B tumour antigen.

Unfortunately little is known of tumour antigens in hamster, although studies in mouse and man have revealed 6 major groups of tumour antigen peptides. These are summarised in Table 8.2. It is likely that hamsters have similar, tumour antigens.

Tumour antigens, or more specifically tumour antigen peptides are found expressed on the Major Histocompatility Complex 1 (MHC 1) between the $\alpha 1$ and $\alpha 2$ subunits where it is secured by 2 anchor residues. This consist of a 9 amino acid peptide that results from the intracellular proteolysis of the tumour antigen protein, the resulting peptide of which is collected in a pre-golgi compartment and transported to the MHC 1. The tumour antigen is not a mutated protein, although

oncogene proteins can function as tumour antigens (Review; Pardoll 1994, Theodore et al 1994).

Table 8.2.: Tumour antigens in Mouse and Man (Pardoll 1994)

Tumour	Antigen	Normal adult tissue distribution	Source of T cells that recognise antigen
Murine P815 mastocytoma	P1A	Testes	Vaccinated mice
Murine Lewis lung carcinoma	Connexin 37	Lungs	Vaccinated mice
Human melanoma	MAGE (1-12)	Testes	Peripheral blood lymphocytes from vaccinated patients
Human melanoma	MELAN-A (MART 1A)	Melanocytes	Peripheral blood lymphocytes . Tumour infiltrating lymphocytes
Human melanoma	gp100	Melanocytes	Tumour infiltrating lymphocytes, draining lymph node.
Human melanoma	Tyrosinase	Melanocytes	Peripheral blood lymphocytes . Tumour infiltrating lymphocytes

Mouse and human tumour antigens fall into 2 main categories. P1A and MAGE family are not expressed in any normal adult tissue except testes, and are possibly developmental antigens re expressed during the process of tumourogenesis. MAGE 1 can be activated by demethylating agents such as 5-azacytidine: the altered methylation state commonly observed in cancers may thus account for their activation. Its relative tumour specificity makes MAGE-1 an excellent potential vaccine target. Subsequent searches have failed to reveal many patients with MAGE 1 reactive T cells, indicating that it represents a non dominant tumour antigen. The remaining melanoma antigens (tyrosinase, gp100, MART) are

differential antigens specific to a melanocyte lineage. gp100 and MART seem to be dominant; a single peptide in each is recognised by T cells from many HLA-A2 patients.

It is important to consider why T cells don't recognise these antigens and why tumours can thus develop. The reason may be that when a neoantigen arises within a tumour, it is tolerated by the immune system equivalently to a tissue specific antigen. The efficiency with which a particular epitope is processed, presented and ultimately recognised by a T cell receptor is a much more critical determinant of immunological reactivity than is neoantigenicity. Since genetically altered proteins seem to be common in cancer cells, they are still a relatively minor source of the total MHC 1 associated peptides. Only if a tumour specific neo-epitope happens to be effectively processed and presented will it qualify as a target for T cell immunity. As transglutaminase is a a post translational modification enzyme that is active in the cytoplasm and at the cell surface, it may be that transglutaminase is acting within processing and presentation pathways to increase tumour antigenicity. In particular, the ability of transglutaminase to crosslink membrane proteins may cause distortions in cell structure and may have a role in increasing presentation of tumour antigens.

In addition, we know that the human HLA-A and HLA-B antigens within the MHC 1 contain 2 glutamine residues and that transglutaminase can specifically couple amines to these in the carboxy terminal region of HLA-B7 and A2 antigens. Such a crosslinking within these antigens may have an effect on altering the immunological status of the cell (Pober and Strominger 1981). Pober and Strominger postulated that in the activated macrophage where transglutaminase activity is raised that transglutaminase alters the MHC status, attracting cytotoxic T cells and helper T cells and thus triggering a second stage immune response. It would therefore seam possible that the MHC in the fibrosarcoma cells is altered in such a manner in the raised transglutaminase clones, and it may be this that is

altering tumour antigen presentation. Unfortunately, little is known about the hamster immune system, except that it is believed to contain only one MHC, and that is relatively simple in comparison to the human MHC's, and that it is unrelated to the HLA antigens. Therefore unless the hamster MHC has antigens capable of being crosslinked in a similar manner to HLA antigens then transglutaminase action within the MHC is not relevant to this situation.

In addition transglutaminase may be involved intimately in receptor mediated endocytosis and antigen presentation to lymphocytes (Teshigawara et al 1985). Teshigawara demonstrated that cells high in transglutaminase were more able to express lymphocyte recognition antigens, and that inhibitors of transglutaminase reversed this ability. This may be important in some of the cell surface antigens presented by neoplastic cells that aid immune recognition by T cells such as CD4, CD8 and CD45 (for review see Julius et al 1993) or apoptotic cells such as CD36 (Roberts et al 1985, Silverstein et al, 1989, Oquendo et al 1988, Sun et al 1989) or in tumour antigen presentation on the MHC I.

T cells constitute 1-5% of all cells in the body. The antigens they recognise are cell surface complexes formed between short peptides (generally 8 - 20 amino acids) and integral membrane proteins encoded by the MHC (Buus et al 1986, Townsend et al 1986, Bjorkman et al 1987). Of the 2 major T cell subsets defined on the basis of CD4 and CD8 expression, $CD4^+$ T cells recognise peptide fragments derived from endocytosed proteins in association with MHC II molecules on the surface of specialised antigen presenting cells (Unanue and Allen 1987, Braciale et al 1987)

The other major T cell subset, and the one of particular interest in relation to tumour antigens, is $CD8^+$ T cells. These cells recognise short peptides that arise within any cell by proteolysis of cellular proteins (Goldberg and Rock 1992) and are then transported to the surface in association with MHC I (Townsend and Bodmer

1989). Since a nucleated cell typically expresses $\approx 10^5$ molecules of each of ≈ 6 different MHC I proteins, then there are perhaps 10^6 peptide MHC I complexes on the surface of any cell representing the several thousand different proteins being synthesised in that cell. Normally, an individuals $CD8^+$ T cells do not respond to complexes of self peptides and self MHC-I. However if a foreign protein as from a viral protein, a mutated protein or even an over expressed protein occupies a sufficient number of MHC I molecules (200 per cell, Christnick et al 1991) $CD8^+$ T cells are activated to lyse the affected cell. Thus $CD8^+$ T cells perform an immune surveillance function, effectively monitoring the contents of all cells by detecting peptides that are sampled by MHC I and displayed at the cell surface. Therefore T cell function could be involved in 3 additional ways to simply recognising the a fore mentioned tumour antigens in the raised transglutaminase clones. If antigen presentation is raised in cells containing high transglutaminase as suggested by Teshigawara et al (1985), then either the tumour antigens or the CD8, CD4 or CD45 antigens could be expressed to a higher degree on the cell surface providing a greater signal for the T cell.

Additionally as the transglutaminase clones contain a human cDNA insert then the immune reaction could simply be recognition of a foreign protein on the MHC I by the T cell. It is therefore important to design experiments to test if human transglutaminase produced in hamster cells is seen as foreign by the hosts immune system before definitive conclusions can be drawn. To test this hypothesis it is necessary to examine serum either from tumour bearing or immunised hamsters for antibodies to human transglutaminase. The major problem here is that transglutaminase is highly conserved between species (80% between human, mouse and guinea pig). Subsequently polyclonal antibodies are likely to cross react with transglutaminase from all species. It is therefore difficult to demonstrate cytotoxic T cell activity against transglutaminase.

Alternatively, as transglutaminase is being expressed by an independent promoter it may be an over expression of the transglutaminase protein causing sufficiently high levels to be associated with MHC I molecules causing a T cell action against the cell. There is data indicating that NK and LAK cells are more active against cells with raised transglutaminase (Knight et al 1993), thus a similar event may be occurring with these cells as with T cells.

This study has demonstrated that Met B tumours contain an inactive transglutaminase that may account for the cells low transglutaminase expression. Expression of the inactive transglutaminase is a result of translational or post translational perturbations. It has demonstrated that both all *trans* retinoic acid and dexamethasone can increase spontaneous apoptosis and transglutaminase expression, although the mechanism of increasing transglutaminase activity is mRNA synthesis dependent with all-*trans* retinoic acid and transglutaminase mRNA synthesis independent with dexamethasone. Transfecting the transglutaminase cDNA into Met B cells does not affect their levels of spontaneous apoptosis and therefore transglutaminase is not a direct regulator of the apoptotic event. Transglutaminase transfected cells have a reduced ability to form tumours when injected into hamster with a strong suggestion that this reduction may be due to increased expression of tumour antigen recognised by T cells. This evidence strongly suggests that transglutaminase may be a potential oncogene.

CHAPTER 9

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