AP-1 regulates a multigenic invasion programme in *v-fos* FBR MuSV transformed fibroblasts

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Abbreviations

APC	adenomatous polyposis coli
BICR	Beatson Institute for Cancer Research
c-	cellular
DIF1	Down In FBRs 1
EST	expressed sequence tag
FRP	Follistatin Related Protein
FrpAP	Frizzled Related Protein AP
G3PDH	glyceraldehyde 3 phosphate dehydrogenase
LOH	loss of heterozygosity
NCBI	National Center for Biotechnology Information
PAGE	polyacrylamide gel electophoresis
PCR	polymerase chain reaction
RT	reverse transcription
TSG	tumour suppressor gene
UV	ultra violet
v-	viral
wt-	wild type

Chemicals

A	adenine,
ATP	adenosine 5′-triphosphate
APS	ammonium persulphate
C	cytosine
cDNA	complementary deoxyribonucleic acid
DEPC	diethylpyrocarbonate
dH_2O	de-ionised water
DMSO	dimethylsulphoxide
DNA	deoxyribonulceic acid
DNAase	deoxyribonuclease
dNTP	3′ deoxyribonucleoside 5′-triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
G	guanine

HEPES	N-(2-hydroxyethyl) piperazine-N′-(2-ethanesulphonic acid)
mRNA	messenger ribonucleic acid
MOPS	3-(N-morpholino) propanesulphonic acid
OAc	acetate
PBS	phosphate buffered saline
PMSF	phenymethylsuphonyl fluoride
RNA	ribonucleic acid
RNA'ase	ribonuclease
SDS	sodium dodecyl sulphate
SSC	sodium chloride, sodium citrate
T	thymine
TAE	tris, acetic acid, ethylenediaminetetra-acetic acid
TEMED	tetramethylenediamine
Tris	2-amino-2-(hydroxymethyl) propane-1,3-diol

Units

A_x	absorbance/ x = wavelength
bp	base pair
Ci	Curie
Da	dalton
g	gram
hr	hour
k	kilo
l	litre
μ	micro
m	milli
M	molar
min	minute
n	nano
°C	degree Celsius
rpm	revolutions per minute
s	second
V	Voltare
v/v	volume for volume
w/v	weight for volume

Single letter amino acid code

Single letter code	Three letter code	Amino acid	DNA codons
A	Ala	Alanine	GCT, GCC, GCA, GCG
C	Cys	Cysteine	TGT, TGC
D	Asp	Aspartic	GAT, GAC
E	Glu	Glutamic	GAA, GAG
F	Phe	Phenylalanine	TTT, TTC
G	Gly	Glycine	GGT, GGC, GGA, GGG
H	His	Histidine	CAT, CAC
I	Ile	Isoleucine	ATT, ATC, ATA
K	Lys	Lysine	AAA, AAG
L	Leu	Leucine	TTG, TTA, CTT, CTC, CTA, CTG
M	Met	Methionine	ATG
N	Asn	Asparagine	AAT, AAC
P	Pro	Proline	CCT, CCC, CCA, CCG
Q	Gln	Glutamine	CAA, CAG
R	Arg	Arginine	CGT, CGC, CGA, CGG, AGA, AGG
S	Ser	Serine	TCT, TCC, TCA, TCG, AGT, AGC
T	Thr	Threonine	ACT, ACC, ACA, ACG
V	Val	Valine	GTT, GTC, GTA, GTG
W	Trp	Tryptophan	TGG
Y	Tyr	Tyrosine	TAT, TAC

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Declaration

I am the sole author of this thesis. All the references have been consulted by myself in the preparation of this manuscript. Unless otherwise acknowledged, all the work presented in this thesis was performed personally.

Abstract

The oncogene *v-fos*, is the transforming agent of the FBJ-MuSV and the FBR-MuSV osteosarcoma virus that elicits cellular responses through activation of the multigenic transcription factor AP-1. The Suppressive Subtractive Hybridisation (SSH), technique has been employed to identify differentially expressed downstream direct and indirect target genes of the AP-1 transcription factor in a rat fibroblast cell system by using FBJ-MuSV *v-fos* transformed 208F fibroblasts and the normal parental fibroblast 208F cell lines.

444 clones were isolated from the up-regulated and down-regulated libraries of which 206 were sequenced on at least one strand. Protein, nucleotide, and EST computational analysis revealed 83% of the sequenced clones represented known genes whereas 17% were novel. A proportion of the up- and down-regulated genes encode proteins which alter ECM, adhesion, motility and transcription and may enhance/suppress invasive phenotype respectively. Many of the clones encode for genes whose function has not been associated with transformation and invasion. The differential expression patterns of the AP-1 direct and indirect target genes have been characterised by Northern Blot analysis in oncogenically and mitogenically transformed cells.

A down-regulated clone matched to the known gene, Follistatin-related protein(FRP), a TGFβ induced gene, which is down-regulated in association with oncogenic transformation in several cancer cell lines and is extinguished in several human tumours. The cDNA was cloned into a 3' Myc tagged expression vector and transient transfections into oncogenically transformed and parental untransformed fibroblasts shows FRP has a peri-nuclear cytoplasmic localization. Stable expression of FRP in FBR cell line does not effect cell proliferation, anchorage-independence or revert transformed morphology. The FRP expressing cells are 70% less invasive than wild type transformants in a matrigel invasion assay highlighting that a down-regulated AP-1 target gene blocks invasion.

A 208F cDNA library was contructed then screened for the full-length cDNA of a novel testis-specific down-regulated clone, DIF1, which matches to 7.5day embryo ectoplacental EST which exhibited homology to the helix-loop-helix transcription factor Alf1. The cDNA was cloned into a 3' Myc tagged expression vector and transient transfections into parental untransformed fibroblasts shows DIF1 has a nuclear localisation. It proved very difficult to transfect oncogenically transformed cells with the DIF1-myc expression vector therefore it was cloned into a GFP fusion vector. Transient transfections into oncogenically transformed cells indicated a cytoplasmic localisation that resulted in a disruption of characteristic cellular morphology.

One down-regulated clone, CD10 exhibited homology to the apoptosis associated frizzled related protein FrpAP, and northern blotting revealed two abundantly expressed transcripts in 208F RNA. The 208F cDNA library was screened for full-length transcripts and the shorter transcript of 1.4Kb was cloned. This was found to have an identical ORF to that of the published FrpAP sequence, but the 3' untranslated region was truncated.

Chapter 1
Introduction

1.1 ONCOGENES AND TUMOUR SUPPRESSOR GENES

Cancer is a leading cause of death in most first and second world countries, manifesting itself from a wide variety of host tissues in various clinical forms. Ongoing advances into the mechanism of the disease has revealed its complexity and elusive nature. Benign tumours, such as warts and moles, grow by simple expansion and often remain encapsulated by a layer of connective tissue and are rarely life threatening. Malignant tumours or cancers arise from the unrestricted progressive growth of a clone of cells, in a particular tissue known as the primary site. The movement of these cells through the circulatory systems can result in secondary tumour formation at distal sites, metastasis. The cells can also invade into local surrounding tissue. Almost all malignant tumours result from the transformation of a single cell, which being free of normal homeostatic controls, proliferates, invades and may metastasise. Transformation is a relatively rare event in the 10^{14} cells a human body contains, and requires a cell or its predecessor to have undergone several independent carcinogenic changes. Therefore exposure to a carcinogen may prime many cells for transformation, but a malignancy may not develop until decades later when one of these cells undergoes a penultimate transformation event.

Cancer is a multi-stage disease and can arise from acquired multiple mutations in two distinct classes of nuclear genes. The proto-oncogenes are positive regulators of cell growth, survival, invasion, and metastasis, whereas tumour suppressor genes (TSGs), are negative regulators. More than 50 different oncogenes and more than twenty different tumour suppressors have been identified, and it has become increasingly apparent that cancer-inducing mutations target conserved signalling pathways. The contributing factors to tumour initiation includes exposure to chemical carcinogens and ionising radiation, infection by oncogenic viruses and familial genetic predisposition. All of these factors can influence the potential of cancer developing through their ability to damage DNA, or affect its replication or repair. In addition, gene products which function to repair DNA damage, are often inactivated during tumourigenesis, although they do not directly function to enhance selective growth, survival or invasion.

The mutations that occur between proto-oncogenes and TSGs are distinct. Proto-oncogenes frequently undergo point mutations, that result in a missense mutation in the gene product and leads to its activation, or a new function. In contrast, TSGs mutations can lead to a loss of function, or can result in a dominant-negative function of the protein. This disparity extends to chromosomal aberrations, such as

those found in tumours, namely deletions, translocations, amplifications and aneuploidy, that result in the activation of oncogenes and the inactivation of TSGs. The different mutational profiles of these two distinct classes of genes accounts for the characteristic dominance of oncogenes, (oncogenic alleles transform cells in the presence of wild-type alleles), compared to the characteristic recessiveness of TSGs, (both alleles of a TSG require inactivation for tumourigenesis). Although the activation of cellular oncogenes permits transformed cells to thrive in the absence of growth stimulus, the inactivation of tumour suppressor genes which function to restrain growth is also fundamental to neoplasia and tumour progression.

Furthermore, recurrent mutations in these activating genes that normally function in the regulation of signalling pathways that incur constitutive activity, coupled with the inactivation of downstream target genes, ascribes a critical and causal functional role in neoplasia. Increasingly these alterations in downstream target gene expression have been consistently linked to tumourigenesis, thus the argument that these have a causal role in cancer becomes more compelling.

1.1.1 Oncogenes

The discovery of oncogenes was prerequisite to the understanding of the molecular mechanisms of tumourigenesis. A summary of the major classes of oncogenes and their identification class is tabulated in Table 1.1.

The retroviral oncogenes are almost exclusively transduced versions of cellular proto-oncogenes whereby their expression and activity is altered (Table 1.1{i}). This was initially identified by the discovery that *v-src*, the transforming agent of the Rous sarcoma virus, was a homologue of *c-src* (Stehelin *et al.*, 1974).

The discovery that proviral insertion mutagenesis could activate oncogenes (Hayward *et al.*, 1981), (Table1.1{ii}), was through activation of the *myc* proto-oncogene in over 80% of ALV-induced chicken bursal lymphomas, and that proviral DNAs were integrated upstream of the lymphoma *myc* genes.

Oncogenes have also been isolated from spontaneous or carcinogen-induced mutations in proto-oncogenes, accounting for sporadically occurring tumours. This was evident from gene transfer experimentation, which suggested that approximately 20% of tumours contain DNA sequences with transforming ability. The isolated DNA sequences included the known oncogenes H-*ras*, K-*ras*, *ros* and *raf*, and also novel oncogenes predominantly of the *src* and *ras* superfamilies (Tabin *et al.*, 1982; Taparowsky *et al.*, 1982; and Santos *et al.*, 1982). These findings substantiated the belief that spontaneous arising neoplasms, are due to mutational activation of proto-

oncogenes. The novel oncogenes identified through gene transfer can be classified into those which are activated in the tumours from which they are derived, and those activated by re-arrangements during the gene transfer process, (Table 1.1{iii}).

The association of oncogenes with chromosomal abnormalities has also led to their identification. The activation of oncogenes by translocation is comparable to insertional mutagenesis whereby oncogene sequences are adjacent to previously separated and unrelated sequences, (Table 1.1{iv}). This can result in the inappropriate expression of the oncogene via a proximal enhancer sequence or through protein fusion products, and is characteristic of cancers of the haematopoietic system (Adams *et al.*, 1986). Chromosomal abnormalities arising from amplifications of genomic DNA has also led to the identification of novel oncogenes (Schwab *et al.*, 1983; Wong *et al.*, 1986).

In addition, further identification of novel oncogenes has been achieved through sequence homology to existing oncogenes. Cross hybridisation with DNA probes to known oncogenes and amplification by PCR using degenerate oligonucleotide primers has been used to isolate new family members from genomic and cDNA libraries (Table 1.1{v}).

i) Oncogenes in acute transforming viruses
ABL, AKT, CBL, CRK, ERB-A, ERB-B, ETS, FES/FPS, FGR, FMS, FOS, JUN, KIT, MIL/RAF, MOS, MYB, MYC, H-*RAS,* K-*RAS REL, ROS, SEA, SIS, SKI, SRC, YES*
ii) Retrovirally integrated oncogenes
AHI1, BMI1, DSI1, EVI1, FIM1, FIS1, FLI1, FLVI1, GIN1, INT1/WNT1, INT2, INT3, INT4/WNT3, LCK, MIS1, MIS2, MIS3, MIS4, MLVI2, MLVI3, PIM1, SPI, TIAM1, TPL2, VIN1
iii) Gene transfer identified oncogenes
activated in the tumours from which they were derived:
N-*RAS, NEU, MET TRK*
activated during gene transfer:
DBL, FGF5, HST, LBC, MAS, B-*RAF, RET, TRE, VAV*
iv) Oncogenes associated with chromosomal aberrations
translocation:

ALL1/MLL/HRX, BCL1/PRAD1/CCND1, BCL2, BCL3, BCR, TAL1, TAL2, TAN1
amplification:
L-*MYC*, N-*MYC, GLI1, ERB-B2, AIB1/SCR1*
v) Oncogenes identified by cross hybridisation or degenerate PCR
*ELK1, ELK2, EPH, ERB-B3, ERB-B4, ERG, FOS*B, *FRA1, FRA2, HCK, JUN*B,
*JUN*D, *LYN,*

Table 1.1

Table of identification of novel oncogenes.

(See Hesketh, 1994 for the majority of these entries).

The proto-oncogenes and TSGs are evolutionary conserved with homologues being discovered in eukaryotes spanning from yeast, worms, flies, frogs and mice to humans. Extensive fundamental research into the function of these genes, as with their oncogenic counterparts in cancer research, has established critical roles in many aspects of cell behaviour. This includes differentiation, proliferation, motility, invasion, replicative senescence and apoptosis. In fact, many of the proto-oncogenes function as components of signal transduction pathways, which transmit extracellular signals that initiate at the cell surface via specific receptor-ligand interactions that are relayed through the cytosol to the nucleus.

Fos encodes a component of the transcription factor AP-1. It is a member of a family of related genes (Cohen and Curran, 1988; Dobrzanski *et al.*, 1991) that have a leucine zipper domain adjacent to a basic DNA binding domain (Landschulz *et al.*, 1988), which heterodimerize with members of the Jun family (Ryder *et al.*, 1989; Hirai et al., 1989) to form the AP-1 transcription factor. All of the Fos-Jun heterodimers bind to the consensus AP-1 binding site, $TGA^{C}/_{G}TCA$, found in the promoter region of AP-1 responsive genes (Angel *et al.*, 1988).

Figure 1.1 presents a schematic overview of the signal transduction pathways that function upstream of AP-1 and includes prot-oncogenes as signalling components. The nuclear transcription factors permit the cell to alter the expression of target genes which elicit a response to the original signal. Proto-oncogenes function at various positions in these pathways, thus abnormal activity can profoundly alter the interpretation of the signalling pathway, contributing to deregulated proliferation and neoplasia. However, the interaction of oncogenes in signal transduction pathways is very complex. The oncogene products can be classified according to their function in signal transduction cascades. The functional classification of the most prominent oncogene products are described in Table 1.2.

Figure 1.1

Activation of AP-1 in fibroblasts through extracellular and intracellular signals, and through oncogenic Ras.

The Integrins signal through the ECM through insoluble ligands which activate Focal Adhesion Kinase (FAK). The extracellular signal of the growth factors functions through Receptor Tyrosine Kinases. These signalling pathways function through the cytosolic Grb-2 (Growth factor receptor-bound protein 2), that binds to the cytosolic guanine nucleotide-releasing factor Sos-1, facilitating the activation of Ras. The Ras or oncogenic Ras then activates Raf-1, which subsequently activates MEK-1 and the phosphorylation cascade to the nucleus via ERKS. The phosphorylation of TCF/Elk-1 in the fos promoter stimulates transcriptional activity, inducing AP-1. The AP-1 induced complexes can initiate transcription through binding to AP-1 sites in the target gene's promoter as shown, or perhaps can prevent transcription through transrepression by binding to the target gene's promoter turning transcription off (not shown).

(Diagram adapted from Blenis, 1993; and Karin, 1995).

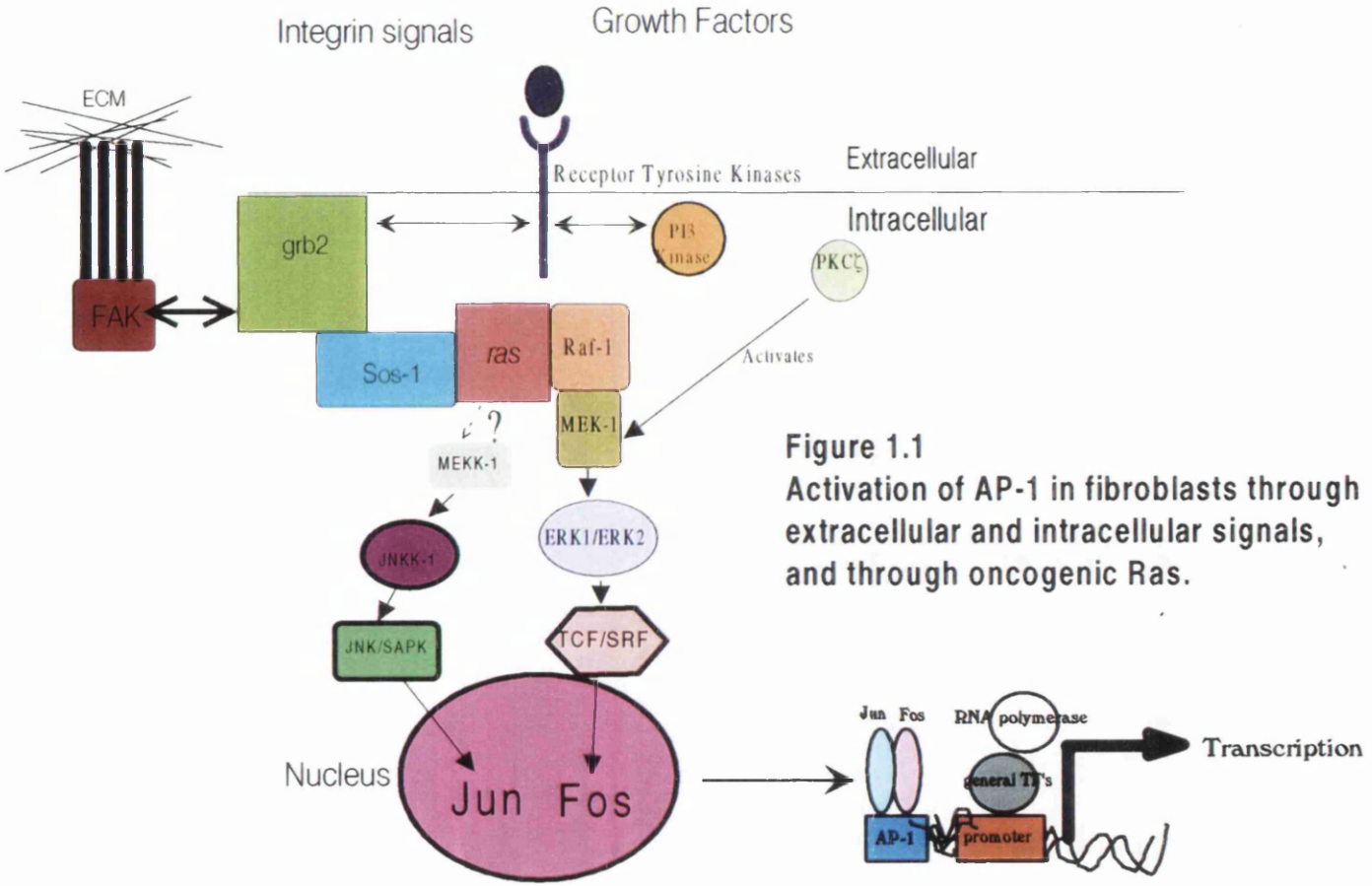


Figure 1.1
Activation of AP-1 in fibroblasts through extracellular and intracellular signals, and through oncogenic Ras.

FUNCTION	ONCOGENE
Ligands	FGF5, INT2, HST1, SHH, SIS/PDGFB, WNT1, WNT3
Receptor tyrosine kinases	EPH, ERB-B/EGFR, FMS, KIT, MET, NEU/HER2/ERB-B-2, RET, ROS, SEA, TRK
Non-receptor tyrosine kinases	INT3/NOTCH4, TAN1/NOTCH1
Non-catalytic receptors ***Membrane associated:*** ***Cytoplasmic:***	 SRC, FGR, FYN, HCK, LCK, LYN, TKL, YES ABL, FPS/FES
Lipid kinases	PI3K
Cytoplasmic regulators of protein activity ***SH2/SH3 Containing adapters:*** ***Guanine nucleotide exchange factors:***	 CRK, NCK, SHC DBL, ECT2, LBC, TIAM1, VAV CBL
Membrane-associated G proteins	H-RAS, K-RAS2, N-RAS, GSP, GIP2
Cytoplasmic protein serine/threonine kinases	AKT, BCR, MEK1/2, MOS, PIM1, RAF/MIL, TPL2
DNA-binding nuclear proteins **(Transcription Factors)**	ALL1/MLL, ERB-A/THRA, ETS1, ETS2, FOS, JUN, MYC, MYB, REL, TAL1, SKI
Transcriptional co-activator **(acetyl transferase)**	AIB1/SCR1

Table 1.2

Tabulated oncoprotein classification pertaining to biochemical role in signal transduction

(Adapted from Hesketh 1994).

The cellular homologue (*c-fos*), of the retroviral oncogene investigated in this study, FBR *v-fos* is highly conserved with 70% homology at the nucleotide level (between mouse and human), (Van Straaten *et al.*, 1983). This high evolutionary conservation suggests an important role in normal cellular physiology. C-Fos is a nuclear protein that is a component of the Activator Protein 1 (AP-1), transcription factor which is the nuclear termini of several signal transduction pathways. Thus, AP-1 activation regulates gene expression in response to activation of the original cellular

stimuli. Its viral counterpart, v-Fos, constitutively activates AP-1, directly regulating gene expression and when inappropriately expressed, transforms cells.

1.1.2 Tumour Suppressor Genes

The TSGs are also known as recessive oncogenes, anti-oncogenes and growth suppressor genes. Several TSGs function by directly opposing the action of oncoproteins. That is they constrain cellular proliferation and survival by impeding mitogenic signalling pathways, blocking cell cycle progression, promoting terminal differentiation and maturation arrest or by inducing apoptosis. TSGs that suppress the invasive and metastatic potential of tumour cells also exist, and are inactivated during tumour progression. In addition, the capping of the replicative life span of a cell is also a target of tumour suppression, and three TSGs, *RB1, TP53 and CDKN2A/p16INK4A* play a role in replicative senescence.

The concept of TSGs was born out of early experiments involving the fusion of normal somatic cells and tumour cells (Harris *et al.*, 1969; Stanbridge *et al.*, 1976), whereby the *normal x tumour* or *less malignancy x more malignant* hybrids exhibited a normal phenotype establishing that malignancy was a recessive trait. These findings were consistent with the hypothesis that tumour cells had lost critical functions as opposed to gaining dominant transforming functions. The genetic basis to cancer was highlighted from the *normal x tumour* hybrids, as the hybrid cells frequently and randomly lost chromosomes, although the segregation of certain chromosomes was predominantly associated with a malignant phenotype (Jonasson *et al.*, 1977; Klinger *et al.*, 1980; Evans *et al.*, 1982). This enabled the mapping of TSGs to chromosomes that most likely contained genes of a suppressive function. Additionally, the concept of tumour suppression was proposed by Knudson's (1971), "two-hit" hypothesis of transformation around the same time in relation to the clinical data pertaining to Retinoblastoma. This hypothesis stated that the sequential mutation of at least two independent genes is essential for transformation of normal retinoblasts to their malignant form. The data collected from the hereditary form revealed that the first hit was from the chromosome 13 deletion, with the second hit a random event in susceptible retinal cells. As the cells in both retinas carry the first hit mutation, the risk of tumour formation is high. In the sporadic form, two random mutations must occur within the same retinoblast, and as either mutation alone is unlikely, there is an extremely low probability for both mutation events to occur in the same cell.

The tumour suppressor genes can be classified into two groups; Class I, in which loss of function results from mutation or deletion of DNA, and Class II, in which

a loss of function is from a regulatory block to expression. The class II suppressor genes are assumed to be regulated by a Class I tumour suppressor. Therefore, they are class I tumour suppressor effector genes. The tumourigenicity of several types of human cancers is enhanced by the inactivation, through mutation of several class I TSG families including p53, Rb and APC (Levine, 1993). The identification and isolation of class I TSGs was through unwieldy pedigree and cytogenetic analyses (Sager, 1989). The down-regulation of class II genes, such as non-muscle α-actinin, tropomysin I, CLP, retinoic acid receptor β, and interferon regulatory factor 1 (Gluck *et al.*, 1993; Hirada *et al.*, 1993; Houel *et al.*, 1993; and Mishra *et al.*, 1994), in tumour cells has indicated a functional role in tumour suppression. In recent years, the discovery of TSGs has dramatically increased with the advent of differential screening (or differential hybridisation), differential display and subtractive hybridisation. Additional TSGs have been identified through using these techniques to identify genes that are down-regulated in tumour cells including the mapsin gene, lysyl oxidase (*rrg*), and *NO3* (Contente *et al.*, 1990; Ozaki and Sakiyama, 1994; Zou *et al.*, 1994).

Reintroduction of a TSG family member into a tumour cell can reverse the transformed phenotype and can block specific characteristics of transformation. This supports the possibility for novel therapies in the treatment of cancer by using a TSG to block specific tumour characteristics such as proliferation or invasion or even to reverse transformation.

The TSGs have been further characterised by Kinzler and Vogelstein (1997), as either "gatekeepers" or "caretakers". The gatekeepers such as *RB1*, *TP53*, and *CDKN2A* among others, control cellular proliferation by directly inhibiting growth or promoting death. Accordingly, restoration of the missing gatekeeper function to cancer cells leads to suppression of tumour growth. In contrast, the caretakers, susceptibility genes that indirectly suppress tumourigenesis function in the maintenance of the genome and include the mismatch repair genes, nucleotide excision repair genes and genes like *ATM*, *BLM*, and *WRN* which maintain chromosome integrity after DNA damage and recombination. The inactivation of a gatekeeper TSG directly manifests a growth advantage to the affected cell. However, tumour initiation is not a direct result of inactivation of a caretaker gene, but the potential of tumour progression being relayed through the increase in the mutation rate of all genes, including gatekeepers, through promoting genomic instability and is equivalent to a constant exposure to mutagens. It is this Mutator phenotype that confers an increased risk of tumourigenesis. The restoration of function of a caretaker

in a transformed cell does not affect its growth as these indirectly acting genes are not required for tumourigenesis.

More recently Kinzler and Vogelstein (1998), have further proposed a second class of caretaker genes by recent studies in the defective genes of juvenile polyposis syndromes (JPS), (Howe *et al.*, 1998), and in ulcerative colitis (UC). The increased cancer susceptibility due to inherited mutations in JPS is the result of an abnormal stromal environment and thus an altered terrain for epithelial cell growth and can be thought of as a "landscaper" effect.

1.2 THE FBJ AND FBR MURINE SARCOMA VIRUSES

1.2.1 Virus isolation

The oncogene *v-fos* was initially identified through experimentation into the induction of osteosarcomas by Finkel and co-workers (Finkel *et al.*, 1966), in which two viral isolates that induced osteosarcomas were isolated from cell-free filtrates of spontaneous or ^{90}Sr induced murine osteosarcomas.

The Finkel-Biskis-Jinkins Murine Sarcoma Virus (FBJ-MuSV), was isolated from a spontaneous osteosarcoma in a 270 day old male CF1/An1 mouse. Sterile extracts prepared from the tumour resulted in spinal osteosarcomas, upon interperitoneal or subcutaneous injection into newborn CF1 mice (Finkel *et al.*, 1966). Subsequent subcutaneous injection of sterile extracts prepared from the spinal osteosarcomas into four newborn CF1 mice litters resulted in osteosarcomas within 71 days, and preparation of more sterile extracts from these osteosarcomas was performed with filtration through 0.45 μm pores. Further injection of crude extracted material and filtrated extracts into CF1 mice, resulted in the outcome of osteosarcoma development at 35 and 61 days respectively (Finkel *et al.*, 1966). These experiments indicated that tumourigenicity was a product of a virus and the subsequent decrease in incubation period was a consequence of an increase in titer. The presence of C-type viral particles were later validated through electron microscopy and the virus was termed FBJ-MuSV (Levy *et al.*, 1973), and subsequent tissue culture experiments demonstrated that the FBJ virus was in fact a mixture of a low titer focus forming virus, FBJ-MSV, and a helper virus, the high titer plaque forming FBJ-MLV virus (Levy *et al.*, 1973).

The other viral isolate was recovered from a ^{90}Sr-induced osteosarcoma in a X/Gf mouse, the Finkel-Biskis-Reilly Murine Sarcoma Virus (FBR-MuSV), and was passaged in a similar manner as the FBJ-MuSV virus (Finkel *et al.*, 1975). The FBR-MuSV was also shown to be a complex of a type-C replicative defective, acutely transforming virus and a replication competent helper virus of a different pseudotype to that of the FBJ-MuSV (Curran and Verma, 1984). The inoculation of newborn mice with FBR-MuSV induced tumours of a similar ilk to those of the FBJ-MuSV (Finkel *et al.*, 1975). In addition, FBJ-MuSV has been shown to morphologically transform all rodent fibroblasts and fibroblast-like cells of mesodermal origin. Furthermore, in tissue culture assays the virus can rapidly transform fibroblast cells which can be further enhanced with corticosteroid treatment (Lee *et al.*, 1979).

Tissue culture experiments revealed that the FBJ-MuSV induces transformation but not immortalisation, whereas FBR-MuSV induces both (Jenuwein *et*

al., 1985). The characteristic of FBR-MuSV in both transformation and immortilisation of cells in tissue culture are not shared by any other retroviral oncogene.

In addition to transforming cells of mesenchymal origin, the *v-fos* oncogene can transform other cell types including those of epithelial origin and lymphocytes (Lee *et al.*, 1993; Valge-Archer *et al.*, 1990). Interestingly, the histological characteristics of *v-fos* induced osteosarcomas indicated that the local invasive cells attached to bone tissue were of mesoderm origin suggesting that fibroblastic cells were the target of *v-fos* oncogene, although electron microscopy studies confirmed the presence of a variety of cell types (Yumoto *et al.*, 1970). It was noted in further studies performed by Ward and Young (1976), that *v-fos* induced tumours are mainly localised in the periosteum, as opposed to the deep bone cortex, the localisation site of human osteosarcomas. Therefore, the histological variation and cellular pleimorphism found in the *v-fos* induced tumours indicated that the type of osteosarcoma formed were of a parosteal type of bone tumour as suggested by Verma (Verma and Graham, 1987).

1.2.2 Viral *fos* and cellular *fos* identification

The FBJ-MuSV was first isolated from the generation and use of a non-producer (NP), cell line (Curran and Teich 1982a), 208F fibroblasts which were a derivative of thioguanine-resistant Rat1 cells (Quade, 1979), transformed with FBJ-MuSV viral complex. The transformed NP cells formed foci and colonies in soft agar, and when injected into synergistic mice, tumour formation ensued. The Tumour Bearing Rats Sera (TBRS), from these mice were used to precipitate material from ^{35}S labeled FBJ-MuSV NP cells, that resulted in a 39kD protein of cellular origin. The isolated protein, c-Jun, was exclusively expressed in the FBJ-MuSV NP cells, and was absent in 208F cell line, and in 208F cells transformed by Kirsten-MuSV, Moloney-MuSV, Abelson-MuSV, or Rausher-MuSV. Furthermore, antisera that recognised structural FBJ-MuSV proteins did not cross-hybridise with the 39kD protein and *in vivo* labeling with subsequent tryptic peptide analysis revealed no structural relationship to the envelope proteins of the FBJ-MuSV (Curran and Teich 1982a).

A later report by Curran and Teich (1982b), identified the transforming component of the FBJ-MuSV through the precipitation of two proteins, one of 39 kD and a novel protein of 55kD, using TBRS. The p55 v-Fos was also specific to the FBJ-MuSV NP cells, and was undetectable in 208F cell line, and the 208F cell line transformed by Kirsten-MuSV, Moloney-MuSV, Abelson-MuSV, or Rausher-MuSV. The separation of ^{35}S labeled fingerprints from cell extracts by two-dimensional gels revealed that the two precipitated proteins were not the same as those from the *gag*

and *env* encoded products (Curran and Teich, 1982b). Peptide mapping of the *in vitro* translation of viral RNA in a reticulocyte lysate and immunoprecipitation confirmed that the p55 v-Fos was specific to the FBJ complex. The phosphorylation of the p55 v-Fos on Serine but not Tyrosine residues and the absence of protein kinase activity was deciphered from phospho-amino acid analysis (Curran and Teich, 1982b).

Reverse transcribed labeled cDNA of viral RNA from FBJ-MuSV complex was utilised in the molecular cloning of the FBJ-MuSV by the screening of Southern blots containing DNA from non-producer FBJ-MuSV transformed cell lines (Curran and Teich, 1982b). The biologically active segment of proviral DNA was identified and isolated and was found to contain an extensive region of non-homology with helper virus DNA which was expressed as a 3.5Kb mRNA in transformed cells. This non-homologous region was similar to a cellular homologue which was conserved in several different vertebrate species (Curran and Teich, 1982b). The putative oncogene was named *v-fos* and the proto-oncogene *c-fos* from *F*BJ derived *O*steo*S*arcoma, (*FOS*).

The cloning of the FBJ-MuSV permitted similar analysis on the FBR viral isolate that identified a 75kDa phosphoprotein, antigenically related to p55 v-Fos. The proviral genome of FBR-MuSV was cloned into a plasmid and shown to be transforming. Analysis of the DNA sequence revealed that the FBR *v-fos* gene was a fusion of *c-fos* sequences and the 5' viral *gag* gene and contained unknown murine cellular sequences at the 3' end, that resulted in the larger p75 v-Fos as opposed to the p55 v-Fos (van Beveren *et al.*, 1984).

1.2.3 Viral *fos* and cellular *fos* structure

The DNA sequence analysis of both *v-fos* oncogenes and the *c-fos* proto-oncogene highlighted their similarity (van Straaten *et al.*, 1983; Curran *et al.*, 1983; van Beveren *et al.*, 1983; and van Beveren *et al.*, 1984), and their comparison is demonstrated schematically by Figure 1.2.

The murine and human *c-fos* genes have a similar intron/exon structure and exhibit near homologous identity at both the nucleotide and amino acid level. The 27 single amino acid differences are throughout the 380 amino acid length of the protein. *C-fos* mRNA encodes a 2.2Kb transcript from the assembly of four exons from a 3.5Kb transcription unit (Curran *et al.*, 1983).

Figure 1.2

Comparison of c-*fos* and both v-*fos* Genes

Structure of the *c-fos* (mouse), gene and FBJ-MuSV and FBR-MuSV proviral DNAs. Top and bottom: 5' and 3' long terminal repeat sequences (LTR), of FBJ- and FBR-MuSV are depicted by open boxes, other noncoding regions are indicated by a solid line. Stippled boxes indicate the portions of the *v-fos* and *gag-fos* genes encoding the viral homologue to *c-fos*, (Middle). The exons are depicted by stippled boxes, introns 5' and 3' untranslated regions are indicated by a solid line. Indicated below or in each exon are the length (in amino acids), of the segment of the *fos* protein it encodes. Also indicated in the figure are the TGA termination codon used by *c-fos*, the TAG termination codon used by *v-fos*, and the location of the TATA box, 5'-cap, CRE, AP-1, DSE, SCM, and poly (A) signal.
(Adapted from Ransone and Verma, 1990).

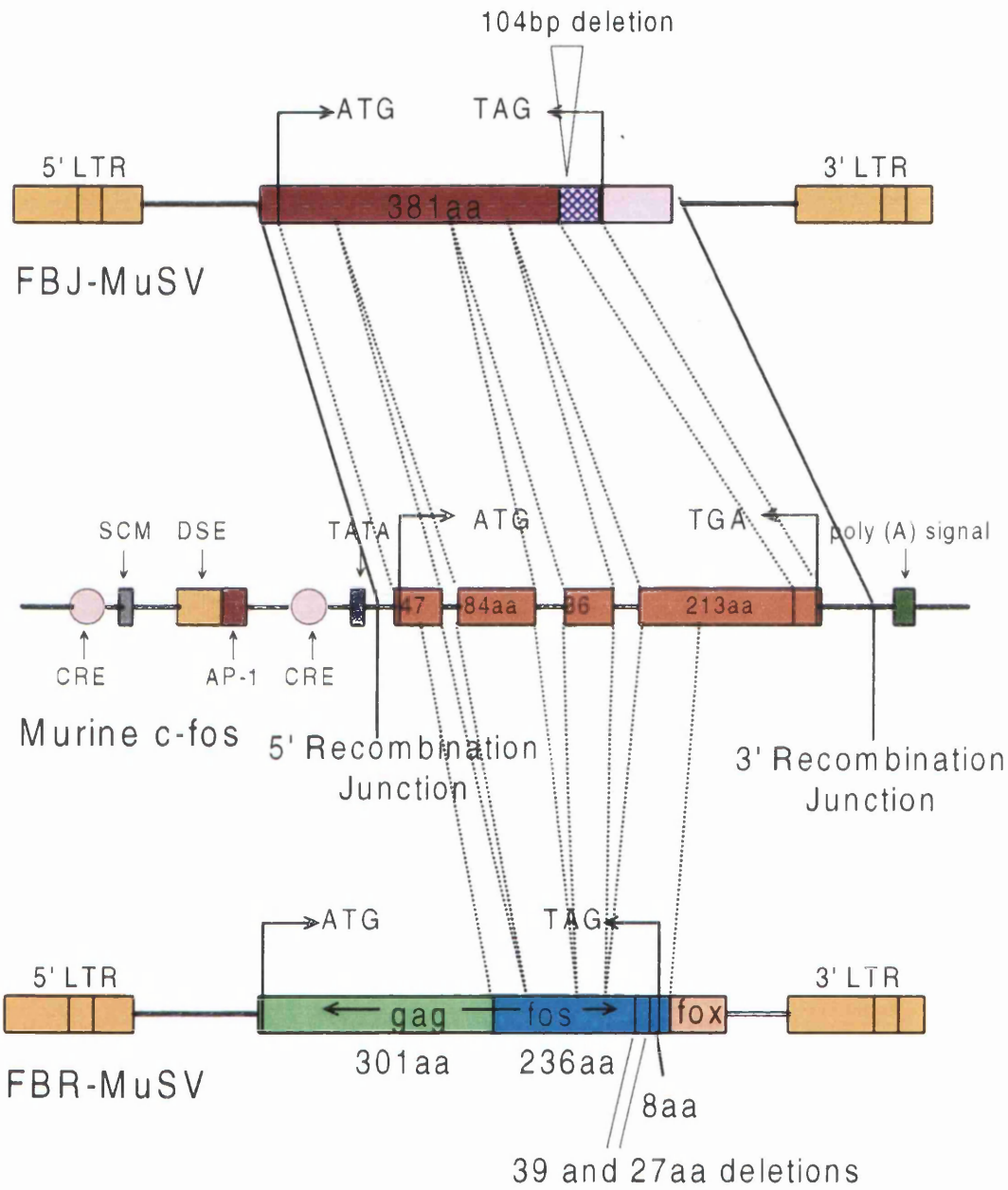


Figure 1.2
Comparison of c-fos and both v-fos Genes

The FBJ-MuSV is 4026bp flanked by two 617bp Long Terminal Repeats (LTR). *V-fos* is substituted for the retroviral *gag-pol-env* genes and contains 1639bp of cellular sequences located in the centre of the viral genome (van Beveren *et al.*, 1983). The open reading frame (ORF), encases these substituted cellular sequences and encodes a 381 amino acid protein (van Beveren *et al.*, 1983). The 48 amino acids at the C-terminus of the human and murine c-Fos proteins are not homologous to those of FBJ *v-fos* (Curran *et al.*, 1983). The FBJ *v-fos* nucleotide sequence has a 104bp deletion at the 3' end of the ORF (van Beveren *et al.*, 1983), that results in a frameshift and a distinct C-terminal 48 amino acid in relation to c-Fos. However, the remainder of the protein is similar to murine *c-fos* with only five amino-acid substitutions (van Beveren *et al.*, 1983).

The FBR-MuSV provirus is somewhat shorter at 3791bp, of which 709bp are derived from the *c-fos* gene and 437bp originated from unidentified cellular sequences (van Straaten *et al.*, 1983). The ORF encodes a *gag-fos* fusion protein, p75 $^{gag\text{-}fos}$. The protein has a 24 amino acid truncation at the N-terminus and a 98 amino acid truncation at the C-terminus as compared to c-Fos (van Straaten *et al.*, 1983). Two small internal deletions of 39 and 27 amino acid are also present. There are five amino acid substitutions and these are different to the five substitutions present in FBJ v-Fos (van Straaten *et al.*, 1983).

1.2.4 Viral *jun* and cellular *jun* identification

Similar to *v-fos*, the *v-jun* oncogene was isolated from a replicative defective, acutely transforming retrovirus, which had no homology to previously identified GTP binding or protein kinase oncogenes and was found to localise at the nucleus (Maki *et al.*, 1987; Vogt *et al.*, 1987). Studies using two-dimensional gel electrophoresis indicated that Fos proteins complexes with a basic 39kDa cellular protein (Franza *et al.*, 1987), that was originally identified by Curran and co-workers (Curran and Teich, 1982a; Curran *et al.*, 1985).

The cellular homologue of *v-jun*, *c-jun* was identified by screening a genomic DNA library with an oligonucleotide designed from the coding sequence of *v-jun* oncogene (Bohmann *et al.*, 1987). A sequence homology at the C-terminal 66 amino acid region between the cellular homologue, c-Jun to the DNA binding domain of yeast transcription factor, GNC4, was observed (Maki *et al.*, 1987; Vogt *et al.*, 1987). In addition, this region was homologous to c-Fos and c-Myc, albeit to a lesser degree (Maki *et al.*, 1987), and had a predicted α-helical character. This suggested that c-Jun, like GCN4 could function as a sequence specific transcription factor, and in fact a

domain swap experiment performed in yeast where the GCN4 DNA binding domain was replaced by the homologous region of c-Jun, resulted in no loss of function (Struhl, 1987). The DNA sequence to which yeast GCN4 binds is very similar to that of the AP-1 consensus binding site (Curran and Franza, 1988), and thus the relationship between c-Jun and the AP-1 transcription factor was investigated. In the experiments conducted by Angel and co-workers (1988), partially purified AP-1 was shown to react with anti-Jun antisera, and that bacterially expressed c-Jun and v-Jun bound to the same DNA sequences as AP-1 (Angel *et al.*, 1988). Furthermore, in a transient transfection assay, v-Jun activated transcription via TPA responsive elements (Angel *et al.*, 1988).

Curran and co-workers demonstrated that Fos family proteins shared a common DNA binding site with AP-1 in the promoters of cellular and viral genes by using DNA-affinity precipitation assays followed by high resolution, two-dimensional electrophoresis (Franza *et al.*, 1988). The ensuing studies involving electrophoretic mobility shift analysis and photoaffinity cross-linking, demonstrated that the FSE could efficiently bind Fos proteins (Rausher *et al.*, 1988a), and that p39 is in fact the *c-jun* proto-oncogene (Rauscher *et al.*, 1988b). The conduction of immunoprecipitation experiments using Fos- and Jun- specific antibodies subsequently identified p39/Jun as an AP-1 associated protein (Sassone-Corsi *et al.*, 1988).

This data provided compelling evidence that Fos and Jun proteins were a major component of the AP-1 transcription factor complex, and as a result of this association could activate transcription *in vitro* and *in vivo*. This suggested a co-operativety between the actions of the Fos and Jun proteins.

1.2.5 Fos and Jun AP-1 family members

Studies of Fos protein complexes using immunoprecipitation and immunoblotting on one and two dimensional gels resulted in the observation of a set of immunologically related proteins, designated Fos related antigens (Franza *et al.*, 1987). Similar to c-Fos, these proteins were induced by serum stimulation but exhibited different expression patterns (Franza *et al.*, 1987; Cohen and Curran, 1988). This indicated that c-Fos belonged to a family of related genes.

*Fos-R*elated *A*ntigen-1, *fra-1*, was the first gene isolated with homology to *c-fos* (Cohen *et al.*, 1989). *Fra-1* was isolated by screening a λgt11 library generated from cells that were serum stimulated for 75 minutes, with antibodies raised to a synthetic peptide derived from a highly conserved *c-fos* sequence that had previously been shown to cross react with several *Fras* (Cohen and Curran, 1988). The sequence

analysis of the cDNA clone that encoded a 35kDa antigen revealed significant homology to c-Fos at the amino acid level, but less homology was exhibited at the nucleotide level (Cohen *et al.*, 1989). The Bzip domain (Refer to Figure 1.3), of the *fra-1* exhibited a 77% homology to *c-fos*, including a conserved cysteine in the basic DNA binding domain (Cohen and Curran, 1988), and the C-terminal 40 amino acid portion of Fra-1 is 65% homologous to c-Fos (Gius *et al.*, 1990). Further studies on Fra-1 observed heterodimerisation with c-Jun, but in keeping with c-Fos, it was unable to dimerise with itself or any other Fos family members (Cohen *et al.*, 1989). Fra-1 is rapidly phosphorylated on serine residues after which it localises to the nucleus (Cohen *et al.*, 1989). In FBR *v-fos* transformed 208F fibroblasts the *Fra-1* mRNA and protein is up-regulated compared to untransformed 208F fibroblasts (Hawker, M^{c}Garry, Ozanne, unpublished observations). The *fra-1* gene has been shown to be positively controlled by AP-1 in a *Fos*-ER expression system in fibroblasts (Bergers *et al*, 1995). In addition, Fra-1 is also found to be up-regulated in *ras*, *raf* and MEK1 transformed cells (Mechta *et al.*, 1997; Cook *et al.*, 1999; Treinies *et al.*, 1999).

The low stringency screening of a human cDNA library with a *c-fos* probe resulted in the cloning of an additional *fos-R*elated *A*ntigen-2, *fra-2.* Transcription of the *fra-2* gene was induced upon phorbol ester (TPA), stimulation of U937 human monocytic cells. (Matsui *et al.*, 1990). The isolation of *Fra-2* from chicken where it encoded a 45kDa antigen, and subsequent experimentation found that *Fra-2* was oncogenic in chick cells (Suzuki *et al.*, 1991). The mouse *fra-2* gene was isolated from genomic cDNA clones encoding the homologue and also induced the transformation of chicken embryo fibroblasts (Foletta *et al.*, 1994).

FosB was discovered by low stringency screening of positive clones isolated from the differential screening of serum stimulated NIH 3T3 fibroblasts using a synthetic oligodeoxynucleotide probe derived from the putative DNA binding site of *c-fos* (Zerial *et al.*, 1989). FosB is a nuclear protein of 388 amino acids with 70% homology to c-Fos , with high conservation in the BZip and C-terminal regions (Zerial *et al.*, 1989). FosB was also induced by serum, heterodimerises with Jun family members, and binds to and activates from AP-1 sites indicating that the Fos family members share biological and biochemical functions (Zerial *et al.*, 1989; Dobranski *et al.*, 1991). In addition, *fosB* can transform rat fibroblasts (Schuermann *et al.*, 1991; Wisdom *et al.*, 1992). A short form of *fosB* was cloned. It results from alternative splicing which generates a protein lacking the C-terminal 101 amino acids (Nakabeppu and Nathans, 1991; Mumberg *et al.*, 1991; Yen *et al.*, 1991; and Dobrzanski *et al.*, 1991). The truncated protein functions as a negative regulator of cellular transformation and suppresses *fos*-induced transformation (Mumberg *et al.*, 1991).

The Jun family member, *junB* was discovered by the screening of serum stimulated cDNA libraries with probes derived from stimulated-cell RNA. *JunD* was isolated by the screening of serum stimulated cDNA libraries with *junB* or *c-jun* probes at moderate to low stringency (Lau and Nathans, 1987; Ryder *et al.*, 1989; and Hirai *et al.*, 1989). JunB and JunD are extensively homologous to c-Jun in the BZip region and in the N-terminal transactivation domain (Ryder *et al.*, 1989). The proteins can homodimerise and form heterodimers with other members of the Fos and Jun families. Similar *to c-jun*, *junB* is inducible by serum and TPA (Lau and Nathans, 1985), whilst *junD* is constitutively expressed throughout the cell cycle in a wide variety of cell types (Ryder *et al.*, 1989; Chiu *et al.*, 1989; and Hirai *et al.*, 1989).

Furthermore, both Jun and Fos family members can form heterodimers with ATF transcription factor family members (Benbrook and Jones, 1990; Hai and Curran, 1991). The ATF family are a set of leucine zipper transcription factors that were identified by virtue of their interacting with DNA sequences which mediate transcriptional activation in response to cAMP or Adenovirus E1A (Ziff, 1990). The cAMP (CRE), response element, TGACGTCA, differs from TPA response promoter element, TGACTCA by one nucleotide (Sassone-Corsi *et al.*, 1990). AP-1 and ATF co-purify on DNA affinity columns and are immunologically related (Hai *et al.*, 1988).

Therefore, AP-1 is not simply a dimerisation of c-Fos *and* c-Jun but can be regarded as a much more intricate transcription factor complex with the feasible binding of several Fos, Jun and ATF family members, each with different DNA binding specificities. Thus, through the various potential homodimeric and heterodimeric complexes that can be formed, AP-1 elicits a high degree of transcriptional control.

In *v-fos* transformed 208F fibroblasts Fra-1, Fra-2, JunD and c-Jun are the predominate AP-1 components. FosB and JunB are detectable but at a lesser extent (M^{c}Garry, Ózanne, unpublished observations).

1.2.6 Function of Fos proteins

The p55 c-Fos protein is composed of distinct functional domains that mediate transactivation, dimerisation with Jun/ATF family members, DNA-binding and cellular transformation. The functional domains are shown in Figure 1.3A.

The N-Terminal Activation domain (N-TA), is an independent domain involved in transactivation and transformation (Jooss *et al.*, 1994). Adjacent to N-TA is the basic/zipper region (BZip region), that functions to dimerise with the Jun family proteins and binds DNA. The bZip domain includes the basic region that contacts DNA and the leucine repeat which forms the characteristic Leucine Zipper helical

wheel that stabilises the protein dimers via a scissor grip model. A heterodimeric DNA binding configuration *of* c-Fos and c-Jun is depicted in Figure 1.3B.

The HOB domains at the C-terminus are conserved between the different Fos family members and function as transactivation domains. The HOB1 motif is deleted in the FBR v-Fos protein (Sutherland *et al.*, 1992), and the TATA-binding motif at the C-terminus of c-Fos is deleted in the FBJ and FBR v-Fos proteins (Metz *et al.*, 1994a; 1994b). The C-TM domain is present in both the c-Fos and FBJ v-Fos proteins, but is deleted in FBR v-Fos. However, the transforming potential is fortified by the cellular *fox* sequences, that partially substitute the C-terminus for the wild-type c-Fos protein (Funk *et al.*, 1997). The studies conducted to demonstrate the function of these regions are further detailed below.

The region between amino acids 41 and 73 at the N-terminus of FosB and FosB/sf were found to be highly conserved and deletion compromised the transforming ability of FosB (Wisdom and Verma, 1993). This indicated that in addition to the BZip region, the N-terminus of Fos family members is required for transformation. Similarly, in the *c-jun* proto-oncogene different regions of the protein were shown to be required for the transcriptional activity, that is amino acids 68-91, which encodes the N-TA (Angel *et al.*, 1989). This region is homologous to the transactivation domain of Fos proteins that were identified through deletion analyses as imperative for transformation (Jooss *et al.*, 1994). It is highly conserved throughout the Fos family including the viral Fos proteins and contains Proline and Glutamine residues commonly found in transactivation domains. This N-terminal domain region homologous between the Fos and Jun family members is regulated by Serine phosphorylation. The Serine 70 amino acid in Fos protein corresponds to the Serine 73 found in the c-Jun protein that is phosphorylated by Stress Activated Protein Kinases (SAPK/JNK), and is required for transactivation and transformation (Alani *et al.*, 1991; Binetruy *et al.*, 1991; Smeal *et al.*, 1991; and Pulverer *et al.*, 1991). The conserved motifs at the N-termini of Fos and Jun proteins that contain the conserved Proline, Glutamine and Serine residues is highlighted in Table 1.3 below.

c-Jun human	ASPELER L IIQ
Fos/aa 66-79	SPDLQWLVQP
c-Fos human	TSPDLQWLVQP
FosB human	TSQDLQWLVQP
Fra-1 rat	TSQELQWMVQP
Fra-2 human	TSQDLQWMVQP
FBJ-MuSV	TSPDLQWLVQP
FBR-MuSV	TSPDLQWLVQP

Table 1.3

Conserved N-termini motifs of Fos and Jun proteins

The conserved N-termini motifs are shown underlined with the Proline, Glutamine and Serine residues highlighted in bold. The Serine residues can potentially be phosphorylated by MAP kinases (Jooss *et al.*, 1994).

This indicates a further complexity of AP-1, by the regulation of the transactivation and transrepression potential of Fos and Jun proteins by *in vivo* phosphorylation.

Fos and Jun family members function co-operatively in transactivation and DNA-binding to an AP-1 binding site, also known as the TRE (TPA-Responsive Element), through their physical interaction (Angel *et al.*, 1988; Chiu *et al.*, 1988; and Sassone-Corsi *et al.*, 1988). Investigations using *in vitro* translation products were conducted to identify the DNA-binding potential of Fos and Jun proteins, and to identify the nature of the dimers formed during *in vitro* translation. Jun was found to bind to the TRE forming dimers with itself and with other Jun family members, JunB and JunD; and as heterodimers with Fos family members (Kouzarides and Ziff, 1988; Halazonetis *et al.*, 1988; Nakabeppu *et al.*, 1988; and Rauscher *et al.*, 1988c). The Fos-Jun heterodimers display an increased binding affinity for the TRE as compared to Jun homodimers indicating further the complexity of AP-1 transforming potential (Schutte *et al.*, 1989a). The Fos family members do not homodimerise.

The Leucine zipper region orchestrates the physical interaction between the Fos and Jun family members and was initially identified in the liver CCAAT enhancer binding protein (Landschulz *et al.*, 1988). The leucine zipper forms a coiled coil in which the Leucine residues are located in a canonical structure spaced at seven amino acid intervals along the polypeptide chain. This structure favours the interactions of

Figure 1.3

Functional domains of *c-fos* and heterodimer DNA binding configuration

A: The p55 c-Fos protein functional domains includes the N-terminal transactivation domain (N-TA, Pink), the Leucine zipper/basic region (bZip, Yellow), the HOB1 and HOB2 and C-TM domains (Blue, Blue/Pattern), and the TATA-binding domain (TBD, Pink). A colour coded key indicating the domains function are shown below.

B: The simplified scissor grip model of c-Fos and c-Jun physical interaction, and their binding to the TRE is depicted. The c-Jun and c-Fos proteins are shown in pink and blue respectively.

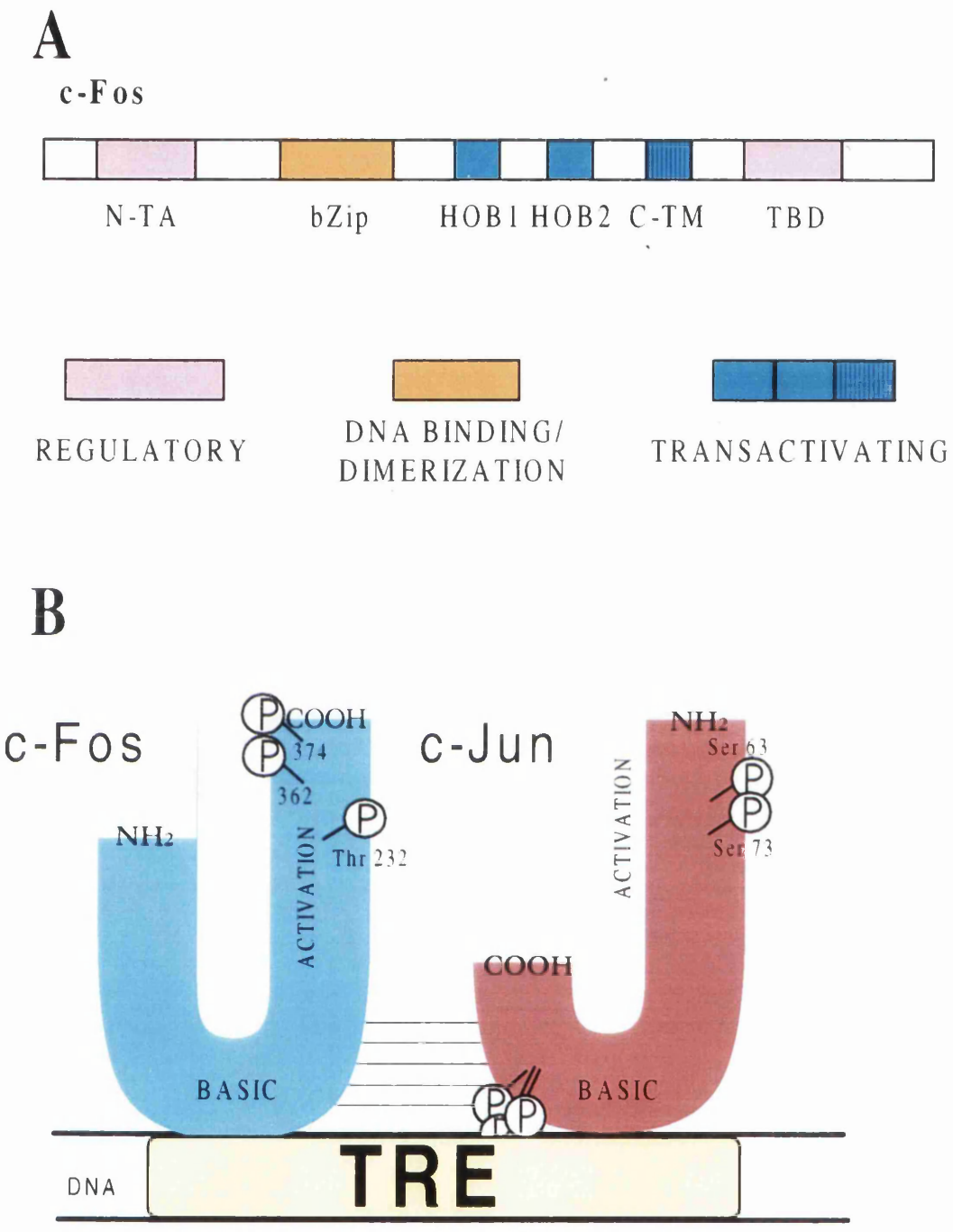


Figure 1.3
Functional Domains of c-Fos and Heterodimer DNA Binding Configuration

leucine residue repeats from two different polypeptides permitting their dimerisation. The Leucine residue repeats are common to the Fos and Jun family members and are also present in other transactivators including GCN4, CREB and C/EBP in which they are found in the same conserved positions. In addition, site-directed mutagenesis directed at the Leucine zipper has also identified this regions importance in DNA-binding (Kouzarides and Ziff, 1988; Gentz *et al.*, 1989; and Neuberg *et al.*, 1989). Thus, the leucine zipper domain is necessary for dimerisation and DNA binding.

The characterisation of the regions of the FBR *v-fos* oncogene detected that the region spanning amino acids 111 to 220 is critical for the induction of transformation. This region encodes the acidic domain, the basic domain which is involved in DNA contact, and the Leucine zipper (Jenuwein and Muller, 1987). Similarly, the corresponding homologous region in the FBJ *v-fos* oncogene that spans amino acids 111 to 206 is also essential for transformation (Yoshida *et al.*, 1989).

Functional analysis of Fos proteins with regards to their interaction with Jun proteins revealed that the majority of the Leucine residues contribute to the formation of an intact, functional zipper. A single mutation in residue Leu-179 in the highly transforming v-Fos protein E300, resulted in a slight decrease in zipper function, whereas mutation of residue Leu-165 completely abolished binding to c-Jun and repressed the transformation ability (Schuermann *et al.*, 1989). Therefore, the Leucine zipper is also required for the *in vitro* induction of transformation.

Additional experimentation involving the mutation of various FBR v-Fos protein domains further confirmed the inhibitive properties of Leucine zipper mutants in transformation (Wick *et al.*, 1992). However, although the transforming properties of FBR *v-fos* was partially suppressed, the proliferative properties of the cell lines expressing the dominant negative mutants remained unaltered (Wick *et al.*, 1992). In addition, the Leucine zipper/Basic region mutants of Fos proteins were also found to inhibit transformation by other oncogenes, and could inhibit EJ Ha-*ras* transformation two-fold. This observation suggested that the retroviral *v-fos* oncogenes are part of the *ras* signal transduction pathway which correlated with previous microinjection studies, whereby injection of *ras* proteins induced *c-fos* expression in vitro (Stacey *et al.*, 1987).

The *c-fos* gene was undetected in quiescent cells but was rapidly induced to high levels after the addition of growth factors or serum, but declined soon after mitogenic stimulation (Greenberg and Ziff, 1984; Kruijer *et al.*, 1984; and Muller *et al.*, 1984). Down-regulation of c-Fos protein levels by degradation is conducted by the C-terminus of the c-Fos protein and not by transrepression of mRNA levels. The FBJ v-Fos protein has a different reading frame in this region resulting from the frame-shift

mutation, and thereby escapes regulatory control (Van Beveren *et al.*, 1983; and Van Straaten *et al.*, 1983). Further studies pertaining to the of 3' sequences of *c-fos* whereby they were replaced with FBJ coding sequences resulted in the induction of transformation (Miller *et al.*, 1984; Meijlink *et al.*, 1985). This indicated that structural modifications to the *c-fos* proto-oncogene can confer oncogenic potential. Additional evidence revealed that the 3' sequences of *c-fos* destabilised the message (Lee *et al.*, 1988; Wilson and Treisman, 1988), and substitution of this region with those of the FBJ *v-fos* oncogene resulted in the stabilisation of the *c-fos/v-fos* mRNA.

An additional function to the transforming potential of FBR *v-fos* is the ability to immortalise, a prerequisite during the passage of rodent cultures towards a neoplastic stage. In contrast, the FBJ v-Fos protein can transform early passage cells, but cannot immortalise them (Jenuwein *et al.*, 1985). The generation of chimeric Fos proteins and extensive detailed analysis of the FBJ-FBR-*c-fos* chimeras revealed that two different alterations determine the transformation potential of the FBR v-Fos protein (Jenuwein and Muller, 1987). The first mutation involved in the induction of immortalisation is an amino acid change at position 138 from a polar Glutamine to a Valine, and when this point mutation was introduced into FBJ v-Fos it confers immortilisation potential. Furthermore, the introduction of this point mutation in *c-fos* results in an increase in transactivation (Lucibello *et al.*, 1991). The presence of another point mutation, a Glutamine 175 to Lysine in the FBJ v-Fos protein results in a decrease in transformation by 80%.

A secondary alteration that contributes to the transforming potential of FBR *v-fos* is the two internal deletions at the C-terminus. The re-introduction of the deletions with the corresponding *c-fos* sequences into FBR v-Fos protein results in a reduction of the transforming ability (Jenuwein and Muller, 1987). Further analysis using a five times TRE construct, that permits the monitoring of the transcriptional activity of Fos proteins, revealed a co-operation between the FBR-specific point mutations and the two C-terminal deletions. The replacement of the deletions failed to affect the transactivation potential, but significantly suppressed transformation (Lucibello *et al.*, 1991). These observations further clarified previous findings whereby the transactivating domain of Fos is located at the C-terminus of the protein (Lucibello *et al.*, 1988; Sassone-Corsi *et al.*, 1988), and an expression plasmid encoding a C-terminally truncated FBR *v-fos* protein inhibited transactivation of a TRE-containing construct (Lucibello *et al.*, 1988).

The HOB1 and the TATA-Binding Domain (TBD), which exert a positive regulatory role in Fos transcriptional activation are deleted in the FBR *v-fos* oncogene (Sutherland *et al.*, 1992; Metz *et al.*, 1994b). The transforming capacity was rescued

by the alternative domains, N-TA and C-TM (Jooss *et al.*, 1994; Funk *et al.*, 1997), and by the addition of C-terminal cellular *fox* sequences (Jenuwein and Muller, 1987). Detailed analysis of the C-terminal region of *c-fos*, *FosB* and FBJ *v-fos* revealed a conserved motif of hydrophobic amino acid residues with the transcriptional activation domain of the viral transactivator VP16 (Funk *et al.*, 1997). The C-TM domain co-operatly functions with the N-TA module to activate transcription.

1.3 FOS BIOLOGICAL MODELS IN CARCINOGENESIS

1.3.1 Cell growth regulation by fos

The induction of *c-fos* when cells transit from G_0 to G_1 indicates a cell cycle function. This assumption was supported by antisense (Holt *et al.*, 1986; Nishikura and Murray, 1987), or antibody micro-injection (Riabowol *et al.*, 1988; Kovary and Bravo, 1991a), experiments that showed a decrease in the number of cells entering S phase under antagonistic *c-fos* expression. Micro-injection of double stranded AP-1 oligonucleotides into primary human diploid fibroblasts prohibited S phase initiation (Riabowol, 1992). The micro-injection of antibodies specific to individual Fos and Jun family members into Swiss 3T3 fibroblasts and subsequent monitoring of DNA synthesis revealed that S phase was reduced in cells injected with combinations of antisera directed against the Fos or Jun families in either serum stimulated or asynchronously growing cells (Kovary and Bravo, 1991b).

These studies provided compelling evidence for a critical role of *c-fos* in inducing the expression of genes that are required for cell cycle progression. However, *c-fos* is also expressed in non-proliferative cells such as neuronal cells whereby induction of *c-fos* in response to a wide variety of signals results in depolarisation (Sager *et al.*, 1989), and the activation of mature macrophages with interferon γ (Higuchi *et al.*, 1988). *C-fos* expression has also been implicated in the *in vitro* differentiation of F9 teratocarcinoma cells, TPA and DMSO induced differentiation of HL-60 cells and in the NGF stimulated differentiation of PC12 cells (Holt *et al.*, 1986; Ito *et al.*, 1989). This suggests that the biological consequence of *c-fos* expression is dependent upon cell type, differentiation status and cellular micro environment. Therefore, *c-fos* through AP-1 and variable heterodimer combinations regulates distinct sets of target genes at different developmental stages resulting in different biological effects.

The recent advent of recombinant DNA technology facilitated the generation of knock-out embryonal stem (ES), cells of *c-fos* (Field *et al.*, 1992; Brusselbach *et al.*, 1995), and *c-jun* (Hilberg and Wagner, 1992).

The proliferative potential of ES *fos* -/- cells remained unaltered in comparison with wild type cells, both in terms of saturation densities and doubling times. Serum induction of quiescent *fos* -/- fibroblast cultures indicated that they retain the same proliferative potential and that they can also spontaneously differentiate *in vitro* similar to the parental wild type *fos* +/+ cells (Field *et al.*, 1992). Further studies exhibited that proliferation and serum re-entry of *fos* -/- cells into the cell cycle was similar to that of

fos +/+ ES and fibroblast-like cells (Brusselbach *et al.*, 1995). The analysis of proliferation using growth curves between *fos* +/+ and *fos* -/- fibroblasts showed that their was no proliferative difference, and FACS analysis revealed that cell-cycle progression and re-entry was not impaired in the *fos* deficient fibroblasts. The parameters of AP-1 activation was also examined and DNA-binding to a TRE consensus, TRE-dependent trans-activation in transfection assays indicated that there was no difference between the *fos* -/- and wild type cells (Brusselbach *et al.*, 1995).

Similar studies conducted using *c-jun* knock-outs (Hilberg and Wagner, 1992), produced similar results. These studies converged upon the idea that the AP-1 components, *c-fos* and *c-jun*, are not essential for cell proliferation *in vitro*, contradicting previously published evidence that suggested cell proliferation was affected when the expression (Holt *et al.*, 1998), or the function of *fos* and *jun* was impaired *in vitro* (Riabowol *et al.*, 1988; Kovary and Bravo 1991a; 1991b).

The *in vivo* targeted disruption of *fos* and *jun* genes suggested a direct role in development and differentiation, indicating an essential function in murine development. Targeted disruption of *c-jun in vivo* results in embryonic lethality and the omission of *fos* is associated with bone defects (Wang *et al.*, 1992; Johnson *et al.*, 1992a).

1.3.2 *In vivo* models of Fos carcinogenesis

The development of transgenic mice carrying the *c-fos* transgene controlled under a regulatable metallothionein promoter (MT), demonstrated that high levels of *fos* could be induced in different cellular backgrounds and these mice did not develop bone neoplasms (Ruther *et al.*, 1985). The targeted expression of a *c-fos* transgene in mice under an H2-K^b MHC promoter impaired the immune system, however no lymphoid or thymic neoplasms developed (Ruther *et al.*, 1988).

Chimeric mice were generated using ES cells transfected with a MT *c-fos* transgene and high expression was maintained during development resulting in the formation of chondrogenic sarcomas irrespective of the degree of chimerism (Wang *et al.*, 1991). An important observation was the specificity of the tumour formation. Although the MT *fos* oncogene was expressed in other tissues there was no tumour development. These results are in contrast with previous findings of the same laboratory as they previously indicated that the target was the osteogenic progenitor cells and not chondroblasts (Ruther *et al.*, 1989). The study conducted by Wang and co-workers (1991), whereby the *fos* genes were expressed ectopically early in

development demonstrated that Fos could transform specific cell types different to those commonly targeted by the *v-fos* oncogene.

The expression of *c-fos* under the control of an H2-K^b MHC promoter coupled with a 3' FBJ-MuSV LTR resulted in osteosarcoma formation with a short latency (Grigoriadis *et al.*, 1993). The tumours grew rapidly in all bones with evidence of osteoblastic activity. They exhibited a high degree of neovascularisation and were highly invasive with an increased mitotic index. Cell lines derived from these tumours were morphologically transformed and displayed the characteristics of osteoblastic gene expression (Grigoriadis *et al.*, 1993). Substitution of *c-fos* for *fosB* or *c-jun* does not result in osteosarcoma formation (Grigoriadis *et al.*, 1993), contradictory to previous evidence that highlighted a transforming role for the *fosB* gene when expressed under the transcription signals of the FBR-MuSV LTR (Schuermann *et al.*, 1991).

The mouse epidermis has been successfully used as a model to investigate the role of oncogenes in neoplastic development (Balmain *et al.*, 1984; Quintanilla *et al.*, 1984; Brown *et al.*, 1986; Greenhalgh and Yuspa 1988). *Fos* oncogenes have been implicated in the formation of epidermal tumours. The introduction of the *v-fos* oncogene into papilloma cell lines expressing an activated Ha-*ras* oncogene resulted in tumour formation when injected into nude mice. In contrast papilloma cell lines expressing E1A or *myc* oncogenes did not form tumours, implying a role for *v-fos* in the generation of these epidermoid malignancies. These investigators proposed and later confirmed that the tumours formed through a co-operation event with *v-ras* and a nuclear oncogene with the properties of a transcriptional activator, such as the *v-fos* oncogene. The co-operative link was established through the injection of keratinocytes infected with *v-ras* and *v-fos* MuSVs into nude mice which induced the formation of squamous cell carcinomas (Greenhalgh *et al.*, 1990).

Collectively, these studies indicate the role of *fos* oncogenes and AP-1 family member components in experimental models of oncogenesis and a role for *ras* and *fos* oncogenes in this *in vivo* model of oncogene co-operation.

The disruption of *c-fos* in mice by homologous recombination (Johnson *et al*, 1992b; Wang *et al.*, 1992), resulted in mice with retarded growth and a low survival rate (Johnson *et al.*, 1992b). In addition, the disruption of the *c-fos* proto-oncogene conferred abnormalities in bone formation, reduction of the cartilaginous tissues and hypertrophy of the chondrocytes, and furthermore, FACS analyses demonstrated an impaired function of haemopoetic cells (Wang *et al.*, 1992).

Cells derived from the fos -/- mice were used to investigate the role of AP-1 in tumourigenesis. The proliferative rate of 3T3-like cells from *fos* -/- mice were not

substantially different from *fos* +/+ mice (Hu *et al.*, 1994), suggesting a redundant role of AP-1 components in cellular proliferation, contradictory to other observations (Holt *et al.*, 1986; Nishikura and Murray, 1987; Riabowol *et al.*, 1988). In *fos* -/- cells the expression of other *fos* family members, *fra-1* and *fra-2* as well as *jun* family members were variable after induction by growth factors and phorbol esters, but the level of AP-1 binding activity remained unchanged (Hu *et al.*, 1994). Transformation studies with *fos* -/- mice showed that there was no significant difference in foci formation induced by oncogenes that function upstream of AP-1 in the signal transduction cascade including *v-raf*, *v-src*, and *v-raf*, as compared to wild type *fos* +/+ cells.

The previously discussed co-operation of *ras* and *fos* oncogenes as an essential factor in the development of epidermal neoplasia was further examined using the *fos* null mice. In these studies, *fos* -/- mice were treated with phorbol esters that resulted in the formation of epidermal tumours. The *fos* -/- cells isolated from these mice were transfected with a virus encoding the *ras* oncogene and grafted onto the skin of recipient mice and no tumours formed (Saez *et al.*, 1995). The result indicated that the *fos* null mutation impairs the initiating signals from the viral *ras* oncogene and that *c-fos* was required for tumourigenicity *in vivo*.

1.4 SIGNALLING PATHWAYS THAT FUNCTION THROUGH AP-1

The induction of *c-fos* and *c-jun* transcription is one of the earliest nuclear responses to a wide variety of extracellular stimuli and is thought to be crucial in mediating cellular proliferation (Curran and Franza, 1988; Herschman, 1991).

A simple diagrammatic representation of the growth factor and oncogene signal transduction pathways that function upstream of AP-1 with regards to EGF and *ras* are detailed in Figure 1.1. The Integrin signalling pathway through the extracellular matrix (ECM), is also highlighted. The signal transduction pathway described shows that the transactivation and down-regulation of AP-1 target genes can function through growth factor receptors, i.e., EGF and oncogenic *ras*.

1.4.1 *ras* signal transduction pathway

The expression of *c-fos* in mammalian cells and ensuing AP-1 activity is regulated by a signal transduction pathway that involves the small GTP-binding protein $p21^{ras}$ and downstream kinases (Karin, 1995). The protein kinase Raf-1 is a direct target of the *ras* proto-oncogene (Leevers *et al.*, 1994), and activated Raf-1 regulates the *c-fos* promoter in a signal-dependent manner (Jamal and Ziff, 1990). The activation of Raf-1 by $p21^{ras}$ results in the phosphorylation of MEK-1, a dual specificity MAP kinase kinase that phosphorylates Mitogen Activated Protein Kinases (MAP Kinases), on Tyrosine and Threonine residues. MAP kinases were originally identified as insulin regulated kinases that were activated by phosphorylation on Tyrosine and Threonine residues (Ray and Sturgill, 1987). The MAP kinases can phosphorylate a host of substrates, and upon mitogenic stimulation the MAP kinases translocate to the nucleus (Chen *et al.*, 1992), where they phosphorylate their target proteins such as the transcriptional activators. Elk-1 is a transcriptional activator that is a member of the Ternary Complex Factors (TCF), complexes with SRF (Hipskind *et al.*, 1991). The phosphorylation of TCF/Elk-1 by MAP kinases in the *c-fos* promoter stimulates transcriptional activity, regulating *c-fos* induction (Karin, 1995). The JNK/SAPK pathway is a stress response system activated by extracellular signals such as growth factors, cytokines and UV irradiation (Fanger *et al.*, 1998). The pathway is activated through the MAPK cascade resulting in the phosphorylation of JNK/SAPK. The JNKs phosphorylate the transcriptional activation domains of the transcription factors ATF2, Elk-1 and c-Jun, increasing their transcriptional activity.

1.4.2 Epidermal growth factor signal transduction pathway

The mitogen stimulation of *c-fos* transcription is a paradigm for a gene regulated via the *ras* pathway. Mitogens such as EGF relay their signals from the cell surface to the nucleus by a biochemical pathway that includes cellular proto-oncogenes as key components. These series of events are triggered by the interaction of EGF with its cognate receptor, c-ErbB resulting in the activation of receptor and non-receptor tyrosine kinases (Pazin and Williams, 1992). The signal being relayed through *c-ras* via accessory proteins such as PtdIns 3-kinase (PI3 kinase), through the above described Raf-1 and MAP kinase cytoplasmic signalling cascade (Bollag and M^{c}Cormick, 1991).

1.4.3 *c-jun* Tam-67 dominant-negative deletion mutant

The *c-jun* proto-oncogene can transform rat-1 and primary embryo cells when it is transcriptionally driven under Mo-MuSV LTR and co-expressed with *ras* oncogene (Schutte *et al.*, 1989a; 1989b).

Deletion analysis of *c-jun* revealed that the N-terminus contains a transactivation domain (Angel *et al.*, 1989). Alani and co-workers (1991), performed investigative transactivation and transformation assays of *c-jun* deletion mutants. These experiments demonstrated a direct correlation between *c-jun* induced transformation and the N-terminal transactivation domain. Similar N-terminal deletion mutant studies conducted with the *v-jun* oncogene reversed transformation induced by *ras*, *src*, Polyoma-mT and *v-fos* oncogenes *in vitro* (Lloyd *et al.*, 1991). The deletion mutant efficiently suppressed transcriptional activation by oncogenes and phorbol esters and furthermore reduced Cathepsin L mRNA abundance, an AP-1 target gene.

The *c-jun* deletion mutant Tam-67 lacks the amino acids 1 to 123 that encodes for the N-terminal transactivation domain of the *c-jun* proto-oncogene. Tam-67 has been shown previously to suppress transformation by SV40, *ras*, and phorbol esters (Brown *et al.*, 1993; Brown *et al.*, 1994). This signified a role for the truncated dominant negative deletion mutant of *c-jun*, Tam-67 in the regulation of oncogenic transformation by the AP-1 transcription factor.

The mechanism of inhibition by *c-jun* deletion mutant, Tam-67 was elucidated using chimeric proteins whereby the leucine zipper of Tam-67 was replaced with the leucine zippers of GCN4 and *c-fos*. The DNA binding, inhibition of *c-jun* induced transactivation and TPA/ras-induced transformation studies of the chimeric protein revealed that only the Tam/Fos protein inhibited AP-1 induced transactivation and

TPA/ras-induced transformation. The Tam/Fos protein is an equally potent inhibitor of transactivation and transformation, with respect to Tam-67 protein. The results of these studies suggests that Tam-67 inhibits AP-1 mediated processes by interacting with endogenous Jun or Fos family members, by which it interferes with AP-1 function, via a quenching mechanism. In contrast, the Tam/GCN4 proteins fail to inhibit the transcriptional activation of an AP-1 reporter gene *in vivo* which indicates that blocking of all the available AP-1 sites would require a very high concentration of Tam/Tam homodimers (Brown *et al.*, 1994). Therefore, the inhibition of AP-1 function by Tam-67 is more easily achieved by quenching the transcription factor activity, rather than blocking the enhancer or promoter elements of the AP-1 responsive genes.

A previously described model for a blocking and quenching mechanism of Tam-67, (Brown *et al.*, 1993), is shown by Figure 1.4. In the blocking mechanism, shown in the top half of Figure 1.4, the Tam-67 homo-dimerises and binds DNA at AP-1 sites, thus blocking the binding of AP-1 proteins, the Jun and Fos family members, to the DNA. The Tam-67 homodimers have no transactivation domain so AP-1 transcription would blocked. In the quenching mechanism, shown in the lower half of Figure 1.4, the Tam-67 dimerises with a wild-type Fos family member to produce an inactive or less active dimer that can bind DNA, but cannot, or can only weakly activate transcription. The effects of the wild-type AP-1 components, the Fos family members, would be quenched, as they would be inactivated through the formation of dimers with Tam-67. In addition, the quenching mechanism also blocks the binding of unbound wild type Fos/Jun pairings to AP-1 sites that are already bound by Tam-67/Fos heterodimer.

Figure 1.4

Tam-67 inhibits activation of AP-1 responsive genes

Potential mechanisms to explain inhibition of AP-1 via Jun and/or Fos function by the dominant-negative *c-jun* mutant, Tam-67. The RNA polymerase and general transcription factors are shown interacting with a target gene's promoter. The Jun and Fos proteins represent the various members of the families of leucine zipper proteins, are shown forming, Tam/Fos and Tam/Tam dimers that are binding DNA at an AP-1 site.

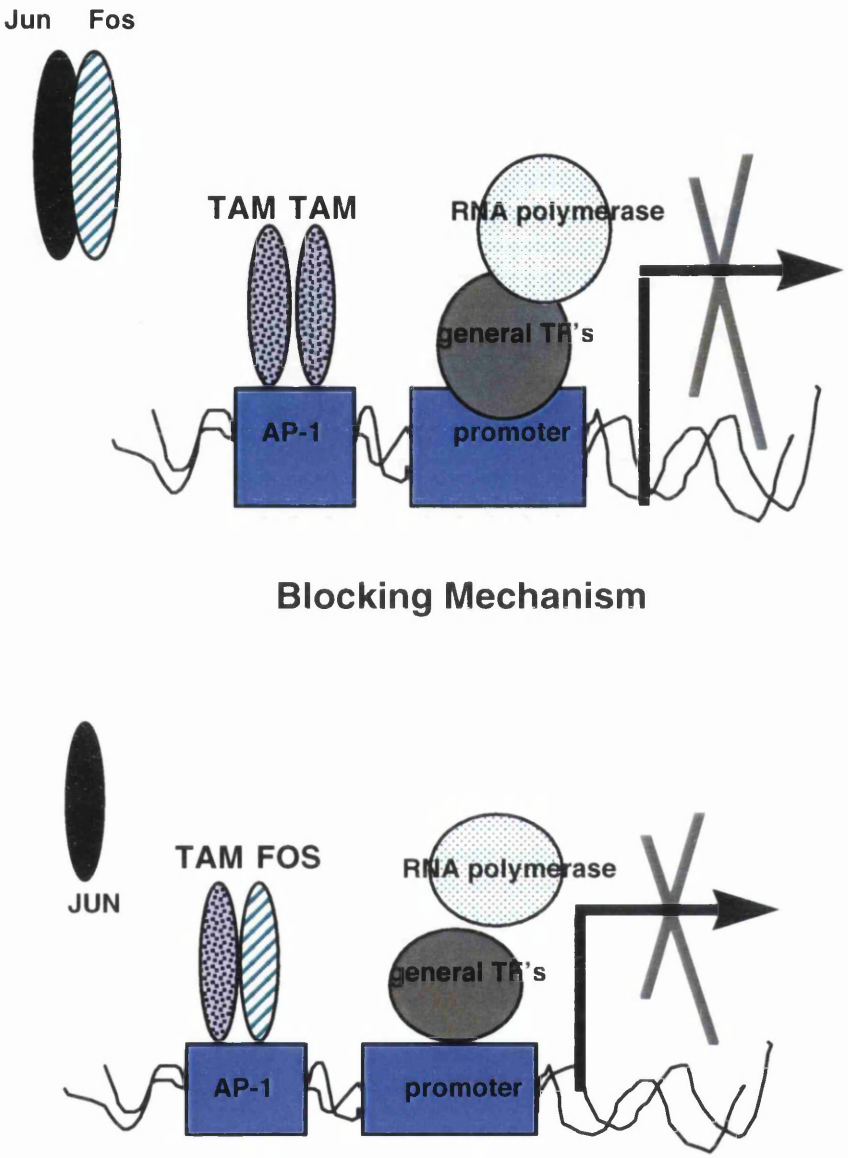


Figure 1.4
Tam67 Inhibits Activation of AP-1 Responsive Genes

1.4 AP-1 DOWNSTREAM TARGET GENES

AP-1 is an essential component in transformation by oncogenes which function up-stream of the growth factor-*ras-raf*-signal transduction pathway. Oncogenic transformation of fibroblasts is largely dependent upon AP-1 activity. Through AP-1, *v-fos* can adapt a variety of cell functions. AP-1 binds to DNA consensus sequences of downstream effector genes thereby controlling transcriptional regulation of effector genes. It was postulated that through this method of regulatory control AP-1 can induce or repress the expression of effector genes responsible for the transformed phenotype directly or indirectly through secondary signal transduction targets. In addition, *v-fos* exerts control upon signal transduction pathways that permits communication between the plasma membrane and the nucleus (Bortner, 1993).

The malignant transformation of cells requires altered patterns of gene expression and the regulation of transcriptional control can be altered by the transcription factor-type oncogenes such as *v-fos* (Bortner 1993). The identities of the genes regulated by the *v-fos* oncogene can provide an insight into the changes at the cellular level that accompany the altered gene expression during the multi-step process of carcinogenesis. It can also identify potential targets for therapeutic intervention (Aoyama and Klemenz, 1993). The extracellular proteases and the components of the ECM are de-regulated by the oncogene *v-fos* and their expression can have important consequences on invasive and metastatic behavior.

Our laboratory (Hennigan *et al.*, 1994; Lamb *et al.*, 1997a; 1997b), and others (Braselmann *et al.*, 1992; Jooss and Muller, 1995; Rupp *et al.*, 1998), have identified several direct and indirect AP-1 target genes which encode regulatory proteins, cytoskeletal and structural proteins several of which contain functional AP-1 binding sites that have been linked with the process of metastasis. This includes Ezrin, CD44, profilin, talin, collagenase, transin, c-jun, fra-1, interleukin-2, cathepsin L, laminin binding protein and mac2, all of which are involved in morphological transformation. A negative regulatory effect is exerted by *v-fos* upon the *c-fos*, and *erg-1* promoters, heat shock 70 promoter, the glucocorticoid receptor and the TGF-β inhibitory element in the transin gene promoter (Jooss and Muller, 1995).

1.5 CELL LINES

The cell lines used herein were all derived from the parental untransformed 208F fibroblast cell line, itself a derivative of fibroblast Rat-1 cells. Figure 1.5 details the morphology of confluent and sub-confluent cell lines used in this study viewed with phase contrast optics at 100x magnification.

The 208F cells (Figure 1.5, [a]), are flat in appearance, undergo density dependent growth arrest and do not form foci at confluence. The FBR cell line (Figure 1.5 [b]), are FBR *v-fos* transformed 208F fibroblasts and are highly refractile, bipolar cells that readily form foci and their growth is not density arrested. 208F cells mitogenically stimulated with EGF growth factor at 24 and 48 hours (not shown), results in transformation of 80% of the culture. These cells are of a bipolar morphology, they form foci and their growth is not density arrested. The RAS cell line (Figure 1.5 [c]), are K-*ras* transformed 208F cells that are of an irregular transformed morphology. They form foci and their growth is not density arrested. The FBR TAM67 cell line (Figure 1.5 [d]), are FBR cells expressing the dominant negative deletion mutant of *c-jun*, Tam-67. These cells are flat revertants.

The transformation characteristics of the described cell lines are summarised in Table 1.4.

The FBR cell line consitutively express *v-fos*, therefore AP-1 activity is also constitutive. The EGF treatment of 208F cell line increases AP-1 activity through the previously described growth factor signal transduction cascade. The RAS cell line increases AP-1 activity through the previously described *ras* oncogene signal transduction pathway. The FBR TAM67 cell line inhibits AP-1 activity.

Figure 1.5

Morphological characteristics of cell lines

This figure details the morphology of confluent and sub-confluent cell lines used in this study viewed with phase contrast optics at 100x magnification. (a), depicts 208F fibroblasts, (b), depicts FBR fibroblasts, (c), depicts FBR TAM67 fibroblasts and (d), depicts RAS fibroblasts at sub-confluent and confluent densities.

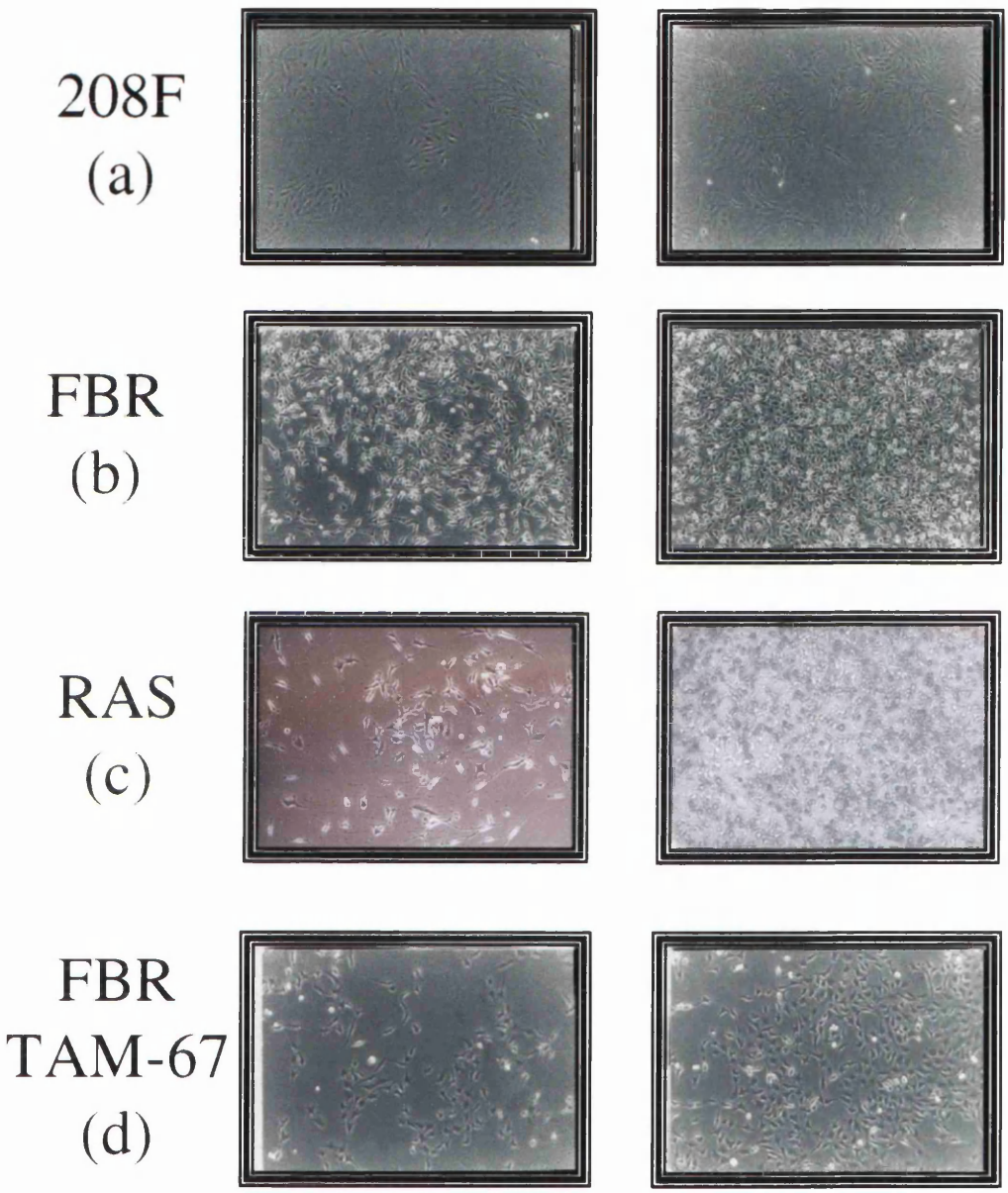


Figure 1.5
Low and High densities of cell lines

Transformation Characteristic	208F	FBR	208F & EGF	RAS	FBR TAM67
Agar Growth	-	+++	++	+++	-
Transformed Morphology	-	+++	++	+++	-
Focus Formation	-	+++	+	+++	-
Tumourigenicity	-	+++	ND	+++	ND
In vitro Invasion	-	+++	+++	++	-

Table 1.4

Transformation characteristics of cell lines

(Muller *et al.*, 1984; Hawker *et al.*, 1993; Hennigan *et al.*, 1994; Lamb *et al.*, 1997b).

1.6 AIMS

The detailed research over the previous twenty years is now progressing from the signal transduction pathways that relay the signal to the nuclear transcriptional regulators, to the identity and function of their target genes. The uncovering of known and novel effector genes, that are probably critical to front-line tumourigenesis, represents a new frontier of tumour molecular and cellular biology.

The overall aim of this project was to determine the function of AP-1 in transformation and invasion by the identification of genes differentially expressed as a consequence of FBR *v-fos* transformation. This would be achieved using the FBR *v-fos* transformed rat fibroblasts as compared to the 208F untransformed parental rat fibroblasts. The aims would be accomplished by:

1: The isolation and identification of up-regulated novel and known AP-1 direct and indirect target genes. This would provide an insight to the biological function of each identified gene with regards to transformation and invasion.

2. The isolation and identification of down-regulated novel AP-1 direct and indirect target genes would indicate the role of AP-1 in down-regulation, potentially through a transrepression mechanism. Furthermore, the biological function of these genes is that they may act as suppressors of transformation and growth.

3: The project aims to characterise the expression of a sub-set of the up-regulated and down-regulated AP-1 targets in transformed phenotypes through which AP-1 is active.

4: The project would focus on up- and down-regulated AP-1 target genes to ascertain their function in transformation and invasion.

Chapter 2

Materials and Methods

2.1 MATERIALS

2.1.1 Chemicals and reagents

All chemicals not individually listed were obtained (AnalaR grade), from BDH Chemicals Ltd., Poole, Dorset, UK. Solutions and buffers were prepared using de-ionized water obtained from a Millipore MilliQ $_{plus}$ system.

CHEMICAL	SOURCE
[α-^{32}P]dCTP~3000Ci/mmol	Amersham International plc., Amersham, Bucks., UK
Agarose, ultrapure, electrophoresis grade	
Bicinchoninic acid solution	Sigma Chemical Co. Ltd.,
Bovine serum albumin (BSA)	Poole, Dorset, UK
Bromophenol blue	
Butan-1-ol	Fisher Scientific, UK Ltd., Loughborough, UK
CsCl	Boehringer Mannheim UK Lewis, East Sussex, UK
CHAPS	
Chloroform	Sigma Chemical Co Ltd
$CuSO_4$	
Deoxyribonucleotides	Promega Corporation Southampton, UK
DEPC	Sigma Chemical Co. Ltd.
Dimethyl formamide	Fluka Chemika-Biochemika AG, Buchs, Switzerland
Dithiothreitol	
EGTA	
Ethanol	James Burrough Ltd., Witham, Essex, UK
Ethidium bromide	Sigma Chemical Co. Ltd
Ficoll	Fisher Scientific UK. Ltd., Loughborough, UK
Formaldehyde(39%)	

Hepes	
Lysozyme	
MES	
MOPS	Biogenesis Ltd.,
RNAzol B	Poole, Dorset, UK
NaV	Sigma Chemical Co. Ltd.
Paraformaldehyde	
Polyacrylamide	
PMSF	Severn Biotech Ltd.,
Propan-1-ol	Kidderminster, UK
PVP	
TEMED	Life Technologies, Ltd.,
Tris	Paisley, UK
	Sigma Chemical Co. Ltd.
Triton-X 100	
Tween 20	Vector Laboratories
Vectashield	Peterborough, UK
Xylene cyanol FF	Sigma Chemical Co. Ltd
Torula yeast RNA type IV	

2.1.2 Enzymes

All DNA modifying enzymes and their buffers, except those listed below, were obtained from Life Technologies Ltd., Paisley, UK.

ENZYME	SOURCE
AmpliTaq DNA Polymerse	PE Applied Biosystems
	Warrington, UK
Calf Alkaline phosphatase	Boehringer Mannheim UK,
	Lewes, East Sussex, UK
DNA'ase free RNA'ase A	Sigma Chemical Co. Ltd.
Klenow Polymerase	
M-MLV Reverse Transcriptase	

Pfu DNA Polymerase	Stratagene Ltd.
T4 DNA ligase	Northumbria Biologicals Ltd. Cramlington, Northumberland, UK

2.1.3 Kits

KIT	SOURCE
ABI PRISM DNA Sequencing Kit	PE Applied Biosystems, Warrington, UK
Advantage cDNA Polymerase Mix	CLONTECH laboratories UK Ltd. Basingstoke, UK
ECL western blotting detection kit	Amersham International plc., Amersham, Buckinghamshire, UK
QiaFilter Midi/Maxi Plasmid Kits	Qiagen Ltd , Crawley, West Sussex, UK
Qiagen Mini Plasmid Kit	Qiagen Ltd , Crawley, West Sussex, UK
Oligolabelling Kit	Pharmacia Biotech Ltd. St. Albans, Herts., UK
PCR-Script Cloning Kit	Stratagene Ltd.
PCR-SELECT cDNA Subtraction Kit	CLONTECH laboratories UK Ltd Basingstoke, UK
PolyATract mRNA isolation system II	Promega Corporation
TA-cloning kit	Invitrogen, NV Leek, Netherlands.
ZAP-cDNA Gigapack III Gold Cloning Kit	Stratagene Ltd.
ZAP-cDNA Synthesis Kit	

2.1.4 General Plasticware

ITEM	SOURCE
Filter pipette tips	Greiner Labortechnik Ltd., Gloucestershire, UK
Falcon tubes	Becton-Dickinson Labware, Plymouth, UK
5ml bijous	Bibby-Sterilin Ltd.,

20ml universals	Staffordshire, UK
microcentrifuge tubes	Elkay,
pipette tips	Galway, Eire

2.1.5 Miscellany

ITEM	SOURCE
S-200 Columns	Pharmacia Biotech Ltd
S-300 Columns	St Albans, Herts., UK
S-400 Columns	
SuperFect Transfection Reagent	Qiagen Ltd , Crawley,

2.1.6 Molecular weight markers

MARKER	SOURCE
Prestained SDS-PAGE standards	Bio-Rad Laboratories, Hercules, CA, USA
ϕX174 DNA/ Hae III fragments	Life Technologies Ltd., Paisley, UK
λ DNA/ Hind III fragments	
100bp DNA ladder	
0.24-9.5 Kb RNA ladder	

2.1.7 Membranes, paper, and X-ray film

ITEM	SOURCE
Hybond nylon membranes	Amersham International plc., Amersham, Buckinghamshire, UK
Immobilon-P	Millipore (UK) Ltd., Watford, Hertfordshire, UK
3MM filter paper	Whatmann International Ltd., Maidstone, Kent, UK
X-ray film (X-OMAT-AR)	Eastman Kodak Co., Rochester, New York, USA

2.1.8 Antibodies

ANTIBODY	SOURCE
anti-mouse IgG (whole molecule)-FITC-conjugate	Sigma Chemical Co. Ltd., Poole, Dorset, UK
anti-myc antibody	Genevieve Stapleton, BICR
horseradish peroxidase conjugated anti-mouse Ig secondary antibody	Amersham International plc., Amersham, Buckinghamshire, UK

2.1.9 Microbial host, media, and supplies

Sterile glassware and Luria-broth (LB), were prepared by BICR central services personnel.

ITEM	SOURCE
Petri dishes 20 x 20 cm Plates Bacto-agar Bacto-peptone Bacto-yeast extract	Bibby-Sterilin Ltd., Staffordshire, UK
DH5a competent E. Coli [supE44 ΔlacU169(φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1]	Life Technologies, Paisley, UK
Ex-Assist	Stratagene Ltd
INVaF' competent E. Coli [F' supE44 ΔlacU169(φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1]	Invitrogen, NV Leek, Netherlands.
SOLR strain [e14-(mcrA),Δ(mcrCB-hsdSMR-mrr)171, sbcC, recB, recJ, umuC@::Tn5(kan^r) uvrC, lac, gyrA96, relA1, thi-1, endA1, I^R[F' proAB lacI^q ZΔM15]Su- (nonsuppressing)	Stratagene Ltd
XL-1 Blue MRF' competent E.coli [Δ(mcrA)183Δ(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac[F' proAB lacI^q ZΔM15 Tn10 (Tet^R)]	Stratagene Ltd. Cambridge, UK

NZY broth	
Tryptone	
Ampicillin	Sigma Chemical Co. Ltd.,
Kanamycin	Poole, Dorset, UK
Tetracycline	

2.1.10 Plasmid vectors

VECTOR	SOURCE
pBluescript SK (+/-)	Stratagene Ltd.
pBSK(+) rFRP	Innogenetics
pCR2.1	Invitrogen, NV Leek, Netherlands
pCR-Script	Stratagene Ltd
pcDNA3.1(-)/Myc-HisA/B	Invitrogen, NV Leek, Netherlands.
pEGFP-N1	Clontech Laboratories UK

2.1.11 Cell culture media and supplies

Sterile glassware, PE, PBS, and water were prepared by BICR central services personnel.

ITEM	SOURCE
Freezing vials	A/S Nunc, Roskilde, Denmark
Round coverslips (19mm)	Merck Ltd., Lutterworth, Leics.,UK
Cell culture plastic dishes	Becton-Dickinson Labware, Plymouth, UK
FCS	Bioclear UK Ltd. Devizes, Wilts, UK
Dimethyl sulphoxide (DMSO)	Fisher Scientific UK. Ltd., Loughborough, Leicestershire, UK
L-glutamine	Life Technologies, Paisley, UK

DMEM	Sigma Chemical Co. Ltd.,
Trypsin	Poole, Dorset, UK
Sodium bicarbonate	
Sodium pyruvate	

2.1.12 Cell lines

208F cell line was obtained from Dr.C.Quade and is a subclone of the Rat-1 fibroblast cell line. FBR cell line was originally obtained from Dr. Tom Curran and are transformed, non-producer 208F's infected with FBR-MuSV. The Ras transformants used in this study were generated by Lynn McGarry at BICR, by transfection of 208F's with c-Ha-*ras* expressing plasmid, Pl1-2*ras*A, obtained from Dr. Dimitrios A Spandidos. The FBR Tam67 revertants used in this study were generated by Kostas D.Katsanakis at the BICR by transfection of FBR cell line with *c-jun* dominant negative mutant, TAM-67 expressing plasmid, pCMV-Tam67, obtained from Dr. Michael Birrer. Cos-7 cells were obtained from Joe Winnie, BICR.

2.2 METHODS

2.2.1 Mammalian cell culture

All cells were maintained in DMEM supplemented with 10% FCS, 0.3% sodium bicarbonate, 1mM sodium pyruvate and 2mM glutamine and were passaged every 3-4 days as a 1:5 or 1:10 split ratio using 0.25%trypsin in PBS, with the exception of FBR. The FBR cell line were aspirated off the culture plate.

All cell lines were stored in DMEM supplemented with 20% FCS, 0.3% sodium bicarbonate, 1mM sodium pyruvate and 2mM glutamine, 10% DMSO as frozen stocks in liquid N_2.

2.2.2 RNA isolation from cultured mammalian cells

Extraction of total RNA from mammalian cells was carried out by following the manufacturer's instructions for RNAzol B. For adherent cells, medium was aspirated from sub-confluent cultures in 10cm petri dish and 2.5ml of RNAzol B was added to lyse the cells which were further perturbed by brisk scraping. Lysed cellular material was transferred to a sterile 12ml polypropylene tube containing 500ml of chloroform. After vigorous shaking the RNAzol B/$CHCl_3$ mix was kept on ice for 5 minutes and then spun in a cold (4^oC), centrifuge at 5,640g for 15 minutes. Approximately 3ml of upper aqueous phase was carefully removed and added to a fresh tube containing 3ml of cold isopropanol, and this was kept on ice for 15 minutes before subsequent centrifugation at 10,000g for 15 minutes in a cold microfuge. The RNA pellet was washed with 5ml of 70% ethanol and traces of ethanol were removed by aspiration. The RNA pellet was then partially air dried before being resuspended in 50μl of DEPC-treated sterile H_20. This total RNA preparation was quantified by UV-spectrophotometry at 260nm and 10μg was run on a Northern gel. The integrity of the 28S and 18S ribosomal RNA species were examined by visual inspection. Intact total RNA was considered suitable for use in the isolation of $polyA^+$ mRNA.

2.2.3 $PolyA^+$ mRNA isolation

$PolyA^+$ mRNA was isolated using the components provided by the Promega PolyATract mRNA isolation kit (small-scale). A sterile 1.5ml polypropylene tube containing up to 1mg of total RNA, in 500ml of Rnase-free H_2O, was heated for 10 minutes at 65^oC. Biotinylated poly d(T), oligonucleotide was quickly added to the RNA

that was mixed gently and allowed to cool to room temperature, during which time the biotinylated polydT annealed to the polyA tail at the 3'-end of mRNAs. Streptavidin magnetic particles were washed thoroughly in low strength SSC buffer and the RNA/biotinylated polydT was added to the particles. The biotinylated poly d(T), with bound mRNA, bound to the streptavidin-coated magnetic particles. Careful washing of the magnetic particles ensured that non-mRNAs were removed from the preparation and mRNAs were finally eluted in 250μl of Rnase-free H_2O. One tenth volume of 3M NaOAc (pH 5.2), and three volumes of 100% ethanol were added to the mRNA which was stored at -70^{O}C for one hour. It was then spun for 20 minutes at 12,000g in a microfuge (4^{O}C), and washed in 70% ethanol. Traces of ethanol was removed by aspiration and the mRNA was partially dried by lyophilisation in a speedivac. The mRNA was solubilised in 10-12μl of Rnase-free H_2O and quantified by UV-spectophotometry at 260nm.

2.2.4 Northern analysis of RNA

Total RNA samples (10μg), in Rnase-free H_2O were mixed to a volume of 15μl with RNA loading buffer (50% formamide, 33.3% formaldehyde, 5% 5X MOPS {0.1M MOPS, 50mM sodium acetate, 5mM EDTA, [pH 8.0]}), and heated for 10 minutes at 65^{O}C. The sample was snap cooled and 0.5μl RNA loading dye (50% Ficoll, 1mM EDTA, 0.4% bromophenol blue, 0.4% xylene cyanol), 0.5μl 10mgml^{-1} ethidium bromide was added. The samples were loaded onto a 1% agarose gel containing 10% 5X Mops and 18% formaldehyde. Electrophoresis was performed 3-4 hours at 100V at 5V cm^{-1} in IX Mops buffer. The RNA was visualised by UV transillumination and photographed before the gel was soaked in de-ionised H_20 for 20 minutes to leach out the formaldehyde.

RNA was transferred to Hybond N^{-} nylon membrane by upward capillary blotting, as described (Maniatis *et al.*,), using 20X SSC (3M NaCl, 0.3M sodium citrate, pH7), solution as transfer buffer. The membrane was UV-crosslinked using a UV Stratalinker 1800 (Stratagene).

Radioactive probe was stripped from the membrane by gentle agitation for 10 minutes in 0.5% SDS solution that had been heated to 100^{O}C. The solution was allowed to cool before the membrane was removed. The membrane was stored wrapped at 4^{O}C.

2.2.5 Suppression Subtractive Hybridisation

The 208F and FBR subtracted libraries were prepared using the components provided by the PCR-Select cDNA Subtraction kit as per manufacturers instructions. The method is outlined below. For each Tester and Driver and Control sample 2 μg poly A^+ mRNA was used as a template for synthesis of first strand cDNA with MMLV reverse transcriptase. After second-strand synthesis *Rsa* I was used to create blunt-ended cDNA in a size range of 0.1-2Kb. The control skeletal muscle tester was prepared by adding 750pg ϕX174/*Hae* III DNA to 1μl cDNA.

The FBR/208F subtraction was performed in both forward and reverse. Therefore, each mRNA species was used as Testers and Drivers. Each Tester cDNA species was prepared by ligating 2μl Adapter 1(10μm), and 2μl Adapter 2(10μm), independently to a 2μl aliquot of a 1:5 dilution cDNA, and were labelled mRNA species Tester 1 and Tester 2 respectively. The ligations were performed overnight at 16°C, after which ligase was heat inactivated in an EDTA/glycogen mix. The mRNA species that were not ligated to adapters were used as Driver cDNA.

The first hybridisation was performed by adding an excess of Driver cDNA to each Tester cDNA that was heat denatured then annealed for 8 hours at 68°C, after which both hybridised Tester samples were mixed and more heat-denatured Driver was added and incubated overnight at 68°C.

The differentially expressed cDNA's from the forward and reverse FBR/208F subtraction and control skeletal muscle/control skeletal muscle ϕX174/*Hae* III subtraction were filled in with a pre-incubation step at 75°C then PCR amplified using a Perkin-Elmer DNA Thermal Cycler 480. The first PCR was performed using PCR primers to the outer Adapter sequences, Primers 1 and 2, for 30 cycles (each cycle comprised 30 seconds at 94°C to denature, 30 seconds at 60°C to anneal and 1 minute 30 seconds at 72°C to extend). The second PCR was performed using PCR primers to the inner Adapter sequences, Nested Primers 1 and 2, using a 1:10 dilution of first PCR reaction as a template for 16 cycles (each cycle comprised 30 seconds at 94°C to denature, 30 seconds at 68°C to anneal and 1 minute 30 seconds at 72°C to extend).

2.2.6 Cloning of PCR products

Components of the TA cloning kit were used to clone secondary PCR reaction products of the subtracted cDNA libraries. The ligation mixes were prepared according to the kit instructions. The TA cloning transformation procedure would usually result in white kanamycin resistant recombinant colonies developing against a background of blue non-recombinant colonies on LB bactoagar plates containing 40μg ml^{-1} kanamycin and X-Gal. Recombinant colonies were picked by sterile toothpick and propagated, with shaking overnight at 37°C, in 3ml of LB medium containing 40μg ml^{-1} kanamycin. The propagated 3ml cultures were then used in further manipulations.

2.2.7 Wiggle PCR of cloned subtracted cDNA library products

One μl of an 3ml overnight bacterial culture was used as a template for PCR as per Perkin-Elmer PCR kit instructions using 1ng ul^{-1} final Primer concentration. The PCR primers used were M13 R and T7 or Nested Primers 1 and 2. The cycling parameters were 3 minutes at 94°C; each cycle of 35 comprised 30 seconds at 95°C to denature, one minute at 55°C to anneal and 1 minute 30 seconds at 72°C to extend; followed by 10 minutes at 72°C.

2.2.8 Southern analysis of DNA

Following electrophoresis of DNA samples, the agarose gel was photographed and DNA was depurinated by soaking the gel in 0.25M HCl for 15 minutes with gentle shaking. The gel was rinsed in de-ionised H_2O and DNA was transferred for 18 hours onto Hybond N^+ nylon membrane by upward capillary blotting using 0.4M NaOH as the transfer buffer. After transfer, the membrane was rinsed in 2X SSC and air-dried before hybridisation. Membranes in sandwich boxes were prehybridised for 4 hours at 65°C with shaking, in buffer containing 5X SSPE (0.9M NaCl, 0.05M sodium phosphate and 5mM EDTA, pH7.4); 5X Denhardt's solution (0.1% BSA, 0.1% PVP and 0.1% Ficoll); 0.5% SDS and 1$mgml^{-1}$ torula yeast RNA. The hybridisation solution was then replaced with fresh solution containing the radio-labelled probe (prepared as in section 2.2,9), at a concentration of 1-3$X10^6$ counts ml^{-1}, and hybridised at 65°C overnight. The following day, the hybridisation buffer removed and replaced with 1X SSC, 0.1% SDS wash buffer for fifteen-minutes at 65°C, followed by 0.1X SSC, 0.1% SDS wash buffer for fifteen-minutes at 65°C. The filters were then wrapped in Saran wrap, and exposed for a period of time, which was influenced by the strength of signal

detected by a hand-held mini-monitor, to Kodak X-OMAT AR film, in cassettes with intensifying screens, at -70 °C.

The radioactive probe was stripped from the membrane by gentle agitation for 10 minutes in 0.5% SDS solution which had been heated to 100^{o}C. The solution was allowed to cool before the membrane was removed. The membrane was stored wrapped at 4^{o}C.

2.2.9 Random-prime synthesis of radioactive probes

The DNA probes, prepared by random-prime, and used in this study were either restriction digest fragments or PCR products of subtracted library fragments that were approximately 200-800bp in length. The reagents required to label these probes by a random-prime technique were provided in the Pharmacia Biotech Oligolabelling kit. Fifty ng of DNA in a 34μl volume were denatured by boiling for 5 minutes and cooled immediately on wet-ice. Ten μl of 5X Reagent mix (a buffered solution containing dATP, dGTP, dTTP and random hexadeoxyribonucleotides), 5μl of [α-^{32}P]dCTP (1.85 MBq), and 1μl of Klenow fragment were added to the denatured DNA and incubated at 37^{o}C for 60 minutes. Free nucleotides were removed using a Sephacryl S-200 HR microspin column. The column was packed by centrifugation at 3K in a tabletop eppendorf 5415c microfuge for one minute. The random-prime reaction was carefully added to the column and a collection tube was fitted below the column. The random-prime labelled probe was collected by centrifugation at 3K for 2 minutes and free nucleotides were retained within the column which was carefully removed (to a solid radioactive waste bin). One μl of the labelled probe was added to 10mls of EcoscintA scintillant and counts per minute were measured in a Beckman LS5000CE scintillation counter. Total counts contained in the probe were then estimated and it was used immediately or stored at -20^{o}C. Random-prime probes were denatured before addition to the hybridisation mix

2.2.10 Synthetic oligonucleotide synthesis and purification

Synthetic oligonucleotides were synthesised by BICR technical services personnel on an Applied Biosystems RNA/DNA synthesiser model 392 or 394, using phosphoramidite chemistry, according to the manufacturers instructions. Oligonucleotides were resuspended in 200μl sterile H_2O prior to quantification by UV-

spectrophotometry at 260nm. Primers were used at 10mM concentration for the PCR and sequencing reactions.

2.2.11 cDNA library synthesis and screening

An 208F cDNA library was constructed in the λ ZAPII vector using the components provided by the ZAP cDNA synthesis kit. Construction, plating and screening of this library was carried out as described in the Stratagene instruction manual and is outlined below.

Five ng of 208F polyA$^+$ mRNA were used as a template for synthesis of first-strand cDNA with StrataScript Rnase H$^-$ reverse transcriptase. After second-strand cDNA synthesis Klenow polymerase was used to create blunt-ended double-stranded cDNA to which *Eco* RI adaptors were ligated and then kinased. *Xho* I digestion then ensured that the double-stranded cDNA was ready, after size fractionation on a Sephacryl S-400 spin column, for ligation into the *Eco* R1/*Xho* I λ ZAPII vector arms in a unidirectional manner. Forty-five ng of cDNA from the first column fraction, that contained the largest cDNAs, 90 ng from the second fraction, 45 ng from the third fraction and 45 ng from the fourth fraction, where each subsequent fraction contained smaller cDNAs were ligated into 1μg of *Eco* RI/*Xho* I λ ZAPII vector.

One μl (1/5th volume), of each completed ligation was packaged in the λ packaging extract provided and 1μl (1/500th volume), of each packaged library was serially diluted in SM buffer {100mM NaCl; 15mM $MgSO_4$; 50mM Tris-HCl (pH 7.5), and 0.01% gelatin}, at 10^0,10^{-1}. Diluted library was mixed with 3ml molten top agar, X-Gal, IPTG and 200μl of XL1-Blue indicator bacteria and plated on 10cm NZY agar plates. In this way, the phage titre of each packaged extract and the ratio of recombinant (white plaques), to non-recombinant (blue plaques), phage could be determined. The primary library from the first column fraction (F1), contained $9.7X10^4$ $pfuml^{-1}$(plaque forming units per ml), the library from the second column fraction (F2), contained $3.1X10^5$ $pfuml^{-1}$, the library from the third column fraction (F3), contained $3.3X10^5$ $pfuml^{-1}$ and the library from the fourth column fraction (F4), contained $8.0X10^4$ $pfuml^{-1}$. These titres indicated that all four libraries, which contained a very low number of non-recombinants, had been packaged successfully.

The remaining 4/5ths volume of each ligation was also packaged as before, and $1X\ 10^6$ pfu from pooled primary library fractions 1-4 were amplified, harvested and titred again. The amplified 208F library contained $7X10^9$ pfu ml$^-$ and was now ready to screen for cDNAs of Clone KD1 and CD10. To screen, $0.25X10^6$ pfus from 208F were

plated on each of four 200mm square NZY agar plates along with 1.8mls of XL-1 plating bacteria and 30mls of molten top agar per plate. Agar plates were incubated overnight at 37^{O}C and chilled for two hours prior to screening which helped to prevent the top agar sticking to the nylon filters. Hybond N^{+} membranes were then layered onto plates containing phage plaques and left for 2 minutes, while a duplicate filter, was later laid on for 4 minutes. Three orientation marks were made with a needle through the membrane, into the agar, and were also marked on the plate. After transfer, the filter was carefully lifted to ensure no dragging of the membrane across the plate, and placed plaque side-up on 3MM filter paper pre-wet with denaturation solution (1.5 M NaCl, 0.5M NaOH), for 2 minutes, and then transferred to 3MM filter paper wetted with neutralisation solution (1.5 M NaCl, 0.5M Tris-HCl, pH 8), for 5 minutes. Finally, the filter was rinsed in 2 X SSC, briefly blotted with 3MM filter paper, air-dried and UV-crosslinked.

Duplicate filters were prehybridised at 65^{O}C in sandwich boxes, with shaking, in a solution consisting of 5X SSPE (0.9M NaCl, 0.05M sodium phosphate and 5mM EDTA, pH 7.4); 5X Denhardt's solution (0.1% BSA, 0.1% PVP and 0.1% Ficoll); 0.5% SDS and 1mg ml^{-1} torula yeast RNA for four hours. The buffer was refreshed and a random prime labelled radioactive probe (see section 2.2.9), at 2X 10^{6} counts ml^{-1} was added and the hyridisation continued overnight at 65^{O}C. The next day, as much of the hybridisation buffer as possible was removed and replaced with 1X SSC, 0.1% SDS wash buffer for fifteen-minutes at 65^{O}C, followed by 0.1X SSC, 0.1% SDS wash buffer for fifteen-minutes at 65^{O}C. The filters were then wrapped in Saran wrap, and exposed for 48-72 hours to Kodak X-OMAT AR film in cassettes with intensifying screens. When developed, duplicate films were orientated against the filters using the needle and plate marks, and positive clones determined by matching strong signals on the films. Duplicate films were then orientated against the marks on the library plates and "positive" plaques were carefully excised from the NZY agar using a scalpel blade. Phage particles were eluted from the agar overnight at 4^{O}C in 1ml of SM buffer so that the screening process could continue for a further three rounds until a "positive" plaque was purified to homogeneity prior to Bluescript phagemid excision.

2.2.12 Bluescript phagemid excision

Bluescript phagemids were excised from λ phage using the method of Vekris (1994). For this method cultures of the bacterial strains XL-1 Blue and SOLR at A_{600}=1 were prepared and equal volumes of both were combined with ExAssist helper phage at 10^{8} pfu ml^{-1} to give rise to a main mix. One hundred and ninety five μl of main mix

per well were dispensed into a 96-well, round bottom, polystyrene microplate. Five μl of homogeneous λ phage suspension was added to the main mix in the microplate which was then incubated for two hours with vigorous shaking at 37^{O}C. During this time λ and ExAssist both infect XL-1 Blue which suppresses amber mutations in gene I and gene II of the helper phage and consequently the Bluescript phagemid can be excised by gene II product and packaged in filamentous phage particles. The Bluescript phagemids infect SOLR and become double-stranded but ExAssist cannot replicate in SOLR, so there is no λ phage contamination. After the incubation, various volumes from the microplate wells were spread on LB agar plates containing ampicillin at 100μg ml^{-1} and kanamycin at 100mg μl^{-1} and incubated overnight at 37^{O}C. Under these selection conditions only SOLR (kan^{R}), containing the excised double-stranded Bluescript phagemid (amp^{R}), grew. These bacterial colonies were then grown in 3ml cultures of LB medium containing ampicillin or kanamycin from which mini-preparations of plasmid DNA were isolated.

2.2.13 Plasmid DNA preparation

Mini-preparations of plasmid DNA were isolated from 3ml bacterial cultures grown overnight in LB medium, containing an appropriate antibiotic, by QIAGEN plasmid mini-purification kit as per manufacturers instructions.

Cultures were pelleted by centrifugation for one minute in a microfuge at 12,000g, resuspended in 250μl of Buffer P1 (50mM Tris-Cl; 10mM EDTA, pH 8.0; and 100mg ml^{-1} RNase A). Bacteria were then lysed by addition of 250μl of Buffer P2 (0.2M NaOH; 1% SDS). Tubes were mixed by brisk inversion and put on ice immediately. Detergent and protein were precipitated by addition of 350μl of ice-cold Buffer P3 (3M KOAc, pH 5.5), with momentary vigorous shaking. The flocculate was removed by centrifugation at 12,000g for ten minutes. Eight hundred and fifty μl of supernatant containing plasmid DNA were decanted into a QIAprep spin column and centrifuged at 12,000g for 1 minute then the QIAprep column was washed by adding 750μl Buffer PE and centrifuged twice at 12,000g for 1 minute after which the supernatant was discarded. The DNA was eluted from the QIAprep spin column by adding 50μl Buffer EB (10mM Tris.Cl, pH8.5).

For midi-scale preparations of plasmid DNA from 100 ml bacterial cultures were grown overnight in LB medium, containing an appropriate antibiotic, by QIAfilter midi-plasmid purification kit as per manufacturers instructions. Briefly, cultures were pelleted by centrifugation for fifteen minutes at 6,000g, resuspended in 10ml of Buffer

P1 (50mM Tris-Cl; 10mM EDTA, pH 8.0; and 100mg ml^{-1} RNase A). Bacteria were then lysed by addition of 10ml of Buffer P2 (0.2M NaOH; 1% SDS). Tubes were mixed by brisk inversion and put on ice immediately, before detergent and protein were precipitated by addition of 10ml of ice-cold Buffer P3 (3M KOAc, pH 5.5), and mixed gently by inverting 4-6 times. The lysate/flocculate was poured into a QIAfilter cartridge and incubated for 10 minutes at room temperature, after which the lysate was pushed through the filter bed using the plunger into the QIAGEN-tip 100, pre-equilibriated with Buffer QBT (750mM NaCl; 50mM MOPS, pH7.0, 15% isopropanol; 0.15% Triton X-100). The QIAGEN-tip 100 was washed twice with 10 ml Buffer QC (1.0mM NaCl; 50mM mops, pH7.0; 15% isopropanol), then the plasmid DNA was eluted using 5 ml Buffer QF (1.25M NaCl, 50mM Tris, TrisCl, pH8.5, 15% isopropanol), then precipitated using 0.7 volumes isopropanol and centrifuged immediately for 30 minutes at 15,000g. The plasmid DNA was washed with 70% ethanol and resuspended in sterile H_2O.

2.2.14 Agarose gel electrophoresis

DNA restriction fragments from plasmids or PCR products were separated on agarose gels and visualised by staining with ethidium bromide and UV-transillumination. Typically, gels were prepared by dissolving between 0.8% and 1.5% electrophoresis grade agarose in 1 X TAE (40mM Tris acetate; 1mM EDTA, pH8.0). After being heated in a microwave to dissolve the agarose, molten gels were cooled to approximately $60^{O}C$ and ethidium bromide added to a final concentration of $500ngml^{-1}$ before being poured into an appropriate gel tank. Once solid, gels were placed into electrophoresis tanks containing 1 X TAE. Samples were mixed with one-fifth volume of gel-loading buffer (15% Ficoll; 0.25% bromophenol blue and 0.25% xylene cyanol FF), prior to being loaded into the wells of the gel. Electrophoresis was performed at $5V\ cm^{-1}$. In order to estimate the size of fragments resolved by electrophoresis, samples were run alongside aliquots of molecular weight marker, either a *Hind* III digest of bacteriophage λ DNA or an *Hae* III digest of bacteriophage ϕX174 DNA. DNA was visualised by UV transillumination and the gel photographed.

2.2.15 Restriction digestion of plasmid DNA

Approximately 250ng of plasmid DNA was digested in a final volume sufficient to dilute the volume of restriction enzyme added at least 10-fold. Plasmid digests were routinely performed using 10 units of enzyme in each reaction and were incubated at the recommended temperature for at least I hour. For digests of DNA requiring the

addition of two restriction enzymes, either a buffer compatible for both enzymes was selected or the DNA was digested by enzyme 1, the digest checked for completion by agarose gel electrophoresis, and then the buffer conditions in the reaction modified to suit enzyme 2.

2.2.16 Purification of DNA fragments from agarose gels

Plasmid DNA was digested as described above, separated on an agarose gel and the fragments visualised under UV-transillumination. Agar slices, containing fragments of interest, were excised from the gel using a scalpel blade. The agar slices were inserted into Promega Wizard prep columns and these were inserted into 1.5 ml tubes and subjected to 5 minutes centrifugation at 12,000g. Approximately 100μl of DNA solution, eluted by centrifugal force, was collected in the 1.5 ml tube and was ready for use in further manipulations.

2.2.17 Ligation of DNA fragments into plasmid DNA

The DNA fragment to be inserted was digested as above to produce blunt ends or complementary sticky ends to the vector, and then isolated by gel electrophoresis and purified as described in section 2.2.16. To prevent the restricted plasmid DNA from re-ligating without an insert, it was first dephosphorylated. Plasmid was digested with the required restriction enzyme in the presence of 5 units calf intestinal alkaline phosphatase. Enzyme was then removed by extraction with an equivalent volume of equilibrated phenol/chloroform and plasmid DNA was then ethanol precipitated. For ligation, 100ng of vector and three times the molar amount of insert DNA were mixed on ice in a reaction containing ligase buffer (66 mM Tris-HCl, pH 7.5; 5mM $MgCl_2$; 1mM rATP; I mM DTT), and T4 DNA ligase (5-10 units), and incubated overnight at $12^{O}C$.

2.2.18 Transformation of bacterial cells with recombinant plasmid DNA

Competent *E. coli* were thawed on ice, and 100μl aliquots transferred to pre-chilled 6ml polypropylene tubes. Approximately 100ng of recombinant plasmid DNA was added to the cells and gently mixed by stirring with a pipette tip. After incubation on ice for 30 minutes, cells were heat-shocked at $42^{O}C$ for 45 seconds and then placed on ice for a further 2 minutes. Four hundred and fifty μl of SOC medium (2%

bacto-tryptone; 0.5% bacto-yeast extract; 20mM glucose; 10mM NaCl and 10mM $MgCl_2$), was then added to the mixture and the cells were incubated at 37°C for 1 hour on an orbital shaker at 225 rpm. A selection of cell volumes were then spread on L-broth plates containing 2% bactoagar supplemented with the appropriate antibiotic. Plates were incubated in an inverted position overnight at 37°C.

2.2.19 Automated chain termination cycle sequencing

Both cloned DNA and PCR products were sequenced using a Biosystems ABI 373A automated DNA sequencer operated as a core service by staff at the BICR. Twenty to one hundred ng of plasmid or 50–100ng of a PCR reaction were mixed with 4mM of sequencing primer and 8μl of 'Dyedeoxy' reaction mix in a total reaction volume made up to 20μl with water. DNA was subjected to 'cycle sequencing' in a DNA thermal cycler for 25 cycles (each cycle comprises 15 seconds at 96°C to denature DNA, 1 second at 50°C for annealing, and 4 minutes at 60°C to extend). The products were ethanol precipitated, washed with 70% ethanol, and dried in a speedivac prior to being re-suspended in loading buffer (95% formamide; 25mM EDTA, pH 8; 1.5mgml^{-1} dextran blue). Samples were then denatured by heating at 94°C, chilled on ice, and subjected to electrophoresis as advised by the manufacturer.

2.2.20 Transient SuperFect transfection of COS-7 cells

Medium was aspirated from COS-7 cells growing at 60-75% confluence in a 10cm culture dish and the adherent monolayer was washed once with PBS. A transfection mix of 742μl serum-free DMEM, 50μl of SuperFect (3mg ml^{-1}), and 8μg of sterile, supercoiled plasmid DNA (1mg ml^{-1}), was vortexed and incubated at room temperature for 10 minutes, before the addition of 5.6ml DMEM supplemented with 10% FCS, and applied to the cells. The cells were then placed in a 37°C incubator for three hours, after which the transfection mix was removed by aspiration and the cells re-fed with DMEM supplemented with 10% FCS. Following a 72 hour incubation at 37°C the cells were harvested for expression analysis (see section 2.2.21).

2.2.21 Western analysis of Myc-tag/ DIF, Myc-tag/ FRP fusion proteins

Medium was aspirated from transiently transfected COS-7 cells and FRP stable transfectant FBR cells and washed twice in ice-cold PBS, and lysed in a solution comprising 10mM Hepes (pH 7.4); 1mM NaV; 2.5mM $MgCl_2$; 1mM EGTA; 10%

glycerol; 100mg ml^{-1} CHAPS and 1mM PMSF, using a disposable cell scraper to assist homogenisation. Lysates were incubated on ice, for 30 minutes and centrifuged at 14000rpm for 20 minutes at 4^0C in a bench-top microfuge to pellet cell debris. The resultant supernatants were designated as the soluble (S), fractions and stored in aliquots at -70 °C until needed.

Protein concentrations were determined by performing a bicinchoninic acid colourimetric assay. Ten μl aliquots of a 1:9 dilution of soluble and insoluble fractions were incubated with 200μl of reaction mix (1 volume of 4% $CuSO_4$ to 50 volumes bicinchonic acid solution), at 37^OC for 30-45 minutes. Absorbency was measured at 590nm wavelength light using a Dynatech MR7000 Spectrophotometer. The standard curve consists of 6 BSA standards (80, 100, 400, 800, 1000, 2000 μg/ml BSA/H_2O concentration).

For detection of Myc-tag/ DIF, Myc-tag/ FRP fusion proteins by immunoblotting, 80μg of proteins were resolved by electrophoresis through an 10% SDS-PAGE pre-cast gel. Samples were prepared for electrophoresis by mixing 80μg of protein with 20% volume of 5X sample buffer (60mM Tris.HCl, pH 6.8; 2% SDS; 0.1% bromophenol blue; 25% glycerol), boiling the samples for 10 minutes, chilling briefly on ice, and then loading into the wells of the stacking gel alongside an aliquot of Broad range or Low range Biorad SDS-PAGE prestained molecular weight standards (205–7.6Kd), (employed for protein molecular weight determination). Gels were run overnight at 40V. Proteins were then transferred from the gel to ImmobilonP membrane via semi-dry electroblotting for l-3 hours using twelve layers of Whatman 3MM paper which had been soaked in blotting buffer (60mM Tris.HCl; 50mM glycine; 1.6mM SDS; 20% methanol). Blots were first blocked for 1 hour at room temperature in PBS containing 5% non-fat milk powder; 0.1% Tween 20 and subsequently incubated in solution with a 1:10 dilution of anti-Myc mouse monoclonal antibody overnight at 4^0C. Following thorough washing with PBS containing 0.1% Tween 20 and incubation with a 1:5000 dilution of a horseradish-peroxidase conjugated anti-mouse Ig secondary antibody for one hour, proteins were visualised using enhanced chemiluminescence (ECL), according to the manufacturer's instructions.

2.2.22 Transient SuperFect transfection of 208F, FBR, RAS and FBR Tam67 cells

Medium was aspirated from cells growing at 60-75% confluence on sterile, 19mm glass coverslips in a 12 well culture plate and the adherent monolayer was washed once with PBS. A transfection mix of 75μl serum-free DMEM, 7.5μl of SuperFect (3mg ml^{-1}), and 1.5μg of sterile, supercoiled plasmid DNA (1mg ml^{-1}), was vortexed and incubated at room temperature for 10 minutes, before the addition of 400μl DMEM supplemented with 10% FCS, and applied to the cells. The cells were then placed in a 37°C incubator for three hours. After this the transfection mix was removed by aspiration and the cells were re-fed with DMEM+10% FCS. Following a 24 hour and 48 hour incubation at 37°C the cells were fixed for indirect-immunofluorescence expression analysis (see section 2.2.23).

2.2.23 Indirect-immunofluorescence of Myc-tag fusion proteins in transiently transfected cells

Medium was removed, by careful aspiration, from transiently transfected 208F, FBR, RAS and FBR Tam 67 cell lines, cultured on 19mm diameter coverslips in 12-well plates, at twenty four hours post-transfection. Cells were washed in PBS and 1ml of 3% paraformaldehyde (pH 7), was added. After a twenty minute incubation at 37°C the PBS wash was repeated and 1ml of PBS/glycine/ 0.01% Triton X-100 permeabilisation buffer was added to the cells over five minutes. Excess permeabilisation buffer was removed by aspiration and the cells were washed extensively in PBS. A neat concentration of mouse monoclonal anti-myc was added and maintained on the cells for one hour at room temperature, then removed by aspiration and three PBS washes. This was followed by the addition of FITC-conjugated anti-mouse IgG, 1:200 dilution and TRITC-conjugated phalloidin 1:1,000 dilution in blocking solution. A one hour incubation followed and excess FITC/TRITC was removed by washing and the coverslips were dried in air, mounted on glass slides with vectashield reagent and sealed with nail polish. The transfected cells were then analysed on a Biorad MRC-600 laser scanning confocal imaging system.

2.2.24 Stable SuperFect transfection of FBR cells with pCDNA3.1A/FRP and pCDNA3.1A

Medium was aspirated from cells growing at 60-75% confluence on 10cm culture dishes and the adherent monolayer was washed once with PBS. A transfection mix of 742μl serum-free DMEM, 50μl of SuperFect (3mg ml^{-1}), and 8μg of sterile, supercoiled plasmid DNA (1mg ml^{-1}), was vortexed and incubated at room temperature for 10 minutes, before the addition of 5.6mls DMEM supplemented with 10% FCS, and applied to the cells. The cells were then placed in a 37^{o}C incubator for 16 hours. After which the transfection mix was removed by aspiration and the cells re-seeded by splitting to one in three with DMEM+10% FCS supplemented with 400μg/ml G418 selection antibiotic. The media was changed every 48 hours. After incubation at 37^{o}C for 10-14 days, the untransfected cells died off and the transfected cells formed colonies. Twelve FRP expressing colonies, and six neo colonies were harvested by ring cloning and propagated. The cells were plated onto sterile glass coverslips and fixed for indirect-immunofluorescence expression analysis (see section 2.2.23), and the proteins from the positive colony, clone 6, were harvested for western blot analysis (see section 2.2.21).

2.2.25. Preparation of conditioned media

Cells were passaged in DMEM supplemented with 10% FCS and grown to subconfluence. The medium was removed and the plates were washed 3 times with PBS. The medium was changed to serum-free DMEM and the dishes were incubated in a tissue culture incubator at 37 ^{0}C for 72 hours. After this time the medium was harvested and the expression of secreted FRP was analysed by western blotting using 30μl neat conditioned media per well (see section 2.2.21).

2.2.26. Determination of cell proliferation

Determination of cell proliferation was performed according to Tavoloni *et al*, (1994), with minor modifications. Cells were trypsinized, counted and plated at 2x10^{4} cell density into duplicate 30mm falcon dishes in DMEM supplemented with 10% FCS. Cells were counted using a cell counting chamber (Neubauer), every day for six days.

2.2.27. Growth in semi-solid medium

Methylcellulose was used to prepare semi-solid growth medium, and was prepared by mixing 3g methylcellulose with 200ml H_2O. The mix was autoclaved and following sterilisation it was allowed to cool and then stirred at 4°C for 24 hours in the cold room and continuously stirred with a magnetic stirrer.

Cells were trypsinized, counted and resuspended in DMEM supplemented with 10% FCS. Aliquots of $2x10^4$ cells were mixed with 10-14 ml of semi-solid medium (200ml H_20 sterile, 3g methylcellulose, as prepared above, 22 ml 10X F10 Ham's Medium, 4ml MEM minimal essential amino acids, 4ml 0.1M Sodium Pyruvate, 5ml Sodium Bicarbonate, 20ml FCS, 5 ml 100X Glutamine), and plated into duplicate 10cm bacteriological grade dishes. -solid medium supplemented with FCS and the G418 selection antibiotic at 400μg/ml for stably transfected clones was replaced every 4 days.

Chapter 3
Isolation of Downstream AP-1 Target Genes by Suppressive Subtractive Hybridisation

3.1 INTRODUCTION

The AP-1 transcription factor not only up-regulates sets of direct and indirect target genes involved in the induction and maintenance of transformation but is also capable of down-regulation. Previous studies in our laboratory (Hennigan *et al.,* 1994; Lamb *et al.,* 1997), and others (Braselmann *et al.,* 1992; Jooss and Muller, 1995; Rupp *et al.,* 1998), have identified several AP-1 target genes that encode regulatory, cytoskeletal and structural proteins containing functional AP-1 binding sites that have been linked with the process of invasion. Invasion is a tightly regulated process used by many types of non-tumour cells when it is necessary for them to migrate across tissue boundaries under a variety of physiological conditions, such as trophoblast implantation, embryogenesis, angiogenesis, wound healing, and immune response. The process of invasion is complex and it requires the co-ordinated functioning of many dynamic cellular processes such as alterations in gene expression, cell surface receptor activation, cell-cell adhesion, cell-extracellular matrix (ECM), adhesion and ECM remodelling by extracellular proteases, actin cytoskeleton rearrangements mediated by Rho-like GTPases and cell motility. The complexity of invasion indicates that it is multigenic process. At least three transcription factors, AP-1, Ets, and NFκB have been implicated in the regulation of invasion. Both AP-1 and Ets are required for the expression of several genes known to be associated with invasion. This suggested that AP-1, in transformed cells, activates a multigenic program, which regulates invasion.

This chapter reports the construction of subtracted cDNA libraries specific for genes expressed following transformation by FBR *v-fos* oncogene. One library is specific for mRNAs up-regulated in FBR cell line, whereas the other is specific for mRNAs that are down-regulated. Both were prepared using the technique Suppression Subtractive Hybridisation (SSH), (Diatchenko *et al.*, 1996). The cDNA libraries were investigated by sequence analysis and Northern blotting (Refer to chapter 4).

This chapter focuses on sequence and computational analysis to identify the differentially expressed genes. The identification leads to function and reveals that a proportion are associated with transformation and invasion.

3.2 RESULTS

3.2.1 Suppressive Subtractive Hybridisation (SSH)

The approach that was taken to construct the subtracted cDNA libraries was SSH. SSH selectively amplifies target cDNA fragments, such as cell line or tissue specific differentially expressed cDNA fragments, whilst simultaneously suppressing non-target cDNA amplification, that is, common cDNA fragments in cell lines or tissues, such as housekeeping genes. An overview of SSH is shown in Figure 3.1.

The technique is primarily based upon Suppression PCR. This alleviates the need for physical separation of cDNAs, common to subtractive hybridisation methods. Instead, the hybridisation procedure selectively suppresses the common cDNA fragments during the amplification step in PCR. In addition, the subtraction method overcomes the problem of differences in mRNA abundance by incorporating a normalised (equalised), hybridisation step which results in high and low abundance transcripts being equally represented. In one subtractive hybridisation round, a 1,000 fold enrichment of differentially expressed cDNAs can be achieved.

3.2.1.2 Construction of Up-regulated and Down-regulated libraries

cDNA Synthesis

The libraries were constructed from reverse transcribed cDNA from mRNA isolated from logarithmically growing FBR, *v-fos* transformed and 208F untransformed parental fibroblast cell lines. The up-regulated subtraction consisted of FBR mRNA as "tester", and 208F mRNA as "driver". The down-regulated subtraction consisted of 208F mRNA as "tester", and FBR mRNA as "driver". Differentially expressed cDNAs, the target genes, are present in the "tester" cDNA but are significantly reduced or absent in the "driver" cDNA population.

Rsa I Digestion and Adaptor Ligation

The ds cDNAs of tester and driver are cleaved with a four-base cutting restriction enzyme, *Rsa* I to yield short, 0.1-2 kb, cDNA fragments, after which the tester cDNA fragments are divided and ligated with two different adapters, 1 and 2, resulting in two populations of tester, 1 and 2. The adapter ends do not have phosphate groups, only permitting the covalent attachment of the longer strand to the 5'-cDNA ends. Sequence of Adapters 1 and 2, and the complimentary PCR Primer and PCR Nested Primers are shown in Figure 3.3.

Figure 3.1

Overview of Suppressive Subtractive Hybridisation

This figure describes, in general, the steps of the SSH procedure. The cDNA in which specific transcripts are to be found is called the "tester cDNA" and the reference cDNA is called "driver cDNA'.

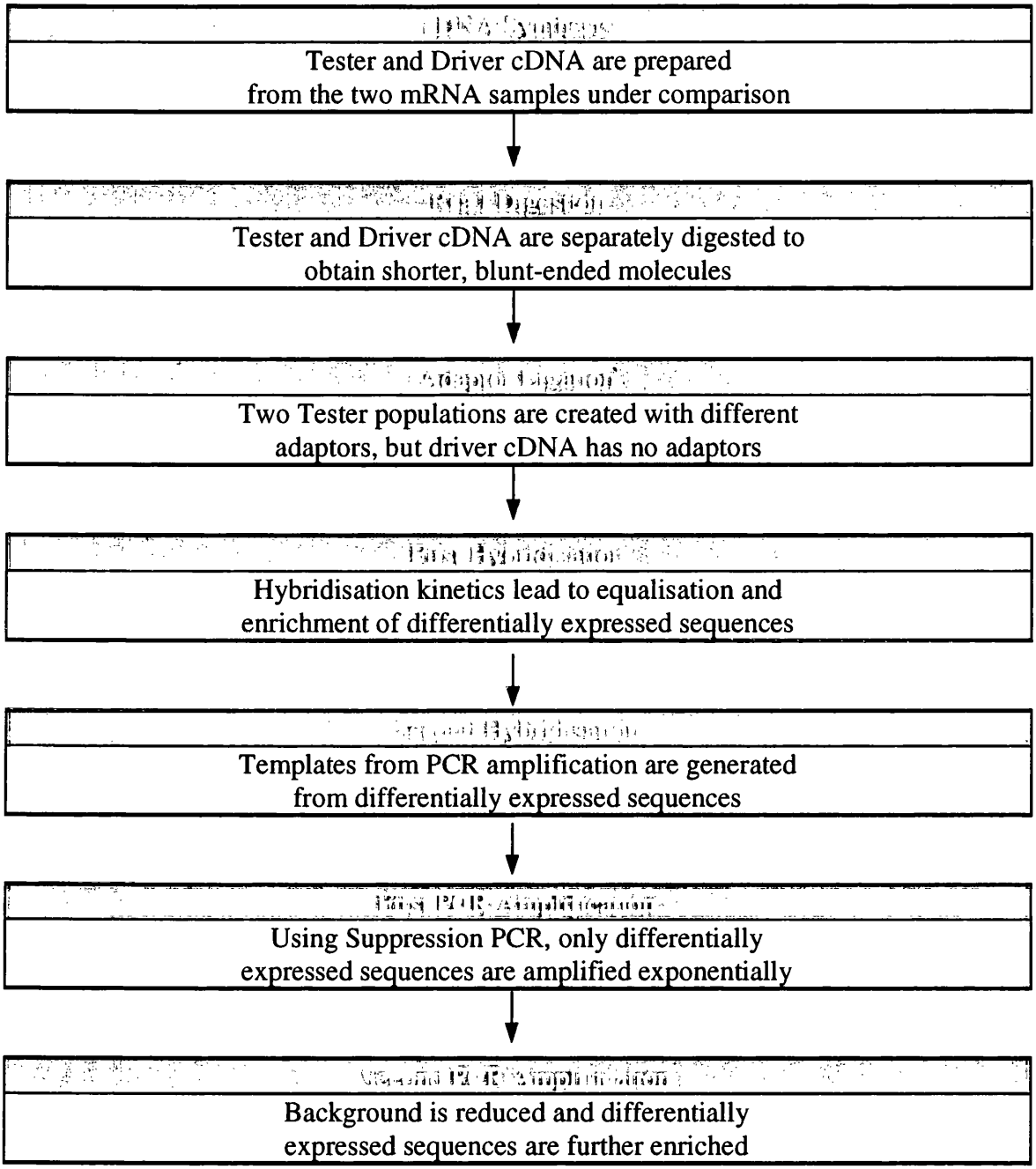

Tester and Driver cDNA are prepared
from the two mRNA samples under comparison
Tester and Driver cDNA are separately digested to
obtain shorter, blunt-ended molecules
Two Tester populations are created with different
adaptors, but driver cDNA has no adaptors
Hybridisation kinetics lead to equalisation and
enrichment of differentially expressed sequences
Templates from PCR amplification are generated
from differentially expressed sequences
Using Suppression PCR, only differentially
expressed sequences are amplified exponentially
Background is reduced and differentially
expressed sequences are further enriched

Figure 3.2

Schematic representation of SSH

Solid lines represent the *Rsa* I- digested tester or driver cDNA. Solid boxes of purple/white represent Adapter 1, with the purple representing PCR Primer 1 sequence and the white nested PCR Primer 1. Solid boxes of red/yellow represent Adapter 2, with the red representing PCR Primer 1 sequence and the yellow nested PCR Primer 1. The type *e* molecules are formed only if the sequence is absent from the driver cDNA as described in text. Refer to text for detailed description of procedure.

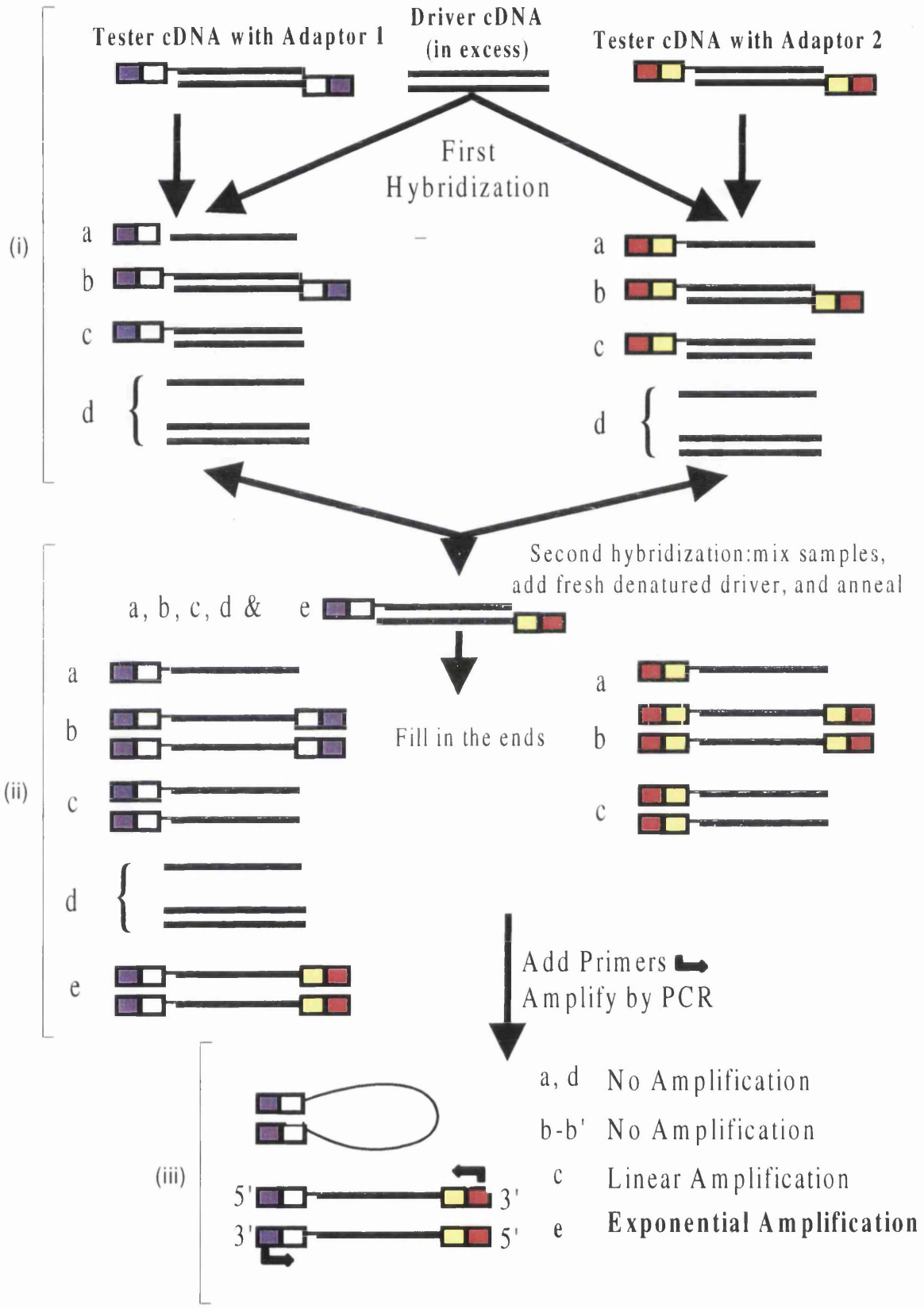


Figure 3.2 Schematic Representation of SSH

Adapter 1

5' CTA ATA CGA CTC ACT ATA GGG CTC GAG CGG CCG CCC GGG CAG GT 3'

3'GG CCC GTC CA 5'

PCR Primer 1 **PCR Nested Primer 1**

5' CTA ATA CGA CTC ACT ATA GGG C 3 5' CTC GAG CGG CCG CCC GGG CAG GT 3'

Adapter 2

5' TGT AGC GTG AAG ACG ACA GAA AGG GCG TGG TGC GGA GGG CGG T 3'

3' G CCT CCC GCC A 5'

PCR Primer 2 **PCR Nested Primer 2**

5' TGT AGC GTG AAG ACG ACA GAA 3' 5' AGG GCG TGG TGC GGA GGG CGG T 3'

Figure 3.3 Adapter, PCR primer and PCR nester primer sequence

First Hybridisation

The SSH technique uses two hybridisations. In the first (refer to Figure 3.2 (i)), an excess of driver is added to each tester sample , denatured and annealed. The ss cDNA tester fraction, (a), (refer to Figure 3.2), is normalised, whereby high and low abundance cDNA concentrations become equal. This occurs as the re-annealing process for homo-hybrid cDNAs, (b), is more efficient and rapid for the high abundance cDNAs, due to the second order kinetics of hybridisation. Also, the ss cDNAs tester fraction, (a), is further enriched in cDNAs for differentially expressed genes, as "common" non-target cDNAs form hetero-hybrids, (c), with the driver population.

Second Hybridisation

The two samples from the first hybridisation are mixed together with the addition of fresh denatured driver in the second hybridisation (refer to Figure 3.2 (ii)). The normalised and subtracted ss tester cDNAs re-associate to form (b), (c), and (e), type hybrids, (refer to Figure 3.2 (ii)). Further addition of the fresh driver during the second hybridisation enriches for type (e), hybrids, the differentially expressed genes, as "common" tester sequences anneal with "common" driver sequences. Newly formed hybrids, (e), have different adaptor sequences at their 5'-ends, one from tester sample 1, the other from tester sample 2. In contrast, hybrids (b), and (c), formed

during the first and second hybridisations have the same adaptor sequences at either end, (refer to Figure 3.2 (ii)). The different adaptor sequences of the subtracted and normalised type (e). hybrids allows preferential amplification using PCR and a pair of primers, P1 and P2, that anneal to the outer part of adaptors 1 and 2 respectively as shown by Figure 3.3. A short extension reaction prior to PCR cycling fills in the sticky ends of the molecules for primer annealing.

First and Second PCR Amplification

The PCR cycles of the hybridisation mixture results in amplification of type (e), hybrids. Type (a), and (d), hybrids do not contain primer binding sites and cannot be amplified. Type (b), hybrids contain long inverted repeats on the adapter ends which form stable pan-handle like structures after each denaturation-annealing PCR step that cannot be use as a template for exponential PCR. This is due to the intramolecular annealing of the long adapter sequences being preferential and more stable than the intramolecular annealing of the short PCR. In addition, type (c), hybrids can only be amplified at a linear rate (refer to Figure 3.2 (iii)). This is the suppression PCR effect.

The differentially expressed cDNAs are exponentially amplified during the PCR reactions as described above. In the first amplification, only the ds cDNAs with two different primer annealing sites, type (e), hybrids are exponentially amplified. In the second amplification, nested PCR further reduces background and enriches for differentially expressed sequences.

Control Subtraction

A control subtracted library was constructed along with the up- and down-regulated library subtractions. The up- and down-regulated and control subtractions are outlined in Figures 3.4.1, 3.4.2 and 3.4.3 respectively. For each subtraction, unsubtracted tester is ligated to adaptors 1 and 2 and PCR amplified as a negative control for subtraction. The control subtraction is performed using skeletal muscle cDNA spiked with the *Hae* III digest (ϕX174 markers), as tester fraction and skeletal muscle only as driver fraction. During the SSH procedure tester skeletal muscle is hybridised to "common" driver control skeletal muscle, allowing the Hae III digest cDNA to form type (e), hybrids that are exponentially amplified. The unsubtracted tester control is a negative control for subtraction.

Figures 3.4.1, 3.4.2, and 3.4.3

Forward subtraction (Up-regulated); Reverse subtraction (Down-regulated); and control subtraction

Each different experimental tester cDNA, the 208F, FBR and control skeletal muscle, were divided into three aliquots and ligated to the appropriate adaptors as shown. These figures also depict the unsubtracted tester control that is ligated to Adaptors 1 and 2 and subsequently PCR'd without the hybridisation steps. The sequential hybridisation steps of the Up-regulated and Down-regulated experiments prior to PCR steps is outlined.

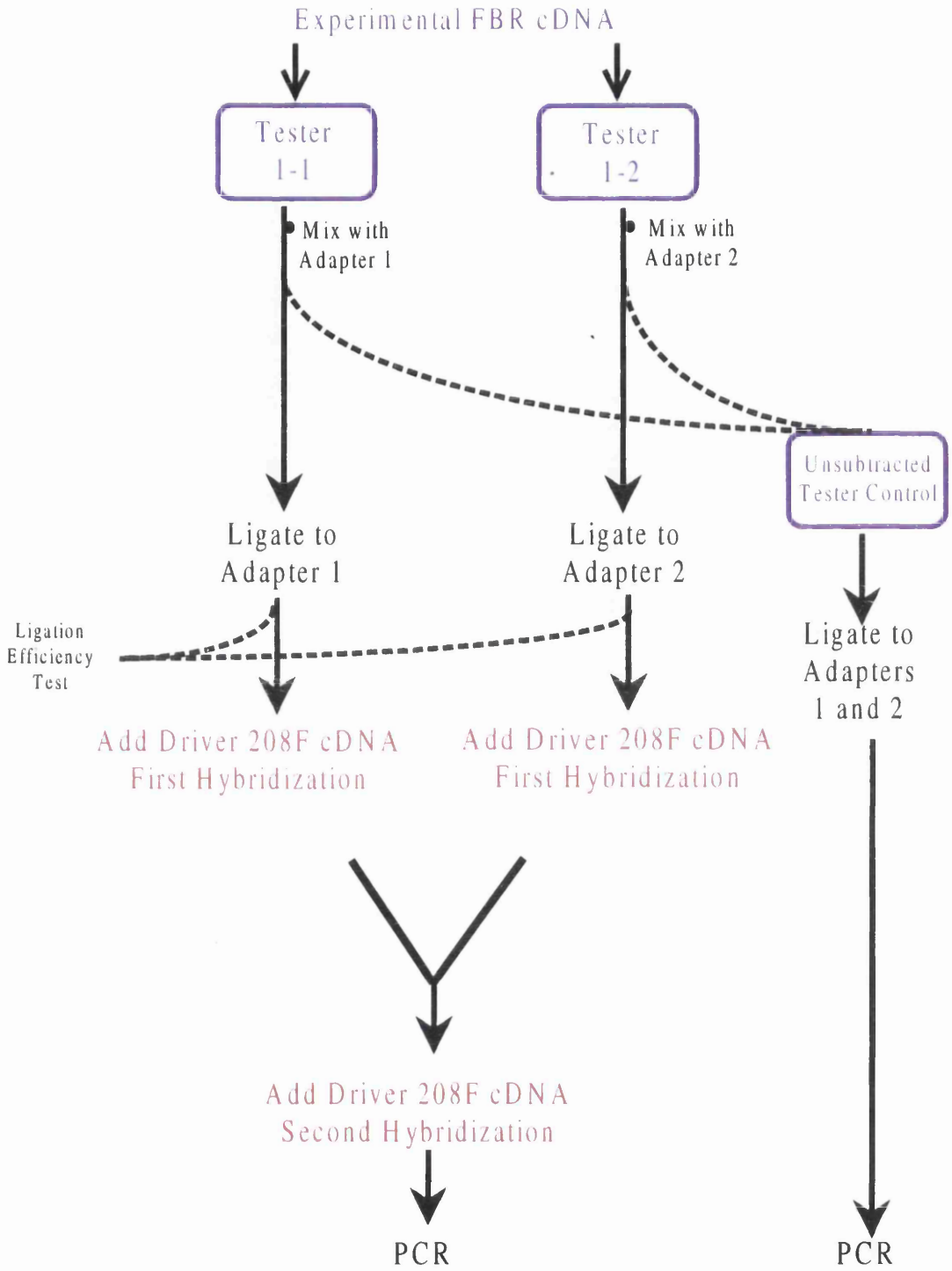


Figure 3.4.1
Up-regulated Subtraction

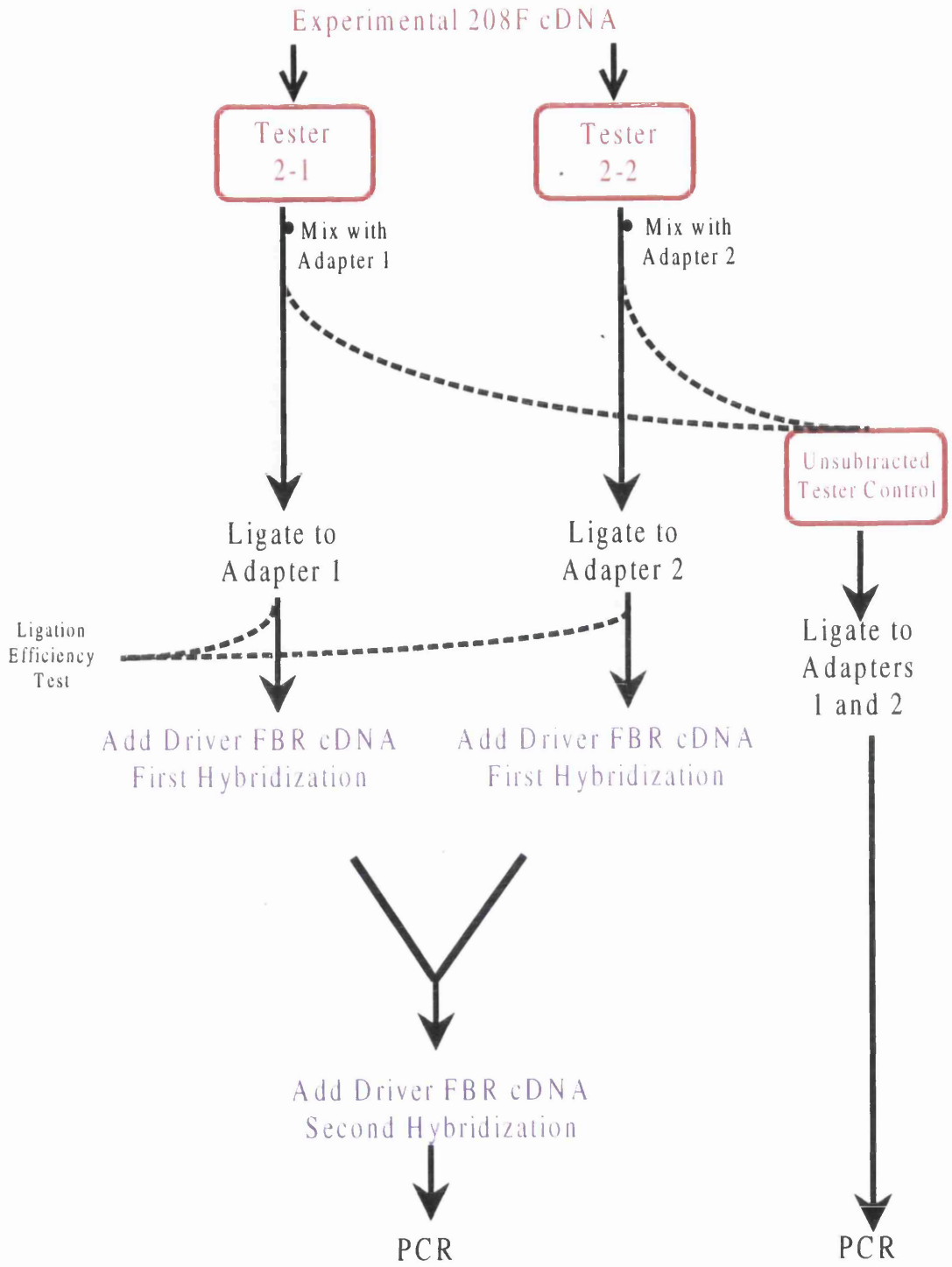


Figure 3.4.2
Down-regulated Subtraction

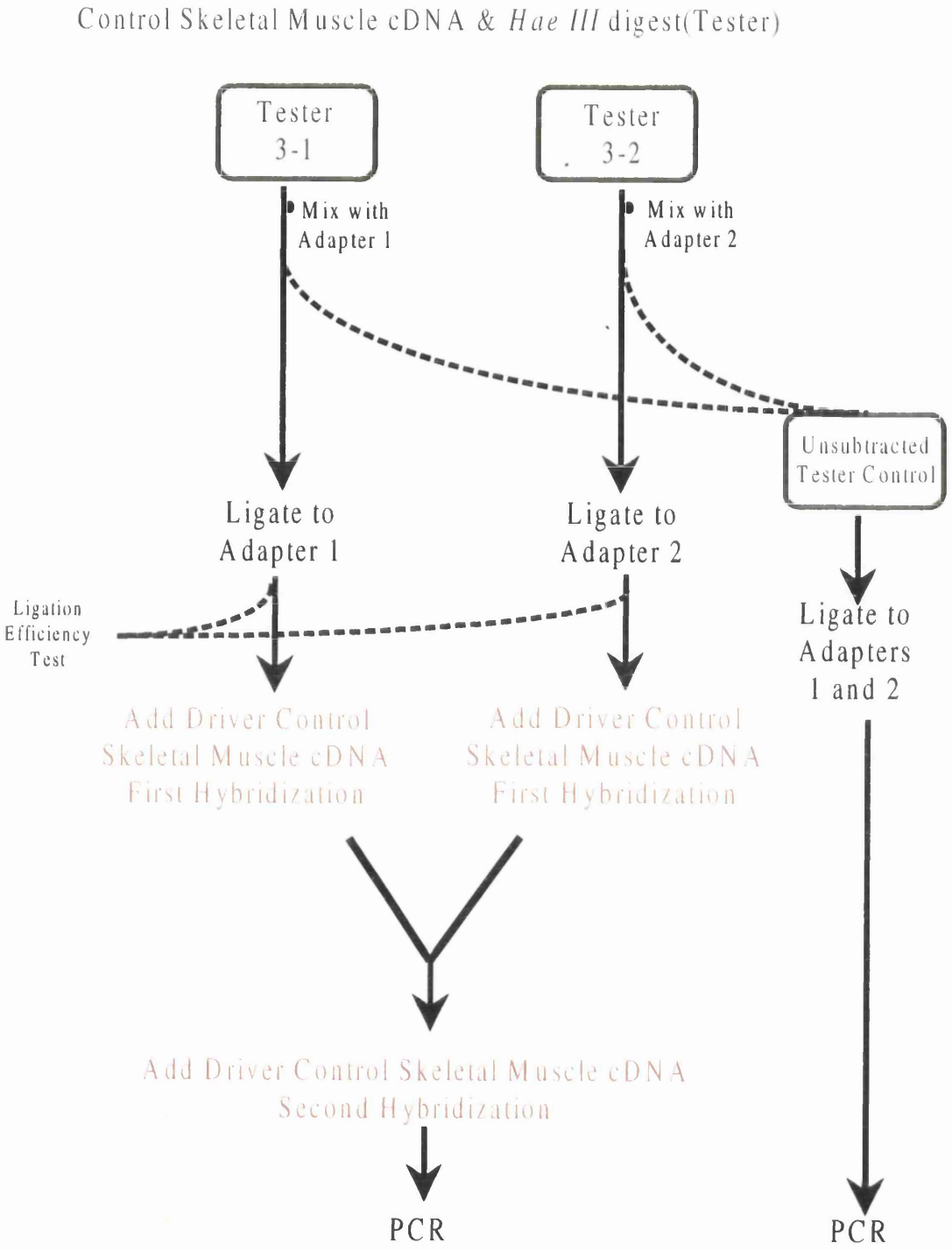


Figure 3.4.3
Control Subtraction

3.2.2 Control analysis of SSH

The differentially expressed cDNAs from the up-regulated, down-regulated and control subtracted libraries (refer to Figure 3.5), are 150-1500 bp in size with the majority approximately 150-600 bp. In the control cDNA library (Figure 3.5, lane 4), the subtracted *Hae* III digest fragments run higher than the corresponding ϕX174 markers (Figure 3.5, lanes 1 & 5), due to the PCR annealing sites on the adaptor sequences. As the control subtraction was performed in conjunction with the up-regulated (Figure 3.5, lane 3), and down-regulated (Figure 3.5, lane 2), subtractions this is demonstrative that the subtraction's were successful. In addition, the banding pattern of the unsubtracted cDNA tester control ligated with both adapters was completely different to the banding pattern of the experimental subtracted cDNA samples (Data not shown). The analysis of subtraction efficiency was performed using PCR and Southern blot hybridisation analysis.

3.2.2.1 PCR Analysis Control of SSH

The PCR analysis of subtraction efficiency for the up-regulated and down-regulated was performed using PCR primers for the house-keeping gene glyceraldehyde phosphate dehydrogenase (GAPDH), and is shown in Figure 3.6. Thirty three PCR cycles were required to produce a GAPDH PCR product for the subtracted up-regulated library, whereas after only 18 cycles of the unsubtracted up-regulated library produced a PCR product. In contrast, even after 33 PCR cycles of the subtracted down-regulated library no product was produced, whereas after only 18 cycles of the unsubtracted down-regulated library a PCR product was produced, albeit of lower abundance than the up-regulated library. This result indicated that the SSH was successful for both of the libraries due to the reduction in abundance of the house-keeping gene GAPDH. However, this result would suggest that the subtraction for the down-regulated library was more efficient than the subtraction for the up-regulated library, but in fact GAPDH has previously been reported as differentially expressed in FBR mRNA as opposed to 208F mRNA during quiescence and growth (Hennigan Ph.D. Dissertation,1993).

Figure 3.5

PCR amplified subtracted cDNA libraries

The secondary PCR products are represented for the down-regulated, up-regulated and control subtracted libraries. Lane 1 & 5: ϕX174/*Hae* III digest size markers. Lane 2: subtracted cDNA library of down-regulated clones. Lane 3: Subtracted cDNA library of up-regulated clones. Lane 4: Control subtracted skeletal muscle tester cDNA library containing 0.2% ϕX174/*Hae* III digested cDNA. Refer to text for a more detailed explanation on library construction.

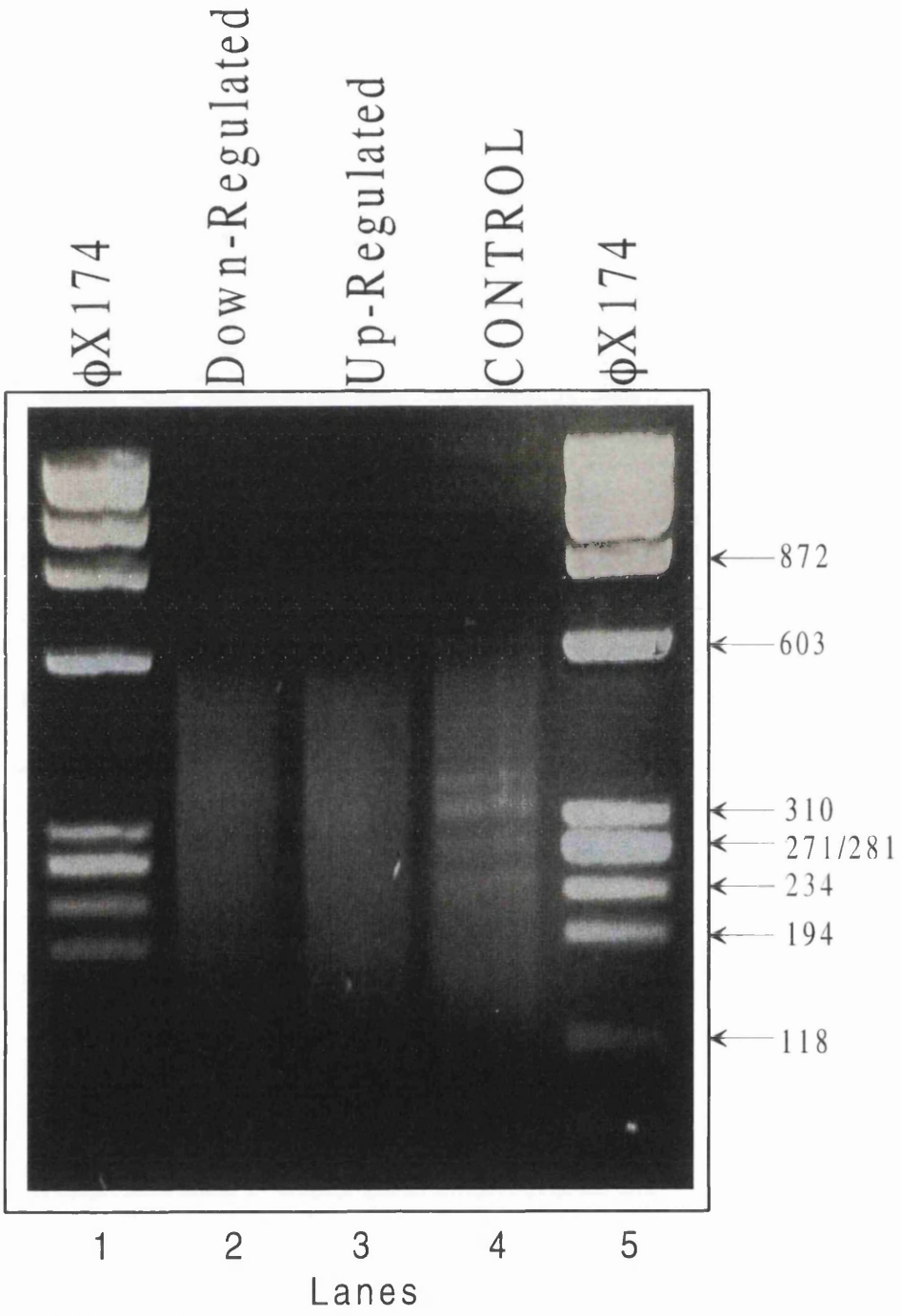


Figure 3.5
PCR Amplified Subtracted cDNA Libraries

Figure 3.6

PCR analysis of subtraction efficiency: reduction in GAPDH abundance

Top: PCR was performed on subtracted, lanes 2-5 and unsubtracted, lanes 6-9, up-regulated cDNA library with GAPDH 3' and 5' primers. Lanes 1 & 10: ϕX174/*Hae* III digest DNA markers; Lanes 2 & 6: 18 cycles; Lanes 3 & 7: 23 cycles; Lanes 4 & 8: 28 cycles; and Lanes 5 & 9: 33 cycles.

Bottom: PCR was performed on subtracted, lanes 2-5 and unsubtracted, lanes 6-9, down-regulated cDNA library with GAPDH 3' and 5' primers. Lanes 1 & 10: ϕX174/*Hae* III digest DNA markers; Lanes 2 & 6: 18 cycles; Lanes 3 & 7: 23 cycles; Lanes 4 & 8: 28 cycles; and Lanes 5 & 9: 33 cycles.

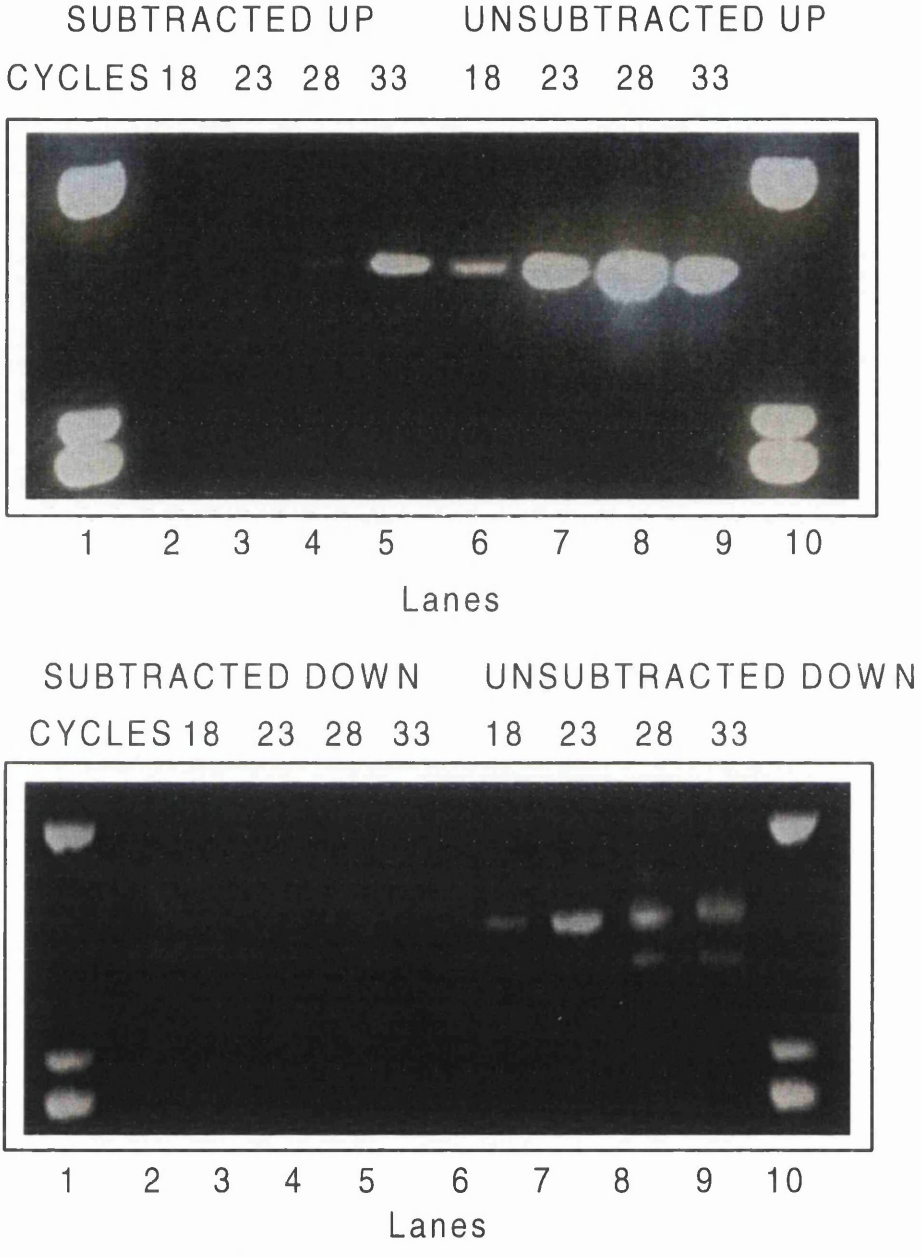


Figure 3.6
PCR Analysis of Subtraction Efficiency: Reduction in GAPDH Abundance

3.2.2.2 Southern Blot Analysis Control of SSH

The subtracted and unsubtracted up-regulated and down-regulated cDNA libraries were hybridised with ^{32}P-labelled up-regulated and down-regulated cDNA libraries independently.

The ^{32}P-labelled cDNA from the up-regulated subtracted library hybridised with the following: (1), itself, (2), the unsubtracted up-regulated library and (3), the unsubtracted down-regulated library, as each of the unsubracted libraries contain common sequences. The up-regulated subtracted cDNA library does not hybridise efficiently with the subtracted down-regulated library as the common sequences have been significantly reduced during the subtraction procedure. The hybridisation of ^{32}P-labelled up-regulated cDNA library to the unsubtracted and subtracted up- and down-regulated libraries is shown by Figure 3.7.1.

The ^{32}P-labelled cDNA from the down-regulated subtracted library hybridised with the following: (1), itself, (2), the unsubtracted down-regulated library and (3), the unsubtracted up-regulated library, as each of the unsubracted libraries contain common sequences. The down-regulated subtracted cDNA library does not hybridise efficiently with the subtracted up-regulated library as the common sequences have been significantly reduced during the subtraction procedure. The hybridisation of ^{32}P - labelled down-regulated cDNA library to the unsubtracted and subtracted up- and down-regulated libraries is shown by Figure 3.7.2.

Figure 3.7.1 and 3.7.2
Southern blot analysis of subtraction efficiency

Two μg DNA from unsubtracted down-regulated library, subtracted down-regulated library, subtracted up-regulated library and unsubtracted up-regulated library were electrophoresed on a 1.5% agarose gel, transferred to nylon filters, and hybridised with either a down-regulated library cDNA probe or an up-regulated library cDNA probe.

Figures 3.7.1 and 3.7.2. Lane 1: unsubtracted down-regulated library; Lane 2: subtracted down-regulated library; Lane 3: subtracted up-regulated library; and Lane 4: unsubtracted up-regulated library. These were probed with up-regulated library cDNA probe and down-regulated library cDNA probe respectively.

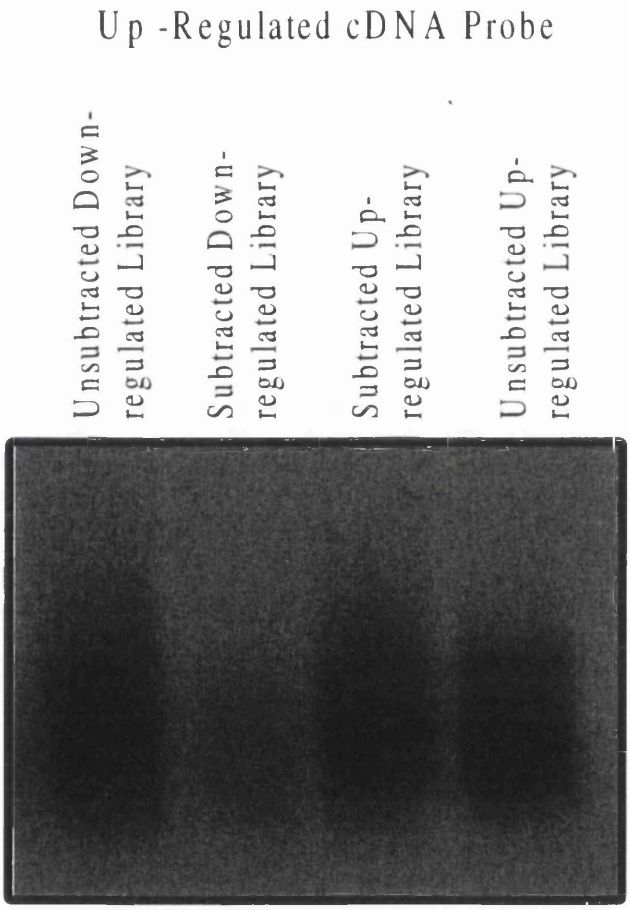


Figure 3.7.1
Southern Blot Analysis of Subtraction Efficiency

Down -Regulated cDNA Probe

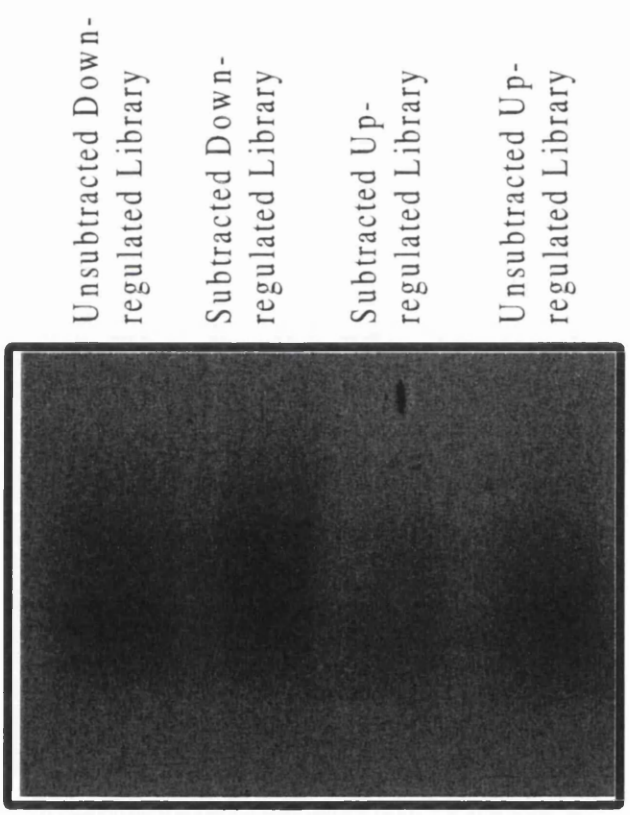


Figure 3.7.2
Southern Blot Analysis of Subtraction Efficiency

3.2.3 Sequencing and Computational homology analysis of differentially expressed cDNAs

Once it was established from the subtraction analysis that the up-regulated and down-regulated libraries were of good quality, the differentially expressed cDNAs were cloned into a T/A cloning vector to sequence individual differentially expressed cDNA fragments, which will be referred to as clones. The details of clone preparation and sequencing is described in chapter 2, sections 2.2.6, 2.2.7 and 2.2.19.

Approximately 500 clones were chosen at random for PCR amplification of the inserts and the products were analysed on agarose gels. Some of the clones had multiple inserts with two or more bands present in the same lane in the agarose gel, or no inserts with no band in the agarose gel lane, whereas some had inserts too short, <200 bp, for automatic sequencing and all of these were discounted. The differentially expressed cDNA clones of a suitable size, 200-800 bp, and of single inserts, were purified from PCR mixture using resin columns. The cDNA fragments were subsequently automatically sequenced using the ABI373 system on both strands using either vector primers T7 and M13 reverse primer or nested primers 1 and 2. Eighty-four clones from the up-regulated library and one hundred and twenty-one clones from the down-regulated library were successfully sequenced.

The sequences of the clones were edited to remove all vector and adaptor sequence and also to remove poor quality sequence. Any sequence of a heterozygous nature was discarded. Some clones represent the same gene as they map to different regions of the same cDNA sequence.

The clone sequences were computationally aligned to the GeneBank nr, and EST (Expressed Sequence Tags), databases using the blastx, and blastn programs to search for homologies to known genes at the protein and DNA level, and also to determine if any of the clones represented novel sequences.

The nr database contains a non redundant merge of Swiss-Prot, PIR, PRF, and proteins derived from GenBank coding sequences and PDB atomic co-ordinates and excludes the EST, STS and GSS divisions. The EST's are short, 300-500 bp sequences from mRNA (cDNA), that are produced during sequencing survey projects. They are representative of a mRNA population in a tissue type or at a particular developmental stage.

The blastx program translates the unknown nucleotide sequence into the six conceptual amino acid reading frames and compares them to known genes. As the clone sequences are of rat origin, this program is useful as there will be more similarity to sequences from other well represented species i.e. human, mouse. Also, the

biochemical significance of a match can be better evaluated at the amino acid level. Initially, all the clone sequences from the up-regulated and down-regulated libraries were submitted to NCBI for alignment to the GenBank nr database using the blastx program to determine if any matched known genes. However, many eukaryotic sequences have long untranslated regions at the 3'-end and smaller segments at the 5'-end and any sequence from these regions would not match a protein when translated in a blastx search. An example of this is clone C13 from the up-regulated library which matches to the untranslated region of Brain-Enriched Membrane-Associated Protein Tyrosine Phosphatase (bem)-3 using blastn, but fails to match any protein in a blastx program search.

Therefore, a blastn search was performed whereby the unknown clone sequences were compared to the GenBank database at the nucleotide level and this detects very high scoring matches. The numbered clones from the down-regulated library that match known genes are detailed in Table 3.1 and the numbered clones from the up-regulated library that match known genes are detailed in Table 3.2.

The clone sequences that failed to match any known genes were classified as novel. They were then further analysed using a blastn program search of the GenBank EST database EST database entries contain details of the EST clone including species, tissue type, developmental stage, cell type, treatment such as NGF, and similarity to a particular region or motif of a known gene that can provide information on the unknown sequence. The clones from the down-regulated library that match EST's are detailed in Table 3.3 and the numbered clones from the up-regulated library that match EST's are detailed in Table 3.4. The percentage identity to the EST's is shown for all matches.

All the further remaining clones were classified as novel, non-informative, having no matches and are detailed by Table 3.5.

Although not all the matches were 100% identical, this can be explained by biological errors in reverse transcribing the mRNA prior to subtraction, *taq* errors during the suppression PCR, clone preparation wiggle PCR and sequencing PCR steps, and also sequencing errors. Furthermore, the identity was not always to a gene of rat origin, but to other species, such as human or murine.

The searches were analysed using local ungapped alignments called high-scoring segment pair (HSP). The Karlin-Altschul statistics provides a mathematical theory of the expected distribution of random HSP scores and determines the probability of a particular sequence aligning in a certain size of database by chance. The calculated numerical value of the database matches enabled stringent probability analysis. In addition all the matches were analysed at least twice manually for regions

of homology and identity.

The database identity of the sequenced clones is tabulated in Figure 3.13. A total of 205 clones were sequenced of which 171 (83%), represent known genes whilst 35 (17%), were novel, with 29 (14%), matching to EST's, and 6 (3%), with no matches. The percentage database identity of the clones representing the up-regulated and down-regulated libraries is tabulated in Table 3.7. Interestingly, the sequenced clones from the up-regulated and down-regulated libraries are of similar distribution in database identities with 88% and 80% matching known genes, 10% and 17% matching EST's respectively, and 2% and 3% respectively had no match. The number of clones and their database distribution is detailed in Table 3.7.

Table 3.1

Blastx and Blastn matches for Down-regulated clones

This Table details all of the down-regulated clones that matched known genes submitted to the Genebank nr databases using the blastx and blastn programs. Refer to text for detailed database description and analysis.

Table 3.1
Blastx matches for Down-regulated clones

CLONE	ACC. No.	BLASTX PUTATIVE IDENTITY
A2	P16636	Lysyl Oxidase
A3	AF052685	Protocadherin 43
A4	Q60561	Ribonucleoside-Diphosphate Reductase M2 Chain
A8	X99144	Underexpressed In Thyroid Tissue After TSH Stimulation
A12	P04937	Fibronectin
A16	P52633	STAT-6
B1	P49722	Proteasome Component C3
B3	U64689	Zygin II
B6	P09653	Tubulin beta-5 Chain
B9	AF012891	Frizzled Related Protein frpAP
B12	P53457	Actin
B13	AF034546	Sorting Nexin 3
B15	1CBI	Apo-Cellular Retinoic Acid Binding Protein I
B16	AB018298	KIAA0755 protein

B18	P05982	NAD(P)H Dehydrogenase
C1	P81178	Aldehyde Dehydrogenase, Mitochondrial
C4	X13055	gamma-Actin
C10	AF012891	Frizzled Related Protein frpAP
C14a	AF041472	Ataxin-2
C14b	P93587	Actin
C15	P53497	Actin
D2	Q63716	Thioredoxin Peroxidase 2 (Heme-Binding 23 kD Protein)
D15	P70645	Bleomycin Hydrolase
E2	P27704	Extracellular Signal-Regulated Kinase 3
E4a	B40033	Protein Kinase ERK3
E5	Q15437	Human Protein Transport Protein Sec23 Homolog Isoform B
E6	Q15437	Human Protein Transport Protein Sec23 Homolog Isoform B
E17	AL021127	NAD(P) Dependent Steroid Dehydrogenase-Like Protein
F8	M32016	Lysosomal Membrane Glycoprotein-Type B
F16	U54999	LGN protein
G4	AF098301	Neural F Box Protein NFB42

G8	P50894	40S Ribosomal Protein S7
G13	S19958	Basic Helix-Loop-Helix Transcription Factor ALF1B
G14	D13645	KIAA0020 protein
G15	S40161	Ribosomal Protein S24
H8	Y12760	Latent TGF-Beta Binding Protein-2 Like Protein
H9	M23697	Plasminogen Activator
H11	U37770	Protein Phosphatase 2A B' Regulatory Subunit Beta1 Isoform
H12	S56599	AP56=acetaminophen-binding protein
H16	AJ005529	Magea5
I5	Q15293	Reticulocalbin 1
I7	Q14157	Hypothetical Protein KIAA0144
I8	P17046	Lysosome-Associated Membrane Glycoprotein 2
J14	P29314	40S Ribosomal Protein S9
J17	J04792	Ornithine Decarboxylase
J18	P16636	Lysyl Oxidase
K2	P04906	Glutathione S-Transferase P

K3	P18757	Cystathionine Gamma-Lyase
K6	Q06730	Zinc Finger Protein 33A
K8	Y12760	Latent TGF-Beta Binding Protein-2 Like Protein
K9	AF030001	Tenascin X
K10	P49718	Mouse DNA Replication Licensing Factor MCM5 (CDC46 Homolog)
K16	D86370	Megakaryocyte Potentiating Factor
L5	Y12760	Latent TGF-Beta Binding Protein-2 Like Protein
L7	U64033	Tera
M1	P27704	Extracellular Signal-Regulated Kinase 3
M4	Q62632	Follistatin-Related Protein (FRP)
M7	Y12760	Latent TGF-Beta Binding Protein-2 Like Protein
M10	AF044195	IkappaB Kinase Complex Associated Protein; IKAP
N1	Q16659	Extracellular Signal-Regulated Kinase 3
N9	J04792	Ornithine Decarboxylase
N10	D86983	Similar to D.Melanogaster Peroxidasin
N11	M77194	Polymerase
N13	M38135)	Cathepsin H

O6	AB011162	KIAA0590 Protein
O7	Q10569	Cleavage And Polyadenylation Specificity Factor
P6	M31552	Retinoic Acid-Binding Protein
P10	AC002397	C3F
P12	Z11995	45kDa protein
P13	Z11995	45kDa protein

Blastn matches for Down-regulated clones

A6	V00680	Rat Mitochondrial Genes Coding For 16S And 12S rRNAs And tRNAs Specific For Valine And Phenylalanine
A10	M31197	Rat Tissue-Type Plasminogen Activator
A17	D14043	Human mRNA For MGC-24
A18	V00680	Rat Mitochondrial Genes Coding For 16S And 12S rRNAs And tRNAs Specific For Valine And Phenylalanine
B5	AF084928	Homo sapiens Erythroblast Macrophage Protein EMP mRNA
B10	AB000822	Mus musculus mRNA For SNAP-23
C7	AC002476	Human PAC Clone DJ318C15 From Xq23
C12	U77829	Rattus norvegicus gas-5 growth arrest homolog non-translated mRNA sequence

D9	M27830	Human 28S Ribosomal RNA Gene
D11	S77494	Lysyl Oxidase {3' Untranslated Region}
E4b	M18209	Mouse Transcription Factor S-II, Clone PSII-2
E12	AF034692	Mus musculus alpha-1-Microglobulin/Bikunin Precursor
F7	D63478	Human mRNA For KIAA0144 Gene
F9	AF052129	Homo sapiens clone 23904 mRNA sequence
F10	AF039843	Homo sapiens Sprouty 2
F12	D17711	Rat mRNA For dC-Stretch Binding Protein (CSBP)
F14	U11038	Rattus norvegicus Lysyl Oxidase mRNA
G6	S63167	3 beta-Hydroxysteroid Dehydrogenase Isomerase Type II.2
I6	M70641	Mouse FISP-12 protein (fisp-12) gene
I9	AF052129	Homo sapiens clone 23904 mRNA sequence
I13	D17711	Rat mRNA For dC-Stretch Binding Protein (CSBP)
J2	AF080568	Rattus norvegicus CTP:Phosphoethanolamine Cytidylyltransferase mRNA
J11	X56133	Rat mRNA For F1-ATPase alpha Subunit
K4	M70642	Mouse FISP-12 Protein (fisp-12)
L2	M60833	Mouse alpha2(VIII) Collagen Gene Exon, Partial Sequence

L3	U22493	Mus musculus Fibrillin-1 (Fbn1) mRNA
O13	X14848	Rattus norvegicus mitochondrial genome
P18	U79287	Human clone 23867 mRNA sequence

Table 3.2

Blastx and Blastn matches for Up-regulated clones

This Table details all of the up-regulated clones that matched known genes submitted to the Genebank nr databases using the blastx and blastn programs. Refer to text for detailed database description and analysis.

Table 3.2
Blastx matches for Up-regulated clones

CLONE	ACC. No.	BLASTX PUTATIVE IDENTITY
A1a	M77194	Polymerase
A3	P02631	Oncomodulin
A5	P00787	Cathepsin B
A7	P07152	Stromelysin-2
A10	P00786	Cathepsin H
A13	M27315	ATPase Subunit 6
A12	P29175	FBR murine osteosarcoma virus gag polyprotein
A15	P04642	L-Lactate Dehydrogenase M Chain
A16	P05503	Cytochrome C Oxidase
A17	P02631	Oncomodulin
B4	P07152	Stromelysin-2
B5	L19932	p68(beta ig-h3)
B8	P07152	Stromelysin-2
B10	P02631	Oncomodulin

B11	Q10758	Keratin, Type Ii Cytoskeletal 8
B12	P02631	Oncomodulin
B14	AJ010566	Cytochrome B
B16	AJ010567	Cytochrome B
B18	U97674	Cytochrome C Oxidase Chain I
C6	Y07738	Vimentin
C9	P29315	Ribonuclease Inhibitor
C11	P31000	Vimentin
C14a	S38501	Cyclin A2
C14b	Q10758	Keratin, Type II Cytoskeletal 8
C15	D13978	Argininosuccinate Lyase
C16	P20961	Plasminogen Activator Inhibitor-1
C17	P02631	Oncomodulin
D4	P07152	Stromelysin-2
D7	Q10758	Keratin, Type II Cytoskeletal 8
D8	AB000628	UDP-GlcNAc:a-1,3-D -mannoside b-1,4-N-Acetylglucosaminyltransferase IV
D12	L19932	p68(beta ig-h3)

E3	U07424	Putative tRNA Synthetase-like Protein
E5	JC1132	Phosphoglycerate Mutase
E6		Kelch related Protein1
E10	P07152	Stromelysin-2
E12a	P08113	Endoplasmin Precursor
F1		Kelch related Protein 1
G9		Kelch related Protein 1
G10		Kelch related Protein 1
I6	P07152	Stromelysin-2
I7	P90838	Hypothetical 55.2 kD Protein F16A11.2 In Chromosome I
I8		Kelch related Protein 1
I10		Kelch related Protein 1
K13	P14793	60S Ribosomal Protein L40 (CEP52)
K15	JC1132	Phosphoglycerate Mutase
L4	P00406	Cytochrome C Oxidase Polypeptide II
L6	P47226	Testin 2
L13	P14793	60S Ribosomal Protein L40 (CEP52)

M4	P05503	Cytochrome C Oxidase Polypeptide I
M12		Kelch related protein 1
N1	S12207	Hypothetical Protein (B2 Element)
N2	D29958	KIAA0116 Protein
N11		Kelch related protein 1
N14		Kelch related protein 1

Blastn matches for Up-regulated clones

A1b	M73706	Mouse Ferritin Large Subunit Gene, Complete cds
A4	M23984	Rat mitochondrial proton/phosphate symporter mRNA
B3	AB004305	Mus musculus mRNA for Mblvr
C1	D45414	Rat mRNA for brain-enriched membrane-associated protein tyrosine phosphatase (BEM)-3
C5	Z82195	Human DNA sequence from PAC 274L7 on chromosome X contains ESTs
C13	D45414	Rat mRNA for brain-enriched membrane-associated protein tyrosine phosphatase (BEM)-3
D5	L19932	Mouse (beta ig-h3) mRNA
D9	X02610	Rat mRNA for non-neuronal enolase (NNE)

D14	J03275	Bovine ADP-ribosylation factor
E12b	X05083	RAT mRNA for oncogene-induced transin-2
F3	U96138	Rattus norvegicus CD44 protein (CD44) mRNA
I3	U72941	Mus musculus Annexin IV mRNA
I5	X05083	RAT mRNA for oncogene-induced transin-2
K16	D83037	Mouse mRNA for 14-3-3 zeta
I16	U72941	Mus musculus Annexin IV mRNA
L2	D30804	Rat mRNA for proteasome subunit RC6-1
L5	X01964	Rat mRNA for lactate dehydrogenase
N3	AF026466	Mus musculus putative neuronal cell adhesion molecule (Punc) gene, alternatively spliced forms, partial cds
N8	D45414	Rat mRNA for brain-enriched membrane-associated protein tyrosine phosphatase (BEM)-3
N12	X14848	Rattus norvegicus mitochondrial genome

Table 3.3

EST Blastn matches for Down-regulated clones

This Table details all of the down-regulated clones that had no match to known genes submitted to the Genebank nr databases using the blastx and blastn programs, but matched EST database entries using blastn program. Refer to text for detailed database description and analysis.

Table 3.3
EST matches for Down-regulated clones

CLONE	ACC. No.	BLASTN PUTATIVE IDENTITY
B17	W70681	Soares mouse embryo NbME13.5 14.5 Mus musculus cDNA clone 390151 5' similar to PIR:JC4173 JC4173 reticulocalbin homolog - human
D5	AA636264	Barstead mouse myotubes MPLRB5 Mus musculus cDNA clone 1120941 5' similar to gb:M65142 Mouse lysyl oxidase
D7	AA794937	Knowles Solter mouse 2 cell Mus musculus cDNA clone 1123937 5'
D9	AA705421	Soares fetal liver spleen 1NFLS S1 Homo sapiens cDNA clone 462182 3'
D10	T03105	Fetal brain, Stratagene Homo sapiens cDNA clone FB24B6 3'end
D13	AA060979	Soares mouse p3NMF19.5 Mus musculus cDNA clone 483033 5'
D16	AA944502	EST200001 Normalized rat embryo, Bento Soares Rattus sp. cDNA clone REMAK19 3' end
E7	AA848367	Normalized rat kidney, Bento Soares Rattus sp. cDNA clone RKIAB57 5' end
E9	AI045249	UI-R-C1-kh-e-09-0-UI.s1 UI-R-C1 Rattus norvegicus cDNA clone UI-R-C1-kh-e-09-0-UI 3'

F4	W70707	Soares mouse embryo NbME13.5 14.5 Mus musculus cDNA clone 390175 5' similar to PIR:JC4173 JC4173 reticulocalbin homolog - human
F6	W14868	Soares mouse p3NMF19.5 Mus musculus cDNA clone 330480 5' similar to gb:D13003 Mus musculus reticulocalbin mRNA
G9	AA946090	Normalized rat lung, Bento Soares Rattus sp. cDNA clone RLUBB14 3' end, mRNA sequence
H1	AI176721	Normalized rat ovary, Bento Soares Rattus sp. cDNA clone ROVBV93 3' end, mRNA sequence
J7	AI237525	Normalized rat placenta, Bento Soares Rattus sp. cDNA clone RPLDA19 3' end, mRNA sequence
J12	AA963284	UI-R-E1-gh-h-08-0-UI.s1 UI-R-E1 Rattus norvegicus cDNA clone UI-R-E1-gh-h-08-0-UI 3'
K1	AA409249	Mouse 7.5 dpc embryo ectoplacental cone cDNA library Mus musculus cDNA clone C0006F12 5'
L12	AA412764	Knowles Solter mouse blastocyst B1 Mus musculus cDNA clone 833738 5'
L13	AA077608	Chromosome 7 Fetal Brain cDNA Library Homo sapiens cDNA clone 7B27F10
M9	AI136538	UI-R-C2p-nq-h-02-0-UI.s1 UI-R-C2p Rattus norvegicus cDNA clone UI-R-C2p-nq-h-02-0-UI 3'
O11	AI226259	Soares mouse mammary gland NMLMG Mus musculus cDNA clone IMAGE:1510548 5'
O12	Z41449	H. sapiens partial cDNA sequence; clone c-zug11

Table 3.4

EST Blastn matches for Up-regulated clones

This Table details all of the up-regulated clones that had no match to known genes submitted to the Genebank nr databases using the blastx and blastn programs, but matched EST database entries using blastn program. Refer to text for detailed database description and analysis.

Table 3.4
EST matches for Up-regulated clones

CLONE	ACC. No.	BLASTN PUTATIVE IDENTITY
A1	AA118663	Soares 2NbMT Mus musculus cDNA clone 573295 5'
A11	AA077608	Chromosome 7 Fetal Brain cDNA Library Homo sapiens cDNA clone 7B27F10
B17	AA008657	Soares mouse embryo NbME13.5 14.5 Mus musculus cDNA clone 439981 5' similar to PIR:S48454 S48454 hypothetical protein - yeast
I4	AI112367	UI-R-Y0-mn-d-02-0-UI.s1 UI-R-Y0 Rattus norvegicus cDNA clone UI-R-Y0-mn-d-02-0-UI 3', mRNA sequence
K10	C77740	Mus musculus 3.5-dpc blastocyst cDNA 3'-end sequence, H.sapiens mRNA for ORF
L8	AA272131	Mus musculus cDNA clone 737061 5' similar to SW:ACT_EUPCR P20360 ACTIN
M5	AI231330	Normalized rat embryo, Bento Soares Rattus sp. cDNA clone REMDI67 3' end, mRNA sequence
N6	H34716	Rat PC-12 cells, NGF-treated (9 days) Rattus sp. cDNA clone RPNCO01 5' end

Table 3.5

Down-regulated and Up-regulated Clones with no matches

This Table details all of the down-regulated and up-regulated clones that had no match to known genes submitted to the Genebank nr databases using the blastx and blastn programs and no matches to the EST database entries using blastn program.

Table 3.5
Down-regulated clones: No matches

C16

E11

F1

K13

Up-regulated clones: No matches

D10

E14

Tables 3.6, 3.7, and 3.8

Analysis tables of sequenced clones

The Genebank nr and EST database analysed down-regulated and up-regulated clones were tabulated in Figure 3.6 according to their database identity. In Table 3.7 the percentage of total down-regulated and up-regulated clones represented in a database is detailed. Table 3.8 details the representative distribution of both the down-regulated and up-regulated clones collectively in each database as number of clones and as a percentage to total clones analysed.

DATABASE IDENTITY	Up-regulated	Down-regulated
TOTAL	84	121
Blastx	54	69
Blastn	20	28
EST	8	20
NONE	2	4

Table 3.6
Table of Up-regulated and Down-regulated Sequenced Clones:Putative Identities

DATABASE IDENTITY	PERCENTAGE Up-regulated	PERCENTAGE Down-regulated
Blastx	64	57
Blastn	24	23
EST	10	17
NONE	2	3

Table 3.7
Up-regulated and Down-regulated Sequenced Clones:
Percentage Putative Database Identities

DATABASE IDENTITY	Number of Clones Sequenced	PERCENTAGE
TOTAL	205	100
Blastx	123	60
Blastn	48	23
EST	28	14
NONE	6	3

Table 3.8
Table of Total Sequenced Clones:
Percentage and Putative Database Identities

3.3 DISCUSSION

The plethora of known genes up- and down-regulated by the AP-1 transcription factor is potentially vast. Therefore to understand the changes that occur when a normal fibroblast becomes transformed, it becomes important to group the genes into sub-sets. The functional classifications of the sub-sets would enable the examination of their possible roles in transformation and invasion. However, the functional classifications to which the genes isolated in this study have been assigned are not strict. In fact, several of the genes would fit into more than one class.

Sequence analysis of the cDNA libraries supports the proposal that AP-1 direct and indirect target genes contribute to the transformed and motile state of FBR cell line. This is more apparent for the genes isolated from the up-regulated library as more is known about the activation of transformation than it's repression. In fact 18 known genes isolated from the up-regulated library have previously been shown to be directly associated with transformation and invasion. This includes cell-cell and cell-ECM molecules, ECM modelling proteins including proteases, cytoskeletal proteins and enhancers of cell motility.

However, many known genes isolated from the up-regulated library have not previously been linked to transformation and invasion indicating that AP-1 does not exclusively function in regulation of transformation. This suggests that AP-1 in FBR's has additional regulatory functions, possibly encompassing cell cycle control and cell survival.

The identified down-regulated genes includes 20 known growth/tumour suppressors and includes components that function to maintain normal phenotype including cytoskeletal proteins and cell adhesion molecules. The down-regulated library contains many genes that have not previously been ascribed with a transformation suppressor function.

The SSH of FBR and 208F was performed to identify transformation and invasion related genes and as expected many were isolated. However, in both libraries there are genes which have no obvious connection with transformation and invasion. This includes genes which encode for vesicle transport proteins such as BLVR, endoplasmin precursor, Sec23 homolog isoform B and lysosomal glycoprotein type B, and metabolic related proteins including L-lactate dehydrogenase M chain, arginosuccinate lyase, thioredoxin peroxidase and phosphoglycerate mutase. Furthermore, several of the genes isolated encode for proteins with no distinct functional group including heme binding protein, reticulocabin, zinc finger protein 33a, ornithine decarboxylase, MRP related ATPase and annexin IX.

The phenotypic differences between normal and transformed cells contribute to the invasive and metastatic properties of malignant cells. Alterations in the phenotype are caused by changes in the level of gene expression of a sub-set of proteins, namely the transformation sensitive proteins, that may be directly or indirectly involved in cell adhesion, attachment and invasion. In addition, many proteins have a fundamental role in the maintenance of a normal phenotype and their loss can permit the unrestricted growth of a tumour cell.

Therefore, transformation is a complex biological process requiring many transformation specific genes to be switched on and off enabling the initiation of remodelling and maintenance of cellular phenotype. This provides the transformed cell with a more rapid growth, loss of contact inhibition, anchorage independence, altered motility, and an increased invasive potential. Thus, conversion from the normal to a transformed cellular state involves the co-ordinated up-regulation and down-regulation of an unknown number of critical genes. The subtracted libraries constructed in this study have identified sets of AP-1 direct and indirect target genes, although not exhaustive, of which many have functions ascertaining to transformation and invasion.

3.3.1 FBR Murine Provirus

One of the clones isolated from the up-regulated library encodes for the FBR Murine provirus. This provides concrete evidence of the differential expression of the cDNA libraries with the expression of the actual transforming agent in the FBR mRNA population.

3.3.2 Extracellular Matrix and Cytoskeleton remodelling in transformation and invasion

The FBR *v-fos* transformed cells are of a highly refractile bipolar morphology and are motile and invasive, whereas the 208F's are non-transformed with a typical fibroblastoid morphology. The morphological changes that occur during Fos transformation includes the different expression of ECM proteins during cytokeletal remodelling.

Cancer metastasis is a complex process and even after the primary tumour is surgically removed, recurrences at distant sites can occur. The process of metastasis usually occurs with local invasion of a transformed cancer cell into local surrounding tissue, infiltration into vascular and lymphatic system, circulation followed by extravasation, and growth at secondary sites (Fidler *et al.,* 1978; Nicolson, 1988; Zetter, 1990; Aznavoorian *et al.,*1993). The initial step of local invasion requires cell motility, the enhancement of which is correlated with metastatic potential in animal

models and a poor prognosis in human cancers (Hosaka *et al.,* 1978; Haemmerli and Strauli, 1981; Partin *et al.,*1989).

The transformed state and invasive nature of the FBR cells provides an *in vitro* model from which transformation specific genes can be isolated.

3.3.3 Changes in expression of Extracellular Matrix and Cytoskeleton components

Genes that effect ECM and cytoskeleton remodelling are well represented in each library. The ECM components Fibronectin and α2 (VIII) Collagen are down-regulated in *v-fos* transformants as are the cytoskeletal proteins actin and tubulin. In addition, integral membrane protein Protocadherin 2 (Pcdh2), is also down-regulated. The cytoskeleton related cytokeratin 8 polypeptide, vimentin and CD44 are found to be up-regulated.

The morphology of the FBR *v-fos* transformed cells is due in part to gross cytoskeleton reorganization and previous studies (Jooss and Muller *et al.*, 1985; Hennigan *et al.*, 1994; Lamb *et al.*, 1997a, 1987b), has revealed that AP-1 transformation changes the cytoskeletal components expression patterns. In *v-fos* transformed fibroblasts the F-actin stress fiber bundles that run in parallel arrays along the ventral surface of the cell and focal contacts are lost during remodelling in favour of F-actin cable structures along the length of the cells into the pseudopod tip that is capped by actin rich bundles. The microtubules form a fine looping filament meshwork that extends to the cell periphery from an organised perinuclear microtubule centre. However, after *v-fos* transformation tubulin organises at the pseudopodial tips into a compressed central filament with a terminal loop structure that stops short of the actin bundle (Hennigan *et al.,* 1994; Spence, unpublished observations).

There are several cellular and extracellular factors that regulate cell motility, but the motility of the cell is directly affected by the bundling and subcellular distribution of the actin cytoskeleton (Stossel, 1993; Lauffenburger and Horwitz, 1996; Cramer *et al.,* 1997, Honda *et al.,* 1998). Therefore, the regulation and re-organisation of the actin cytoskeleton such as that in FBR *v-fos* cells may contribute to the increased invasive capability of these cells.

The ECM is a complex matrix of scaffolding, consisting of proteins and proteoglycans that provides structural rigidity in cells. *In vivo*, specific ECM structures function to structurally support and compartmentalise tissues (Matrisian 1990). The ECM components includes Type I-VIII collagens, laminin, fibronectin, gelatins and heparin sulphate proteoglycan.

The cytoskeleton is a cytoplasmic network of microfilaments, microtubules and

intermediate filaments of tubulin, vimentin and actin that also provides the cell with a structural support and rigidity to maintain their shape. However it must be noted that although cells have rigid support, they are not stationary, but are in fact motile and dynamic.

The microtubules and actin microfilament cytoskeletal proteins are essential for cellular processes such as cell growth and division, motility, signalling and the maintenance of cell shape. The dynamics of microtubules and actin in transformation and tumourigenesis earmarks these molecules as potential therapeutic targets in cancer treatments (Jordan and Wilson, 1998).

The protocadherin 43 that is found down-regulated in FBR *v-fos* transformants is localised at cell-cell contact sites and functions as a cell-cell adhesion molecule (Sano *et al.*,1993). The cadherins mediate calcium dependent cell-cell adhesion and contain a large extracellular domain at the N-terminus and a small cytoplasmic domain at the C-terminus that are connected by a transmembrane region. The extracellular domain consists of five motifs, each containing a cadherin-specific sequence, whilst the cytoplasmic domain regulates cadherins through interactions with cytoskeletal proteins (Sago, *et al.*, 1995). The mammalian E-cadherin and Drosophila fat function a suppressors of tumour invasion (Chen and Obrink, 1991; Frixen *et al.*, 1991; Mahoney *et al.*, 1991; Vleminckx *et al.*, 1991).

The transmembrane protein, CD44, up-regulated in FBR *v-fos* transformants is a cell surface hyaluronan receptor but can interact with other ECM components (Bennett *et al.,* 1995; Jalkanen and Jalkanen, 1992; Weber *et al.,* 1996). The various isoforms are formed through alternative splicing and post-translational modifications in the extracellular domain of the native protein (Haynes *et al.,* 1991). Overexpression of CD44 isoforms is associated with the metastatic behaviour of several human tumours (Mayer *et al.,* 1993; Mulder *et al.,* 1994), and CD44 has also been linked to tumour progression by enhancing melanoma cell motility and invasion (Birch *et al.,* 1991; Faassen *et al.,* 1992; Thomas *et al.,* 1993). Furthermore, CD44 can confer tumour growth and metastatic potential in *in vivo* models (Gunthert *et al.,* 1991; Guo *et al.,* 1994). The expression of CD44 is known to be regulated by AP-1 (Hofmann , 1993), and was previously shown in our laboratory to be required both for the *in vitro* invasion of Fos transformed and growth factor treated 208Fs using the matrigel invasion assay (Lamb *et al.,* 1997a). Recently MMP9, which requires AP-1 for its expression and is up-regulated in many transformed cells and tumour derived cell lines, has been shown to associate with CD44. CD44 associates with a proteolytic form of MMP-9 at the cell surface and this complex promotes cell-mediated Collagen IV degradation *in vitro* and mediates tumour cell invasion of G8 myoblast monolayers. In addition, disruption of

CD44/MMP-9 cluster formation, by overexpression of soluble or truncated cell surface CD44 inhibits tumour invasiveness *in vivo* (Yu and Stamenkovic, 1998). Furthermore Okamoto *et al.*, (1999), reported that in cancer cells CD44 was cleaved at the membrane-proximal region of the ectodomain. This cleavage was shown to be mediated by a membrane-associated metalloprotease expressed in cancer cells, and that a tissue inhibitor of metalloprotease-1 (TIMP-1), as well as metalloprotease inhibitors, inhibit CD44 cleavage in a cell-free assay (Okamoto *et al.*, 1999). Thus CD44 cleavage is regulated by an intricate balance between some proteases and their inhibitors.

The down-regulation of the cytoskeletal components actin and tubulin in FBR's may be due to the gross cytoskeletal re-arrangements that occurs upon transformation by *v-fos*. The deregulation of protocadherin 43 and potential loss of cell-cell adhesion is perhaps contributory to the increased cell motility of the FBR cell line. The up-regulation of CD44 and the protease MMP-10 is consistent with the motile behaviour and invasive characteristics of the FBR cell line.

3.3.4 Matrix Metalloproteinases and the ECM

In the FBR *v-fos* transformed cells there is an up-regulation of Beta ig-h3; MMP-10, also known as transin, oncogene-induced transin and stomelysin2, and their degradation targets, the ECM proteogylcans Fibronectin; Type 1 Collagen and α2(VIII)Collagen, are found to be down-regulated. This is consistent with the morphological remodelling changes that occur upon v-fos transformation of 208F fibroblasts.

The invasion of a transformed cell into normal surrounding tissues is regarded as a tissue remodelling process in which the ECM and surrounding normal tissue is proteolytically degraded by the proteases including the Matrix Metalloproteinases, MMPs (Johnsen *et al.*, 1998). The proteolytic remodelling of the ECM in oncogenic transformation is known to affect the adhesion, motility and invasiveness of the cell (Bortner *et al.*,1993). *In vivo*, the MMPs are involved in proteolytic degradation during tissue remodelling processes such as wound healing, post-lactation mammary gland involution and trophoblast invasion during implantation (Johnsen, *et al.*, 1998).

The MMPs are a family of tightly regulated enzymes that degrade ECM and basement membrane components. They are zinc ion containing proteinases that are secreted and require activation for proteolytic activity whereby they degrade one or more components of the ECM. Inhibition is conferred by chelating agents and also by TIMPS, that is specific tissue inhibitors of metalloproteinases (Matrisian, 1990). MMPs are characterised into subclasses as to their substrate specificity, Collagenases: MMP-

1, MMP-8, MMP-13, Gelatinases (Type IV Collagenases): MMP-2, MMP-9, Stromelysins: MMP-3, MMP-10, Furin-recognition site MMPs: MMP-11 including membrane bound type MMP-14, MMP-15, MMP-16, MMP-17, and the newly described: Enamolsin (Matrisian, 1990; Shapiro, 1998). The MMPs selectively degrade the various basement membrane and ECM components, Fibronectin, Laminin, Enactin, Proteoglycans and Type I-VII, and X Collagens. The Stromelysin2 (MMP10), promotor region contains a functional AP-1 site (Kerr *et al.,* 1990), and thus is regulated by AP-1.

3.3.5 ECM and Cell Adhesions

In this study several ECM components and cell adhesion proteins have been found to be down-regulated. This includes LTBP-2, Fibrillin, Fibronectin, Tenascin-X, and α2(VIII)Collagen. The ECM stabalizing enzyme Lysyl Oxidase was also found to be down-regulated.

The collagens are vital ECM components, with type I showing tumour suppressor activity. The down-regulation of important ECM components such collagens and tenascin-X as would facilitate cell shape changes and increased cell motility as observed in FBRs. The down-regulation of the cell-cell adhesion proteins LTBP-2 like protein, Fibrillin-1, and Fibronectin plus the ECM stabilizing enzyme Lysyl Oxidase, in FBR *v-fos* transformants may enhance morphological transformation and motility.

Cell adhesion links cells to each other or to the ECM, establishing intracellular adhesion plaques with cytoskeletal components that are organised into large complexes. These large cell adhesion complexes activate intracellular signalling pathways that regulate cell morphology, migration, gene expression, growth and differentiation (Yamada and Geiger, 1997; Burridge *et al.*, 1997). This adhesion dependent cell-signalling is critically important in preventing tumourogenesis of normal cells. In fact, several adhesion molecules have tumour suppressor activity and function by maintaining cell-cell and cell-ECM adhesions thereby inhibiting cell motility. *In vivo* this blocks migration and invasion into surrounding tissues that results in metastasis (Boudreau and Bissel, 1998; Tlsty, 1998).

The ECM microfibrils contain several structural proteins including Fibrillins, LTBPs and microfibral-associated glycoproteins (Mecham *et al.*, 1995). The LTBT-2 that is found down-regulated in FBR *v-fos* transformants is a member of the Fibrillin-LTBP gene family (Sakai *et al.,* 1986). The Fibrillins and LTBPs have similar domain structures and are mainly composed of EGF and 8-cysteine repeats, the former a common feature of many extracellular proteins. Most of the EGF like repeats contain

the consensus sequence for calcium binding (Handford *et al.,* 1991; Gibson *et al.,* 1995), that stabilises protein structure against proteolytic degradation and also stabilises protein-protein interactions (Reinhardt *et al.,* 1997; Fehon *et al.,* 1990; Rao *et al.,* 1995). The latter binds via disulphide bridges to heterologous proteins and the third 8-Cys repeat of LTBP-1 mediates the binding to latent TGF-β (Sahariner *et al.,* 1996).

The TGF-β family of proteins regulate the growth, differentiation, adhesion and migration of many cell types (Roberts *et al.*, 1990, Massague, 1990,; Moses *et al.,* 1990). It inhibits the growth of most cell types and stimulates the production of ECM proteins such as Fibronectin, Collagen, and Tenascin (Chiefetz *et al.,* 1990) The TGF-βs are secreted from cells as a latent complex that is comprised of the homodimeric mature growth factor, TGF-β latency-associated protein and a LTBP. LTBP-2 down-regulation in FBRs may be due to the growth inhibition function of this gene. Furthermore, LTBP-2 inhibits ECM protein production. Interestingly the ECM proteins that provide structural rigidity, Fibronectin and the α2 (VIII) Collagen are also down-regulated in FBRs.

Fibrillins of which there are two forms 1 and 2, are major components of the ECM. The fibrillin-1 module, D25 segment (the 25th module of fibrillin-1 D segment), and LTBP-2 (Moren *et al.,* 1994), both contain the cell adhesion motif arginyl-glycyl-aspartyl (RGD), that mediates integrin-dependent cell adhesion (D'Arrigo *et al.,* 1998), to the ECM ligands via integrins. Both Fibrillin-1 and LTBP-2 are susceptible to proteolytic processing and release from the ECM (Hyytiainen *et al.,* 1998). A characteristic of transformation is the loss of cell adhesion. Thus the down-regulation of Fibrillin-1 and LTBP-2 in FBRs probably lends to the de-regulation of cell adhesion in addition to other functions.

The insoluble ECM component Fibronectin is a key modulator in embryogenesis, wound healing, homeostasis and thrombosis. It is comprised of two monomeric subunits attached by two disulphide bonds at the carboxy terminus and is a mosaic protein made up with the modular units Fn1, Fn2 and Fn3. The modules form functional domains that contain other ECM protein binding sites and cell-surface receptors for integrins and blood proteins and through these sites Fibronectin functions as a ECM cell-cell adhesion protein through these sites(Potts and Campbell, 1994).

Tenascin-X is an large ECM glycoprotein (Matsumoto *et al.,* 1992a, 1992b; Bristow *et al.,* 1993). *In vivo* tumourigenic assays in nude mice have shown Tenascin-X to be down-regulated during tumourigenesis (Sakai *et al.,* 1996). The down-regulation of Fibronectin, ECM-cell-cell adhesion protein and Tenascin-X, ECM glycoprotein emphasises the de-regulation of cell adhesion molecules in *v-fos*

transformation.

Lysyl Oxidase was previously cloned as the *ras* recision gene, *rrg* (Contente *et al.,* 1990). The human gene has recently been mapped to chromosome 5q23 (Szabo *et al.,* 1997), a region of loss in non-functioning pituitary adenomas (Daniely *et al.,* 1998), and in pancreatic tumours (Achille *et al.,* 1998). It is a copper amino oxidase that initiates covalent cross-linking between and within the sub-units of collagen and elastin thereby stabilizing the ECM (Smith-Mungo and Kagan, 1998). The enzyme Lysyl Oxidase is a Class II suppressor gene (Lee *et al.,* 1991). In FBRs it is down-regulated. Lysyl Oxidase expression is also down-regulated in Ki-*ras* and EJ-*ras* transformed fibroblasts and spontaneous revertant clones have a restoration of Lysyl Oxidase expression levels. This restoration of expression suppresses anchorage-independent growth effect as opposed to morphological change to a flatter cell shape (Hajnal *et al.,* 1993; Oberhuber *et al.,* 1995). It is also down-regulated in several malignantly transformed human cell lines (Hamalainen *et al.,* 1995). Recently, an Interferon Regulatory Factor- 1 (IRF-1), response element was found in the Lysyl Oxidase gene promoter. The IRF-1 transcription factor and its antagonistic repressor IRF-2 function as regulators of the IFN system, and in addition IRF-1 functions as a tumour suppressor (Sok-Pin Tan, *et al.,* 1996). The regulation of IRF-1 and its down-regulation in transformation would result in the loss of Lysyl Oxidase expression, which could contribute to the transformation process. Analysis of IRF-1 expression in FBR cell line by northern blot detailed a marked down-regulation of IRF-1, however, western analysis of protein extracts were inconclusive. Several Lysyl Oxidase-related genes have recently been cloned (Kim *et al.,* 1995; Saito *et al.,* 1997), with one gene functioning in cellular adhesion and senescence (Saito *et al.,* 1997).

Beta ig-h3 is a Transforming Growth Factor-β-(TGFβ), responsive gene which contains four regions of the RGD motif, a modulator of cell attachment, and a motif similar to an active binding domain of laminin (Skonier *et al.,* 1994). It has been found down-regulated in mesenchymal tumour cells (Shenker and Trueb, 1998), and decreased tumourigenicity is observed when TGF-β transfected CHO cells were injected into nude mice, although no growth inhibition was detected (Skonier *et al.,* 1994). Beta ig-h3 can also function as an anti-adhesion molecule and has been shown to prevent the attachment of several cell types to tissue culture dishes as post-translational modification removes the RGD sequences (Skonier *et al.,* 1994), although in contrast it has been shown to promote the adhesion of dermal fibroblasts (LeBaron *et al.,* 1995). The up-regulation of Beta ig-h3 in *v-fos* transformed cells is most likely related to the protein's anti-adhesion properties, where it may contribute to increased cell motility and invasiveness.

The Type I Collagen contains the polypeptides α1(I) and α2(I) in a ratio of 2:1 and are strictly coordinately regulated and expressed in a tissue-specific manner. The down-regulation of α1(I)Collagen has previously been reported in *ras* transformed fibroblasts (Liau *et al.*, 1985). The transformation by *ras* blocked the expression of the α1(I)Collagen through an intronic AP-1 site (Slack *et al.*, 1995). The stable transfection of α2(I)Collagen in K-*ras* transformed fibroblasts results in a flatter cell morphology with increased substratum adherence, a reduction of colony formation in soft agar, slower proliferation kinetics and suppression of tumourigenicity in nude mice (Travers *et al.*, 1996). The transformation by *ras* requires constitutive AP-1 activity and if blocked, reversion of transformation occurs (Lloyd *et al.,* 1991; Granger-Schnarr *et al.,* 1992; Wick *et al.*, 1992). In SV40 transformed human fibroblasts α2(I)Procollagen synthesis is ablated (Parker *et al.*, 1989).

Collectively, the down-regulation of these key proteins that function to maintain the normal phenotype through structural constraints, cell adhesion properties or via tumour suppressor activity is therefore likely to be a prerequisite to transformation, and cell motility in the FBRs.

This would appear to be a co-ordinated down-regulation of genes that function as ECM and adhesion proteins perhaps suggesting that AP-1 functions as a multigenic switch.

3.3.6 Proteases

Cathepsin B and Cathepsin L (Hennigan *et al.,* 1994), are up-regulated in FBR cell line but Cathepsin H clone was isolated from the down-regulated library. Tissue-type Plasminogen Activator (tPA), and the protease inhibitor Sorting Nexin 3 are down-regulated. The protease component RC6-1 is found to be up-regulated whilst C3 is down-regulated.

The Cathepsins are aspartyl or cysteine proteases that are localized in lysosomes of normal cells, but their expression and release are dramatically increased in transformed cells. The activity, secretion and membrane association of Cathepsin B is increased in malignant tumours particularly in cells at the invasive leading edge. Cathepsin B functions by degrading basement membrane and ECM components (Berquin and Sloane, 1996). The substrates for Cathepsin L also includes the ECM components, Laminin, Fibronectin and Collagen but an acidic pH is required for activity (Mason *et al.,* 1985). Cathepsin L is overexpressed in many human cancers (Chauhan *et al.,* 1991), and expression in Ha-*ras* transformed NIH 3T3s, murine B16 and human A2058 melanoma cells correlates with metastatic ability (Denhardt *et al.,* 1987; Rozhin *et al.,* 1989). Overexpression of Cathepsin L in NIH 3T3 cells does not result in

transformation (Kane *et al.,* 1988). The up-regulation of Cathepsin B cysteine protease in FBRs is perhaps correlated with their function in degradation of basement membrane and ECM components (Berquin and Sloane, 1996), an important step in transformation and cell motility.

The secreted protease Sorting Nexin 3 mediates the binding, internalization and degradation of regulatory serine proteases and thus functions as a protease inhibitor (Knauer *et al.,* 1983). The deregulation of Sorting Nexin 3, coupled with an increase in protease expression, as in FBR *v-fos* transformants, would perhaps prevent the degradation and activity of proteases that are necessary for the maintenance of transformed phenotype and invasiveness.

In FBR *v-fos* transformants there is a down-regulation of tPA, and an up-regulation of Plasminogen Activator Inhibitor-1. PAI-1 is an AP-1 target gene (Keeton *et al.*, 1991), which is often up-regulated in conjunction with urokinase-plasminogen activator. uPA has not yet been isolated in the library, but it is a known target of AP-1 and is implicated in the invasion by many tumour cells (Niedbala and Sartorelli, 1990). Non-neuronal enolase also functions in conjunction with plasminogen activator as a cell surface receptor for uPA (Miles *et al.*, 1991). However tPA has been found down-regulated in a variety of tumours and may not be associated with invasive behaviour of cells.

The changes in expression of proteasome components in FBRs, the up-regulation of RC6-1 and the down regulation of C3 suggests that protease specificity may also be changed in transformed cells. This possibly adds another level of gene regulation to transformation as changes in proteasome components may alter the stability of specific proteins.

3.3.7 Genes with Growth/ Tumour Suppressor function

The down-regulation of CRBPI in human breast cancer cells has recently been reported (Jing *et al.,* 1996), resulting in the deregulation of RA metabolism, thereby abolishing it's anti-proliferative effects. In FBRs CRBPI is also down-regulated although the effect of this growth inhibitor in *v-fos* transformation has not yet been addressed. In FBRs several other genes with a growth/tumour suppressor function have been found to be down-regulated. This includes Necdin, PP2A and Sprouty.

The biological functions of Vitamin A (retinol), requires the metabolic conversion to retinoic acid (RA), which is regulated by the cellular retinoic acid binding protein I (CRBPI). The retinoic acid signalling pathway regulates cell proliferation and differentiation and inactivation of one or more proteins in the pathway can induce carcinogenesis. The actions of RA is through the nuclear receptor families RAR and RXR (Giguere *et al.,* 1987; Petkovich *et al.,* 1987; Krust *et al.,* 1989; Mangelsdorf *et*

al., 1990; Mangelsdorf *et al.,* 1992), that form RAR:RXR heterodimers and RXR homodimers (Leid *et al.,* 1992; Marks *et al.,* 1992). These receptors function as transcription factors, directly regulating gene expression and indirectly through other transcription factors. In a variety of cell types AP-1 activity is inhibited by RA treatment (Schule *et al.,* 1991; Pan *et al.,* 1992; Perez *et al.,* 1994; Van de Klundert *et al.,* 1995; Chen *et al.,* 1995).

The post-mitotic neuronal nuclear protein, Necdin was first isolated from a subtracted cDNA library of neurally differentiated murine embryonal carcinoma cells (Maruyama *et al.,* 1991). It was found to be down-regulated in FBR *v-fos* transformants. Necdin is expressed in all post-mitotic neurons and is contstitutively expressed from early embryonal stages until adulthood (Uetsuki *et al.,* 1996). The differentiated post-mitotic neurons never undergo cell division and are permanently quiescent. Previous studies have implicated the Retinoblastoma protein (Rb), a growth suppressor, with neuronal differentiation-associated growth arrest (Lee *et al.,* 1992). Ectopic expression of Necdin strongly suppresses the growth of NIH3T3 cells (Hayashi *et al.,* 1995), suggesting that it may act as a growth suppressor in post-mitotic neurons.

The nuclear tumour suppressor proteins Rb and p53 interact with small DNA tumour viruses, SV40, human papillomavirus, and Adenovirus transforming proteins (Weinberg, 1991), that target cellular growth suppressors in normal cells. Rb and Necdin both bind independently to the SV40 large T antigen, Adenovirus E1A and also to the transcription factor E2F1, that promotes cell cycle progression. Furthermore, Necdin can functionally replace Rb as a growth suppressor in Rb-deficient SAOS-2 cells, indicating that Necdin acts a growth suppressor similarly to Rb (Taniura *et al.,* 1998).

The Protein Phosphatase 2A (PP2A), is one of the most abundant intracellular protein phosphatases and is a hetereotrimer containing a catalytic (C), subunit, a structural (A), subunit and a variable regulatory (B), subunit. The substrate specificity and activity of PP2A is largely determined by regulatory B subunit. Three unrelated B gene families, each encoding several phosphatase regulatory genes have been identified.

In many human tumours, including lung, colon, breast, cervical, head and neck, ovarian and also melanomas there is a loss of heterozygosity (LOH), at chromosome 11q22-24. The re-introduction of normal chromosome 11 or a derivative (X;11), chromosome containing 11pter-q23 can reverse the tumourigenic potential of lung, breast and cervical carcinoma derived tumour cells when injected into nude mice (Saxon *et al.,* 1986; Satoh *et al.,* 1993; Negrini *et al.,* 1994). This data indicated the

location of one or more tumour suppressor genes at this locus and PP2A was subsequently found to map to this region of LOH. In this study PPP2R1B gene somatic alterations were found in 15% of primary lung tumours, 6% of lung tumour-derived cell lines and 15% of primary colon tumours (Wang *et al.,* 1998), and this strongly suggested tumour suppressor activity.

The PP2A protein regulates the MAPK cascade (Santoro *et al.,* 1998), and is believed to suppress growth by antagonising protein kinase oncoproteins and its loss has been linked to carcinogenesis. It also functions as a G2/mitosis cell cycle checkpoint control (Lee *et al.,* 1991), that is disrupted by a HOX11 oncogene interaction with PP2A (Kawabe *et al.,* 1997). PP2A can inhibit telomerase activity in the nucleus (Zhao, 1997), and is a target of chemical tumour promoters (Kawabe *et al.,* 1997). The DNA tumour viruses Polyomavirus T antigen and SV40 small t antigen binds PP2A-A subunit and displaces subunit B thereby inhibiting phosphatase activity and mediating viral transformation (Pallas *et al.,* 1990; Sontag *et al.,* 1997). In addition, the Casein kinase 2α inhibition of cellular transformation by *ras* oncogene is via an increase in PP2A activity (Heriche *et al.,* 1997).

In FBR *v-fos* transformants there is a down-regulation of PP2A Bα3 isoform. This is perhaps due to it's function as a regulator of the MAPK signalling cascade which can lead to AP-1 activation, or perhaps due to its antagonistic function against protein kinase oncoproteins.

The Sprouty protein belongs to a new family with a highly conserved cysteine-rich domain that is a membrane-associated or secreted signalling protein that function as antagonistic inhibitors of FGF. Recent studies of sprouty in *Drosophila* airway developmental patterning using sprouty mutants (Hacohen *et al.,* 1998), discovered that it inhibits branch budding in trachea by antagonising the Bnl FGF pathway. The mammalian Sprouty homologues are involved with FGF signalling pathways in developing tissues and they function as an inhibitor of FGF. Several recently discovered branching inhibitors have recently been shown to be tumour growth suppressors and they function by blocking angiogenesis (Hanahan and Folkman, 1996).

Recently, the murine homologue of *Drosophila* Sprouty was cloned and shown to have a conserved function in negatively modulating respiratory organogenesis (Tefft *et al.*, 1999).

Casci and co-workers (1999), discovered that Spouty is an intracellular inhibitor of *ras* signalling in *Drosophila*. In contrast to Hacohen and co-workers (1998), who reported that Sprouty was extracellular, Casci and co-workers (1999), discovered that Sprouty is an intracellular protein that associated with the inner surface of the plasma

membrane. Furthermore, Sprouty was found to bind to two intracellular components of the *ras* pathway, Drk1 (homologous to mammalian Grb2), and Gap1. Sprouty controls Ras/MAPK signalling directly by inhibiting signal transduction between the receptor and Ras. The intracellular localisation of Sprouty permits the inhibition of the intracellular signal transduction pathway and thus acts upon several receptor tyrosine kinases (RTK), (Casci *et al.*, 1999). Interestingly, the expression of Sprouty is dependent upon the Ras/MAPK pathway's activity, therefore suggesting negative feedback (Hacohen *et al.*, 1998; Casci *et al.*, 1999).

Several human homologues of Sprouty have been cloned (Hacohen *et al.*, 1998), and a recent study (Tefft *et al.*, 1999), has reported Sprouty to be an antagonistic inhibitor of FGF signaling in the mammalian system. Presumably murine Sprouty also functions as an inhibitor of *ras* signaling.

3.3.8 Regulatory proteins

The regulatory proteins 4-3-3ζ, and Oncomodulin were found to be up-regulated in FBRs. SNAP-23 and Zygin 2 were found to be down-regulated.

The 14-3-3 proteins are a highly conserved family of acidic proteins and several mammalian isoforms have been found, β, γ, ε, ζ, η and τ and are expressed in a tissue-specific manner. *In vivo*, these proteins form homo- and hetero-dimers (Jones *et al.*, 1995), and contain an annexin-like domain at their centre (Liu *et al.*, 1995). Crystal structure analysis reveals a dimeric structure consisting of α-helical bundles arranged around an amphipathic groove. Invariant residues line the groove implicating a ligand-binding surface on each subunit and this cleft potentially functions as a binding site to alter bound protein interactions (Lui *et al.*, 1995; Xiao *et al.*, 1995). Multiple functions of 14-3-3 proteins have been reported including activation of enzymatic neurotransmitter synthesis, interaction of IGF receptor (Furlanetto *et al.*, 1997), interactions with c-Cbl in a yeast two hybrid screen (Robertson *et al.*, 1997), and the regulation of Protein Kinase C (PKC), activity (Aitken *et al.*, 1992). PKC is required for focal adhesion formation in fibroblasts (Woods and Crouchman, 1992).

The binding protein 14-3-3ζ is a phosphoserine (pSer)-binding protein that interacts directly with oncogene products and components of the mitogenic and apoptotic signalling pathways suggesting a role in growth regulation. *In vitro*, Raf-1 binds and forms a complex with 14-3-3 (Li *et al.*, 1995), in the cytosol and Raf-1 is displaced at the plasma membrane for recruitment and activation by Ras, after which Raf-1 is recycled to the cytosol by 14-3-3 (Roy *et al.*, 1998). The 14-3-3 proteins also interact with MEKK1, -2 and -3, but do not affect kinase activity (Fanger *et al.*, 1988). This data suggests that 14-3-3 proteins regulate signal transduction by acting as a

scaffold to localise protein kinase activity rather than activating or inhibiting kinase activity.

The up-regulation of a 14-3-3ζ protein in FBR *v-fos* transformants is presumably two-fold. Firstly it functions in the regulation of PKC thereby decreases cell adhesion contacts and increasing motility. Secondly, it also functions in trafficking the Raf-1 and MEKK-1, -2 and -3 proteins which are downstream effectors of Ras in the mitogenic signalling pathway whereby *c-fos* and *c-jun* are induced and AP-1 is activated. However in FBRs, not only is *c-fos* transrepressed but the Raf-1 pathway is switched off (K.Katsanakis, unpublished observations), and perhaps the up-regulation of 14-3-3ζ is caused by the accumulation of these redundant trafficking molecules.

The transport and exocytosis of proteins along the secretory pathway in eukaryotic cells requires the docking and fusion of the cytoplasmic vesicles to the cell membranes via the trimeric complex of vesicle-associated membrane complex protein (VAMP), syntaxin and a SNAP protein (Sollner *et al.,* 1993). The synaptosomal-associated protein-23kD, (SNAP23), was identified as a syntaxin 4-binding protein in a yeast two-hybrid system screen of a human B lymphocyte cDNA library and is an isoform of one of the SNARE proteins SNAP-25 (Ravichandran *et al.,* 1996). In various cell types different isoforms of VAMP and syntaxin have been reported (Bennett *et al.,* 1993; McMahon *et al.,* 1993; Calakos *et al.,* 1994; Rossetto *et al.,* 1996; Ravichandran *et al.,* 1996), however SNAP-25 expression is exclusive to neuronal tissues (Bark *et al.,* 1995), whereas in contrast SNAP-23 is widely expressed in most tissues (Ravichandran *et al.,* 1996). SNAP-23 is also induced by various cytokines *in vitro* (Morikawa *et al.,* 1998). A major signal transduction pathway that functions downstream of the cytokines receptors is the Ras-Raf-MAPK cascade that induces *c-fos* and *c-jun* (Satoh *et al.,* 1992; Duronia *et al.,* 1992; Schlessinger and Ullrich, 1992). Recent deletion analysis studies of the bc subunit of cytokine receptors indicated that SNAP-23 was a downstream target of the Ras-Raf-MAPK pathway (Morikawa *et al.,* 1998). The down-regulation of SNAP-23 in FBRs may be due to the down-regulation of the Raf-MAPK pathway (K.Katsanakas, unpublished observations), and therefore a downstream target of this pathway, SNAP-23 is also down-regulated.

The Zygin 2 protein is also known as FEZ2 (fasciculation and elongation protein). The proteins of the FEZ family, FEZ1, FEZ2 and C.elegans unc-76 have poor similarity to other proteins and represent a new protein class involved in axonal outgrowth. The N-terminal regions of the proteins are highly acidic and structural analysis revealed that large regions of the protein would form amphipathic helices, although there are no identifiable motifs. The FEZ family members are believed to be intracellular due to the absence of a signal sequence or transmembrane domain and

the cellular localisation of the unc-76 protein is in the cell bodies and axons of most neurons. Studies to determine the function of unc-76 using mutants indicated two possible functions for the FEZ family members consistent with the intracellular localisation and fasicle-specific defects of the mutant protein in developing C.elegans. The unc-76 protein may have a structural role in the formation and maintenance of fasicles through an intracellular association with a cell surface adhesion molecule, axonal membrane or the cytoskeleton. Otherwise, the unc-76 protein could transduce signals from cell-surface molecules to the intracellular machinery that regulates axonal extension and adhesion (Bloom and Horvitz, 1997). A recent two-hybrid interaction screen in yeast with a rat homologue of FEZ1 was shown to bind the C1 regulatory domain of protein kinase C types ζ and ε, and these rat homologues of FEZ1 and FEZ2, Zygin I and II were identified as synaptotagmin-binding proteins. As previously discussed, PKC is involved in the modulation of adhesion contacts and membrane structure, whilst the synaptogtagmins are involved in membrane docking and fusion in nonneuronal cells. These interactions coupled with the unc-76 mutant data would suggest that FEZ family members are important for the regulation of cellular morphology. The functions of Zygin II pertaining to increased cell surface adhesion, the maintenance of cytoskeleton, coupled with regulation of outgrowth would correspond with the down-regulation found in FBR *v-fos* transformed cells.

Oncomodulin, is a small, acidic calcium binding protein and was initially discovered in rat heptoma (MacManus, 1979), and is up-regulated in AP-1 transformed fibroblasts. It's expression is also found in blastocyst and placental cytotrophoblasts (Brewer and MacManus, 1985; Brewer and MacManus, 1987), and is elevated in a wide variety of rodent and human tumours but no expression has been detected in normal rodent or human adult tissues (MacManus *et al*, 1984; Durkin *et al.,* 1983). Recently, it has been identified as Parvalbumin β-isoform and it's expression in the outer hair cells of the organ of corti of mouse, gerbil, and rat has been reported (Sakagichi *et al.,* 1998). Although the function of Oncomodulin has yet to be elucidated, it has been discovered that in chemically transformed fibroblasts an increase in transcript and protein levels at the G1/S boundary and this would suggest a regulatory Ca^{2+}-dependant role in the cell cycle (Blum and Berchtold, 1994). Recent studies on cells transfected with Parvalbumin resulted in morphological shape changes and an increase in cell motility (Andressen *et al.,* 1995), and the presence of Oncomodulin in tumours and in invasive cytotrophoblasts suggests a function of Ca^{2+}-dependant motility important for invasive behaviour (Pauls *et al.,* 1996). It is postulated that the up-regulation of Oncomodulin in FBR *v-fos* transformants contributes to cell motility and invasiveness.

3.3.9 Cell Cycle Regulatory proteins

MCM5.CDC46 is down-regulated and Cyclin A2 is up-regulated in FBR *v-fos* transformants.

The cell cycle in eukaryotic cells strictly limits the duplication of the genome to once per cycle. The replication of DNA is initiated from sequences called origins which ensures the duplication of chromosomes and a block to replication until the segregation of chromosomes during mitosis occurs. The conserved minichromosome maintenance (MCM), gene family are necessary for DNA replication and can modify or license replication-incompetent chromatin to a replication-competent state. They are constitutively expressed in the nucleus but only bind chromatin in late mitosis to early S phase as DNA replication progresses (Kimura *et al.,* 1994; Kubota *et al.,* 1996; Romanowski *et al.,* 1996; Coue *et al.,* 1996). The MCM5/CDC46 DNA replication-licensing factor bound to chromatin during G1 blocks DNA replication and the kinase CDC7 is required to bypass or inactivate the block (Hardy *et al.,* 1997).

The cell cycle is monitored by a complex biochemical process whereby Cyclin Dependent Kinases (CDKs), are sequentially activated by a variety of cyclins. The consecutive activation of cyclin/CDK complexes permits the progression of the cell through the cell cycle. The protein cyclin A2 accumulates in somatic cells from the end of G1 until the metaphase of mitosis. Cyclin A2 binds to and activates CDK2 in G1 and S phases and CDK1 in G2 and M (reviewed by Nurse, 1994).

The isolation of cell cycle regulatory genes from the up-regulated and down-regulated libraries would suggest that AP-1 may function in the regulation of cell cycle control and thus cell survival.

3.3.10 Metabolism and Ribosomal proteins

The differential expression of the metabolic, ribosomal and protein synthesis proteins in tumour cells has previously been documented (Zhang *et al.,* 1997; Gress *et al.,* 1996). In FBR *v-fos* transformants there is an up-regulation of lactate dehydrogenase, glyceraldehyde-3-phosphate (Hennigan *et al.,* 1994), dehydrogenase, and cytochrome oxidase is observed and this has previously been reported in a pancreatic cancer-specific expression profile (Gress *et al.,* 1996). This increase in aerobic glycolysis pathway proteins is assumed to be a results of increased proliferation and energy metabolism of transformed cells. In contrast, several metabolic, in particular the dehydrogenases and ribosomal proteins are also down-regulated.

Reverse Northern hybridisation experiments (data not shown), revealed that most ribosomal clones were not differentially expressed which would indicate that they 19 ribosomal protein was previously reported in *v-fos* transformants, tumour cells (Chiao *et al.,* 1992), pancreatic tumours (Gress *et al.,* 1996), and in gastrointestinal tumours (Zhang *et al.,* 1997).

3.3.11 EST Homologies to proteins

The analysis of clones that do not match any genes submitted in the Genebank nr database were subject to analysis to EST database entries. The EST sequences provided more information about the clone and can hint at a possible function. Several of the down-regulated clones exhibited homology to EST entries with homology to reticulocablin homologue, a gene already found in this library. Reticulocalbin (RCN), is a member of the EF-hand Ca(2+)-binding protein family and is a luminal protein of the endoplasmic reticulum (ER), (Ozawa and Muramatsu, 1993). Although RCN has six repeats of a domain containing an EF-hand motif, the varying degrees of divergence of the amino acid sequences of these domains from the EF-hand consensus sequences suggested that some domains might have lost their Ca(2+)-binding capability and adopted new functions. Another down-regulated clone is similar to lysyl oxidase.

3.3.12 Summary

The changes in expression of the transformation specific proteins upon transformation by *v-fos* would implicate AP-1 as the activation switch to initiate and maintain the transformed state.

Interestingly, the transformation specific proteins isolated in this study not only includes modulators of the ECM and cytoskeleton such as proteases, MMPs and cell adhesion molecules but also regulatory proteins and also growth/tumour suppressor proteins. Once a cell has become fully transformed the alterations in gene expression reflect the new steady state integration of changes in signal transduction pathways and transcription factor activity responsible for the maintenance of transformation.

However, many of the genes isolated in this study encode for proteins that have not previously been associated with transformation and invasion. This includes proteins associated with protein synthesis, metabolism, vesicle transport, transcription, signal transduction and cell cycle regulatory proteins. This would indicate that AP-1 transcription factor controls additional cellular processes other than transformation and invasion.

Chapter 4

Characterisation Of *v-fos* Suppressive Subtractive Hybridisation cDNA Library Clones

4.1 INTRODUCTION

The sequenced clones from the up-regulated and down-regulated cDNA libraries were assumed to be differentially expressed between FBR and 208F cell lines based upon the subtraction controls performed. Northern blot analysis was used to characterise the sequenced clones to verify and quantify their differential expression. It would also reveal the size and splicing patterns of the novel transcripts. The differential expression of the clones was extended beyond 208F versus FBR to include characterised signal transduction pathways that function upstream of AP-1, to induce or inhibit function. This would test the association of the sequenced clones with transformation pathways that function through AP-1, such as the *ras* oncogene and EGF growth factor. This included 208F fibroblasts treated with EGF (Epidermal Growth Factor), and 208F fibroblasts stably transformed with Kirsten-*ras* (RAS). FBR fibroblasts expressing the dominant-negative *jun* deletion mutant, Tam-67, were also analysed to determine which sequenced clones required AP-1 activity for their differential expression.

This chapter reports the expression patterns of a representative fraction of sequenced clones from the up- and down-regulated cDNA libraries, that may function as transformation effectors of AP-1.

4.2 CHARACTERISATION OF EXPRESSION

4.2.1 Northern Blot analysis

Northern blot analysis was performed using 10 μg total RNA samples from logarithmically growing 208Fs; FBRs; RAS; EGF treated 208Fs, (refer to methods); and FBR-Tam67 partial revertants. The Northern blots were probed with various cDNA clones isolated from the up-regulated and down-regulated libraries.

The loading levels were determined from photographs of the RNA gels prior to transfer to nylon membranes and by probing with cDNA for the 7S ribosomal RNA, (refer to Figure 4.1).

Twenty-two clones from the down-regulated cDNA library were characterised by Northern blot analysis, nineteen of which are reported in this Chapter. Their expression and loading controls are shown in Figures 4.2-4.11. The remaining three down-regulated clones are detailed in subsequent chapters. Fourteen sequenced clones from the up-regulated cDNA library were characterised by Northern blot analysis, all of which are reported in this Chapter. Their expression and loading controls are shown in Figures 4.12-4.18. Exposure times of the hybridised northern blot to X-ray film is included as an indication of the relative abundance of a cDNA clone in a messenger RNA population.

Quantitation of expression indicates the differentially expressed sequenced clones represent abundant and rare mRNAs, based upon densitometric analysis (data not shown), and the time of exposure. In addition, the different expression patterns and sizes of the mRNAs revealed by northern analysis indicated that the cDNAs represent distinct mRNAs as would be expected due to the normalisation step in the libraries' construction.

Figure 4.1

Ethidium Bromide Stained RNA Gel prior to Northern Blot Transfer

The gel photograph prior to transfer shows the RNA ladder markers in the first lane and the corresponding size on a cm ruler. The subsequent lanes show 10μg of total RNA from the various cell lines used as detailed above the lanes.

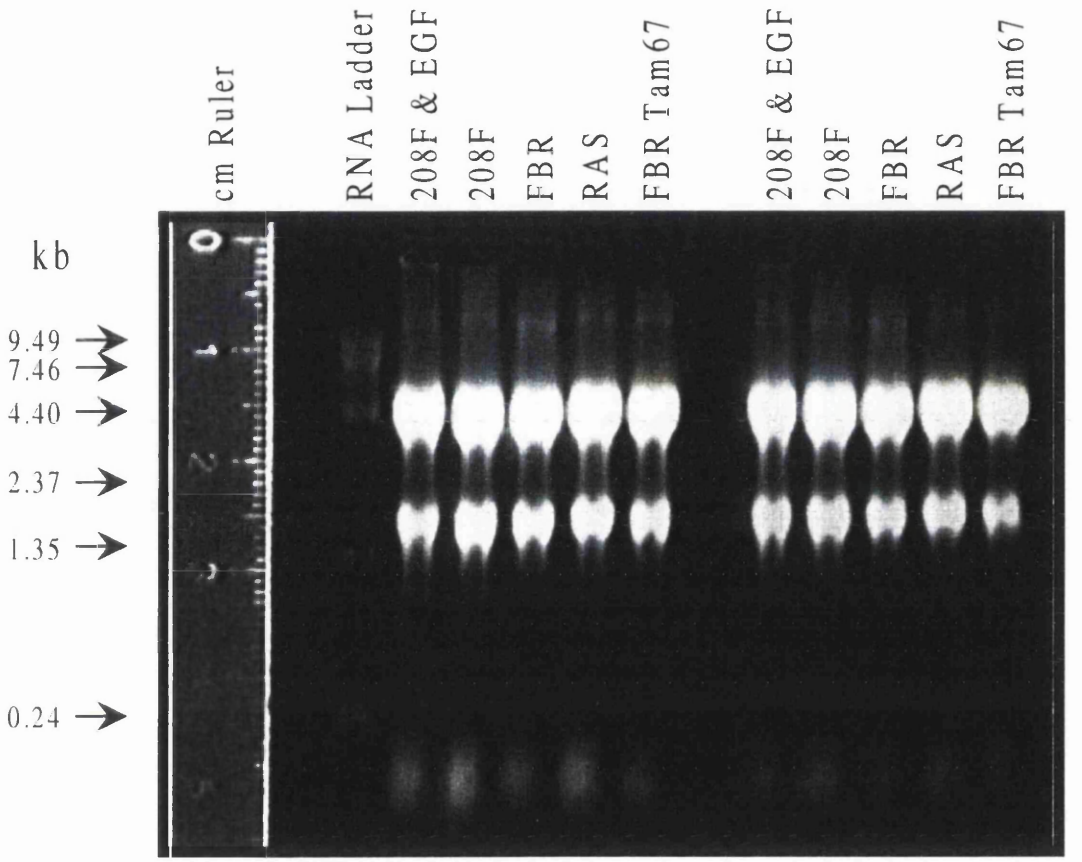
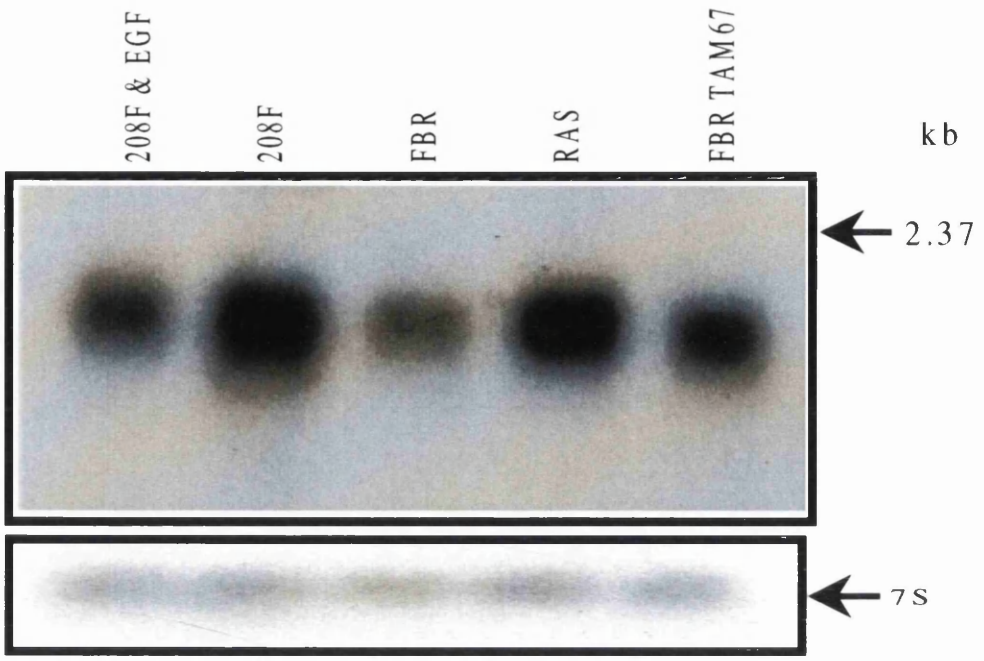


Figure 4.1
Ethidium bromide stained RNA gel prior to Northern Blot Transfer

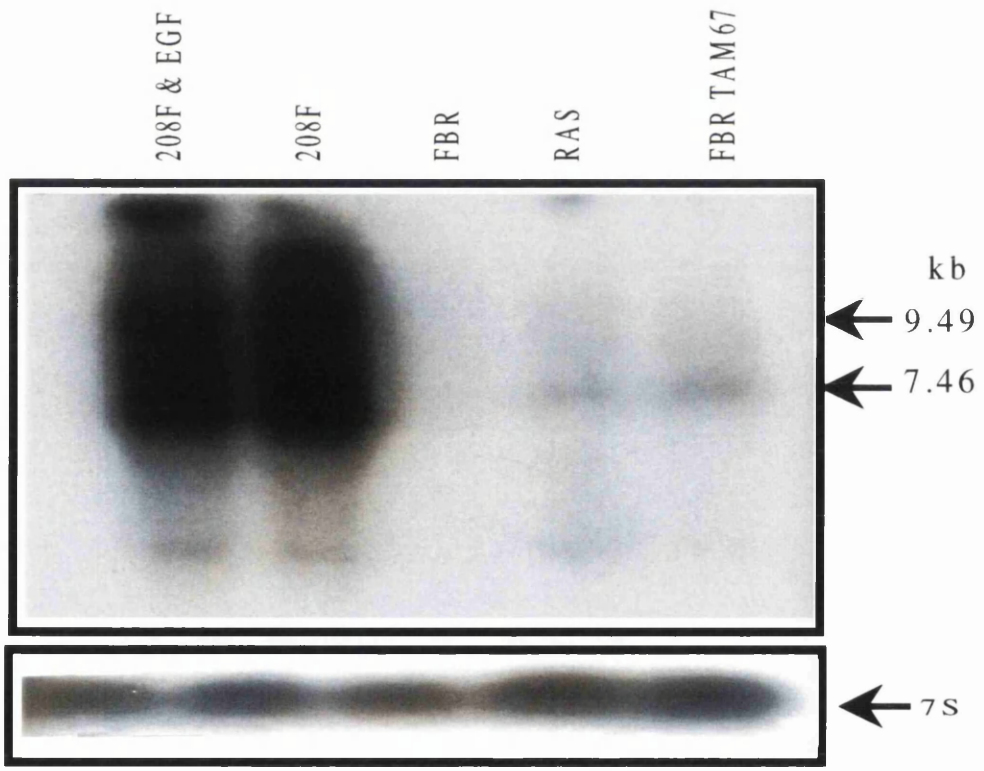
Figure 4.2-4.18

Northern Blots and 7S Control

These figures detail the scanned X-ray film of the exposed Northern blots and the corresponding scanned X-ray film of the exposed 7S Ribosomal loading control. The size of the mRNA transcript is indicated by arrows with regards to the RNA ladder in kilobases. The clone nomenclature, database identity and exposure times of the Northern blots in days are detailed below each blot in the figures.

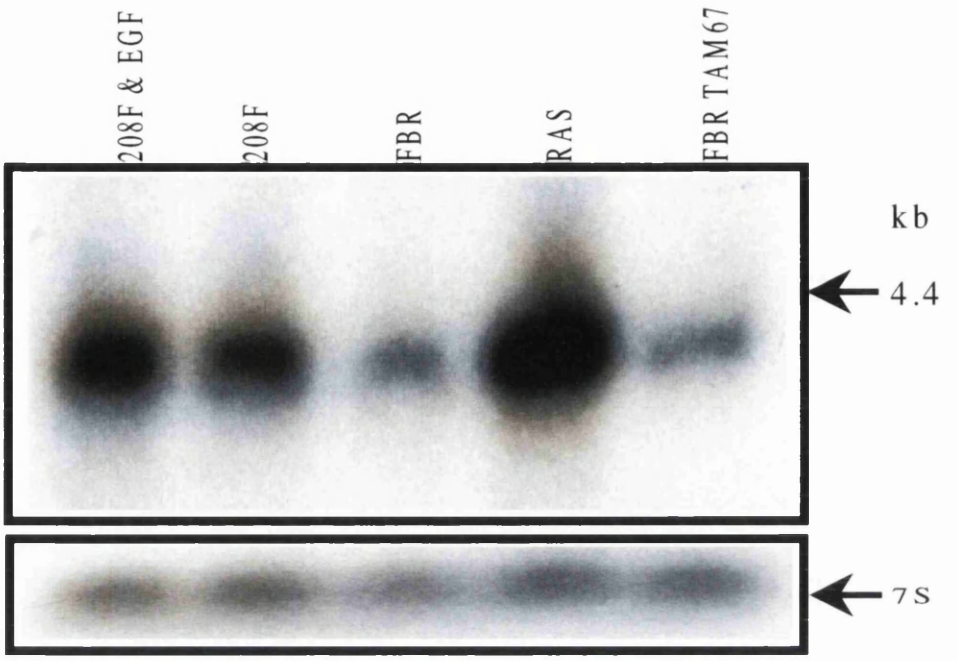


Clone AD3, Protocadherin 43(2), 3 Day Exposure

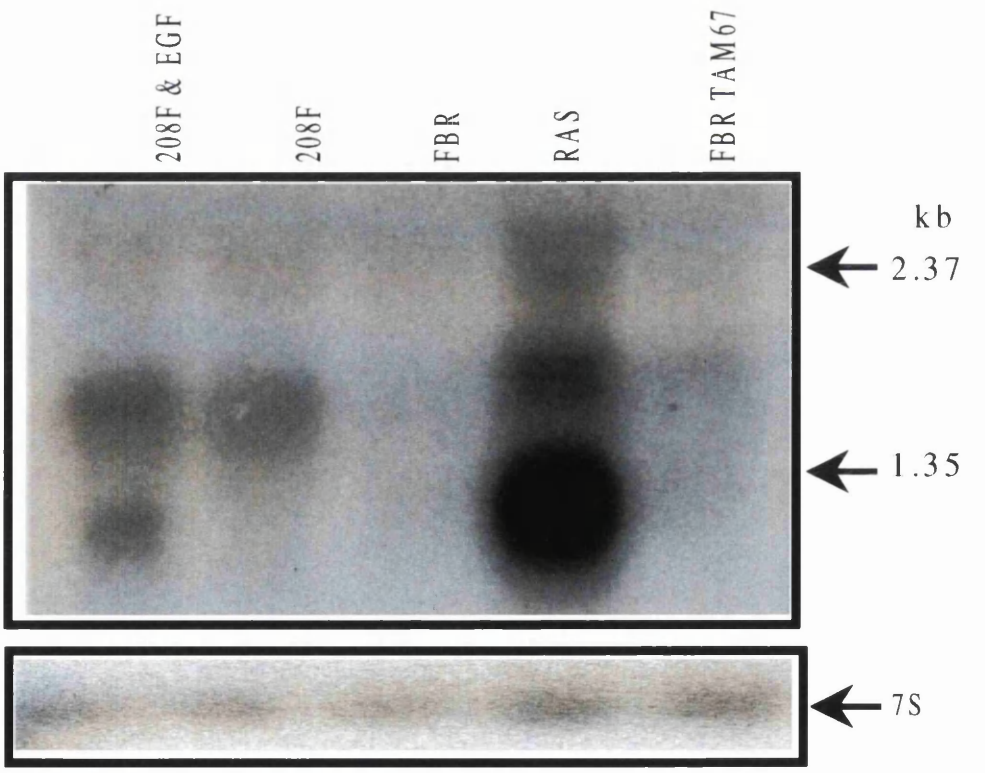


Clone A12, Fibronectin, 1Day Exposure

Figure 4.2
Northern Blots and 7S contol

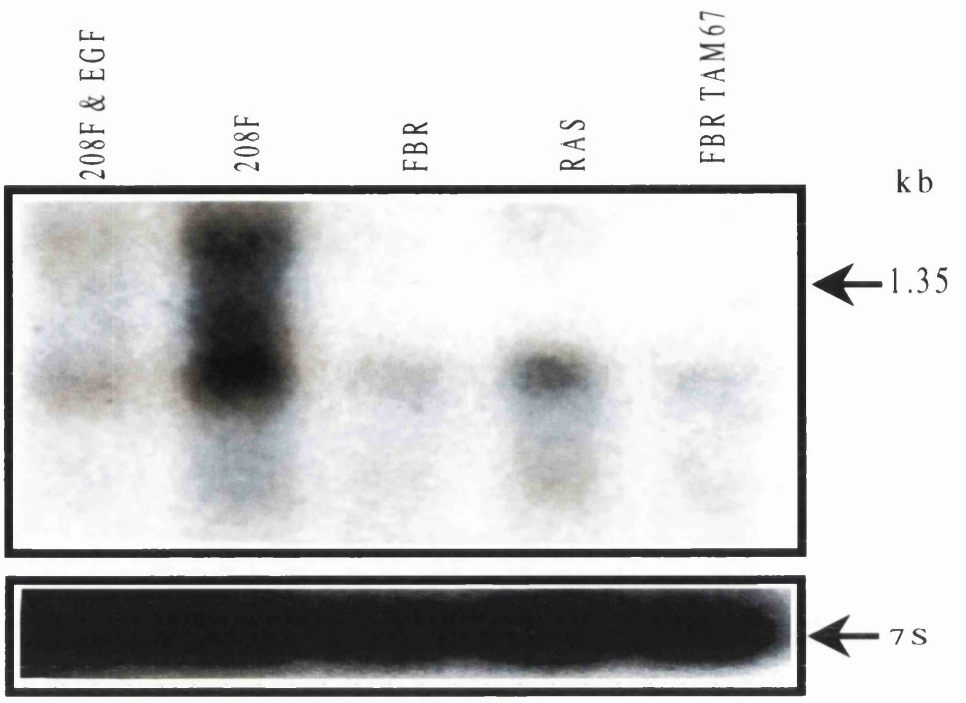


Clone AD16, STAT-6, 3 Day Exposure

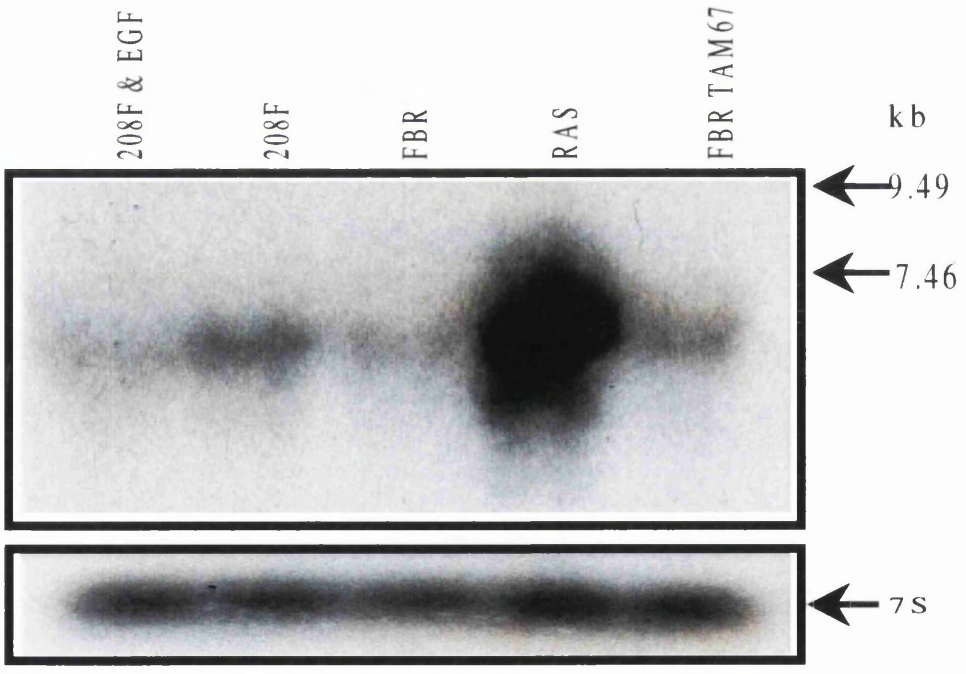


Clone BD3, Zygin II, 3Day Exposure

Figure 4.3
Northern Blots and 7S contol

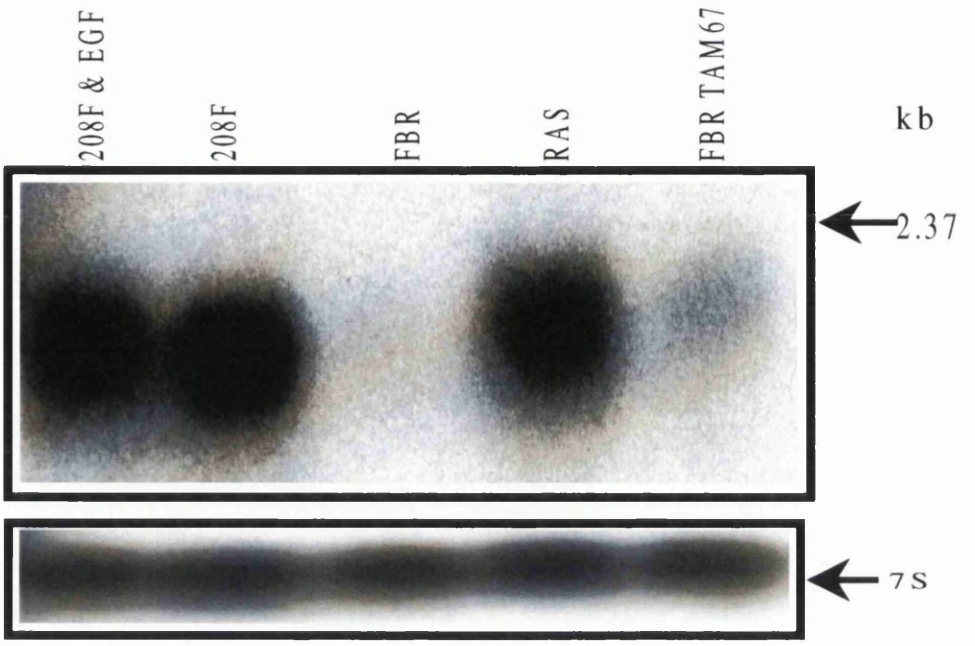


Clone DD13, Novel, 3 Day Exposure

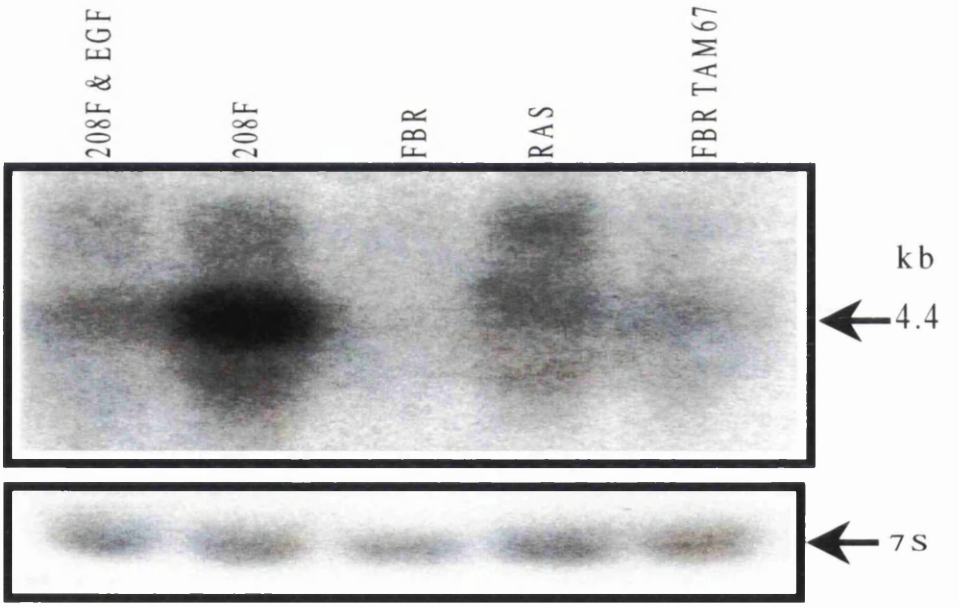


Clone ED2, ERK3, 1Day Exposure

Figure 4.4
Northern Blots and 7S contol

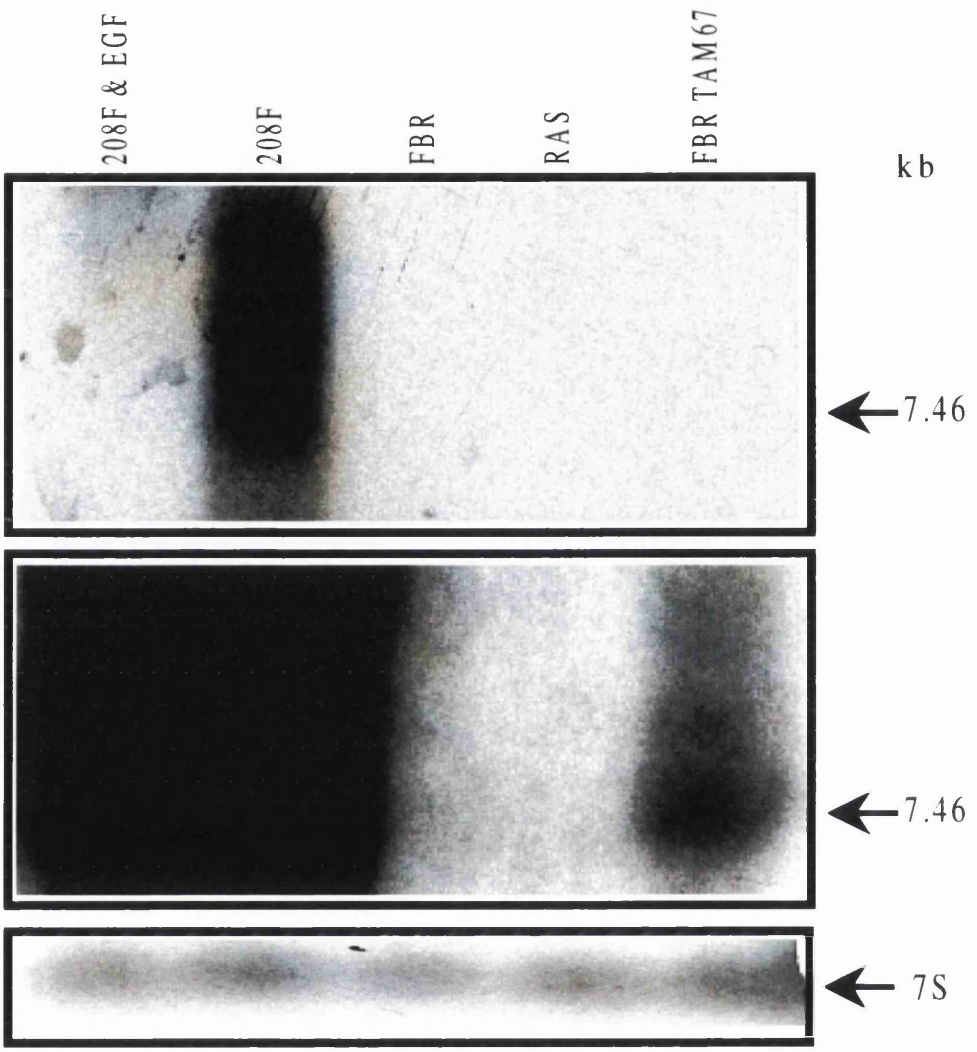


Clone IU3, Novel, 2 Day Exposure



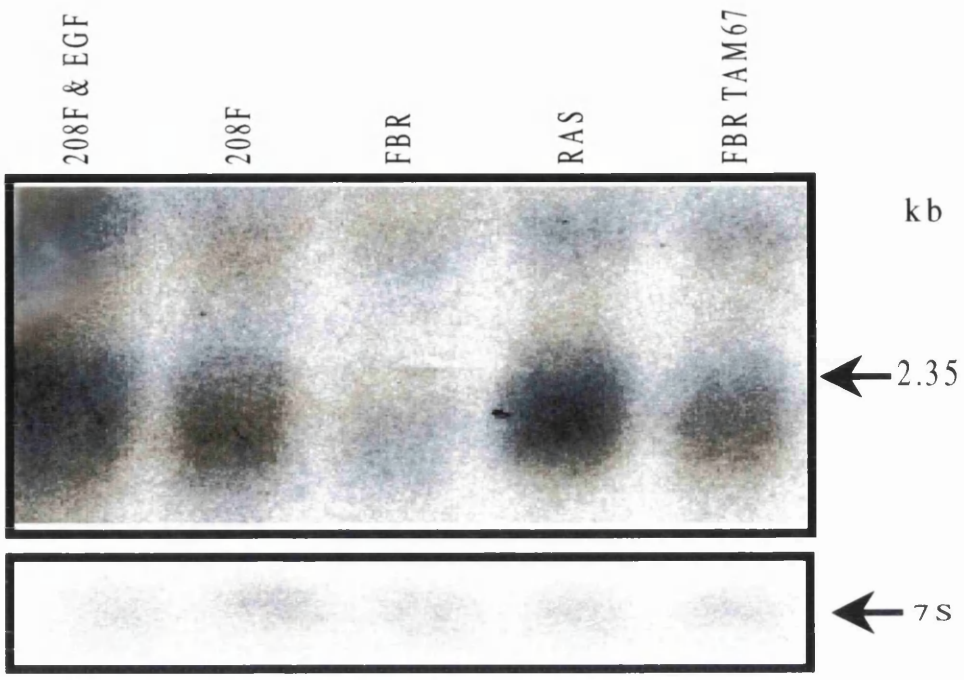
Clone ED12, Novel, 3 Day Exposure

Figure 4.5
Northern Blots and 7S contol

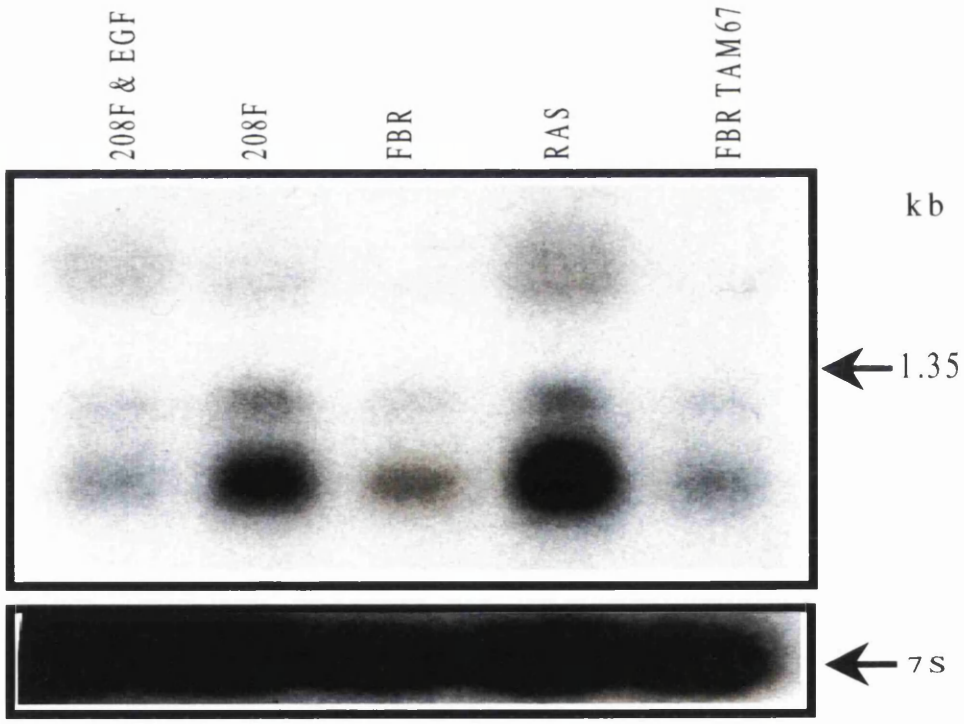


Clone GD2 Novel, 4 Hour Exposure, above;
and 3 Day exposure, below

Figure 4.6
Northern Blots and 7S contol

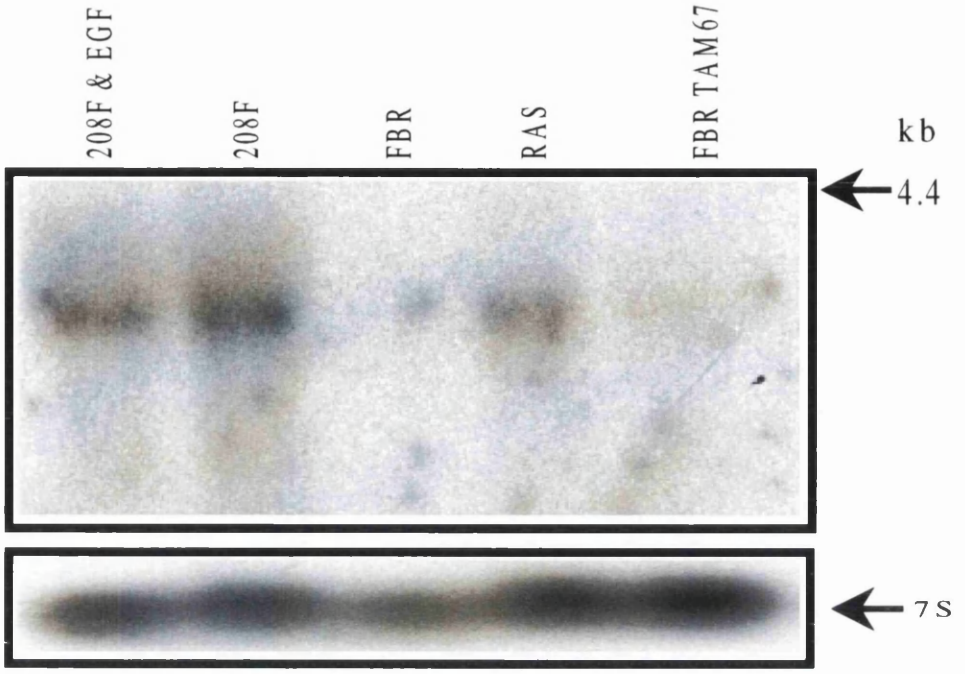


Clone JD2, Caspase 11, 3 Day Exposure

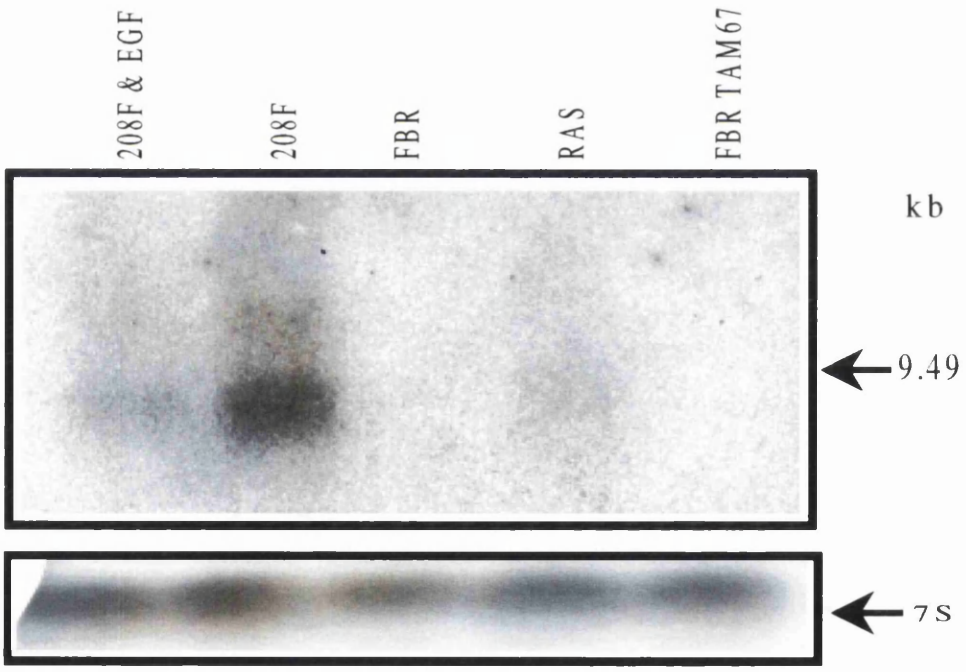


Clone JD12/15 Novel, 1 Day Exposure

Figure 4.7
Northern Blots and 7S contol

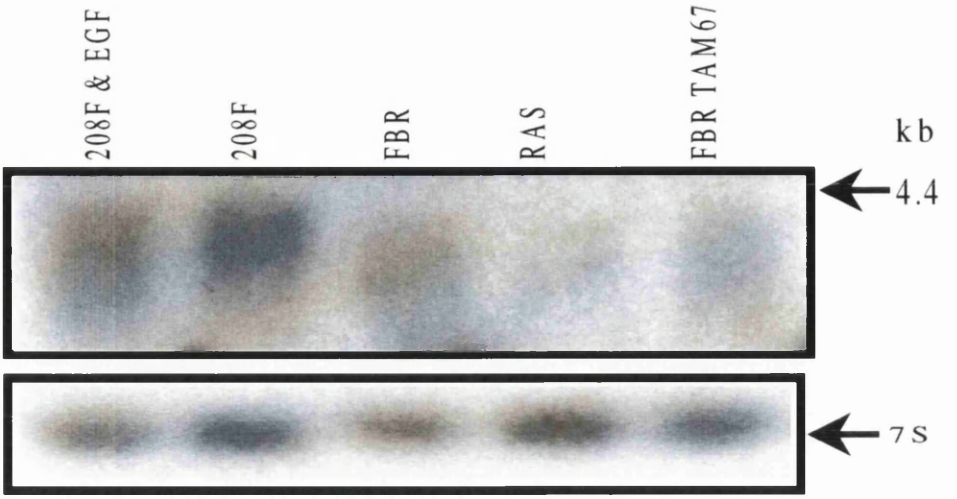


Clone JD18, Lysyl Oxidase, 3 Day Exposure

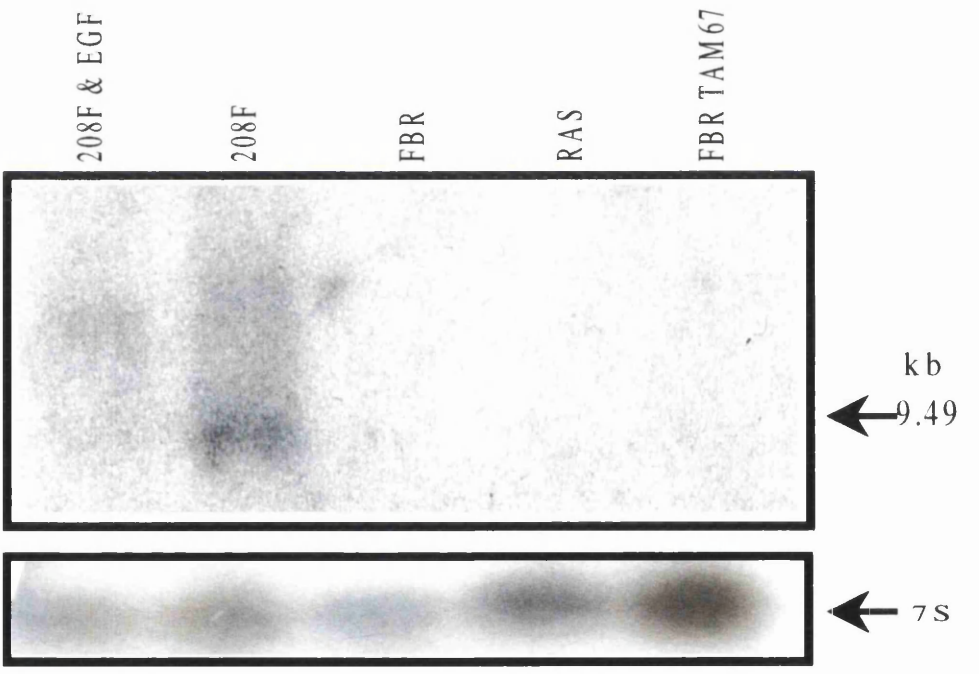


Clone KD8, Latent TGFβ Binding Protein, 13 Day Exposure

Figure 4.8
Northern Blots and 7S contol

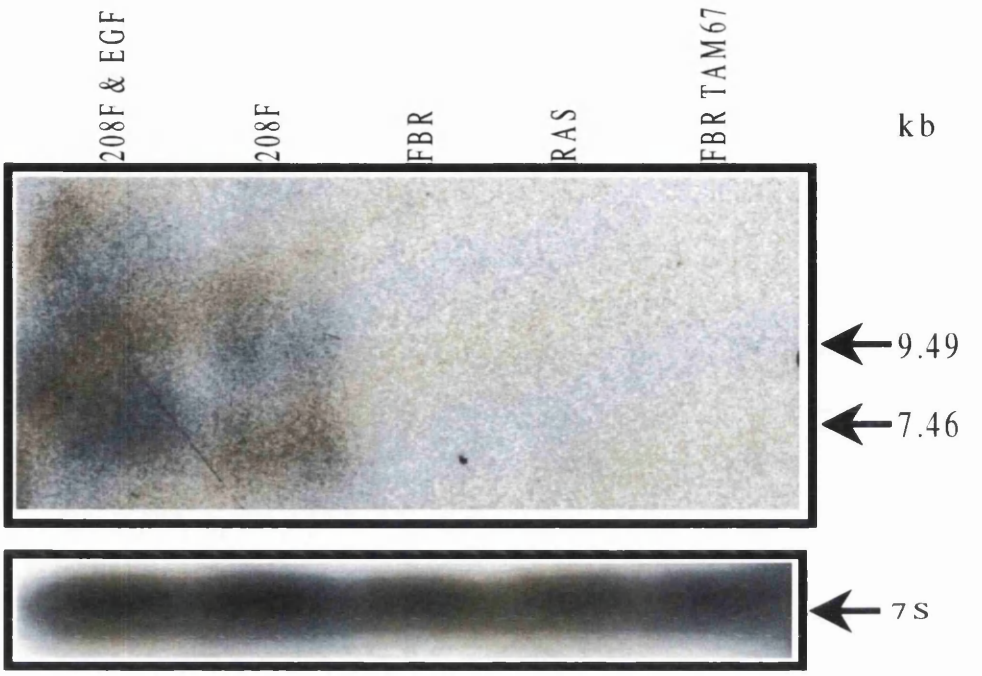


Clone KD10, MCM 5/CDC46, 3 Day Exposure

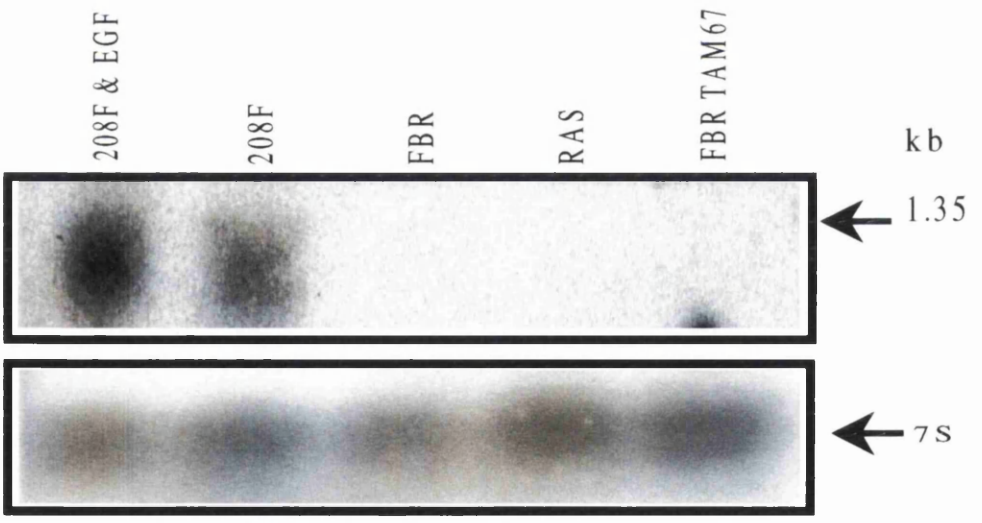


Clone LD3, Novel, 2 Day Exposure

Figure 4.9
Northern Blots and 7S contol

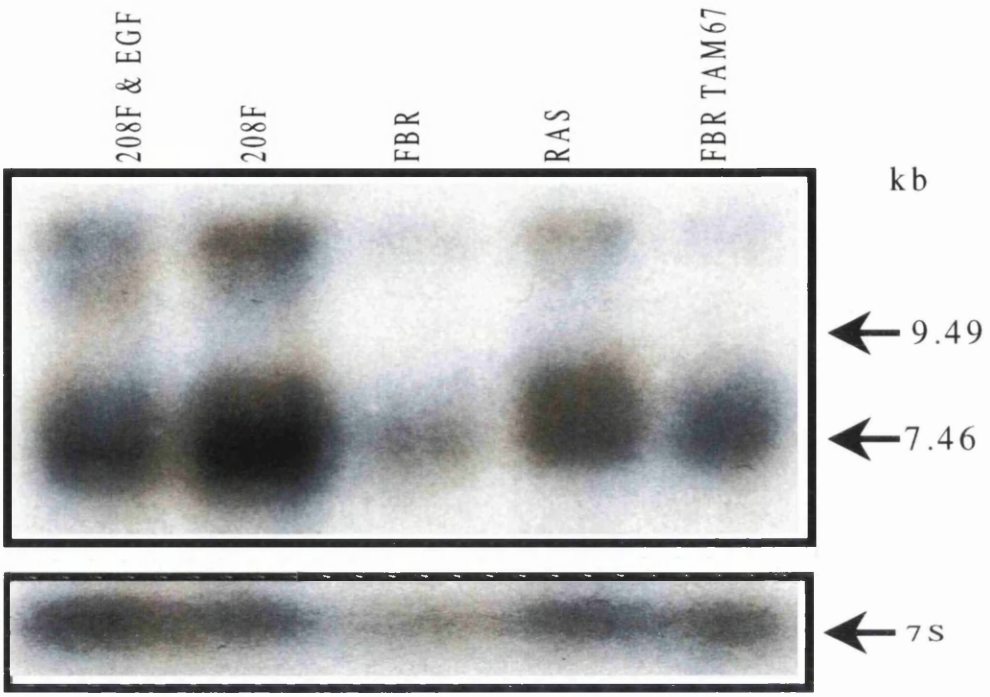


Clone OD2, Novel., 13 Day Exposure

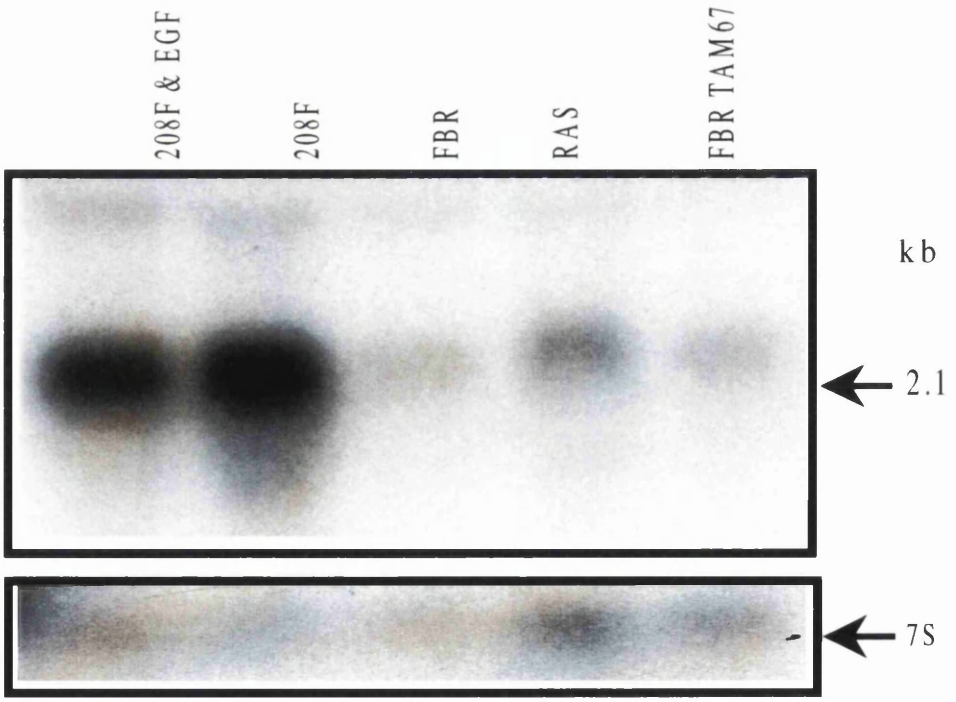


Clone PD6, CRBPI, 1 Day Exposure

Figure 4.10
Northern Blots and 7S contol

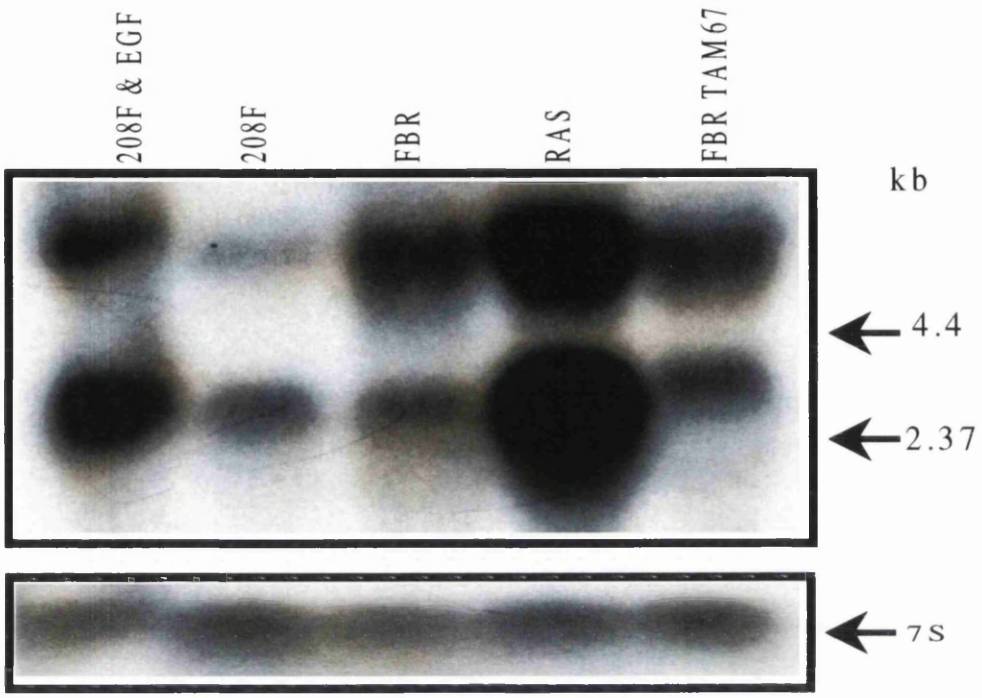


Clone PD18, Novel, 2 Day Exposure

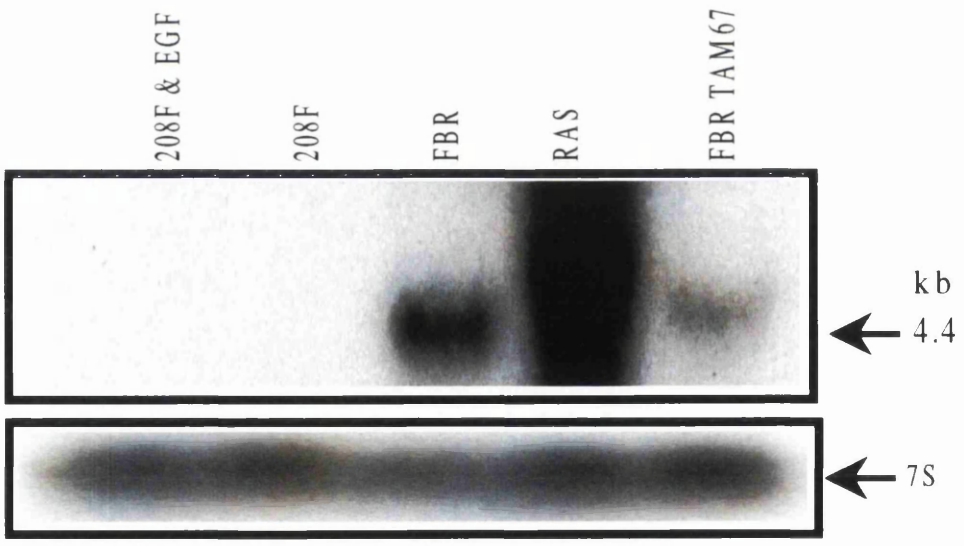


IRF-1, 2 Day Exposure

Figure 4.11
Northern Blots and 7S contol

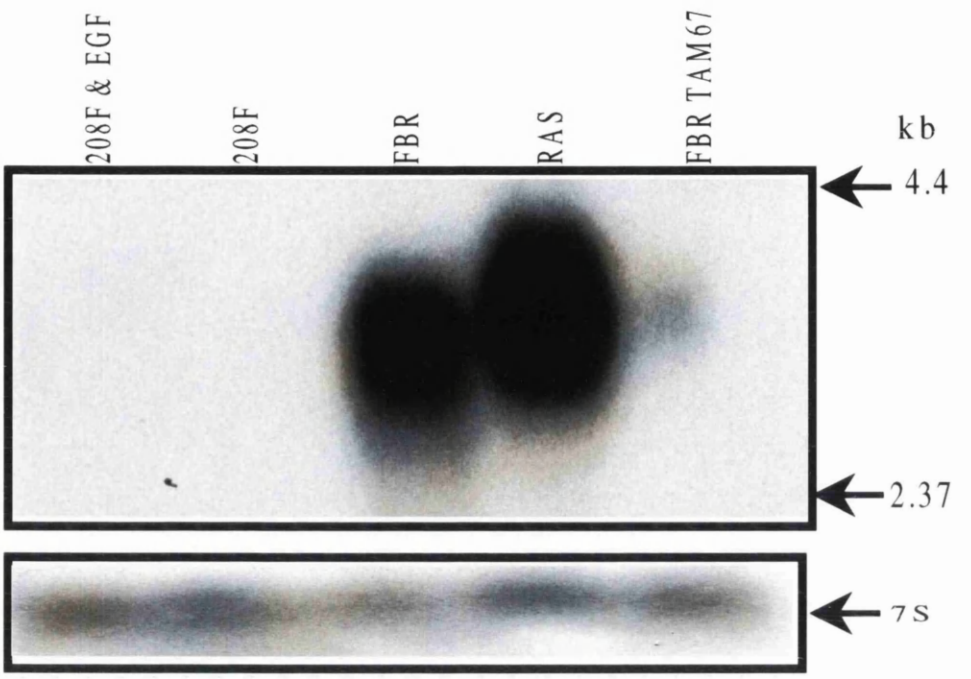


Clone AU5, Cathepsin B, 3 Day Exposure

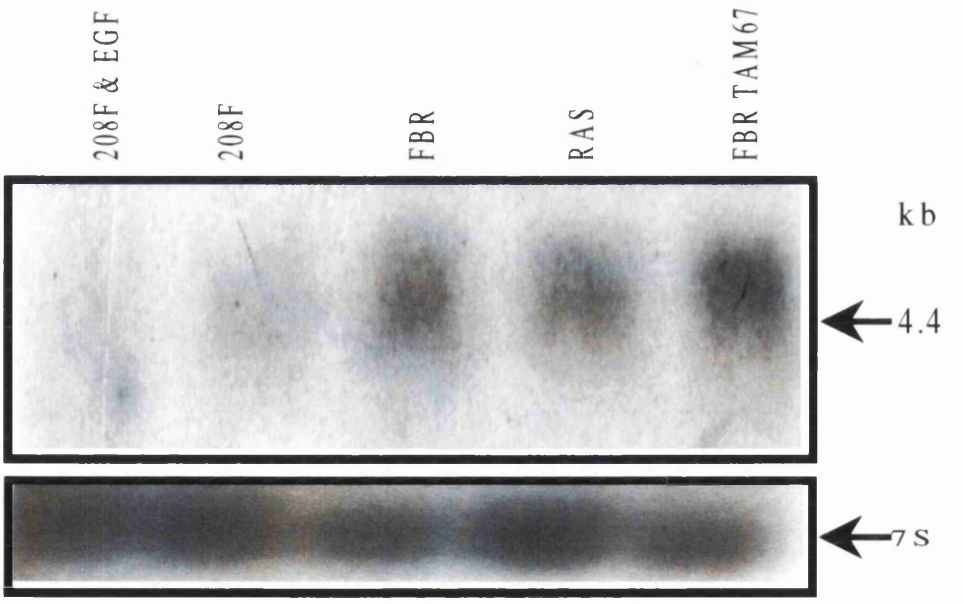


Clone AU12, FBR-gag-fos, 3 Day Exposure

Figure 4.12
Northern Blots and 7S contol

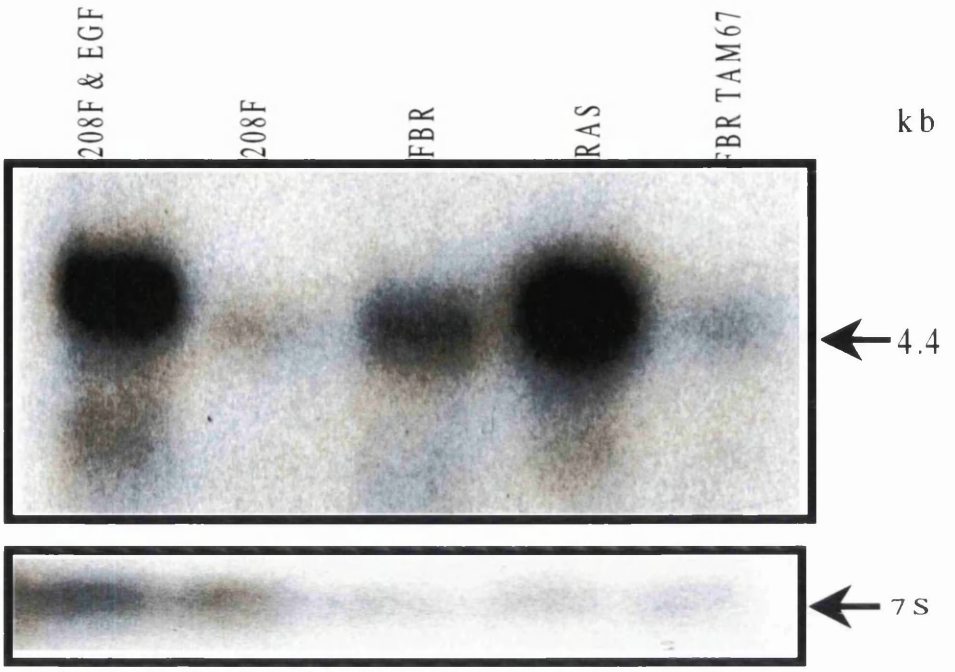


Clone BU4, MMP-10, 3 Hour Exposure

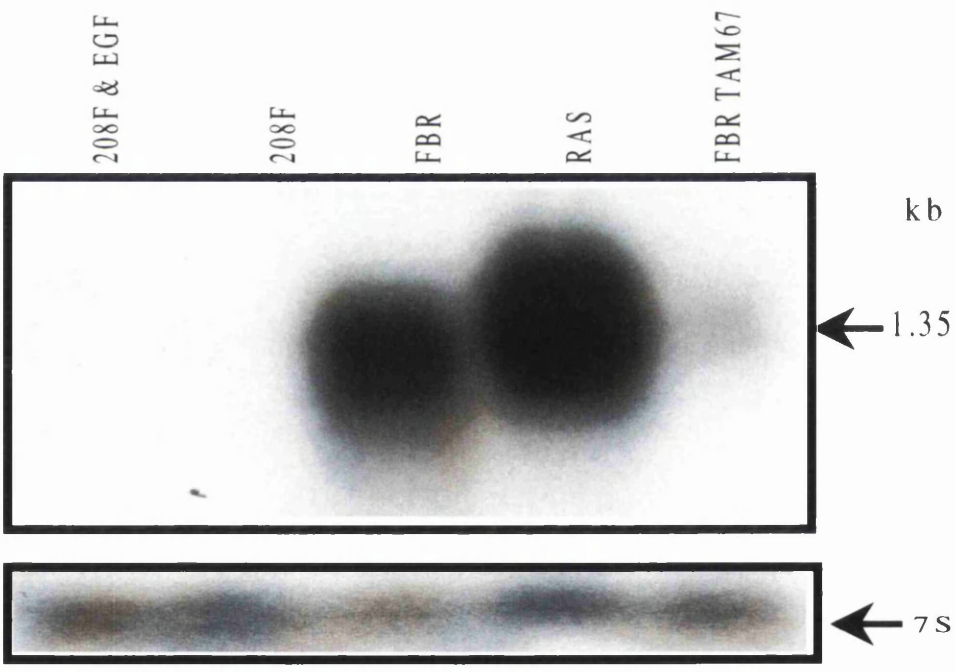


Clone BU17, Human Nucleotide Binding Protein

Figure 4.13
Northern Blots and 7S contol

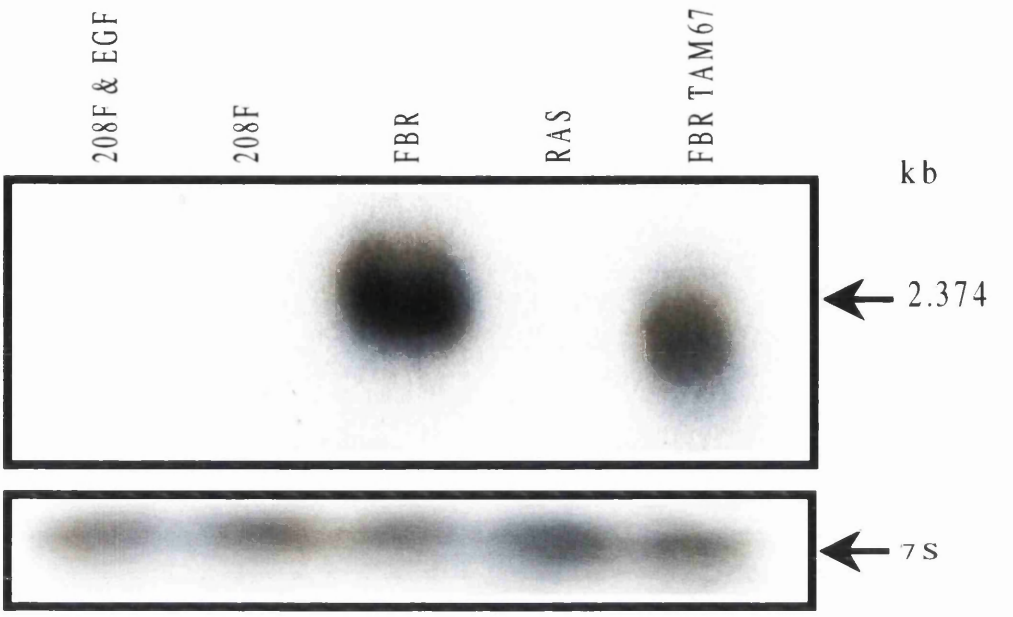


Clone CU16, PAI-1, 3 Day Exposure

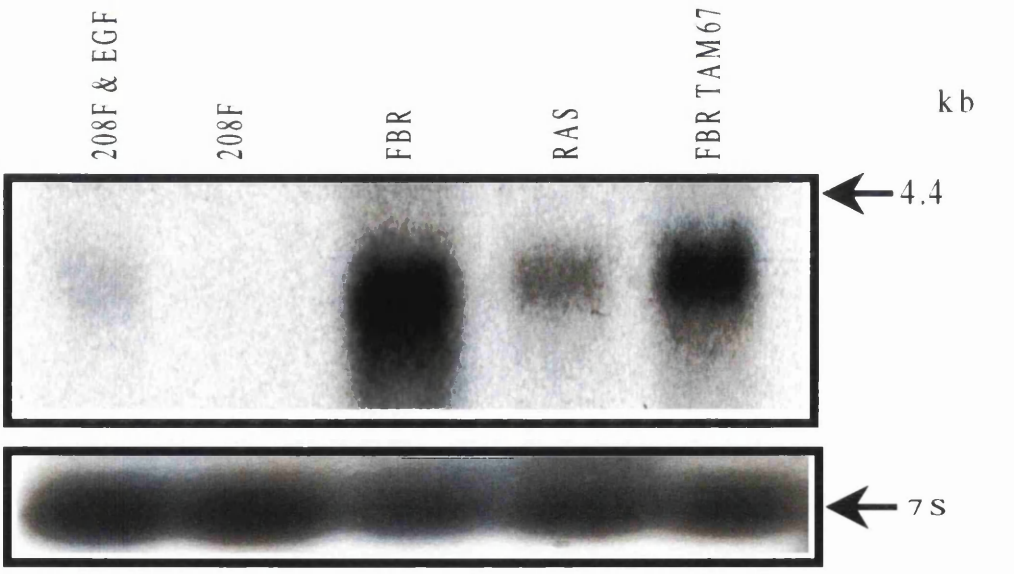


Clone CU17, Oncomodulin, 2 Day Exposure

Figure 4.14
Northern Blots and 7S contol

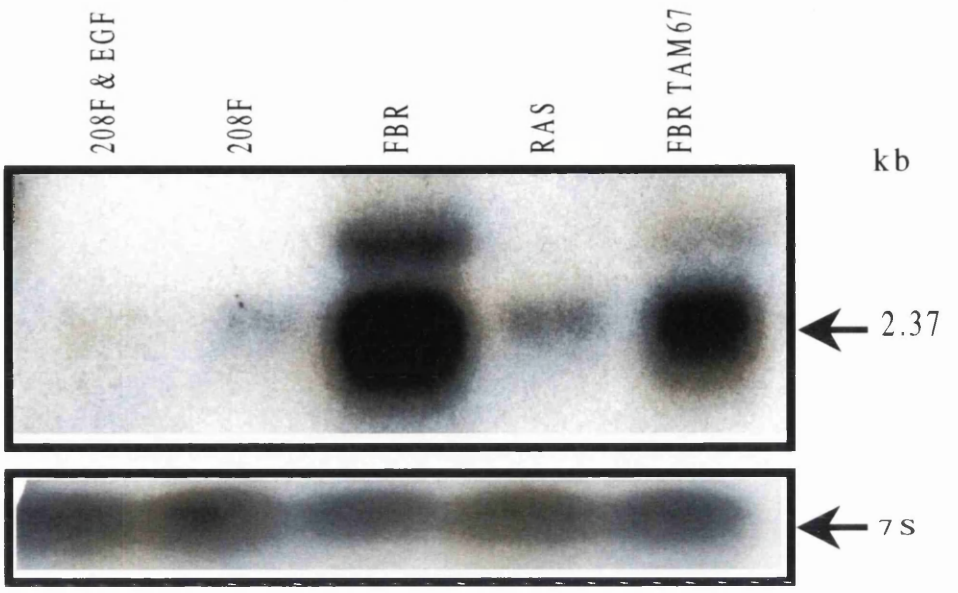


DU7, Cytokeratin 8 polypeptide, 1 Day Exposure

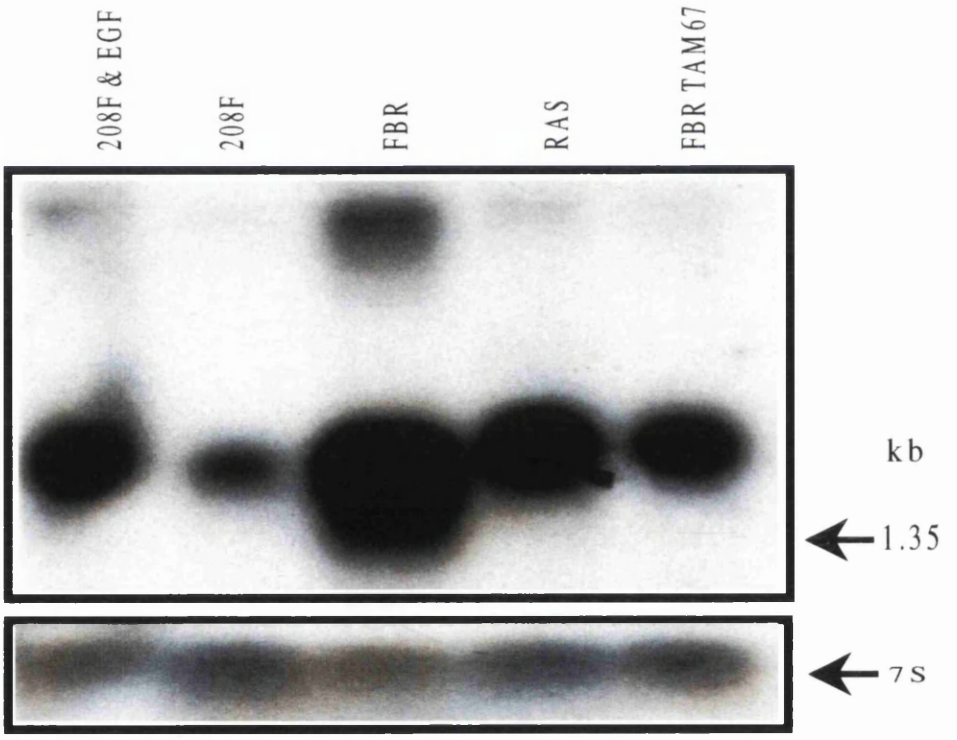


Clone DU12, β ig-h3, 2 Day Exposure

Figure 4.15
Northern Blots and 7S contol

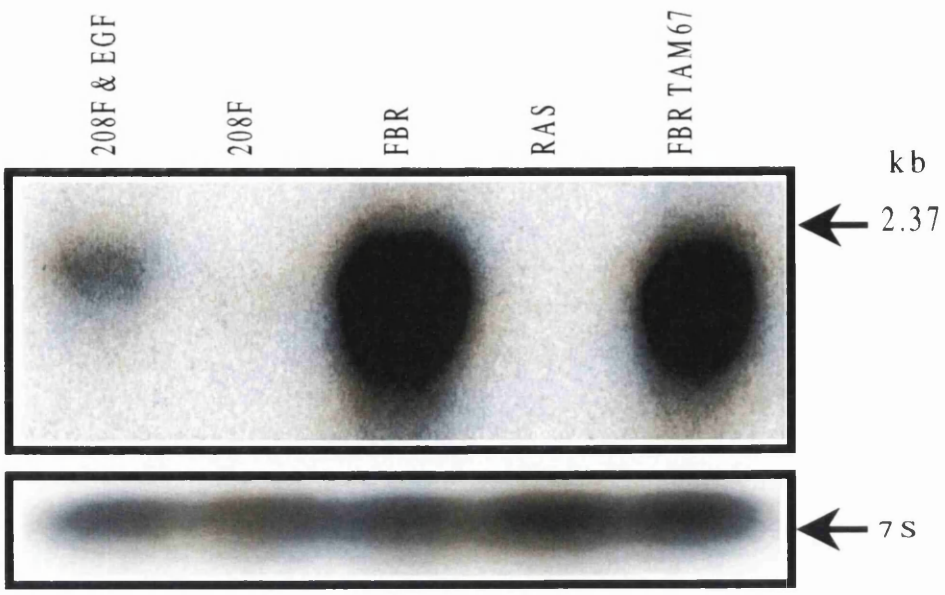


Clone FU1, Kelch form, 2 Day Exposure

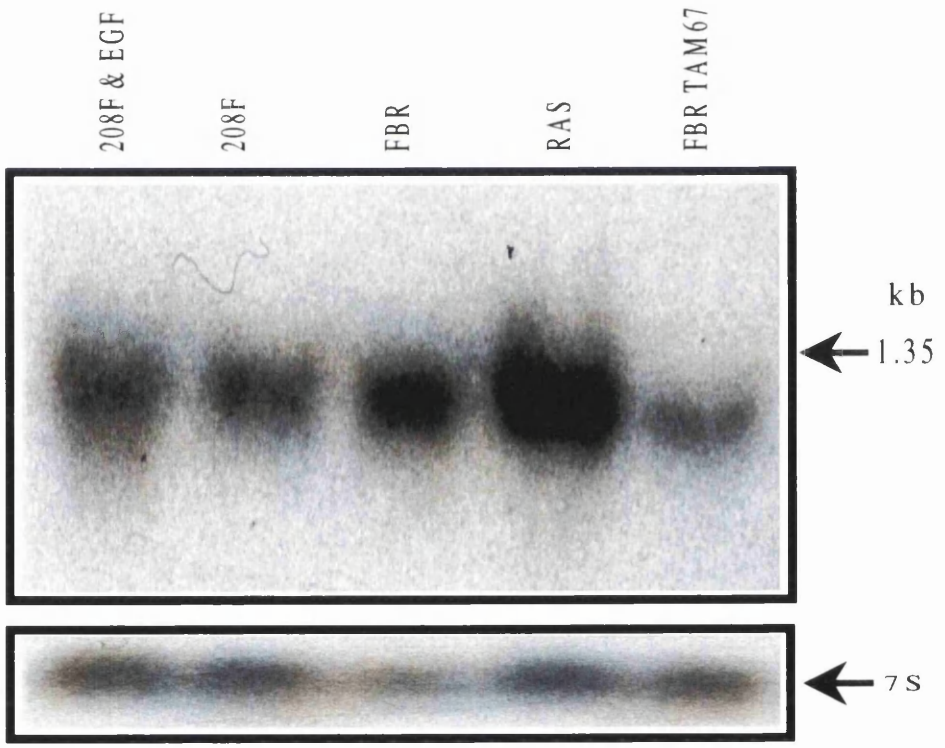


Clone GU8, Mac 2, 2 Day Exposure

Figure 4.16
Northern Blots and 7S contol

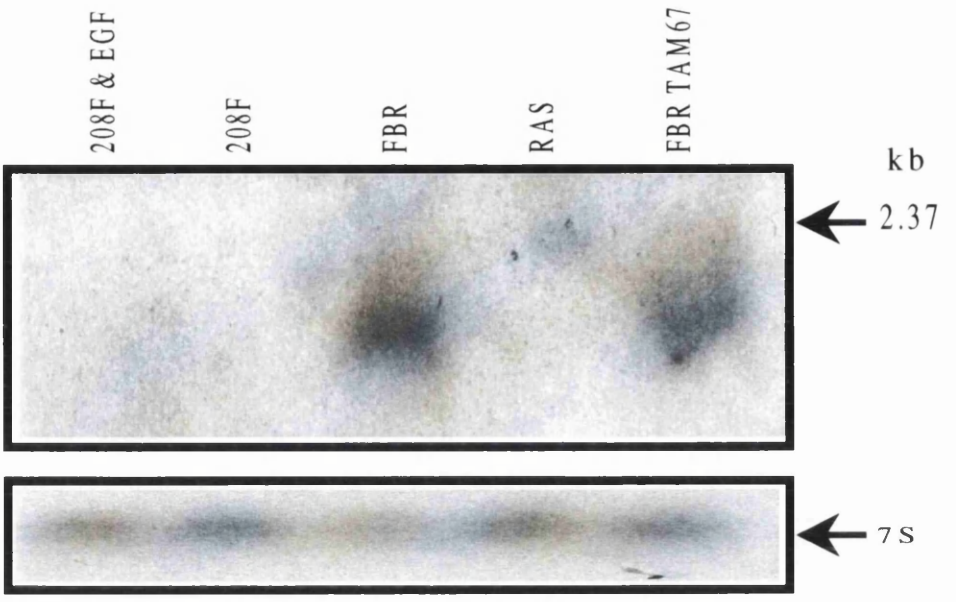


Clone IU1 Novel, 2 Day Exposure

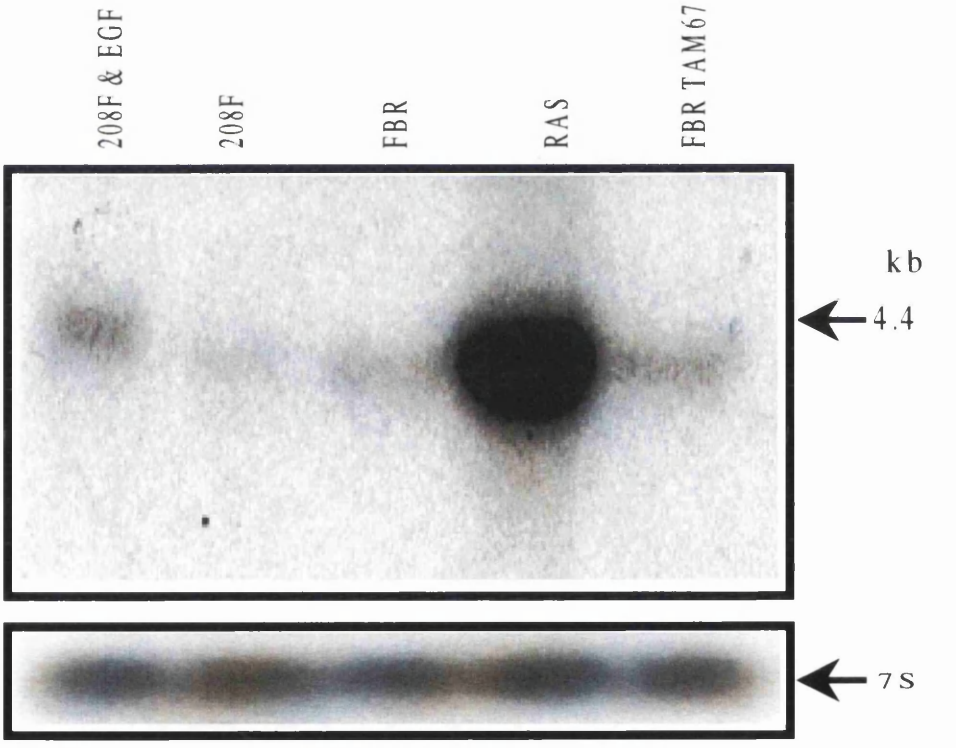


Clone LU5, Lactate Dehydrogenase, 2 Day Exposure

Figure 4.17
Northern Blots and 7S contol



Clone MU5, Novel, 14 Day Exposure



Clone NU8, BEM-3, 7 Day Exposure

Figure 4.18
Northern Blots and 7S contol

4.2.2 Relative differential expression

The differential expression of a few of the clones is easy to compare from the Northern blot figures However, the comparison of expression patterns between all of the blots is more difficult due to the number of clones characterised. The quantitation of expression is tabulated in Tables 4.1 and 4.2 respectively. This indicates the relative abundance of the differentially expressed sequenced clones by scoring.

The scoring system was compiled from the relative differential expression of a clone in a Northern blot (refer to Figures 4.2-4.18). Hybridisation kinetics and exposure times of the Northern blots are not equal. Thus, the comparative expression of several clones in an RNA species is not appropriate. The different gradiations of the scoring system was as follows:

Black :	High Expression
Dark Grey:	Moderate Expression
Light Grey	Low Expression
White	Negligible Expression

Tables 4.1-4.2

Differential Expression Patterns of Down-regulated and Up-regulated Clones Analysed by Northern Blot

Table 4.1 details the down-regulated genes. Table in 4.2 details the up-regulated genes. The clone name and database identity is in the first lane, followed horizontally by the expression pattern in the various cell lines tested. The cell lines are as previously described (refer to chapter 1).

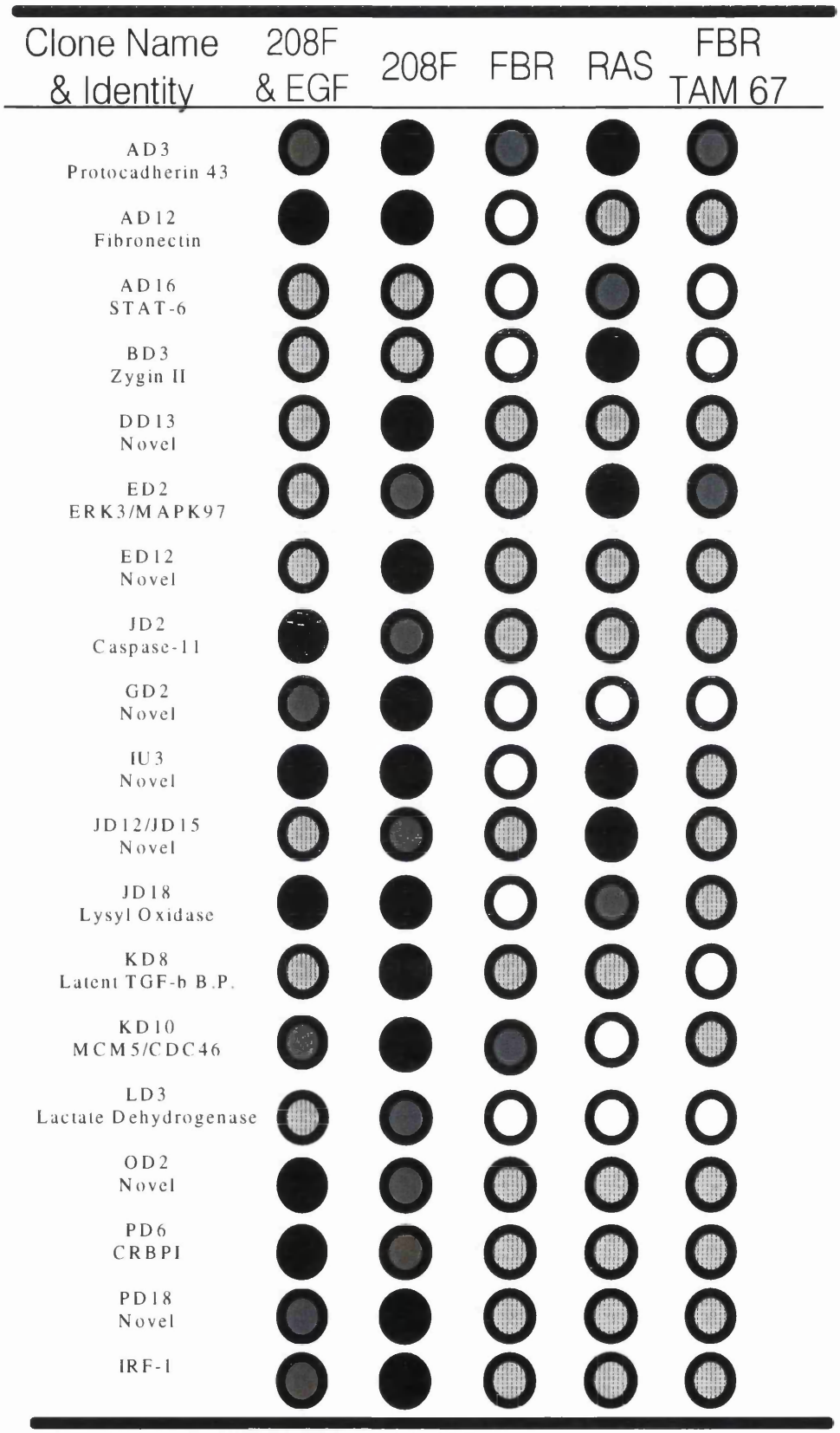


Table 4.1
Differential Expression of Down-Regulated Clones Analysed by Northern Blot

Clone Name & Identity	208F & EGF	208F	FBR	RAS	FBR TAM 67
AU5 Cathepsin B					
AU12 FBR-gag-Fos					
BU4 MMP-10					
BU17 Human N.B.P.					
CU16 PAI-1					
CU17 Oncomodulin					
DU7 Cytokeratin 8					
DU12 b-ig-h3					
FU1 Krp1					
GU8 Mac2					
IU1 Novel					
LU5 Novel					
MU5 Novel					
NU8 BEM-3					

Table 4.2
Differential Expression of Up-Regulated Clones Analysed by Northern Blot

4.3 DISCUSSION

The Northern blot experiments determined the differential expression of sequenced clones between the FBR *v-fos* transformed fibroblasts and 208F parental fibroblasts, and in other AP-1 dependent transformed cell types as previously described in chapter 1. Sequence analysis of the clones demonstrated that many of the identified genes have been previously associated with aspects of transformation.

The expression patterns of a representative fraction of the sequenced clones isolated from the down-regulated library revealed that all were down-regulated in FBRs and 74% were also down-regulated in K-*ras* transformed 208Fs, 58% were also down-regulated in 208Fs stimulated with EGF. In FBRs Tam-67, only 32% were down-regulated.

The expression patterns of a representative fraction of the sequenced clones isolated from the up-regulated library revealed that all were up-regulated in FBRs and 79% were also up-regulated in K-*ras* transformed 208Fs, and 36% were up-regulated in 208Fs stimulated with EGF. In FBR Tam-67 revertants, 79% were down-regulated when compared to FBRs.

Most strikingly, all but one of the clones examined by northern blot analysis were differentially expressed between the 208Fs and FBRs. Therefore, the Northern data presented in this chapter coupled with the library controls performed provides credence that all of the genes in the up-regulated and down-regulated libraries are differentially expressed, with possibly a few exceptions.

The expression analysis confirms the complexity of change in gene expression upon transformation by *fos* and *ras* oncogenes, as demonstrated by the sequence analysis that essentially all of the genes in the libraries are differentially expressed. In some instances, the expression change in some of the cell lines examined for a particular clone is only partial. Furthermore, not all genes down-regulated by *v-fos* are also down-regulated by K-*ras* and EGF stimulation. Likewise, not all genes up-regulated in *v-fos* transformants are also up-regulated in K-*ras* and EGF transformants. It was presumed that some of the isolated clones would be specific for *v-fos* transformation.

One up-regulated clone, AU12 encodes for the FBR-gag-fos polyprotein of the FBR-MuSV, and northern analysis reveals it is up-regulated in FBR RNA and also in K-*ras* RNA. The K-*ras* transformed cells are made through retroviral infections in the 208F cell line. Therefore, isolation of this cDNA further confirms the differential expression between the subtracted 208F and FBR cell lines.

The clones predominantly down-regulated by *v-fos* includes AD3, protocadherin 43; AD16, STAT-6; IU3, Novel; and JD2, Caspase 11. The clones predominantly up-regulated by *v-fos* includes DU7, Cytokeratin 8 polypeptide; DU12, βig-h3; FU1, Krp1; IU1, Novel; and MU5, Novel.

As previously discussed in chapter 1, the growth factor signal transduction pathway, such as that elicited through EGF stimulation, can up-regulate and down-regulate putative AP-1 target genes. Several of the clones are up-regulated in *v-fos* and K-*ras* oncogene transformants, but not in EGF transformed cells, such as AU12, FBR-gag-fos-gag polyprotein; BU4, MMP-10; BU17, human nucleotide binding protein; CU17, Oncomodulin; and FU1,Krp1. Interestingly, only one clone; IU1, Novel, is up-regulated in EGF transformed 208Fs, that is not also up-regulated in K-*ras* transformants. Several of the clones, AD12, Fibronectin; JD18, Lysyl Oxidase; KD10, MCM5/CDC46; LD3, Novel; OD2, Novel; PD6, CRBPI and IRF-1 are not as dramatically down-regulated in EGF transformed cells, as in *v-fos* and K-*ras* oncogene transformants. This difference in expression patterns for some of the clones analysed is accountable. EGF transformation is inducible and transient with 70-80% of the cell population transformed and not all of the EGF signal transduction pathway targets are Fos/Jun family member AP-1 target genes. In addition, full transformation by Fos requires three days in a regulated system as opposed to the forty-eight hours post-stimulation of EGF. Furthermore, transcription factors and signalling pathway intermediates that are direct targets of AP-1 will have their own set of target genes and thus not all of the identified clones will be direct AP-1 targets.

Although there are differences in activation of AP-1, morphology, and invasive behaviour with the K-*ras* transformants being less invasive in an *in vitro* invasion assay, 74% of the clones analysed are also down-regulated in K-*ras* and 79% are also up-regulated in K-*ras* transformants. However, several of the down-regulated clones are not down-regulated by K-*ras* transformation, and in a few cases, AD16, STAT-6; BD3, Zygin II; ED2, ERK-3; and JD12/15, Novel; the expression is even higher than that found in wild type, the 208F cells. Evidently, from this analysis, not all of the *v-fos* down-regulated genes are also down-regulated through K-*ras* transformation. The RAS cells are autocrine and produce their own growth factors, such as TGF α. Therefore, the signalling pathways activated in these RAS cells such as the Jun Kinase pathway; and the Ras/MAPK cascade, can lead to the targeting of various effector genes. This includes transcription factors, for example Ets. Also, the transcription factors and signalling pathway components that are direct targets of AP-1 K-*ras* oncogene will have their own set of target genes which may be different to those

of the *v-fos* oncogene clones that were analysed. In contrast, the FBR cells are not autocrine. Thus in comparison, the signal transduction cascades that function upstream of AP-1 in RAS cells is complex. Additionally, post-transcriptional modifications may also account for the differences in expression for approximately 20% of the clones analysed.

The Jun dominant-negative deletion mutant Tam-67 is described in chapter 1. Briefly, Tam-67 lacks the transactivation domain and can dimerise with itself and with Fos and Jun family members to form a stable complex. Tam-67 can bind DNA with the same affinity as wild-type Jun and Fos family members but it cannot activate the transcription of the AP-1 responsive gene, collagenase. Tam-67 inhibits TPA/*ras*-induced transformation, SV40 T/*ras* transformation and also prevents transformation of rat embryo cells in co-operation with activated *ras* (Brown *et al.*, 1993).

This data is consistent with the findings from the northern blot analysis that Tam-67 in FBR cells fully or partially inhibits expression of 70-80% of the AP-1 regulated clones. Interestingly, the Northern data reveals that most of the FBR down-regulated clones are similarly down-regulated in FBR Tam-67 cells. Therefore, Tam-67 does not reverse the down-regulation effect exerted by *v-fos*, suggesting a mechanism of transcriptional repression.

With respect to the FBR Tam-67 cells, the quenching model of transcriptional repression is most appropriate. This can be substantiated on two accounts. Firstly, the quenching mechanism would dampen the effect of AP-1 and there would be partial inhibition of up-regulation and down-regulation as shown by the northern data. Secondly, Tam/Tam homodimers are not preferential binding partners in FBR Tam-67 cells (M^{c}Garry, 1998, unpublished observations), and thus the blocking mechanism would not pre-dominate.

Interestingly, the expression of the down-regulated genes in Tam-67 cells is not required to maintain the reverted phenotype of these cells. Previous studies in our laboratory shows that a decrease in wild type levels of CD44 is not required for morphological reversion in FBR *v-fos* transformed cells. However blocking CD44 expression blocks invasion in an *in vitro* invasion assay (Lamb *et al.*, 1997a). Furthermore, a down-regulated clone, FRP (refer to chapter 5), when re-introduced into FBR cell line does not induce morphological reversion. However FRP expression blocks invasion in an *in vitro* invasion assay. This highlights the complexity of transformation and indicates that each gene may have some role in suppressing or enhancing transformation or invasion.

The mechanism of transcriptional repression by AP-1 is not well understood. In *fos* transformants AP-1 may repress transcriptional activation through methylation

activity. A three-fold increase in *dnmt1* expression after 48 hours was recently reported in a *c-fos* conditional expression system based on LacI whereby *c-fos* is rapidly repressed in the presence of IPTG and is rapidly induced in it's absence (Bakin and Curran, 1999). In this system transformation requires 2-3 days.

Dnmt1 functions as a catalyst in the transfer of methyl groups from S-adenosyl methionine to the C-5 position of cytosines in DNA. An increase in expression has previously been observed in tumour cells (Wu *et al.*, 1993), and overexpression in fibroblasts results in transformation (Wu *et al.*, 1993). It has been reported that DNA methylation is linked to gene silencing, and overexpression of *dnmt1* can lead to progressive methylation of several genes (Baylin *et al.*, 1998). The methylation of DNA can repress gene expression through the condensation of chromatin. This occurs when the methylated sites on the DNA binds with 5-methylcytosine binding protein (MeCP2), which complexes with Sin3A and histone deacetylase (HDAC), and this resulting complex subsequently decreases histone acetylation, thus compacting chromatin structure (Nan *et al.*, 1998; Jones *et al.*, 1998).

The *dnmt1* gene is believed to be a direct target of AP-1. The experimental evidence to support this includes an increase in transcription rate in a *c-fos*-induced nuclear run-on assay (Bakin and Curran, 1999), a dominant-negative *c-jun* reduces *dnmt1* expression in *ras*-transformed cells (MacLeod *et al.*, 1995; Rouleau *et al.*, 1995), and finally, the *dnmt1* gene has AP-1 sites that activate transcription in a *ras*- and *jun*-dependant manner (MacLeod *et al.*, 1995; Rouleau *et al.*, 1995; Yoder *et al.*, 1996; and Tucker *et al.*, 1996). In addition, an inhibitor of HDAC, Trichostatin A, causes reversion of *fos* transformation (Bakin and Curran, 1999), suggesting that *fos* transformation is mediated by elevated expression of *dnmt1*, which represses gene expression.

Furthermore, the up-regulated cDNA library clones have been further sequenced by Dr.J.Winnie. Two clones, SAP18 and RbAp46 were isolated. Northern blotting reveals an up-regulation of SAP18 in *v-fos* and K-*ras* transformants (Winnie, unpublished data). The understanding of transcriptional repression has recently been significantly advanced by the demonstration of a link between transcriptional repressors and histone deacetylases. mSin3-accociated polypeptide, SAP18, was isolated in an affinity purification experiment of mSin3-containing complexes, and GST pull-down assays revealed that SAP18 directly interacts with mSin3 *in vitro* (Zhang *et al.*, 1997). Further experimentation demonstrated that SAP18 exists in a complex with mSin3 and HDAC1 *in vivo* and exerts transcriptional repressive activity. The mSin3/HDAC1- mediated transcriptional repression involves the modification of core histones and is enhanced by SAP18 (Zhang *et al.*, 1997).

Development is dependent upon the up-regulation of certain genes and also by the repression of others. Recently, the murine homologue of SAP18 was cloned and it was demonstrated that the RNA was expressed with stage and lineage specificity in the haematopoietic hierarchy, and that alternative polyadenylation sites are used. This suggested a role for SAP18 in transcriptional repression during haematopoietic development (Boehmelt *et al.*, 1998).

The RbAp46 protein was first identified as a major protein from HeLa cell lysate that specifically bound to an Rb affinity column (Huang *et al.*, 1991). Recently, it has been demonstrated that RbAp46 complexes with the human histone deacetylases, HDAC1, HDAC2 and RbAp48 in the mSin3 complex (Zhang *et al.*, 1987). This complex was shown to be functionally important for mSin3-mediated repression of transcription (Zhang *et al.*, 1997).

Therefore, with respect to the Northern data presented in this chapter, the down-regulation of AP-1 target genes in the FBR *v-fos* transformants is possibly due to *dnmt1* DNA methylation leading to transcriptional repression, and/or SAP18/RbAp46/mSin3/HDAC1 complex transcriptional repression.

The analysis of the expression data of the clones by Northern blotting details that 20-25% of the clones are *v-fos* specific, whilst the remaining 75-80% are additionally regulated through the EGF and K-*ras* oncogene signal transduction pathways that function upstream of AP-1. This further emphasizes the important function of the proteins encoded by the sequenced clones in. The expression of the remaining 20-30% is possibly because the genes do not have an AP-1 responsive element in their promoter region, but are in fact secondary targets of AP-1 responsive genes.

Chapter 5

Role of Follistatin-related Protein in AP-1 Transformation

5.1 INTRODUCTION

Characterisation of many of the clones from the down-regulated library by Northern blotting (refer to chapter 4), and sequence analysis (refer to chapter 3), identified genes for further analysis. One such clone MD4, was identified as rat Follistatin-related Protein (FRP), originally identified as a TGF-β inducible gene.

The transforming growth factor (TGF)β1 acts as a growth inhibitor in mouse osteoblastic MC3T3-E1 cells. FRP mouse homologue TSC-36 was isolated in a differential screen for latent TGF-β1 induced genes which included the *ras*-recision gene lysyl oxidase (Shibanuma *et al.,* 1993). The expression of TSC-36 was found reduced in v-Ki-*ras* transformed MC3T3 cells or transformed NIH3T3, but its expression recovered to normal levels in a flat revertants of *ras*-transformed cells (Shibanuma *et al.,* 1993). Tissue distribution studies of TSC-36 in adult mouse organs revealed expression in heart and lung, and further analysis in adult lung sections detected expression in alveolar cells, but not in bronchial epithelium or blood vessels (Mashimo, *et al.,* 1997). TSC-36 was also found to be down-regulated in *v-ras* and *v-myc* transformed cell lines, but not fibroblasts transformed by *v-src*, *v-raf or v-abl*. No expression was detectable in nine human cancer cell lines tested as compared to immortalized non-tumourigenic human fibroblasts and this suggested a role in tumour suppression (Mashimo, *et al.,* 1997).

Subsequently, the rat homologue, named FRP was partially co-purified with superoxide dimutase as an abundant protein in the conditioned media of C_6 glioma cell line (Zwijsen *et al.,* 1994). The cDNA and protein were isolated. Antiserum was raised against the C-terminal predicted peptide of mouse TSC-36 and Western analysis detected a polypeptide with a M_r 38kDa in the culture media of MC3T3 cells (Shibanuma *et al.,* 1993). The secreted glycoprotein was shown to have no effect on the inhibitory action of TGFβ1 on CCL-64 cell growth (Zwijsen *et al.,* 1994). Sequence searches at the protein level revealed homology to the follistatin-like motif of follistatin and agrin (Zwijsen *et al.,* 1994).

The FRP protein sequence similarity to follistatin-like domains of follistatin and agrin and SPARC (Shibanuma *et al.,* 1993, Zwijsen *et al.,* 1994), is shown by Figure 5.1. The comparison of partial amino acid sequences of rat FRP with rat follistatin precursor 1, rat Agrin and rat SPARC is shown by Figure 1, panel A. The amino acid sequence alignments reveals that the cysteine distribution is similar and is shown by Figure 1 panel B. The amino acid identity of FRP to follistatin-like domain of follistatin is 21% over a length of 320 amino acids. Sequence identity of FRP to the follistatin-

like domain of agrin is 47% over a length of 47 amino acids and this similarity is situated within the sixth follistatin-like domain of agrin (amino acids 430-496). The identity of FRP to the follistatin-like domain of SPARC is 16% over a length of 301 amino acids.

This chapter reports the Northern blot expression of clone MD4, FRP and the cloning of the open reading frame (ORF), into an expression vector with a 3' Myc Tag. Transient transfections into 208F, FBR, RAS and Tam-67 cell lines were performed in an attempt to ascertain the role of FRP in suppressing transformation and invasion. This was further examined by the generation of a FRP stably expressing FBR clone, and a FBR neo clone and they were characterised using a proliferation assay, growth in soft agar, and by measuring their invasive capacity in an *in vitro* invasion assay.

5.2 RESULTS

5.2.1 Northern Blot Analysis

Northern Blotting revealed the expression patterns of FRP in the three previously described model systems which activate AP-1 activity and the results are shown by Figure 5.2. A transcript of FRP is easily detectable in 208F, whilst in FBR it is undetectable. In RAS the FRP transcript is reduced by two-fold. In EGF treated 208Fs, which morphologically transforms 70-90% of the cell population, results in FRP transcript being marginally down-regulated. In FBR Tam-67, the transcript is barely detectable.

5.2.2 Cloning FRP into 3' Myc tagged Expression Vector

Clone MD4 maps to the amino acid positions 172-306 of FRP (data not shown). FRP rat sequence in vector pBSK (+) rFRP was a gift from Dr.A.Zwijsen, Innogenetics, Belgium. The complete rat nucleic acid sequence and protein translation of the complete open reading frame of FRP is shown in Figure 5.3 and the potential *N*-glycosylation sites are highlighted in bold. FRP open reading frame was cloned into pcDNA3.1Myc-His A as described in Chapter 2, Methods and is outlined schematically in Figure 5.4. The polyhistidine tag would facilitate the purification of recombinant protein from conditioned media if necessary.

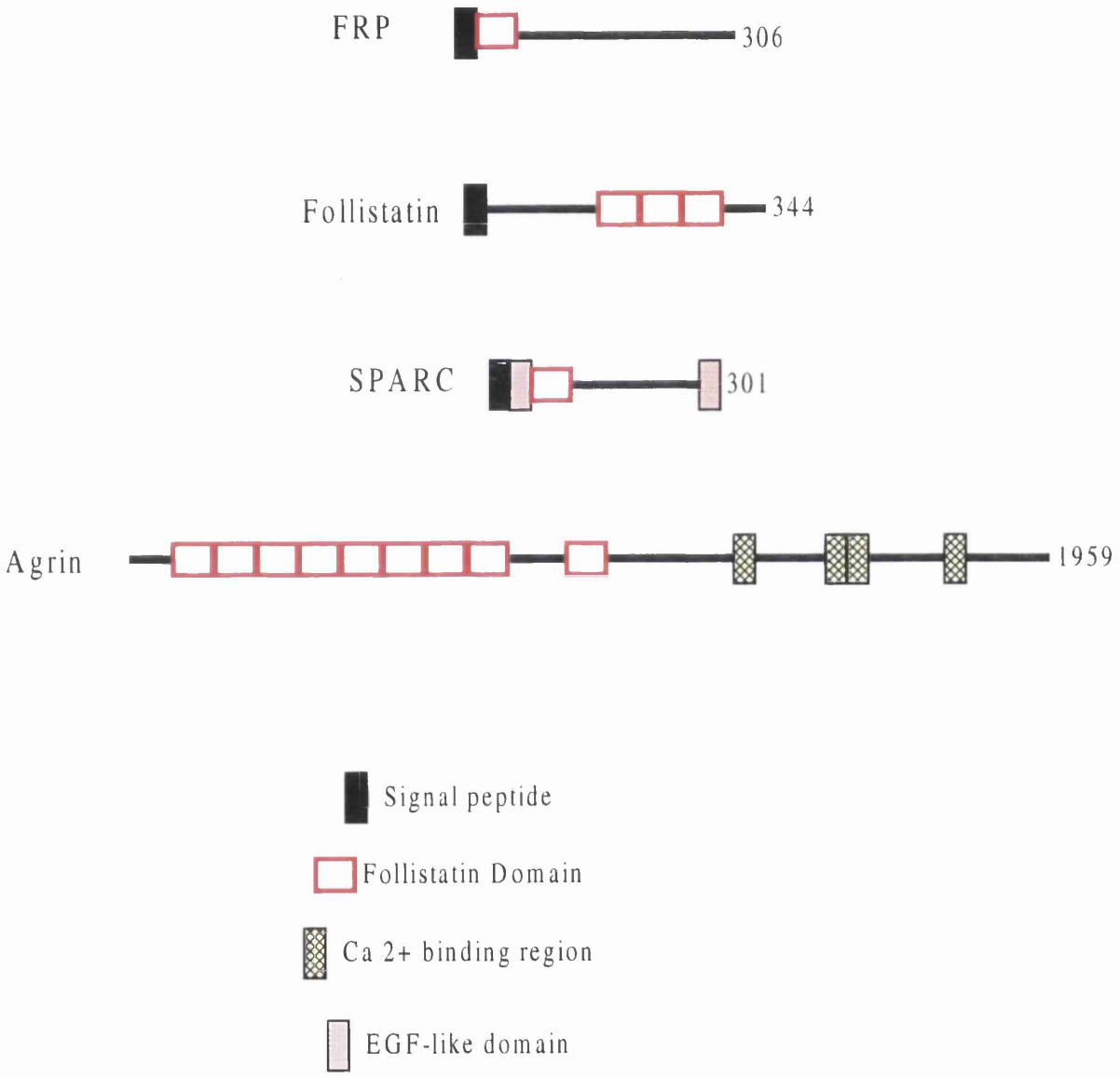
Figure 5.1

Sequence similarity alignments of FRP with other follistatin-module containing proteins

A: This figure details and compares the structure of the FRP protein's functional domains to Follistatin domain proteins, Follistatin, SPARC and Agrin, which is highlighted by a red box. Other features of the protein's are also detailed, signal peptide by a black box, Calcium binding region by a yellow /black cross box, and an EGF-like domain by a pink box.

B: This figure details the amino acid sequence alignment of FRP to Follistatin domain proteins, Follistatin, SPARC and Agrin. The highly conserved regions, 90%, are presented in red typeface, and the lower conserved regions, 50%, are presented in blue typeface.

A


FRP
306
Follistatin
344
SPARC
301
Agrin
1959
Signal peptide
Follistatin Domain
Ca 2+ binding region
EGF-like domain

B

Consensus levels: high=90%RED low=50%BLUE

```
           1                                                    50
      FRP
FOLLISTATIN  MVCARHQPGG LCLLLLLLCQ FMEDRSAQAG NCWLRQAKNG RCQVLYKTEL
     SPARC                              MRA WIFFLLCLAG RALAAPQTEA
     AGRIN
 Consensus

           51                                                   100
      FRP                  MWKRW LALALVTIAL VHGEEE---Q RSKSK-ICAN
FOLLISTATIN  SKEECCSTGR LSTSWTEEDV NDNTLFKWMI FNGGAP---N CIPCKETCEN
     SPARC   AEEMVAEETV VEETGLPVGA NPVQVEMGEF EEGAEETPEE VVACNNPCQN
     AGRIN

           101                                                  150
      FRP   VFCGAGRECA VTEKGEPTCL CIEQCK---- -PHKRPVCGS NGKTYLNHCE
FOLLISTATIN  VDCGPGKKCR MNKKNKPRCV CAPDCS---N ITWKGPVCGL DGKTYRNECA
     SPARC   HHCKHGKVCD -GESNTPMCV CQDPTSCPAP IGEFEKVCSN DNKTFDSSCH
     AGRIN                    CV CPSEC----- VESAQPVCGS DGHTYASECE

           151                                                  200
      FRP   LHRDACLTGS KIQVDYDGHC KE--KKSVSP SASPVVCYQA NRDELRRRII
FOLLISTATIN  LLKARCKEQP ELEVQYQGKC KKTCRDVFCP GSSTCVVDQT NNAYCVTC--
     SPARC   FFATKCT--- -LEGTKKGH- -KLHLDYIGP CKYIAPCLDS ELTEFPLRMR
     AGRIN   LHVHACTHQI SLYVASAGHC

           201                                                  250
      FRP   QWLEAEIIPD GWFSKGSNYS EILDKYFKSF DNGDSHLDSS EFLKFVEQNE
FOLLISTATIN  ----NRICPE PSSSEQSLCG NDGVTYSSA- ----CHLRKA TCL-LGRSIG
     SPARC   DWLKNVLVTL YERDEGNNLL TEKQKLRVK- -------KIH ENEKRLEAGD
     AGRIN

           251                                                  300
      FRP   TAVNITAYPN QENNKLLRGL CVDALIELSD ENADWKLSFQ EFLKCLNPSF
FOLLISTATIN  LAYEGKCIKA KSCEDIQCGG GKKCLWDFKV GRGRCSLCDE -----LCPDS
     SPARC   HPVELLARDF EKNYNMYIFP VHWQFGQLDQ HPIDGYLSHT E----LAP--
     AGRIN

           301                                                  350
      FRP   NPPEKKCALE DETYADGAET EVDCNRCVCS CGHWVCTAMT CDGKNQKGVQ
FOLLISTATIN  KSDEPVCASD NATYAS---- --ECAMKEAA CSSGVLLEVK HSG-SCNSIS
     SPARC   -LRAPLIPME HCTTRFFETC DLDNDKYIAL EEWAGCFGIK EQDINKDLVI
     AGRIN

           351                                382
      FRP   THTEEEMTRY AQELQKHQGT AEKTKKVNTK EI
FOLLISTATIN  EETEEEEEEE DQDYSFPISS TLEW
     SPARC
     AGRIN
```

Figure 5.2

Northern Blot of Clone MD4, FRP

This figure details the one day exposure X-ray film of a Northern blot probed with Clone MD4, Follistatin-related protein, in the top panel. The bottom panel details the corresponding scanned X-ray film of the 7S ribosomal loading control. Arrows with regards to the RNA ladder in kilobases indicate the size of the mRNA transcript. The size of the cloned mouse homologue, TSC-36, was 2.8Kb (Shibanuma *et al.,* 1993).

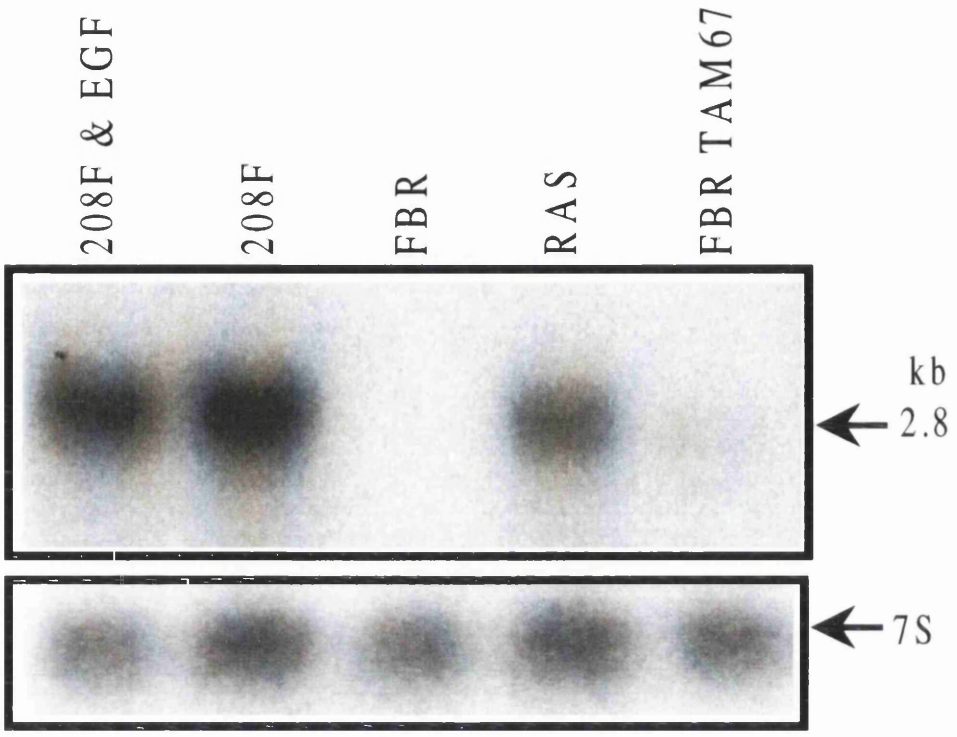


Figure 5.2
Clone MD4, Rat Follistatin-related Protein,
Northern Blot, 1 Day Exposure and 7S Control

5.2.3 Transient Transfection of Cos 7 cells with Myc tagged FRP

The expression of FRP from the pcDNA3.1A/FRP vector and pcDNA3.1A control empty vector was determined by transfection of Cos 7 cells and isolation of soluble protein extracts 72 hours post-transfection. The soluble proteins were analysed by Western Blotting using mouse anti-Myc as the primary antibody, and HRP-conjugated anti-mouse antibody, as secondary. The resulting expression of FRP is shown by Figure 5.5, which confirmed that the expression vector was functional and coded for a protein of the correct size. The previously reported protein molecular mass was of rat FRP was 40-48kDa (Zwijsen *et al.*,1994), whilst the mouse homologue TSC-36 was reported as 38kDa (Shibanuma, *et al.,* 1993). The Myc-tagged protein was approximately 40Kda, of which approximately 2.5Kda was the Myc-tag.

5.2.4 Transient Transfection of 208F, FBR, RAS AND Tam-67 Cell Lines with Myc tagged FRP

The 208F, FBR, RAS and Tam-67 cell lines were transiently transfected with the above described Myc tagged FRP expression vector and fixed twenty four hours post-transfection. The cells were stained by indirect immunofluorescence using an anti-myc primary mouse antibody, followed by an anti-mouse FITC labelled secondary antibody. In conjunction, the cells were counter-stained with rhodamine-phalloidin (TRITC), to visualise F-actin. The methodology is described in Chapter 2, Section 2.2.23. The merged pictures of FRP-myc (Green), and F-actin (Red), cellular location, in each of the transiently transfected cell lines are shown by Figure 5.6.

The phalloidin staining of 208F shows a normal fibroblastoid actin structure with prominent stress fiber arrays that run in parallel along the ventral surface of the cell (See arrow, Figure 5.7, panel (e)). The localisation of the FRP in two representatives of transfected 208F cells is shown in Figure 5.7. The FRP is of a peri-nuclear, cytoplasmic localisation with a slight punctate expression, characteristic of a secreted protein in the ER and golgi apparatus, particularly in Panels (b), and (g). Actin rich membrane ruffles can sometimes be seen in the FRP transfected and untransfected 208F cells (not shown). These findings are consistent with previous studies of 208F actin structure (Hennigan, 1993). This staining pattern of actin architecture is shown in both the transfected and adjoining untransfected FRP cells, is shown by Figure 5.7.

Figure 5.3

Nucleic acid and ORF protein sequence of rat Follistatin-related Protein

The figure shows the published nucleotide sequence of rat FRP cDNA, which is 1370bp long. The amino acid sequence predicted from the open reading frame starting from the first ATG Kozak initiation signal at positions 64-981 is also shown. The eukaryotic signal sequence between amino acid residue 18 and 19 results in EEEQRS as the start of the N-terminal sequence and is underlined. The three potential N-glycosylation sites (Asn-Xaa-Ser/Thr), at amino acid positions 124, 155 and 160 are in bold.

```
        10                  30                  50
         .                   .                   .                   .
ctggcctccaactcactgcttccatcctgcccagtgtcctctcgagtcccggacccgagc

        70                  90                  110
         .                   .                   .                   .
acgatgtggaaacgctggctggcgctcgcgctggtgaccatcgccctggtccacggcgag
   M  W  K  R  W  L  A  L  A  L  V  T  I  A  L  V  H  G  E

       130                 150                 170
         .                   .                   .                   .
gaggaacaaagaagcaaatccaagatctgcgccaatgtgttttgtggagctggccgggaa
E  E  Q  R  S  K  S  K  I  C  A  N  V  F  C  G  A  G  R  E

       190                 210                 230
         .                   .                   .                   .
tgcgccgtcacggagaaggggagccaacgtgcctctgcattgagcaatgcaaacctcac
C  A  V  T  E  K  G  E  P  T  C  L  C  I  E  Q  C  K  P  H

       250                 270                 290
         .                   .                   .                   .
aagaggcctgtgtgtggcagtaatggcaagacctacctcaaccattgtgaacttcacaga
K  R  P  V  C  G  S  N  G  K  T  Y  L  N  H  C  E  L  H  R

       310                 330                 350
         .                   .                   .                   .
gacgcctgcctcactggatccaagatccaggttgattatgatgggcactgcaaagaaaag
D  A  C  L  T  G  S  K  I  Q  V  D  Y  D  G  H  C  K  E  K

       370                 390                 410
         .                   .                   .                   .
aagtctgtgagtccatccgccagccccgttgtctgctatcaggctaaccgtgatgagctg
K  S  V  S  P  S  A  S  P  V  V  C  Y  Q  A  N  R  D  E  L

       430                 450                 470
         .                   .                   .                   .
cggcgccggatcatccagtggctggaagccgagatcattccagatggctggttctctaaa
R  R  R  I  I  Q  W  L  E  A  E  I  I  P  D  G  W  F  S  K

       490                 510                 530
         .                   .                   .                   .
ggcagtaactacagtgagatcctagacaagtactttaagagctttgataatggtgactct
G  S  N  Y  S  E  I  L  D  K  Y  F  K  S  F  D  N  G  D  S

       550                 570                 590
         .                   .                   .                   .
cacctggactccagcgaattcctgaaattcgtggagcagaatgaaacagccgtcaacatc
H  L  D  S  S  E  F  L  K  F  V  E  Q  N  E  T  A  V  N  I

       610                 630                 650
         .                   .                   .                   .
accgcttaccccaatcaggagaacaacaaactgctcagaggcctctgtgttgatgccctc
T  A  Y  P  N  Q  E  N  N  K  L  L  R  G  L  C  V  D  A  L

       670                 690                 710
         .                   .                   .                   .
attgaactgtccgatgagaacgctgactggaaactcagcttccaagagttcctcaagtgc
I  E  L  S  D  E  N  A  D  W  K  L  S  F  Q  E  F  L  K  C

       730                 750                 770
         .                   .                   .                   .
ctcaacccatccttcaaccctcctgagaagaagtgcgccctggaggacgaaacctatgca
L  N  P  S  F  N  P  P  E  K  K  C  A  L  E  D  E  T  Y  A

       790                 810                 830
         .                   .                   .                   .
gatggagctgagaccgaggtggactgcaatcgctgtgtctgttcctgtggacactgggtc
D  G  A  E  T  E  V  D  C  N  R  C  V  C  S  C  G  H  W  V

       850                 870                 890
         .                   .                   .                   .
tgcacagcgatgacctgtgatggaaagaatcagaaggggtccagacccacacagaggag
C  T  A  M  T  C  D  G  K  N  Q  K  G  V  Q  T  H  T  E  E

       910                 930                 950
         .                   .                   .                   .
gagatgacgagatatgcccaggaactccagaagcaccagggaacagcagaaaagaccaag
E  M  T  R  Y  A  Q  E  L  Q  K  H  Q  G  T  A  E  K  T  K
```

```
       970                 990                1010
         .         .         .         .         .         .
aaggtgaacaccaaagagatctaagaagaggcacgtagcacctcatctggaacccagcac
K  V  N  T  K  E  I  *

      1030                1050               1070
         .         .         .         .         .         .
ctcctcttcagcgctaagcccagtatacagcgtctgtggcaatcaccgaatcaccagtat

      1090                1110               1130
         .         .         .         .         .         .
ttgcttgtacggcagcaaatcttatctgtttgttttgcaataaaggaagtgagggtggct

      1150                1170               1190
         .         .         .         .         .         .
ggctagccagggcaggcaggccacaactttcacttctaggaatgctttaagagacactaa

      1210                1230               1250
         .         .         .         .         .         .
agggcaccttggggcaggaggcgagtatccggttggcagaggagcagaggcaggtctgaa

      1270                1290               1310
         .         .         .         .         .         .
tgaaacctttctggggtcagctgtgaggatacaacaggaaaagcatgtgatgttaggggg

      1330                1350               1370
         .         .         .         .         .
aacactgagctggccctgctggaggaaatagggggagcttggtggggagg
```

Figure 5.3
Nucleic acid and ORF of rat Follistatin-related Protein

Figure 5.4

Rat Follistatin-related Protein primers and cloning strategy into pcDNA3.1 Myc-His A expression vector

A: The primer sequences used for the isolation of the FRP ORF by PCR are described. They were designed to include the Kozak translational start site but not the translational stop site of the ORF to enable the 3' Myc-His tagging of the protein, with Kpn I restriction sites prior to the start and at the end of the sequence to facilitate insertion, in frame, into the pcDNA3.1Myc-His A expression vector. The additional three bases at either end of the Kpn I sites are to safeguard the essential sequences from degradation.

B: Cloning strategy. PCR of the cDNA in the pBSK (+) rFRP vector using Pfu polymerase, limited PCR mistakes and generated blunt-ended products. These were cloned into pCRScript vector by ligation. The pCRScript vector with FRP cDNA was restriction digested using Kpn I and the resulting cDNA fragment was ligated into pre-digested and alkaline phosphatase treated pcDNA3.1 Myc-His A. The FRP insert and joined expression vector was sequenced to confirm that the FRP was in the correct orientation and in frame with no PCR mistakes (data not shown).

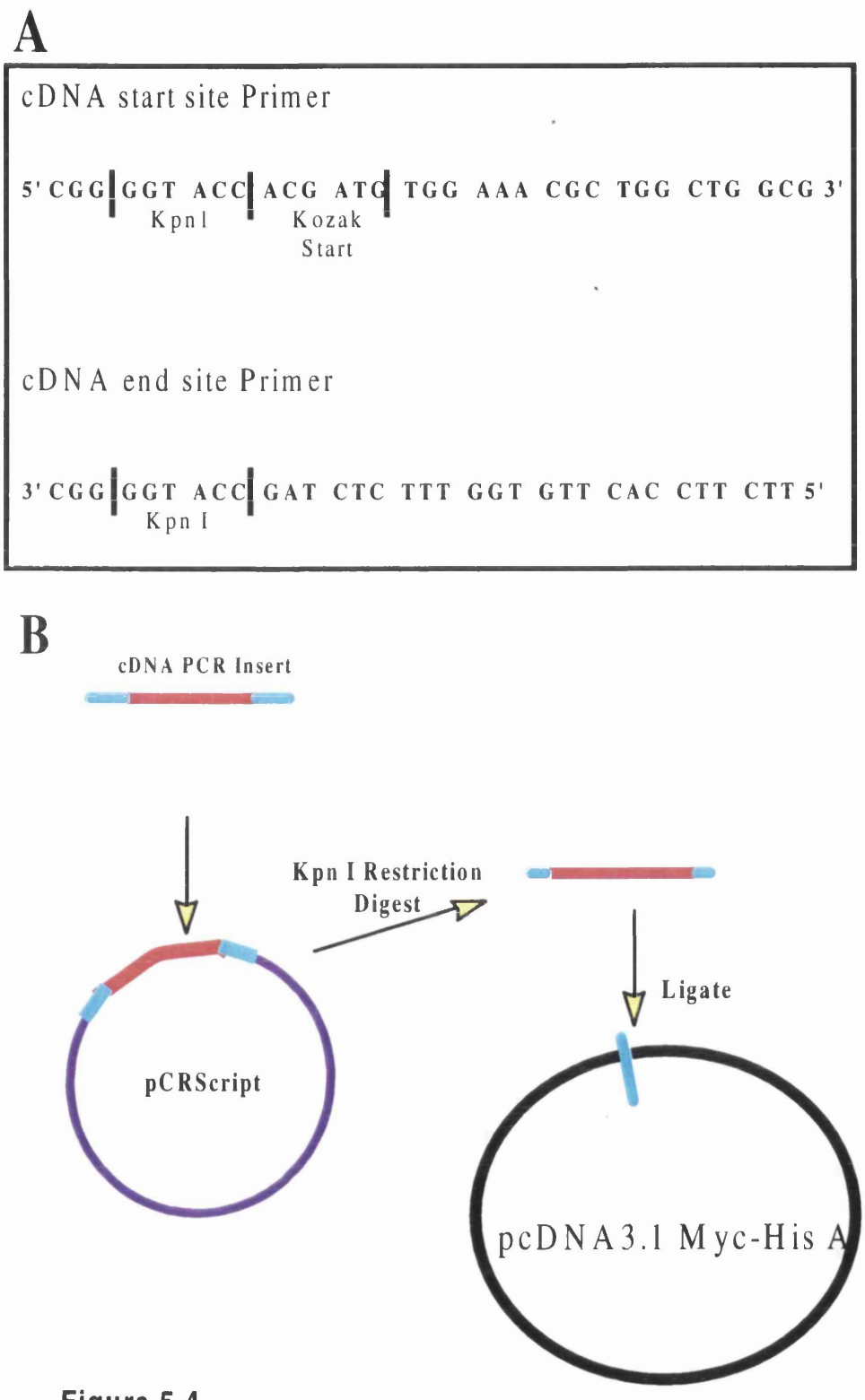


Figure 5.4
Rat Follistatin-related Protein Primers and Cloning Strategy into pcDNA3.1 Myc-His A Expression Vector

Figure 5.5

Protein detection by Western blot analysis of pcDNA3.1 (-) Myc-His A/FRP and pcDNA3.1 (-) Myc-His A after transient transfection in Cos7 cells.

This figure details the protein detection of FRP by Western Blotting after transient transfection in Cos7 cells with pcDNA3.1 (-) Myc-His A/FRP and pcDNA3.1 (-) Myc-His A using an anti-Myc antibody. The empty vector alone resulted in no expression whilst the FRP cDNA in an expression vector resulted in a band of approximately 40kDa.

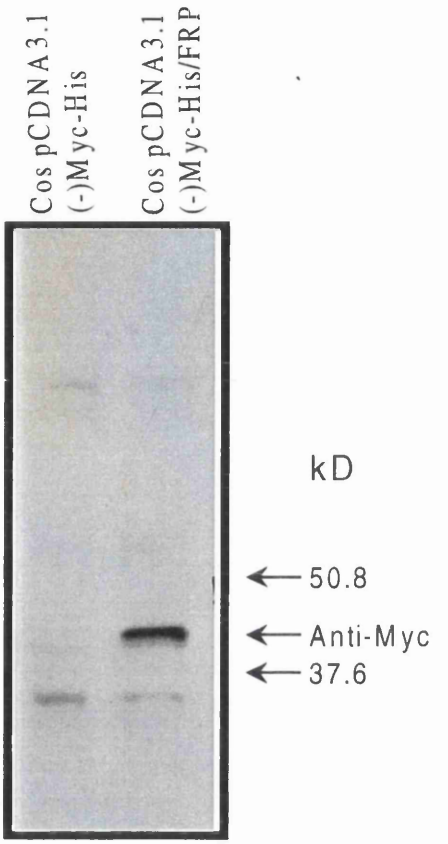


Figure 5.5
Protein detection by Western Blot analysis of pcDNA3.1(-) Myc-His A/FRP and pcDNA3.1 (-) A after Transient Transfection in Cos7 cells

Figure 5.6

Transient transfections in 208F, FBR, RAS and Tam-67 cell lines: Merged pictures of FRP and F-actin staining

The indirect staining of FRP is shown in green and the F-actin is shown in red. Panels (a), and (b), are two different 208F cells indirectly stained for FRP and F-actin, (c), and (d), are two different FBR cells indirectly stained for FRP and F-actin, (e), and (f), are two different RAS cells indirectly stained for FRP and F-actin, and (g), and (h), are two different FBR Tam-67 cells indirectly stained for FRP, and F-actin.

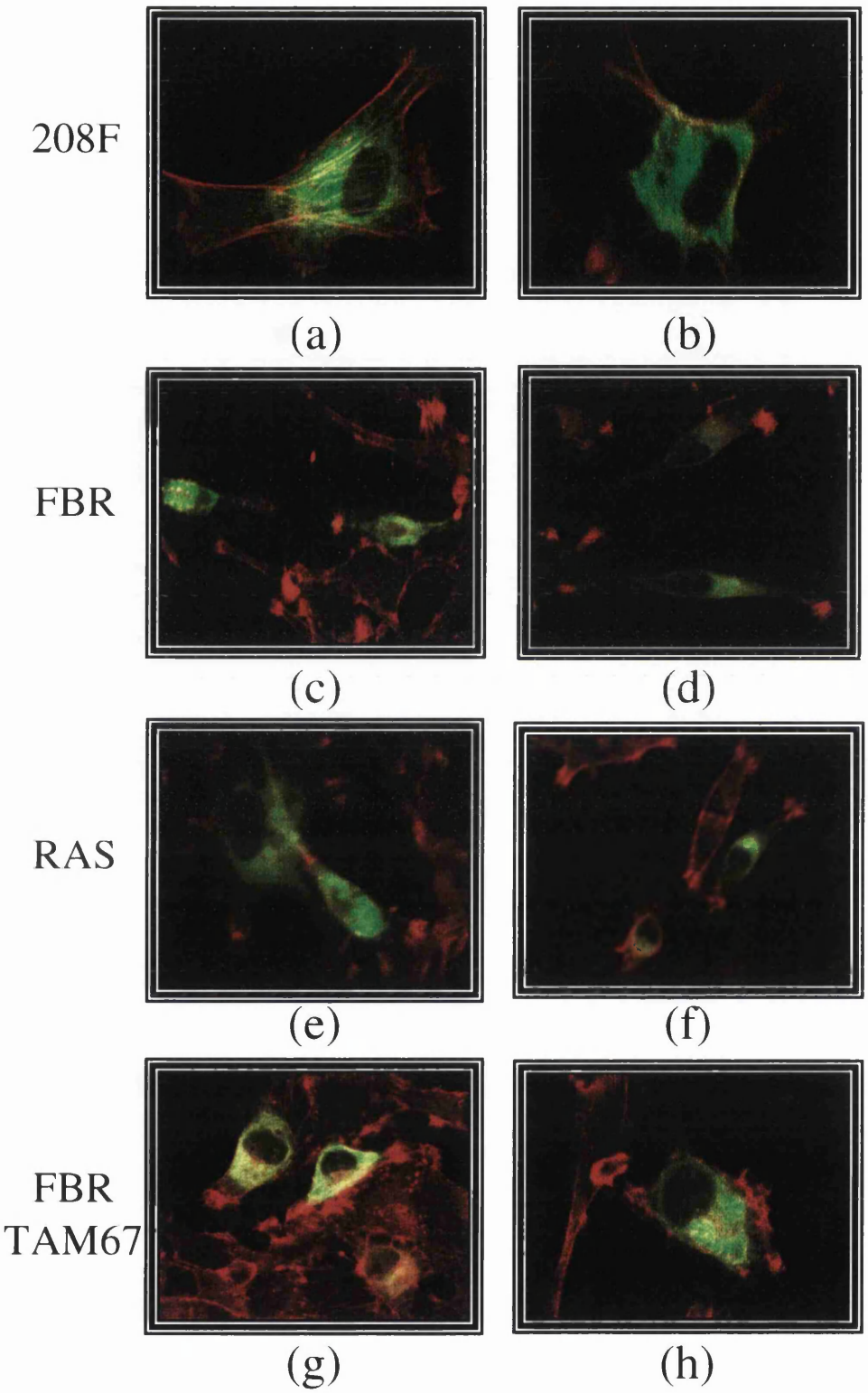


Figure 5.6
Transient Transfections in cell lines:Merged pictures of Anti-Myc FRP and F-Actin staining

The FBR cells have a very different morphology to the 208Fs as they are highly refractile, bipolar cells with no stress fibers. The F-actin runs as thick cables along the length of the bipolar cell and is concentrated in the cortex and pseudopodia, which are capped by actin rich bundles (See arrows, Figure 5.8, panel (a)). The cell on the right in Figure 5.8, Panel (a), (b), (c), and (d), although transiently transfected with FRP is a good example of the phalloidin staining of FBRs. The actin staining pattern of both the transfected and adjoining untransfected FBR *v-fos* transformed FRP cells is shown by Figure 5.8. The FRP stained cell is on the left of the panels, under FRP. The localisation of the FRP in transfected FBR cells are shown by Figure 5.8. The expression of FRP in all of the transfected cells examined was of a peri-nuclear, cytoplasmic localisation with a slight punctate expression, particularly in Panels (a)-(d), but the expression in the two cells in Panels(e)-(h), is very high and not punctate. This punctate appearance is most likely the expression of FRP associated with the ER and golgi bodies during secretion. The expression in FBR FRP transfected cells is proximal to the nucleus in the cell body. As the figure exemplifies, no expression was seen at the pseudopod tips. The transient transfection of FRP in FBR cells does not revert the bipolar, transformed phenotype.

The localisation of the FRP in two transfected RAS cells is shown in Figure 5.9. The expression in the RAS cell line was again mostly peri-nuclear and of a punctate appearance as the lower transfected cell in Panels (a)-(e), and both of the transfected cells in Panels (e)-(h), in Figure 5.9 highlight. The RAS cell line has a very different morphology to that of the fibroblastoid parental cells. The RAS cells in a cultured population are of an irregular morphology. A cell type commonly present is of a somewhat flat irregular triangular or rectangular shape, with multiple short actin rich protruding edges (See arrows, Figure 5.9, panel (a)). It is rich in actin bundles and sometimes has an actin rich ruffling edge (See arrow, Figure 5.9, panel (b)). This cell type can also have weak stress fiber bundles along the ventrical of the cell. Another cell type, with a shorter and broader bipolar morphology (See Figure 5.9, panels (e)-(h)), has F-actin staining along the edges of the cell with thick actin rich ruffling bundles at either end of the cell and weak stress fibers running in parallel along the length of the cell (See arrows, Figure 5.9, panel (f)).

The FBR Tam-67 cells have a partially reverted phenotype similar to 208Fs. They are of a flat morphology with prominent stress fibers that run in parallel arrays along the ventral surface of the cell, but also have actin rich ruffling edges at one or both edges of the cell (See arrows, Figure 5.10, panel (e)). This actin staining pattern of FBR Tam-67s is seen in both the transfected and adjoining untransfected FRP

Figure 5.7

208F Transiently Transfected with myc tagged FRP expression vector and indirectly stained for FRP and F-actin

The panels (a)-(d), and (e)-(h), are sectioned Z-steps from the bottom of the cell upwards at 0.5 μm increments of transiently transfected 208F cells. The FRP stained cell is on the left of the panels, under FRP, and the same cell stained for F-actin, under F-actin, is on the right. Other cultured but untransfected cells are also shown in the F-actin stained right half of the panel.

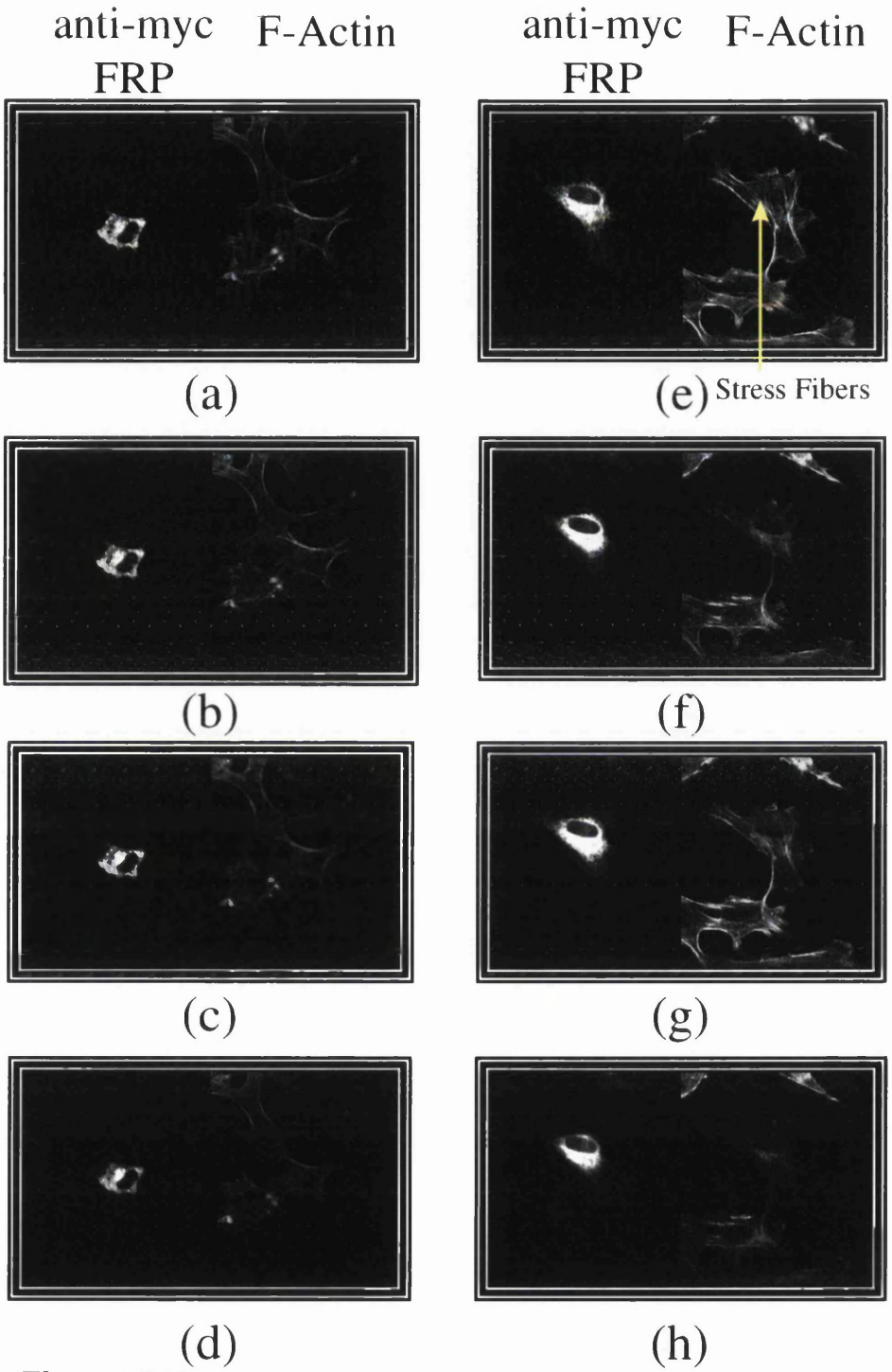


Figure 5.7
208F FRP Transient Transfection: Anti-Myc FRP and F-Actin staining

Figure 5.8

FBR cells Transiently Transfected with myc tagged FRP expression vector and indirectly stained for FRP and F-actin

The panels (a)-(d), and (e)-(h), are sectioned Z-steps from the bottom of the cell upwards at 0.5 μm increments of transiently transfected FBR cells. The FRP stained cell is on the left of the panels, under FRP, and the same cell stained for F-actin, under F-actin, is on the right. Other cultured but untransfected cells are also shown in the F-actin stained right half of the panel.

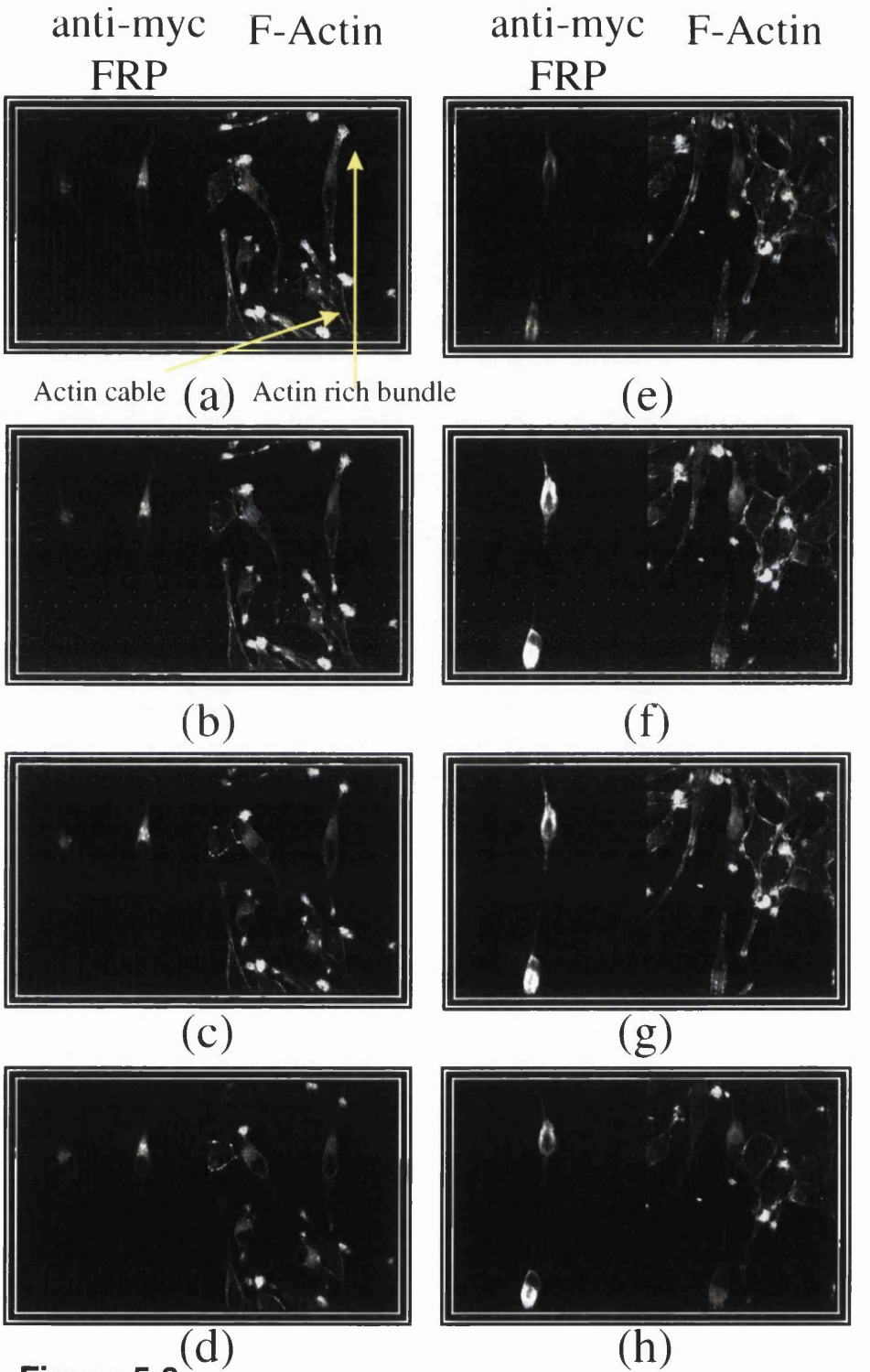


Figure 5.8
FBR FRP Transient Transfection: Anti-Myc FRP and F-Actin staining

Figure 5.9

RAS cells transiently transfected with myc tagged FRP expression vector and indirectly stained for FRP and F-actin

The panels (a)-(d), and (e)-(h), are sectioned Z-steps from the bottom of the cell upwards at 0.5 μm increments of transiently transfected RAS cells. The FRP stained cell is on the left of the panels, under FRP, and the same cell stained for F-actin, under F-actin, is on the right. Other cultured but untransfected cells are also shown in the F-actin stained right half of the panel.

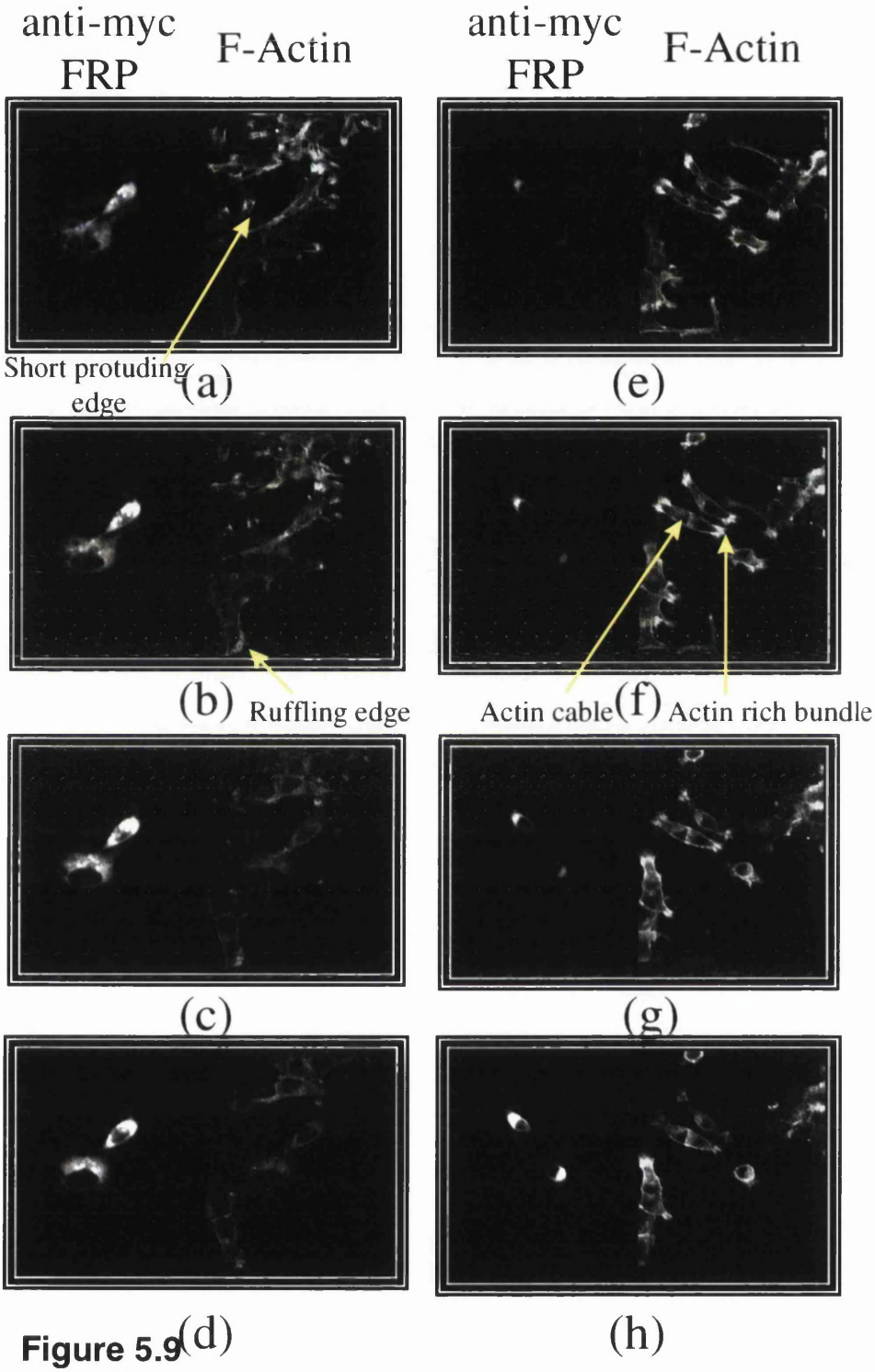


Figure 5.9
RAS FRP Transient Transfection: Anti-Myc FRP and F-Actin staining

cells and is shown by Figure 5.10. The localisation of the FRP in two representatives of transfected FBR Tam-67 cells is shown by Figure 5.10. The expression of FRP in all of the transfected cells examined was of a cytoplasmic localisation, proximal to the nucleus.

FRP is localised peri-nuclear in the cytoplasm with a punctate expression in all of the cell lines. A feature of secreted proteins is the localisation in the ER and golgi bodies. The cytoskeletal arrangements of actin was maintained in all of the FRP transfected cell lines.. No morphological reversion of the transformed phenotype is observed in any of the FRP transiently transfected cells or in the 208Fs or FBR-Tam67 cells. Control transfections were performed using the expression vector without a gene and with a myc-tagged β-gal reporter gene. The empty expression vector did not stain for myc, whilst the myc tagged β-gal reporter also localised cytoplasmically. The staining patterns for F-actin in the control transfected cell lines was the same as in untransfected cells (Data not shown).

5.2.5 Stable Transfection of FBR Cell Line with Myc tagged FRP

To further investigate the effect of expression of FRP-myc in FBR cell line a stable clone was generated. The stable cell lines were derived to determine the effect of long term FRP expression changes in morphology, proliferation rate, anchorage independent growth and invasiveness.

The stable expression of FRP in FBR cell line was achieved as described in Chapter 2, section 2.2.24, using the previously described pcDNA3.1-Myc-His A/FRP expression vector. A neomycin resistant FBR clone, Neo, was also isolated using the empty vector described pcDNA3.1-Myc-His A. After transfection and G418 selection, twelve FRP expressing clones and six Neo clones were propagated. The homogeneous expression of FRP in the different clones was analysed initially immunocytochemically using indirect immunofluorescence. Although all of the clones contained cells that expressed myc-tagged FRP, only in clone six, named FRP Clone 6, were all the cells expressing myc-tagged FRP. Figure 5.11 exemplifies the expression of myc-tagged FRP in FRP Clone 6 and also demonstrates the absence of expression in Neo Clone 2. The F-Actin staining of the FRP expressing and non-expressing FBR clones is also shown.

The FRP Clone 6 and Neo Clone 2 were further analysed by Western Blotting to further confirm expression in soluble protein extracts and in the conditioned media.

Figure 5.10

FBR Tam-67 cells transiently transfected with myc tagged FRP expression vector and indirectly stained for FRP and F-actin

The panels (a)-(d), are pictures of the same FBR-Tam-67 cells sectioned in Z-steps from the bottom of the cell upwards at 0.5 μm increments. The panels (e)-(h), are pictures of the same FBR Tam-67 cells sectioned in Z-steps from the bottom of the cell upwards at 0.5 μm increments. The FRP stained cell is on the left of the panels, under FRP, and the same cell stained for F-actin, under F-actin, is on the right. Other cultured but untransfected cells are also shown in the F-actin stained right half of the panel with a punctate expression, particularly in Panels (a)-(d). The expression in the two cells in Panels(e)-(h), is more pronounced.

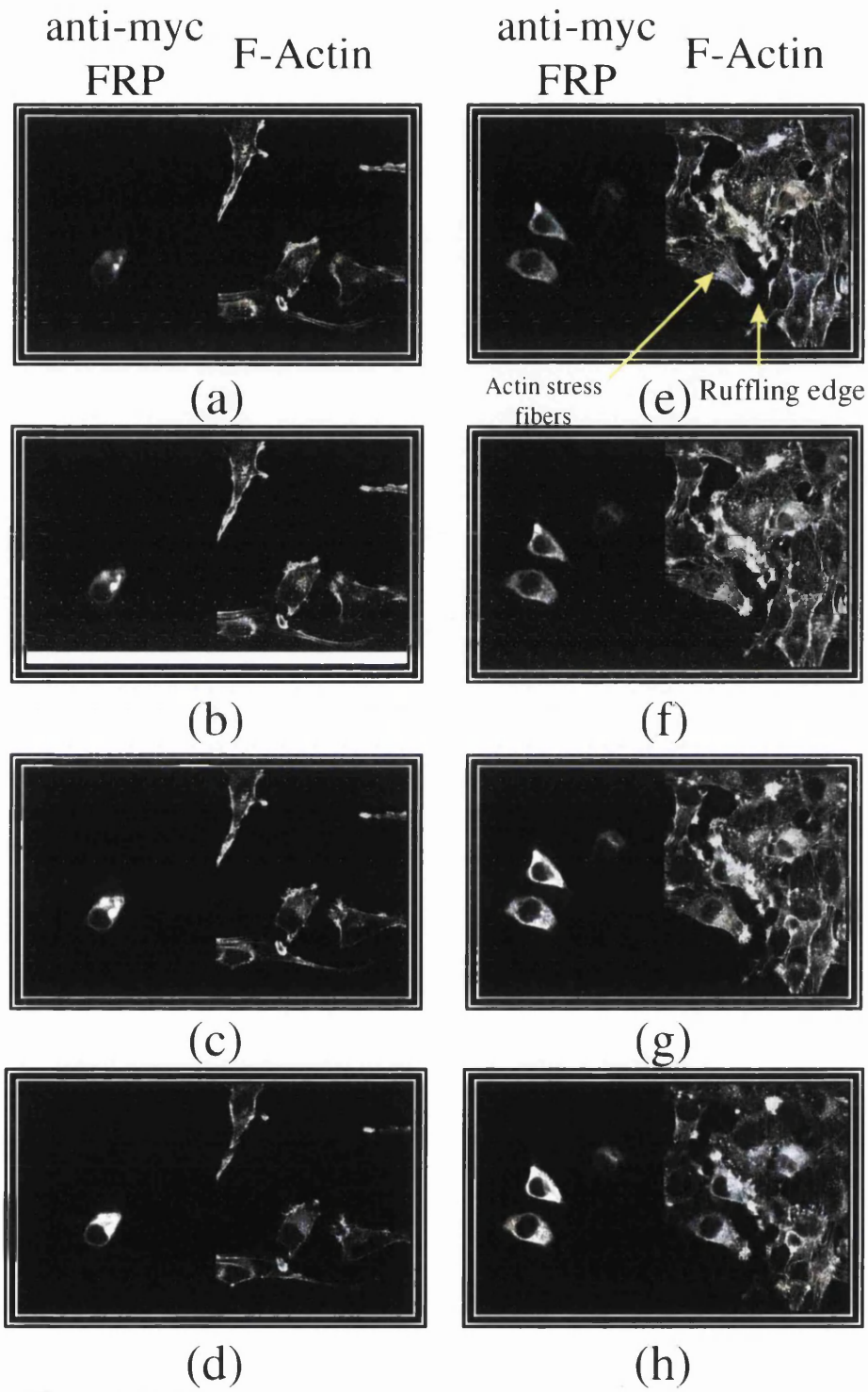


Figure 5.10
FBR Tam-67 FRP Transient Transfection: Anti-Myc FRP and F-Actin staining

This ensured that the correct size of protein was being produced and secreted. The soluble proteins were extracted from cells growing in media supplemented with 10% serum, and conditioned media from the clones cultured in serum-free conditions for four days. The detection of protein by Western blotting was determined using a primary mouse Myc antibody and a secondary HRP-conjugated anti-mouse antibody. The resulting expression of FRP-myc is shown by Figure 5.12 which confirmed that the FRP-myc protein was indeed expressed and secreted in FRP Clone 6 and was of the correct molecular mass.

The cDNA sequence of rat FRP predicts an open reading frame from the first Kozak ATG initiation signal at positions 64-981. This results in a polypeptide of 306 amino acids with a calculated molecular mass of 34.6kDa and an isoelectric point of 5.2. Computer analysis of the amino acid sequence predicts an eukaryotic signal sequence with a cleavage site between residues 18 and 19 (positions -1 and 1). that suggest it is a secreted protein. The translation starting with the N-terminal amino acid sequence of EEEQRS results in a mature product of 32.5kDa and the protein contains three potential N-glycosylation sites (Asn-Xaa-Ser/Thr), at amino acid positions 124, 155 and 160, and 20 Cys residues. Recombinant expression of rat FRP in Cos1 cells pulse labeled with [^{35}S] cysteine and subsequent SDS/PAGE analysis revealed a diffuse molecular mass pattern of 40-48kDa75kDa (Zwijsen *et al.*, 1994). The concentration of Cos1 cells expressing FRP conditioned media by hydroxyapatite chromatography and partially purified by heparin-Sepharose chromatography and Superdex G75 gel-filtration chromatography resulted in FRP elution in a fraction of 55-75kDa (Zwijsen *et al.*, 1994). Thus, the native molecular mass of FRP is 55-75kDa.

5.2.6 Characterisation of FBR FRP cell line

5.2.6.1 Morphological characteristics

The morphological characteristics of the FRP Clone 6 were examined and compared to the typical bipolar FBR like Neo Clone 2 cells, with regards to loss of the bipolar shape and retraction of pseudopod extensions into a flatter cell morphology. No obvious differences were detected. Figure 5.13 depicts the cultured cells photographed using a Nikon Diaphot camera. No apparent change in morphology at confluence, or sub-confluence was observed. The cells exhibit a typical FBR-like refractile bipolar morphology with no shortening of the pseudopodia extensions.

Figure 5.11

FBR Neo Clone 2 and FRP Clone 6 cells indirectly stained for FRP and F-actin

The panels (a), (b), and panels (c), (d), are pictures of the same FRP Clone 6 cells, with the FRP expression shown by panels (a), and (c), with the corresponding F-Actin staining shown by panels (b), and (d), respectively. The panels (e), and (f), are pictures of the same FBR Neo Clone 2 cells, with the absence of FRP expression shown by panel (e), with the corresponding F-Actin staining shown by panel (f).

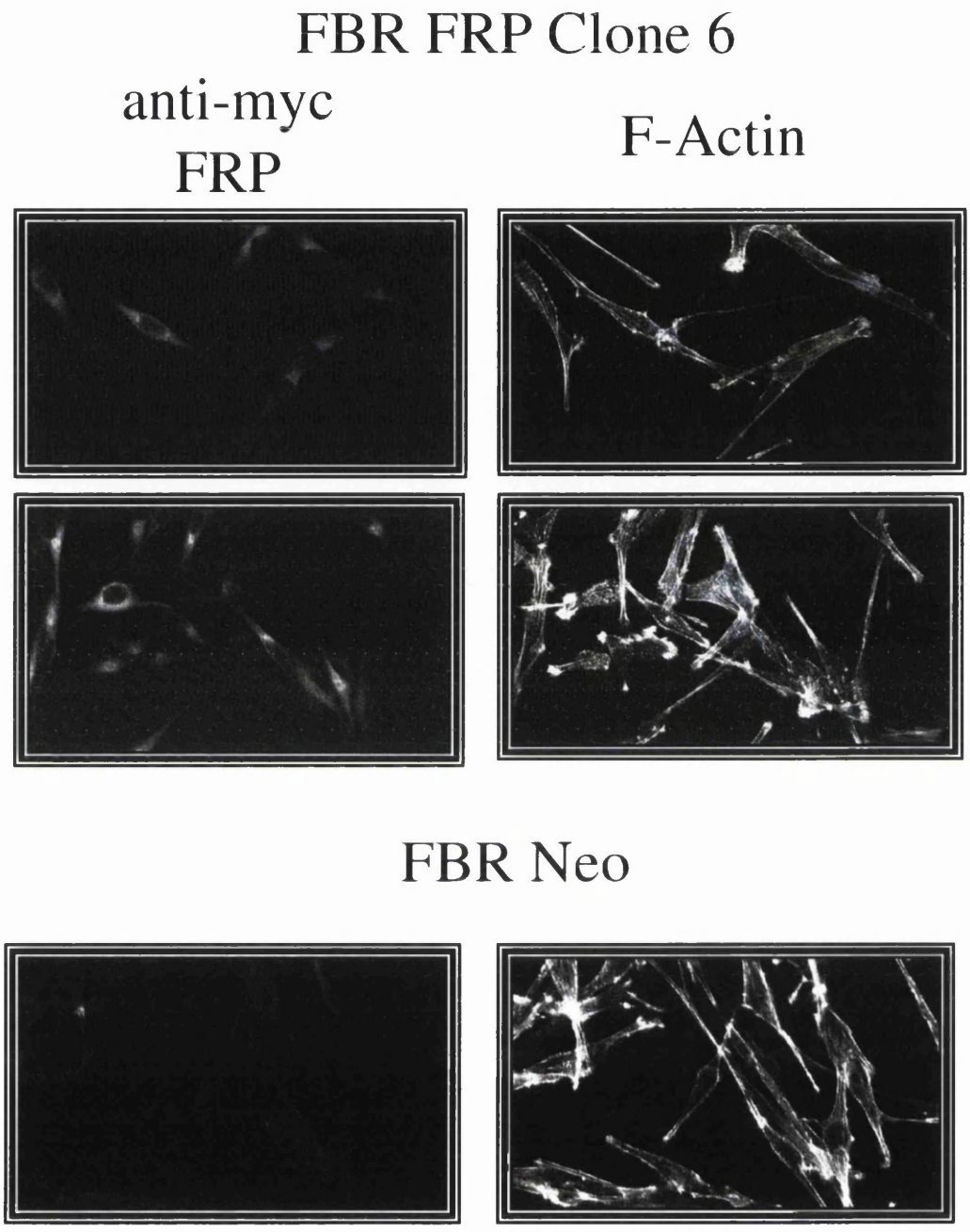


Figure 5.11
FBR Stable expressing FRP and Neo Clone

Figure 5.12

Protein detection by Western blot analysis of FBR Neo Clone 2 and FRP Clone 6

This figure details the protein detection of FRP by Western blotting in FBR Neo Clone 2 and FRP Clone 6 using an anti-Myc antibody. Panel A in the top of the figure shows that using soluble protein from exponentially growing cells, the Neo Clone 2 has no expression whilst the FRP Clone 6 has protein expression of a specific band around 40 kDa. Panel B in the bottom of the figure shows that using conditioned media from quiescent cells, the Neo Clone 2 has no expression whilst the FRP Clone 6 has an excreted native protein of a band around 55 kDa.

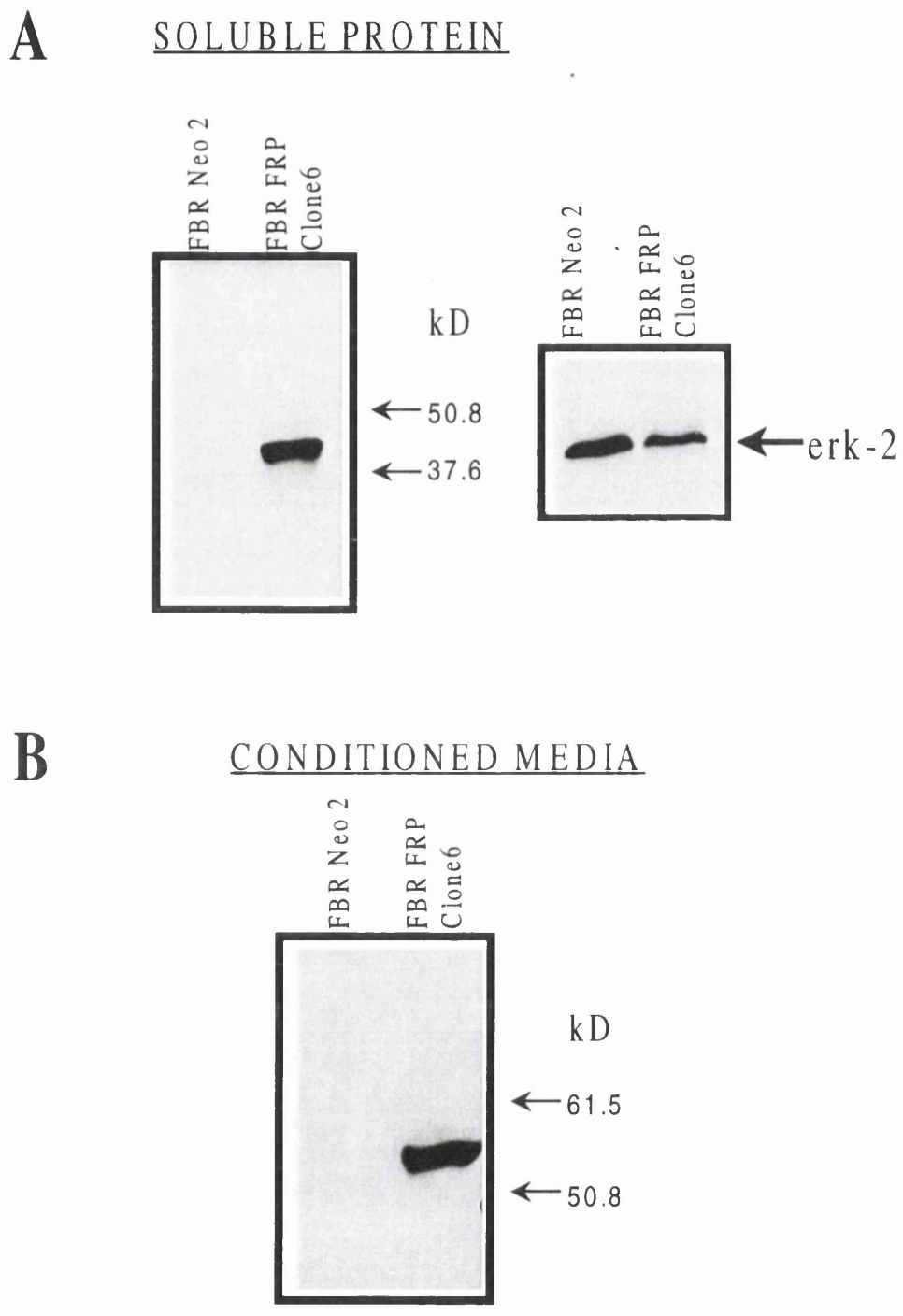


Figure 5.12
Protein detection by Western Blot analysis of FBR NeoClone 2 and FRP Clone 6

Figure 5.13

Morphological Characteristics of FRP Clone 6 and FBR Neo 2

The stable cell lines were grown in DMEM supplemented with 10% FCS and photographed with a Nikon inverted Diaphot microscope. Panel (a), shows the FBR Neo Clone 2 cells at a high density and panel (b), at a lower cell density. Panel (c), shows the FBR FRP Clone 6 cells at a high density and panel (d), at a lower cell density.

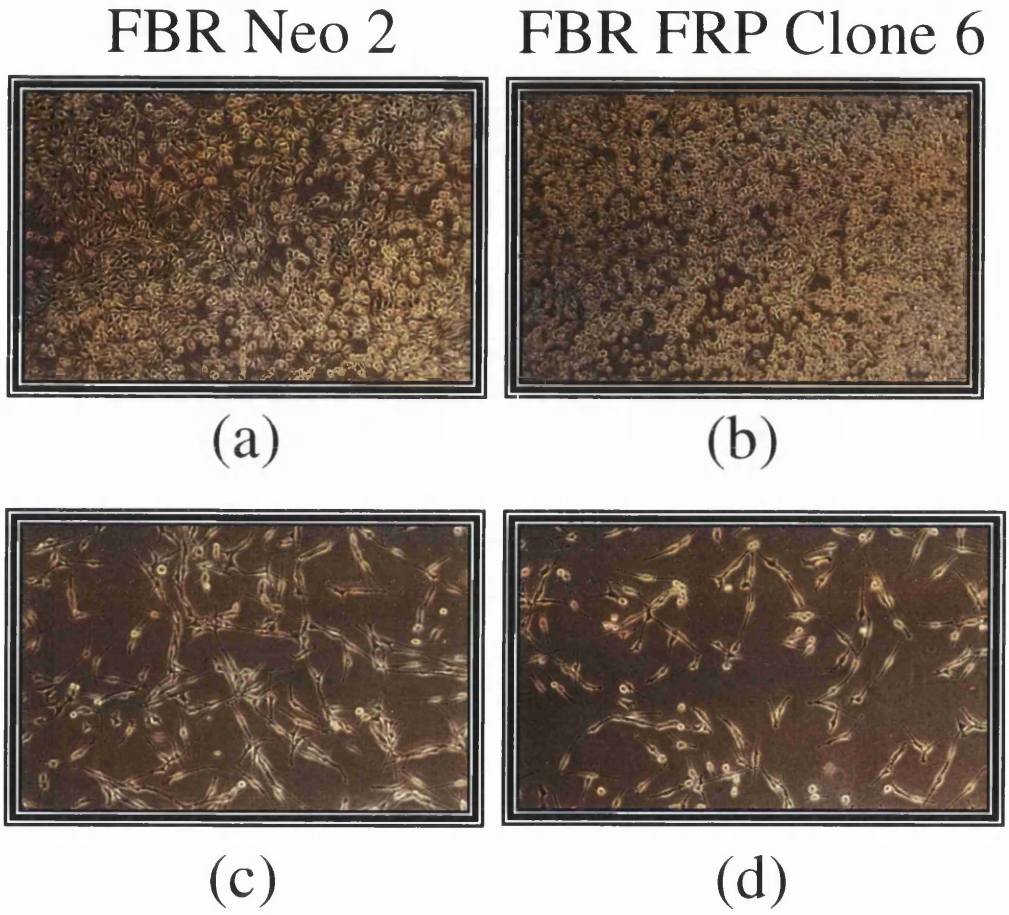


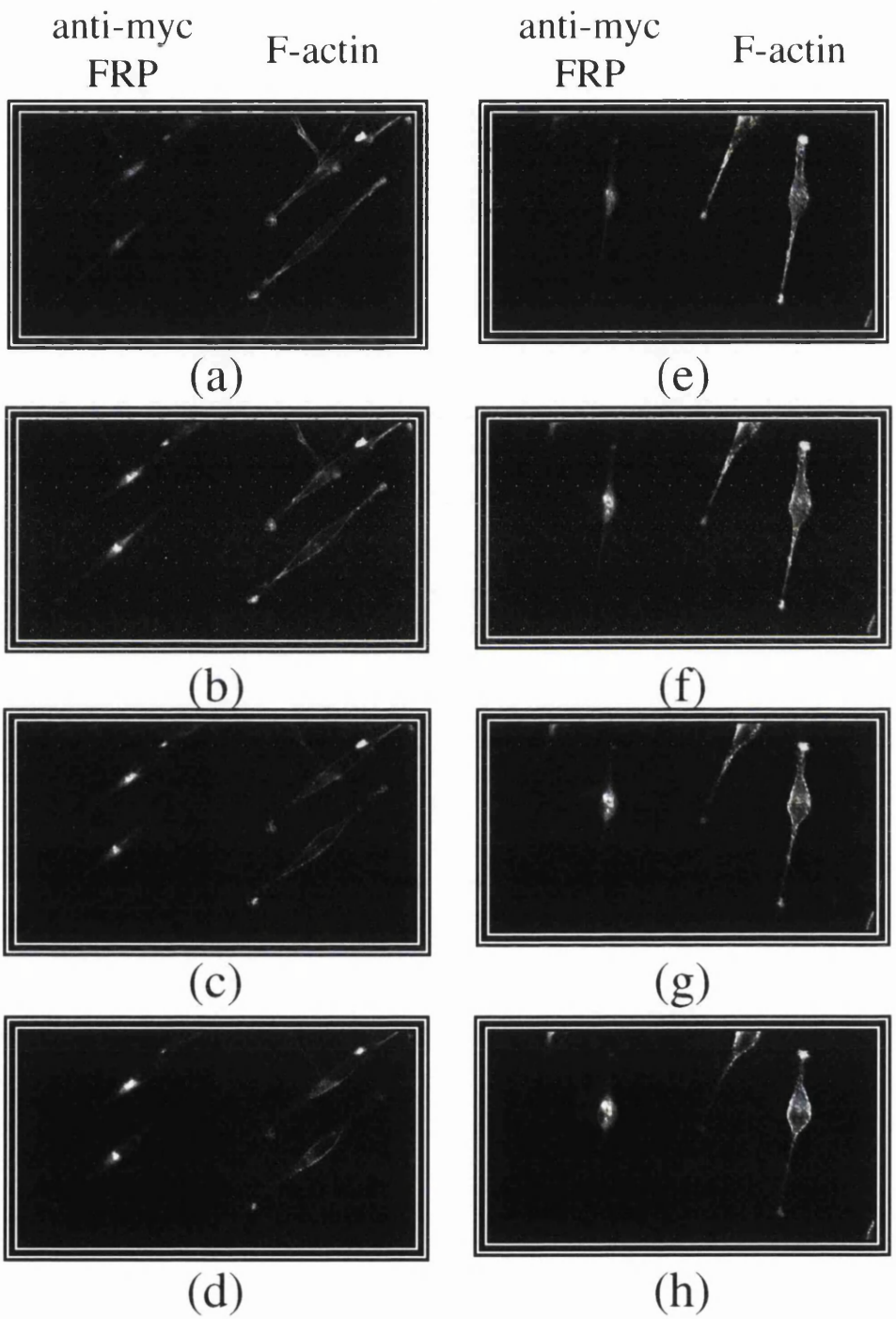
Figure 5.13
Morphological Characteristics of
FBR Neo Clone 2 and FBR FRP Clone 6

Figure 5.14

FBR FRP Clone 6 and FBR Neo Clone 2 indirectly stained for FRP and F-actin

The confocal images shown by the panels (a)-(d), (e)-(h), and (i)-(l), are three different FRP Clone 6 cells, sectioned in Z-steps from the bottom of the cells upwards at 0.5 μm increments. The panels (m)-(p), represent FBR Neo Clone 2 cells sectioned from the bottom of the cell upwards at 100 micometer increments. The FRP stained cells are on the left of the panels, under FRP, and the same cells stained for F-actin, under F-actin, is on the right.

FBR FRP Clone 6



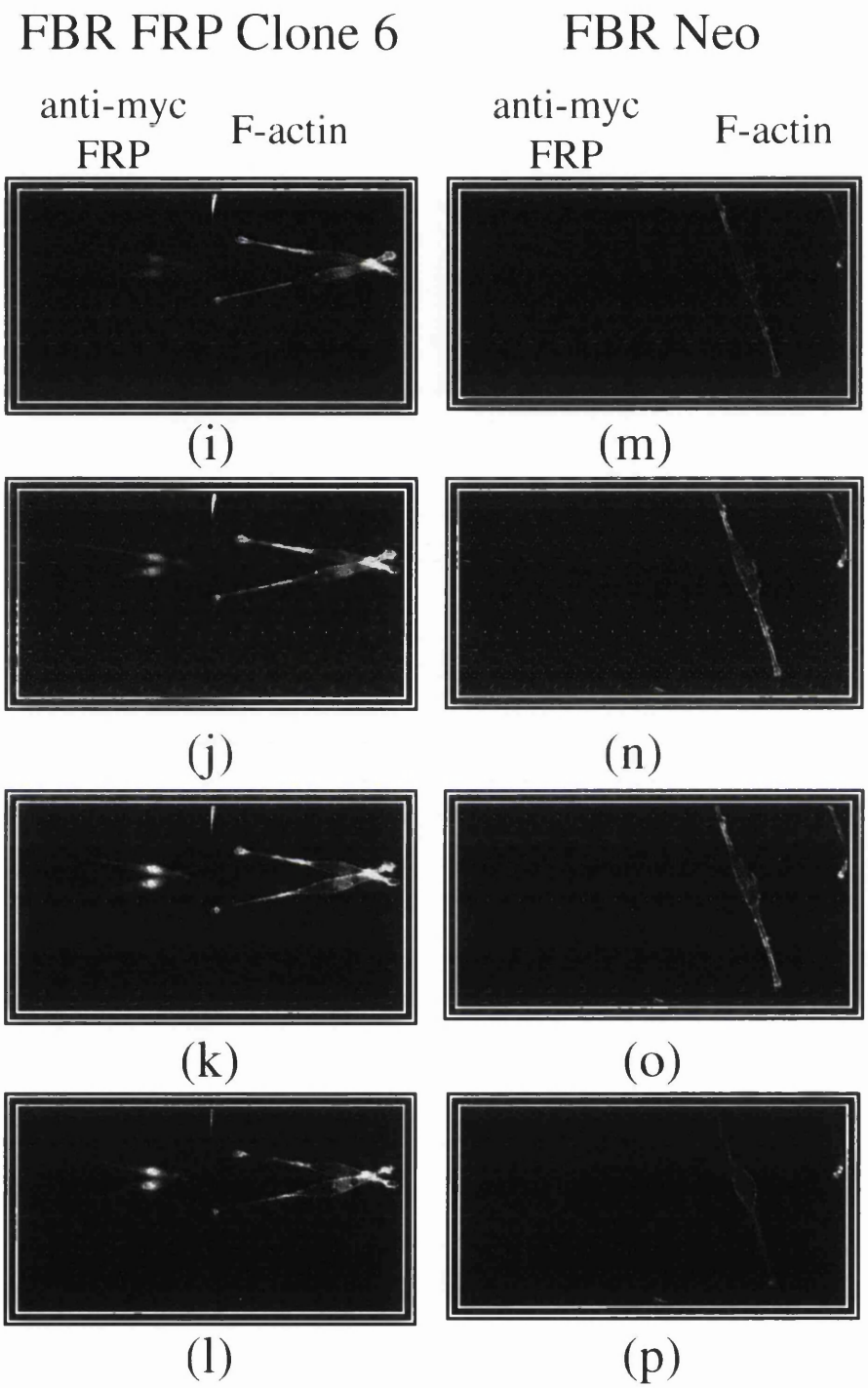


Figure 5.14
FBR FRP Clone 6 and FBR Neo Clone 2 indirectly stained for FRP and F-actin

Cells were further examined by immunofluorescence to examine F-actin structure and to confirm the expression of FRP-myc. The expression of myc-tagged FRP and F-actin in three representative fields of FRP Clone 6 and one representative field of Neo Clone 2 is shown by Figure 5.14. Detailed examination of the FRP Clone 6 reveals that although FRP is expressed, there is no change in the F-Actin distribution. In the Neo Clone 2 the F-actin locates as thick cables that run along the length of the bipolar cell and is concentrated in the cortex and pseudopodia, that are capped by actin rich bundles. The merged pictures of myc tagged FRP (Green), and F-actin (Red), localisation for FRP Clone 6 cells is shown by Figure 5.15. FRP is localised cytoplasmically in all of the cells examined whilst the cytoskeletal distribution pattern of F-Actin is maintained. However, within the cellular population of FRP Clone 6, a subclone population with a flatter morphology, which at first appeared to be a reverted cell type, existed. The merged pictures of myc tagged FRP (Green), and F-actin (Red), localisation for FRP Clone 6 cells Figure 5.16 shows that these flatter cells are in fact undergoing cell division.

5.2.6.2 Proliferation rate and colony formation in soft agar

FRP is down-regulated in *v-fos* and K-*ras* transformed 208F fibroblasts, in *v-myc* transformed NIH3T3 cells and also in various human tumour cells (Mashimo *et al.*, 1997). Therefore, it was possible that FRP exhibited a tumour suppressor function through negative growth regulation. The proliferation rate of the FBR Neo Clone 2 was compared to the proliferation rate of FRP Clone 6 over six days to ascertain whether FRP suppresses the growth of the *v-fos* transformed cell line. The results of the proliferation rate analysis are shown by Table 5.1. There is no significant difference in the rate of proliferation in the cells examined and furthermore, the cells were unable to proliferate in low serum, (0.5%), conditions.

DAYS	TOTAL CELLS FBR Neo Clone 2	TOTAL CELLS FBR FRP Clone 6
0	5×10^3	5×10^3
1	1.8×10^4	2.0×10^4
3	2.5×10^4	2.8×10^4
4	3.2×10^4	3.3×10^4
6	7.9×10^4	8.7×10^4

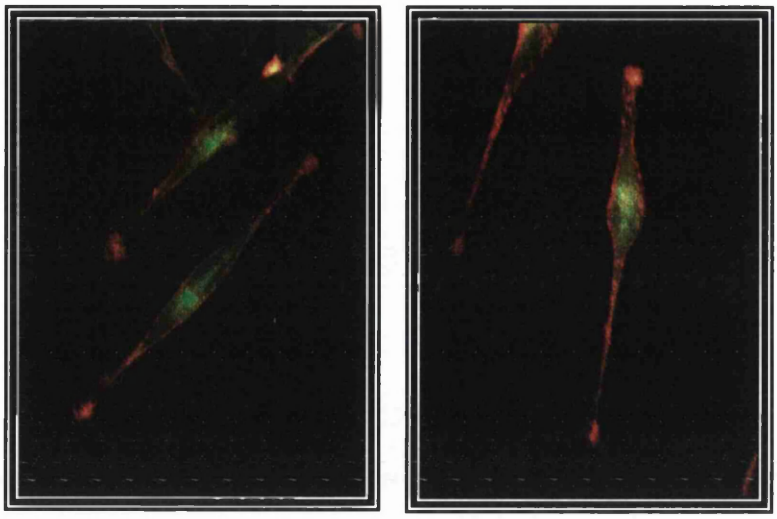
Table 5.1 Proliferation Rates of FBR Neo Clone 2 and FBR FRP Clone 6

Figure 5.15

Merged pictures of FRP and F-actin staining

The indirect staining of FRP is shown in green and the F-actin is shown in red. Panels (a), and (b), and (c), are different cells of FRP Clone 6 population and panel (d), shows a representative cell of FBR Neo Clone 2.

FBR FRP Clone 6



(a) (b)

FBR Neo 2 Clone

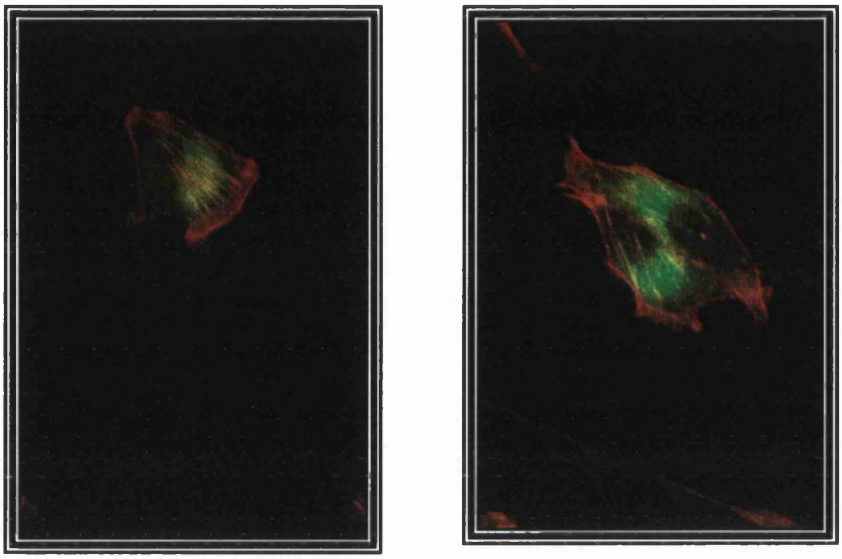
(c)

Figure 5.15
Merged pictures of anti-myc and F-Actin staining in FBR Stable expressing FRP and Neo Clone

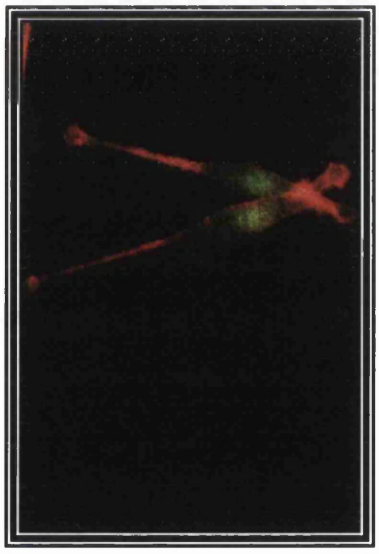
Figure 5.16

Merged pictures of FRP and F-actin staining

The indirect staining of FRP is shown in green and the F-actin is shown in red. Panel (a), shows a FRP Clone 6 cell undergoing division, panel (b), shows two cells after mitosis and panel (c), shows the bipolar phenotype of two cells after mitosis



(a) (b)



(c)

Figure 5.16
Merged pictures of anti-myc FRP and F-actin staining FBR FRP Clone 6 during Cell Division

Previous work in this laboratory has characterised FBRs as anchorage independent, whereas 208Fs are unable to grow in semi-solid media. Flat revertants isolated from FBJ-MuSV and FBR-MuSV transformed cell lines failed to form colonies in semi-solid medium and do not form tumours after injection into nude mice (Zarbl *et al.*, 1997; Wisdom and Verma, 1991). The anchorage-independent growth of the FBR FRP Clone 6 and FBR Neo Clone 2 was examined by determining their ability to form colonies in methylcellulose over 18 days. The number of colonies formed were counted in ten representative fields of 0.25 cm^2 per 10cm petri dish. The number of colonies per plate and their morphology are shown by Figure 5.17 which highlights the ability of both the FBR Neo Clone 2 and FBR FRP Clone 6 to grow under anchorage-independent conditions and additionally the colonies formed in both of the clones examined were of similar size and distribution.

5.2.6.3 Response of FRB neo and FBR FRP to Matrigel Invasion Assay

Invasion occurs when cells from the primary tumour site migrate through the cellular basement membrane components into surrounding tissues resulting in metastasis. A quantitative *in vitro* inverse invasion assay that determines the ability of cells to migrate across a membrane and then into a thick layer of the reconstituted ECM, reduced-growth-factor Matrigel, can be used to measure invasion (Hennigan *et al.*, 1994). In this assay, FBR cells are constitutively invasive (growth factor independent), whereas 208F parental cells are conditionally invasive being dependent upon growth factor stimulation, i.e. EGF, PDGF (Lamb *et al.*, 1997a).

The *in vitro* invasion assay was performed using FBR cell line as compared to FBR clone 6, and also using FBR neo 2 as compared to FBR clone 6. Cells were plated at equal density, 1 x 10^4 per well, and incubated for three days, after which the cells were fixed in the matrigel and the nucleus stained with propidium iodide. The invasion of the cells through the matrigel was quantified using confocal microscopy by examining the number of cells at ten μm intervals above the filter. As shown in Figure 5.18 FRP can suppress *in vitro* invasion of FBR cells. In this experiment the invasion of FBR FRP clone 6 cells are impaired by 70% as compared to FBRs and FBR neo clone 2.

Figure 5.17

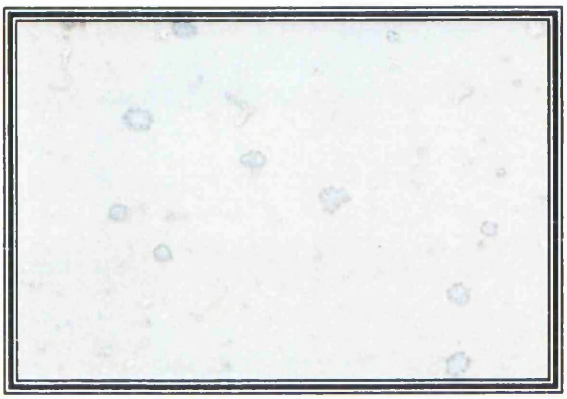
Comparative Growth of FBR Neo Clone 2 and FBR FRP Clone 6 in Methylcellulose

Cells were grown in methylcellulose containing 10% FCS for 18 days, and photographed with an inverted Nikon Diaphot microscope at 100x magnification. In section A, panel (a), the FBR Neo Clone 2 and panel (b), the FBR FRP Clone 6 represents colony size and distribution of the cell colonies.

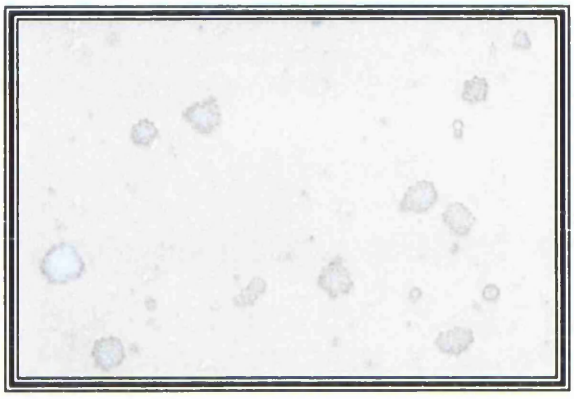
In section B the number of colonies per 10cm dish in duplicate for each of the clones examined is tabulated.

A Morphological Characteristics

FBR Neo Clone 2



FBR FRP Clone 6



B Table of Colony Growth

Clone	Number of Colonies per 10cm Petri Dish
FBR Neo 2	3780/3379
FBR FRP 6	3635/3428

Figure 5.17
FBR Stable expressing FRP and Neo Clone: Growth in Methylcellulose

Figure 5.18

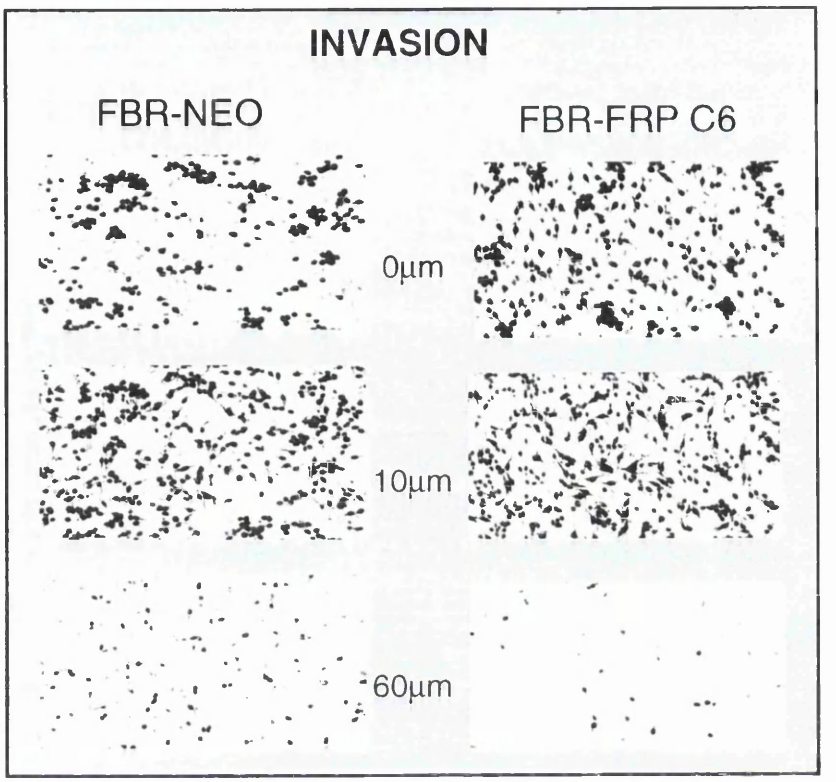
***In vitro* Inverse Invasion Assay of FBR Neo 2 and FBR FRP Clone 6**

A: The migration of FBRs and FBR FRP clone 6 is detailed. The confocal images are of propidium iodide-stained cell nuclei above the filter. The various micrometer points above the filter in the *in vitro* invasion assay is shown.

B: The bar graphs exemplifies the relative number of cells invading through the matrigel, as quantified with a Bio-Rad program (Cosmos), as percentage of total cells of FBR neo clone 2 and FBR FRP clone 6.

(This experiment was performed by Prof.Brad Ozanne)

A



B

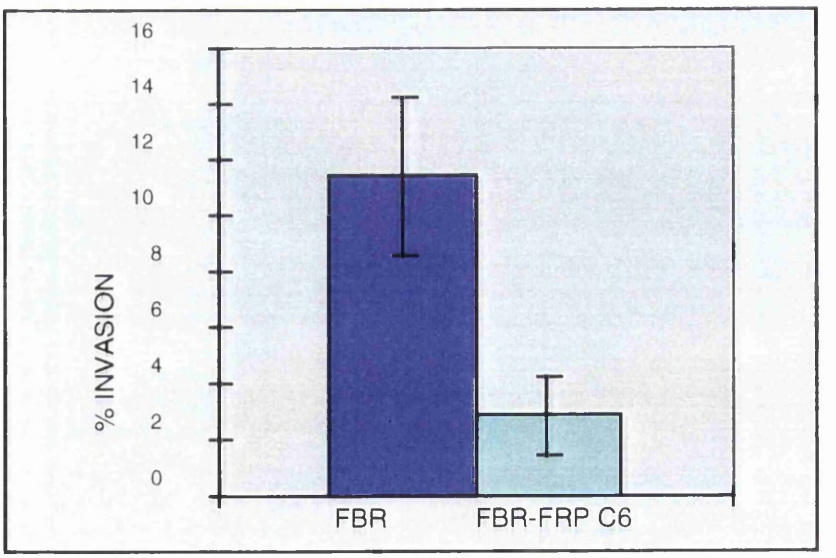


Figure 5.18
In vitro Inverse Invasion Assay of
FBR Neo 2 and FBR FRP Clone 6

5.3 Discussion

FRP is a secreted glycoprotein (Zwijsen *et al.,* 1994), and 24 hours post transient transfection followed by indirect immunoflourescence using anti-myc primary antibody locates FRP peri-nuclear in the cytoplasm in all of the cell lines examined, 208F, FBR, RAS, and FBR Tam-67. In most of the cells the expression was of a strong punctate appearance, similar to other overexpressed secreted proteins. This punctate appearance is most likely the secretion of the protein from the ER to the golgi bodies translocating the protein out of the cell. No expression was detected in the nucleus, at the cell membrane or at the tips of the FBR pseudopod extensions in any of the transiently transfected cells. No commercial antibody was available to locate protein expression in these cells, although no cellular protein has previously been detectable (Shibanuma *et al.*, 1993).

The biological functions of FRP are hinted at by the extinguished expression in *ras*-transformed cells that is recovered to a normal level in flat revertants plus the fact that TSC-36 was isolated as a TGF-β1effector gene (Shibanuma *et al.,* 1993), itself a potent inhibitor of growth in various cell types. In addition, FRP expression is also down-regulated in *v-ras* and *v-myc* transformants and also in human cancer cell lines (Mashimo *et al.,* 1997), suggesting a role in the negative regulation of tumour progression. The sequence similarity of FRP with the follistatin modules of follistatin (Shibanuma *et al.,* 1993; Zwijsen *et al.,* 1994), that have a structural motif for growth-factor binding, would also suggest a function in the growth regulation of cells.

Follistatin (reviewed by Phillips and de Krester, 1998), was first discovered during ovarian inhibin and activin purification from follicular fluid, where it was identified as suppressing FSH secretion, but with properties distinct from inhibin. However, follistatin is not only an activin-binding protein but can also bind to other members of the TGF-β superfamily, namely BMP-4 and BMP-7 (Fainsod *et al.*, 1997; Yamashita *et al.*, 1995), and also the serum protein α2-macroglobulin (Phillips *et al.*, 1997). Characterisation of the follistatin gene promoter found it to contain an AP-1 site (Miyanaga and Shimasaki, 1993).

The follistatin family (reviewed by Phillips and de Krester, 1998), of which there are thirteen, are based upon the presence of a highly conserved module sequence and are not based upon follistatin-like activity. The modular structure has sequence similarity with EGF, and with the Kazal family of enzyme inhibitors, such as ovomucoid. In most of the family members an insertion in the ovomucoid-like inhibitory loop ablates the protease activity in the follistatin domain.

The follistatin modules of agrin, to which FRP exhibits sequence identity (Shibanuma *et al.*, 1993; Zwijsen *et al.*, 1994), have a proposed growth factor affinity with a suggested role in the regulation of the activity of growth factors from the TGFβ and PDGF families (Patthy and Nikolics, 1993).

SPARC, also known as osteonectin, BM-40 and 43K protein regulates cellular-matrix interactions during vasculation, morphogenesis, wound healing and tumourigenesis (reviewed Lane *et al.*, 1994; Motamed and Sage, 1997). However ascribing the various functions of SPARC to defined protein modules has mapped biological function outwith the follistatin domains (Hasselaar and Sage, 1992; Lane *et al.*, 1994).

However, the homology that exists between FRP, Follistain, SPARC, and Agrin is limited. The common domain that all the proteins have is follistatin, whilst the remainder of the sequences are unrelated.

The sequence similarities of the mouse TSC-36 homologue, rat and human FRPs, plus their induction by TGFβ, suggests a growth-factor binding capacity and a biological function in the modulation of action of some growth factors on cell proliferation and differentiation.

To examine the possible role and function of FRP in *v-fos* transformants a stable cell clone expressing FRP was isolated after transfection with pcDNA3.1 Myc-His/FRP conferring G418, neomycin resistance. A stable cell clone conferring neomycin resistance was also isolated after transfection with pcDNA3.1 Myc-His vector alone.

The expression of FRP was confirmed immunocytochemically and by Western blot analysis. The cellular location of FRP in the stably expressing FBR clone was similar to that found in transient expression, with peri-nuclear cytoplasmic expression and no detectable expression in the nucleus, cell membrane or in the pseudopod extensions and tips in any cells examined. In several of the stable transfected cells the expression was of a strong punctate appearance, albeit at a lower intensity than the transiently transfected cells. As shown by Western blot analysis, a protein of approximately 41kDa was detected in Triton-X soluble protein extracts, whilst the protein detected in serum-free conditioned media was of approximately 55kDa.

Revertant cell lines of the transformed phenotype have a flat morphology mainly due to alterations in their cytoskeletal components (Weber *et al.*, 1975; Pollack *et al.*, 1975). Although most of the FBR FRP Clone 6 cells remained transformed in culture, with a characteristic FBR bipolar phenotype under phase contrast microscopy, a small portion of the cells appeared revertant with a flat morphology. Therefore, the

F-actin cytoskeleton architecture was studied as it would possibly reveal a correlation between oncogenic transformation and the appearance of cytoskeletal actin, to serve as a marker for reversion. In the bipolar phenotype cells, there was no change in the thick actin cables running along the length of the cells or the actin rich bundles at the pseudopod tip. However, it became apparent upon F-actin staining that the cell population with a flatter morphology were in fact undergoing cell division. Thus FRP expression does not morphologically revert *v-fos* transformants. A previous report of constitutive expression of FRP homologue, mouse TSC-36, in MC3T3 cells also detailed that the morphology was not significantly affected (Mashimo *et al.,* 1997).

FBR-MuSV and FBJ-MuSV transformed 208F cells are anchorage independent and can grow in soft agar, whereas 208F cells cannot (Hennigan *et al.,* 1994). In contrast, flat revertants of FBR-MuSV and FBJ-MuSV isolated from transformed cell lines do not form colonies in semi-solid medium or form tumours upon injection into nude mice (Zarbl *et al.,* 1997; Wisdom and Verma, 1990). The colony formation of FBR FRP Clone 6 and FBR neo clone in methylcellulose is of no significant difference with similar colony numbers of similar sizes forming. Therefore FRP expression does not affect anchorage independence of FBR cells.

As FRP sequence identity to Follistatin and follistatin like domains of Agrin suggested FRP had a role in the negative regulation of growth and tumour progression, the growth characteristics of the FBR FRP clone 6 cells were analysed in a proliferation assay. FRP expression did not affect proliferation of FBR cells in comparison to the FBR neo clone, in line with previous reportings of no significant growth changes in MC3T3 cells overexpressing FRP mouse homologue TSC-36 (Mashimo *et al.*, 1997). The FRP expressing clone maintains a transformed phenotype in serum-free media is not density inhibited and forms multiple layers of cells at confluence. These findings in conjunction with that of others (Mashimo *et al.*, 1997), dictates that FRP does not regulate cell growth, anchorage-independent growth or revert the transformed bipolar phenotype of FBR-MuSV transformed 208F fibroblasts.

However, the isolation of the FRP homologue TSC-36 after TGF-β induced stimulation (Shibanuma *et al.*, 1993), and ablation of expression in several human cancer cell lines, and in *v-ras* and *v-myc* transformed mouse fibroblasts (Mashimo *et al.*, 1997), indicated that down-regulation is required for tumour progression.

Another aspect of transformation that correlates with tumourigenesis is increased cell motility and invasion. The invasiveness of FBR and 208F cells was previously measured using a quantitative *in vitro* invasion assay which determines the

ability of cells to migrate across a membrane and into a thick layer of reduced growth factor Matrigel, reconstituted ECM (Hennigan *et al.,* 1994). In this assay, 208F fibroblasts are conditionally invasive requiring growth factors such as EGF, PDGF to invade efficiently, whereas FBRs are constitutively invasive (Hennigan *et al.,* 1994; Lamb *et al.,* 1997a).

AP-1 in transformed cells activates a multigenic invasion program. This rationale would dictate that genes which are found down-regulated by AP-1 may be involved in suppressing invasive behaviour as well as other transformation associated characteristics. Previous work in our laboratory details that expression of the hyaluronan receptor, CD44, is required for invasion of 208Fs and FBRs in an *in vitro* invasion assay. The blocking of CD44 expression by antisense oligonucleotides renders the FBR cells non-invasive. Additionally an increase in CD44 expression is dependent upon AP-1 activity in FBRs and in EGF stimulated 208Fs (Lamb *et al.*, 1997a). Furthermore, Ezrin, which associated with CD44 is up-regulated in *v-fos* transformants, and is required for pseudopod extension and cell motility which are two important aspects of invasion (Jooss and Muller, 1995; Lamb *et al.*, 1997b). Work by others has recently shown MMP-9, an AP-1 transactivated gene, which is up-regulated in many transformed cells and tumour derived cell lines associates with CD44 and this association is necessary for invasion (Yu and Stamenkovic, 1998). Therefore, the role of some of the *v-fos* up-regulated genes were examined and have been shown to be involved in invasive behaviour and transformation.

The most striking observation from the characterisation studies conducted in this study is that expression of FRP severely impaired invasion by 70 % in FBR cells. Interestingly, this dispels a previous correlation between the transformed bipolar morphology and the ability to invade (Hennigan, Ph.D. Dissertation, 1993). In our laboratory the motility of FBRs has been studied using time lapse phase contrast confocal microscopy and it is well documented that the cell extends an elongated pseudopod tip at one end with subsequent attachment of this leading projection, followed by nuclear translocation and finally the retraction of the tailing end, resulting in translocation of the entire cell (Hennigan, Ph.D. Dissertation, 1993; Lamb, personal communication). The locomotive state of the cells are similar when moving up through the polycarbonate filter pores and through the matrigel. The FBRs when inside or on top of the matrigel are more extremely bipolar than those in routine culture (Hennigan, Ph.D. Dissertation, 1993). In contrast, the invasive 208F cells that have migrated through the polycarbonate filter pores are of a fibroblastoid morphology. Invariably, conditionally invading 208Fs inside matrigel are bipolar, which suggested a correlation between the bipolar shape and invasiveness (Hennigan Ph.D. Dissertation, 1993).

However, the FRP expressing clone are almost exclusively bipolar and yet the invasive capability is severely retarded. In addition blocked expression of CD44 by antisense oligonucleotides in FBRs , does not induce phenotypic changes, but blocks invasion.

The FRP expressing cell line is not failing to invade through morphological limitations, growth inhibition, or anchorage dependence. Therefore, the mechanism by which FRP blocks invasion is by other means.

The proposed function of FRP has previously been associated with tumour suppression, and the sequence homologies to Follistatin and SPARC indicated that the FRP mouse homologue TSC-36 has an ability to associate with other extracellular proteins (Shibanuma *et al.,* 1993; Mashimo *et al.,* 1997). A recent report examined the link of FRP homologue mouse TSC-36 with ECM binding whereby it was examined whether TSC-36 protein bound heparin sulphate and collagens. The results show that TSC-36 protein did not bind type I and type II collagen, activin A, TGF β1, inhibin A , or BMP4/7, but had a low affinity to sulfate-cellulofine. This indicated that the binding affinity of TSC-36 is different to that of Follistatin and SPARC, although gel-filtration analysis highlighted a potential association with other, as yet unidentified, extracellular proteins (Mashimo *et al.*, 1997).

This tentative link with extracellular protein binding would suggest that in 208F cells, FRP functions as an extracellular autocrine binding protein with tumour suppressor activity. The mechanism by which it functions is unknown but the homologous regions with follistatin modules of follistatin, agrin and SPARC would suggest a conserved binding domain. The high number of cysteine residues (6.5%), and their distribution into two separate regions in the sequence indicates the presence of several disulphide bridges that would stabilise the protein conformation. PROSITE sequence comparisons and two-dimensional PAGE analysis reveals extensive post-translational modifications including several phosphorylation sites, six of which are CKII phosphorylation sites, the other a protein kinase C phosphorylation site. The signalling pathway may function through these phosphorylation sites in the protein. In FBRs the AP-1 transrepressed FRP and ablated tumour suppressor signalling pathway would be switched off. The over expression of FRP in FBRs would result in a block of invasion through this extracellular binding to matrix proteins and subsequent signalling pathway.

In summary, the presented studies into the effects of FRP in FBR *v-fos* transformants has revealed that it is not involved in cell morphology, anchorage-dependent growth or proliferation. However, it does play a significant role in suppressing invasion.

Chapter 6

Cloning and Characterisation of two Novel Down-regulated cDNAs in AP-1 Transformation

6.1 INTRODUCTION

In the down-regulated library many of the genes encode proteins whose function have previously been associated with various aspects of transformation. Several have known or suggested tumour suppressor activity, and many others have functions which alter ECM, cytoskeleton and transcription as discussed in chapter 3.

The expression analysis of these down-regulated gene clones by Northern blotting in oncogenic and mitogenic transformed cells as previously described in chapter 4, enabled selection of clones for further study. Two such clones were selected, KD1 and CD10. KD1 is a novel clone with homology to an EST sequence that exhibited a weak similarity to the helix-loop-helix transcription factor, Alf1. Sequence analysis of the clone CD10, revealed homology to a *frizzled* related protein, FrpAP.

This chapter reports the Northern blot expression, tissue distribution and cloning of the full-length KD1 clone, named as "Down In FBRs" (DIF1) transcript from a 208F cDNA library, sequencing and subsequent computational protein analysis. It also details the cloning of DIF1 open reading frame into an expression vector with a 3' Myc Tag, and also into an expression vector with a 3' GFP fusion protein and subsequent transient transfections into 208F, FBR, and RAS cell lines, to determine its affect and localisation in these cells.

Clone CD10 shares 100% identity to FrpAP, *frizzled* family member at the protein and DNA level over a short region. A full-length cDNA clone was isolated and its nucleic acid sequence revealed it to be near identical to FrpAP.. The Northern blot expression and tissue distribution of clone CD10 is reported in this chapter.

This chapter also reports the Northern blot expression, tissue distribution and cloning of the full-length FrpAp cDNA from a 208F cDNA library, sequencing and computational protein analysis.

The extracellular signalling molecules function as inducers of cell proliferation, migration, differentiation, and also in tissue morphogenesis during normal development. Moreover, they also function in aberrant growth regulatory pathways associated with neoplastic development. The *Wnt* family of glycoproteins are one such molecule involved in these processes. The *Wnt* gene products have long been recognised as one of the major players of developmentally important signalling molecules. Their signalling functions are vital to adult and embryonic pattern formation in Drosophila, in the establishment of the dorso-ventral axis in Xenopus embryos and also in the development of the central nervous system in mice, whereby targeted

disruption of the *Wnt*-1 gene results in serious defects of the midbrain and cerebellum, are reviewed (Cadigan and Nusse, 1997).

The identification of *Frizzled* family members of cell surface proteins function as *Wnt* receptors or as components of a *Wnt* receptor complex was initially discovered through the *Drosophila frizzled* (*Dfz*), gene. The experimental evidence was provided by mutations in *Dfz.* This caused tissue or planar polarity defects whereby alignment of the epithelial cells in the wing blade was disrupted, resulting in wing hairs pointing in several directions, and they also have disruptions of bristles on the notum and legs (Alder, 1992). In addition, a subsequently related gene, *Dfz2*, when expressed in various cell lines by transfection, bind *wingless*, the *Drosophila* homologue of *Wnt*, on their cell surface. Furthermore, stable transfection of *Dfz2* into nonresponsive *wingless* cells, which also lack *Dfz2* expression, enables the accumulation of *armidillo* protein, the β-catenin homologue, in an *wingless*-dependent manner (Bhanot *et al.*, 1996; Wang *et al.*, 1996; Wang *et al.*, 1997).

The *Frizzled* family members are integral membrane proteins characterised by their large extracellular portion at the NH_2 terminal with highly conserved cysteine-rich domain (CRD), that is the putative binding site for *Wnt* ligands (Bhanot *et al.*, 1996). They also contain several putative transmembrane domains and a cytoplasmic tail (Wang *et al.*, 1996; Vinson *et al.*, 1989).

The complex interaction between the various members of the *Wnt* and *Frizzled* families (Nusse and Varmus, 1992; Wang *et al.*, 1996; Bhanot *et al.*, 1996; Yang-Snyder *et al.*, 1996; and He *et al.*, 1997), is further complicated by additional regulatory proteins that can potentially modulate *Wnt* signalling during developmental stages and in specific tissues. Several *Frizzled* sub-family members which lack the seven-pass transmembrane regions but retain the signal sequence and CRD domain have been cloned and are referred to as the sFRP family, secreted *frizzled* related protein (Rattner *et al.*, 1997). Each of these encode a protein with all of the characteristic features of the original *Drosophila frizzled* protein with the exception of the transmembrane regions, suggesting that they are secreted. The CRD regions of *frizzled* gene products are of special importance as they are ligand binding sites for *Wnt* gene products (Bhanot *et al.*, 1996; He, *et al.*, 1997; and Leyns *et al.*, 1997). The sFRP family are interesting molecules as they are secreted and resemble growth factors and cytokines. Their expression is tissue specific and is dependent upon the physiological state of the cells. The homology the sFRPs exhibit to the family of transmembrane receptors, the *frizzled* proteins is unusual for a family of secreted proteins (Melkonyan *et al.*, 1997).

The *Wnts* interact with members of the *Frizzled* family, that transduce the signal to the cytoplasm (Bhanot *et al.*, 1996), and activate dishevelled, which promotes the inactivation of glycogen synthase kinase 3 (GSK3). This in turn suppresses the activity of β-catenin by promoting its degradation, whilst the inactivation of GSK3 in response to *wnt* signalling leads to the accumulation of cytosolic β-catenin pools. β-catenin interacts with HMG-box transcription factors altering their DNA-bending properties, thus contributing to specific gene expression (Cavallo *et al.*, 1997).

In addition, several components of the *Wnt* signalling are implicated in cancers. In fact the first *Wnt* gene discovered, mouse *Wnt-1* was identified due to it's property of forming mouse mammary tumours when expressed ectopically due to proviral insertion (Nusse and Varmus, 1982). Several *wnt* genes when overexpressed in mice can transform epithelial cells *in vitro* (Wong *et al.*, 1994), and in humans, increased expression of *wnt2, wnt5a, wnt7b* and *wnt 10b* have been found in proliferative breast lesions and breast cancer (Huguet *et al.*, 1994; Lejeune *et al.*, 1995; Bui *et al.*, 1997). In colorectal tumours and in some melanomas, mutations in either APC (truncating the protein), (Munemitsu *et al.*, 1995), or β-catenin (stabilising it), can lead to an increased activity of β-catenin/Tcf-4 transcription complexes (Korinek *et al.* 1997; Morin *et al.*, 1997; Rubinfeld *et al.*, 1997), which can potentially promote carcinogenesis. In Rat-1 fibroblasts, the parental line from which 208F cell line was derived, *Wnt-1* induces serum-independent cellular proliferation and changes in morphology(Young *et al.*, 1998). The *Wnt* signal transduction pathway is activated. The resulting induction and accumulation of cytosolic β-catenin and subsequent Tcf/Lef transcriptional activation controls down-stream *Wnt* target genes. In context with the findings of others with regards to deregulation of *Wnt* signalling pathway and carcinogenesis, the proliferative responses of Rat-1 fibroblasts to *Wnt-1* is through β-catenin and Tcf/Lef transcriptional activity (Young *et al.*, 1998). A schematic representation of the carcinogenesis *Wnt* signalling pathway is shown in Figure 6.

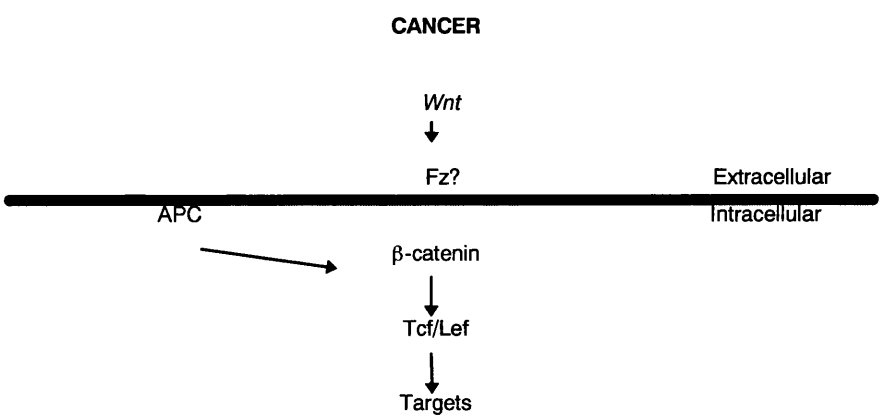


Figure 6 *Wnt* pathway in carcinogenesis

6.2 RESULTS

6.2.1 Sequence analysis

Clone KD1, was identified through the previously detailed database searches in chapter 3 as a novel sequence with homology to an EST, mouse 7.5 dpc embryo ecoplacental clone. Figure 6.1 details the homology to the EST clone.

```
>gb|AA409249|AA409249 EST01172 Mouse 7.5 dpc embryo ectoplacental cone cDNA
        library Mus musculus cDNA clone C0006F12 5'
        Length = 470

 Minus Strand HSPs:

 Score = 109 (30.1 bits), Expect = 3.8e-22, Sum P(2) = 3.8e-22
 Identities = 25/29 (86%), Positives = 25/29 (86%), Strand = Minus / Plus

Query:  234 AGGATGGAACCTACTGACAACAGCCAGGA 206
Sbjct:  297 AAGATGGAACCTACTGAGAACAACCAGAA 325

 Score = 348 (96.2 bits), Expect = 3.8e-22, Sum P(2) = 3.8e-22
 Identities = 82/99 (82%), Positives = 82/99 (82%), Strand = Minus / Plus

Query:  199 AACCTCTGGGACATCCCCCAGGCCCAAATCGAAGATAGGGGCCCCGAGGATGCCCAAGGC 140
Sbjct:  330 AACCTATGGGACATCCCTCGGGCCCAAACTGAAGAAAGGGGNCCCGGGAGTANCCAGGAC 389

Query:  139 CCAATAGCTGAGGCAGGGGAGGTGGAAGCCATTGCCACC 101
Sbjct:  390 CCTATAGCTGAGGCAGAGGAGGTGGAAGCCATTGCCAAC 428
```

Figure 6.1 EST Homologies of Clone KD1

6.2.2 Northern blot and tissue blot analysis

The expression pattern of clone KD1 was analysed by Northern blotting. The RNA was isolated from previously described cell lines, 208F and EGF, 208F, FBR, RAS and FBR TAM-67. The northern blot was hybridised with ^{32}P labelled KD1 clone (refer to Figure 6.2A). An RNA species of approximately 1.9Kb was detected in 208F mRNA, but no transcript was seen in the FBR mRNA. This data confirms that clone KD1 is down-regulated in FBR *v-fos* transformed cells. As shown no expression of the mRNA transcript was found in FBRs or in 208F cells treated with EGF. There is a two-fold down-regulation in K-*ras* transformed cell line. In FBR Tam-67 cells the decrease in expression is partial.

Figure 6.2

Northern blot and rat Tissue blot of clone KD1

A: This figure details the three day exposure X-ray film of Northern blot probed with Clone KD1, in the bottom panel the corresponding scanned X-ray film of the 7S Ribosomal loading control is shown. The Northern blot for KD1 was performed three times with the same result as that shown.

B: The expression of KD1 in rat tissues is detailed. The actin loading control is shown on the right hand side. The tissue blot was performed only once using the Clontech rat tissue blot. The size of the mRNA transcript is indicated by arrows with regards to the RNA ladder in kilobases and was approximately 1.7Kb.

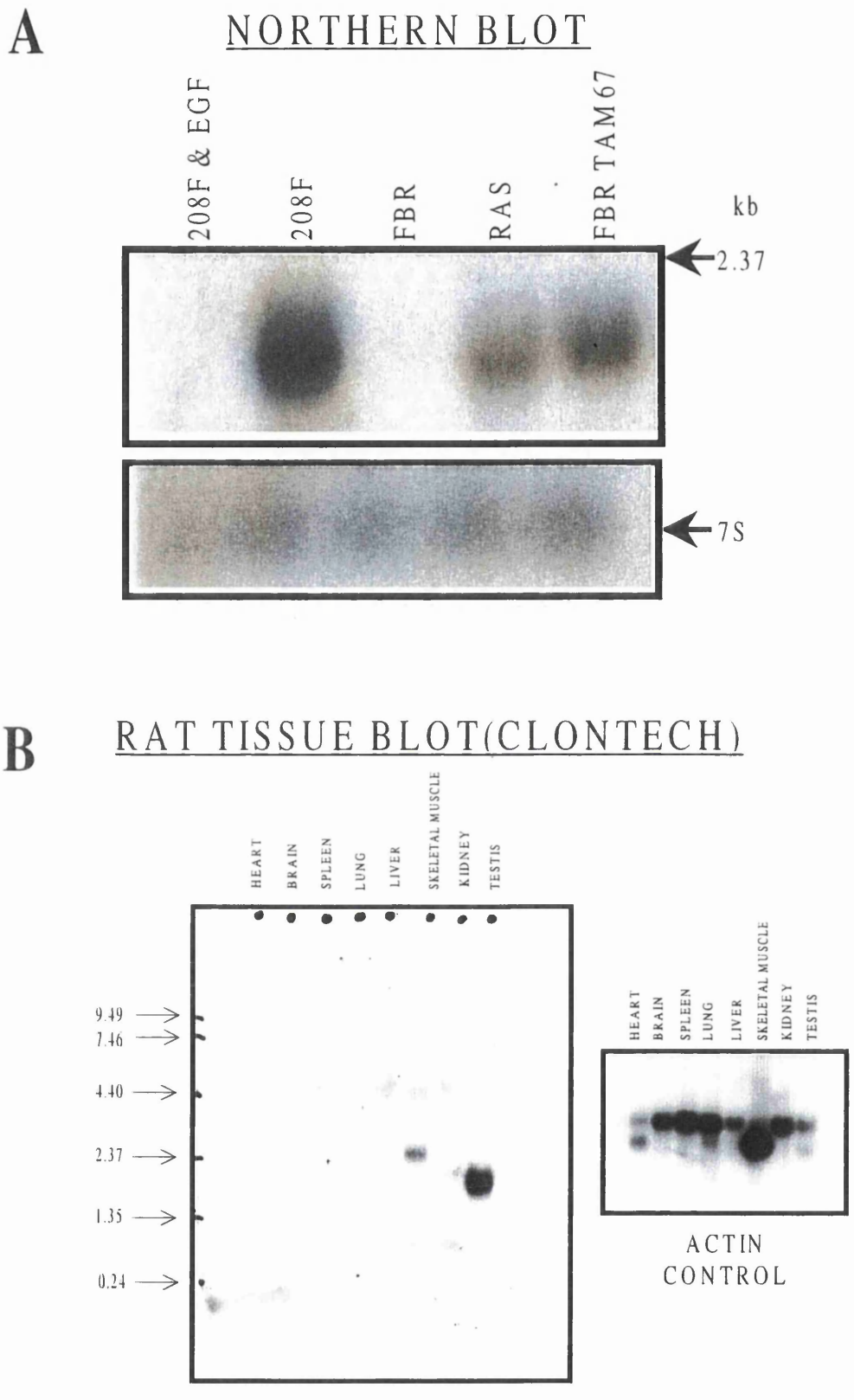


Figure 6.2
Northern Blot and Rat Tissue Blot of Clone KD1

To determine which tissues KD1 was expressed, a rat tissue blot was hybridised with ^{32}P labelled clone KD1. An RNA species of 1.9Kb was detected in the testis, suggesting that its expression is testis specific. It should be noted that the RNA species of 2.2Kb in the muscle tissue is from another experiment.

6.2.2

6.2.2.1 Library screening and sequence analysis of full-length cDNA of DIF1

To isolate a full length cDNA clone of KD1, a library derived from 208F mRNA was screened with KD1. Approximately 1 x 10^6 plaques were screened (refer to Section 2.2.11). Three rounds of sequential screening of positive plaques resulted in the isolation of ten single positive plaques. The pBluescript cDNAs were excised from phage (refer to section 2.2.12), and analysed using restriction mapping and automatic DNA sequencing. The resulting sequences were aligned using GCG GELASSEMBLE package to overlap the sequences to determine the full-length transcript. The full-length transcript was named DIF1. The complete nucleic acid sequence and putative amino acid sequence of DIF1 is shown by Figure 6.3. Nucleic acid sequence analysis revealed an ATG at position 345, located within a consensus translation initiation site (Kozak, 1987), and therefore this is predicted to be the first methionine. The coding region (903bp), is flanked by a 5' untranslated region and a 3' untranslated region containing a polyadenylation signal as shown. The DIF1 cDNA encodes a predicted polypeptide of 300 amino acids.

6.2.2.2 Prosite analysis of ORF protein translation of DIF1

DNA and protein BLAST searches against the various databases failed to identify any significant homologies to known genes. No homologies to transcription factor families was detected. Thus ascribing DIF1 to a protein family and function proved difficult. However, specialised databases can be searched to detect the presence of a motif or sequence pattern in a query sequence that can reveal distant relationships. Therefore, the ORF protein translation of DIF1 was subject to protein families database of alignments (Pfam) and HMMs and also PROSITE analysis to determine if DIF1 protein sequence had identity to any known motifs or protein patterns. These are shown by Table 6.1.

Figure 6.3

Nucleotide and predicted amino acid sequence of DIF1 cDNA

The nucleotides are numbered above the nucleic acid sequence. Amino acids are indicated in single letter code with the first predicted methionine at position 345. This is located with a translation initiation site AAG**ATG**G (Kozak, 1987). The termination codon is denoted by an asterisk at position 1248.

```
        10                  30                  50
AAGGGTGAGGTCTACTCAGCAGGCTCTGCATCTGCGGACACATAATTCAGTGCTGGCTCA

        70                  90                  110
CCAGGCTTTCTGAATCCAAGGACTTGGGGGACTCAAACCCTTTTGTAAGGGTCCTCGGAA

       130                 150                 170
GGGGAAGGTGAACCTGTACAGTGTGTGGTATCACCAAACAGCAGACAGAGCTACCAGAGG

       190                 210                 230
GAATTGTGTAGTATCTTCCAACAATCCAGGCATTAGTCTAAAGGAGCTGAAGGACTTGAC

       250                 270                 290
TCAAGTCATCAGAGGAAGGATCTGGTACATCAGTGTGAAGGTTTGTGAATCTTCATCCCT

       310                 330                 350
GTGACCTGAACAGTGTCTTGCCTGCTCTTTCCAAGGAAACTCAAGATGGAACCTACTGAC
                                                M  E  P  T  D

       370                 390                 410
AACAGCCAGAACTTCAACCTCTGGGACATCCCCCAGGCCCAAATCGAAGATAGGGGCCCC
N  S  Q  N  F  N  L  W  D  I  P  Q  A  Q  I  E  D  R  G  P

       430                 450                 470
GAGGATGCCCAAGGCCCAATAGCTGAGGCAGGGGAGGTGGAAGCCATTGCCACCATATCA
E  D  A  Q  G  P  I  A  E  A  G  E  V  E  A  I  A  T  I  S

       490                 510                 530
GGGGATGTGTCTGCTGGAGAAGAACCTAGTTCTCCTCAGAGAGCCTCCTCTCCTCCCACT
G  D  V  S  A  G  E  E  P  S  S  P  Q  R  A  S  S  P  P  T

       550                 570                 590
GCTATGGGTTTCATTGGAGAGGCATCATTTGGCAATCCTCTAGCAGGGATGCCTGGTACT
A  M  G  F  I  G  E  A  S  F  G  N  P  L  A  G  M  P  G  T

       610                 630                 650
GAATTCCCTGTTCATGGTGAACTAAATGGAAAGATAGTTGATTTGGTGAGATTCTTGCTA
E  F  P  V  H  G  E  L  N  G  K  I  V  D  L  V  R  F  L  L

       670                 690                 710
GTCAAGTTTAGAAGGATGGAGATAACCAGTAAGGAAGAAATGATCCATAGGACCATGAGA
V  K  F  R  R  M  E  I  T  S  K  E  E  M  I  H  R  T  M  R

       730                 750                 770
GATTATGAGGAGCACTACTCTGTGATCTTTAGTAAGGCCGCTGAGTGCATGAAGCTGATT
D  Y  E  E  H  Y  S  V  I  F  S  K  A  A  E  C  M  K  L  I

       790                 810                 830
TTTGGGGTTGACATGATGGAAGTGGACCCTTTTGTCCACTCCTATTTCCTTTACCCTGCT
F  G  V  D  M  M  E  V  D  P  F  V  H  S  Y  F  L  Y  P  A

       850                 870                 890
CTGGGGATCACCTATGATGGGATGCTACATGGAGTTGTAGGCGTACCCAAGACAGGTCTG
L  G  I  T  Y  D  G  M  L  H  G  V  V  G  V  P  K  T  G  L

       910                 930                 950
GTTATAATTGTCCTGTGCATCATCTTTATAGAGGACAATTGTGTCAGTGAGGAGGTATTC
V  I  I  V  L  C  I  I  F  I  E  D  N  C  V  S  E  E  V  F

       970                 990                1010
TGGCATGTAATGAATAGCCTAGGAATGTATGCTGGAGTCGATCATTTCATATTTGGGGAT
W  H  V  M  N  S  L  G  M  Y  A  G  V  D  H  F  I  F  G  D

      1030                1050                1070
```

```
CCCAGGAGTCTCATCACTGAAGACTTTGTGCAGGAAGGTTATGTGGAATACAGGCAGGTG
P  R  S  L  I  T  E  D  F  V  Q  E  G  Y  V  E  Y  R  Q  V

      1090                1110                1130
CCCAACAGCCATCCTCCTCGCTTTGAGTTCCTGTGGGGCCCAAGGGCGTATGCTGAAACC
P  N  S  H  P  P  R  F  E  F  L  W  G  P  R  A  Y  A  E  T

      1150                1170                1190
ACCAAGATGAAAATCCTGGAGTTTTATGCCAGCATCGTTAGGCAGGATCCCAGATCCTAC
T  K  M  K  I  L  E  F  Y  A  S  I  V  R  Q  D  P  R  S  Y

      1210                1230                1250
CCTGAGAAGTATGCAGAGGCTTTGAGGGAAGAACAAGAGAGGGCCTAGGCCAGAAAAGCC
P  E  K  Y  A  E  A  L  R  E  E  Q  E  R  A  *

      1270                1290                1310
CAGCAGATAATAGATATGACTGCTCTGACCAGCACAATATGTAGTAAACTCAACAGTTTA

      1330                1350                1370
TGGTCAGGCAGAACAGTAAAATAAGTGGTAGACCCCCAAATGAGAGCTATTGGAAAGAGA

      1390                1410                1430
GTGTAACCCATTCCTTTCTTACTGTTCTGTTTGGGTGACTTAGGCTCATAATTTTTGAAC

      1450                1470                1490
ATTGTTAACTTTTACTATAAGGCTCATTTACTTGAGAATTTTAAAATTAAAAATGACATC

      1510                1530                1550
AGTAATGCCTGTGTTGATTTAGGTCAGGAGAATTTTGTTATCATAAAAACAAACAGTTTT

      1570                1590                1610
CTAATTTGTAATATGTCACACAATCTGATACCATCAAAGTTGGAATTTCCTGGACAACAG

      1630                1650                1670
AAAACATACTGGCATTAATATAAAAAACAGAATAAAATAAAATTTTTGTTGTGTAAAAAA

      1690
AAAAAAAAAAAAAAAA
```

Figure 6.3
Nucleotide and predicted amino acid sequence of DIF1 cDNA

The location of the protein patterns in the DIF1 protein sequence is highlighted by Figure 6.4. Figure 6.4 also details the Pfam alignment to the Arginase domain at positions 143-155.

PROTEIN PATTERN	POSITION OF MATCH
Protein kinase C phosphorylation site	1 114-116 TSK 2 123-125 TMR 3 265-267 TTK
Casein kinase II phosphorylation site	1 49-52 SAGE 2 114-117 TSKE 3 115-118 SKEE 4 123-126 TMRD 5 284-287 SYPE
Tyrosine kinase phosphorylation site	1 125-131 RDYEEHY
N-myristoylation site	1 76-81 GNPLAG 2 81-86 GMPGTE 3 172-177 GMLHGV 4 179-184 GVPKTG 5 213-218 GMYAGV
Myc-type, "helix-loop-helix' dimerisation domain signature	1 181-189 PKTGLVIIV

Table 6.1 PROSITE protein patterns of DIF1

The predicted molecular weight of DIF1 is 33.8kDa, theoretical pI is 4.65 and the amino acid composition contains 15.7% negatively charged residues (Asp & Glu), and 8% positively charged residues (Arg & Lys), as determined by ExPASy protparam analysis. No nuclear localisation sequences were detected.

The full-length DIF1 clone exhibits no homology to any known sequences through blastx or blastn searches but is identical to an EST database entry, EST222935 Normalized rat spleen, Bento Soares Rattus sp. cDNA clone RSPCE20 3' end, sequence. The homology of DIF1 to EST222935 is shown in Figure 6.5 below.

Figure 6.4

PROSITE and Pfam analysis of predicted amino acid sequence of DIF1 cDNA

A: Amino acids are numbered on the left beginning at the first methionine. The Protein kinase C phosphorylation sites are shown in red, Casein kinase II phosphorylation sites are shown in bold, Tyrosine kinase phosphorylation site is shown in italics, N-myristoylation sites are shown in blue, and Myc-type helix-loop-helix dimerisation domain signature is shown underlined.

B: Homology of DIF1 (KD1), amino acid positions 143-155 to the Arginase motif. The identical residues are shown by single amino acid code and similar residues are shown by +.

A

```
  1 MEPTDNSQNF NLWDIPQAQI EDRGPEDAQG PIAEAGEVEA IATISGDVSA
 51 GEEPSSPQRA SSPPTAMGFI GEASFGNPLA GMPGTEFPVH GELNGKIVDL
101 VRFLLVKFRR MEITSKEEMI HRTMRDYEEH YSVIFSKAAE CMKLIFGVDM
151 MEVDPFVHSY FLYPALGITY DGMLHGVVGV PKTGLVIIVL CIIFIEDNCV
201 SEEVFWHVMN SLGMYAGVDH FIFGDPRSLI TEDFVQEGYV EYRQVPNSHP
251 PRFEFLWGPR AYAETTKMKI LEFYASIVRQ DPRSYPEKYA EALREEQERA
301 *
```

B

Alignment of arginase vs QuerySeq/143-155

```
                    *->GslvglDvVEVNP<-*
                       +++ g+D +EV+P
QuerySeq    143        KLIFGVDMMEVDP     155
```

Figure 6.4
PROSITE and Pfam analysis of predicted amino acid sequence of DIF1 cDNA

Score = 1845 (509.8 bits), Expect = 5.9e-205, Sum P(2) = 5.9e-205
Identities = 369/369 (100%), Positives = 369/369 (100%), Strand = Minus / Plus

Query: 1681 TTTTTTTACACAACAAAAATTTTATTTTATTCTGTTTTTTATATTAATGCCAGTATGTTT 1622
Sbjct: 1 TTTTTTTACACAACAAAAATTTTATTTTATTCTGTTTTTTATATTAATGCCAGTATGTTT 60

Query: 1621 TCTGTTGTCCAGGAAATTCCAACTTTGATGGTATCAGATTGTGTGACATATTACAAATTA 1562
Sbjct: 61 TCTGTTGTCCAGGAAATTCCAACTTTGATGGTATCAGATTGTGTGACATATTACAAATTA 120

Query: 1561 GAAAACTGTTTGTTTTTATGATAACAAAATTCTCCTGACCTAAATCAACACAGGCATTAC 1502
Sbjct : 121 GAAAACTGTTTGTTTTTATGATAACAAAATTCTCCTGACCTAAATCAACACAGGCATTAC 180

Query: 1501 TGATGTCATTTTTAATTTTAAAATTCTCAAGTAAATGAGCCTTATAGTAAAAGTTAACAA 1442
Sbjct: 181 TGATGTCATTTTTAATTTTAAAATTCTCAAGTAAATGAGCCTTATAGTAAAAGTTAACAA 240

Query: 1441 TGTTCAAAAATTATGAGCCTAAGTCACCCAAACAGAACAGTAAGAAAGGAATGGGTTACA 1382
Sbjct: 241 TGTTCAAAAATTATGAGCCTAAGTCACCCAAACAGAACAGTAAGAAAGGAATGGGTTACA 300

Query: 1381 CTCTCTTTCCAATAGCTCTCATTTGGGGGTCTACCACTTATTTTACTGTTCTGCCTGACC 1322
Sbjct: 301 CTCTCTTTCCAATAGCTCTCATTTGGGGGTCTACCACTTATTTTACTGTTCTGCCTGACC 360

Query: 1321 ATAAACTGT 1313
Sbjct: 361 ATAAACTGT 369

Score = 840 (232.1 bits), Expect = 5.9e-205, Sum P(2) = 5.9e-205
Identities = 169/171 (98%), Positives = 169/171 (98%), Strand = Minus / Plus

Query: 1312 TGAGTTTACTACATATTGTGCTGGTCAGAGCAGTCATATCTATTATCTGCTGGGCTTTTC 1253
Sbjct: 369 TGAGTTTACTACATATTGTGCTGGTCAGAGCAGTCATATCTATTATCTGCTGGGCTTTTC 428

Query: 1252 TGGCCTAGGCCCTCTCTTGTTCTTCCCTCAAAGCCTCTGCATACTTCTCAGGGTAGGATC 1193
Sbjct: 429 TGGCCTAAGCCCTCTCTTGTTCTTCCCTCAAAGCCTCTGCATACTTCTCAGGGTANGATC 488

Query: 1192 TGGGATCCTGCCTAACGATGCTGGCATAAAACTCCAGGATTTTCATCTTGG 1142
Sbjct: 489 TGGGATCCTGCCTAACGATGCTGGCATAAAACTCCAGGATTTTCATCTTGG 539

Figure 6.5 Homology of DIF1 to EST222935

6.2.3 Cloning DIF1 into 3' Myc tagged expression vector and GFP fusion protein expression vector

The open reading frame of DIF1 was amplified by PCR using the primers shown in Figure 6.6. The fragment was cloned into pcDNA3.1Myc-His B and pEGFP-N1 to produce DIF1-myc and DIF1-GFP, respectively. It is outlined schematically along with the primers used, which included the start, but not the stop site, in Figure 6.6.

Figure 6.6

DIF1 PCR primers and cloning strategy into pcDNA3.1 Myc-His B, and pEGFP-N1 expression vectors

A: This figure describes the primer sequences used to PCR amplify the complete DIF1 ORF. They were designed to include the following: Kozak start site but not the stop site of the ORF to enable the 3' Myc-His tagging of the protein; Xho I restriction sites prior to the start and at the end of the sequence to facilitate insertion, in frame, into the pcDNA3.1Myc-His B, and pEGFP-N1 expression vectors.

B: The cloning strategy is highlighted whereby initial PCR of the cDNA using Pfu, limited PCR mistakes and generated blunt-ended products that were cloned into pCRScript vector. The pCRScript vector with KD1 inserted was then restriction digested with Xho I and the resulting cDNA fragment was ligated into the Xho I site of pcDNA3.1 Myc-His B and also pEGFP-N1

A

cDNA start site Primer

5' GCC CTC GAG AAG ATG GAA CCT ACT GAC AAC AGC CAG AAC 3

Xho I Kozak Start

cDNA end site Primer

3' GCC CTC GAG GGC GGC CCT CTC TTG TTC TTC CCT 5'

Xho I

B

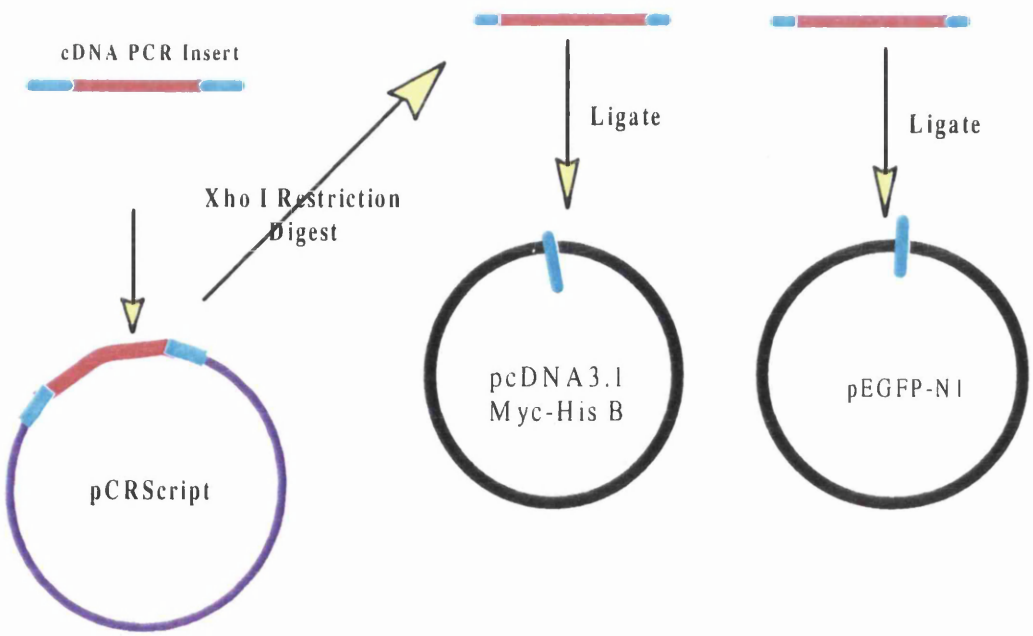


Figure 6.6
DIF1 Protein Primers and Cloning Stratefy into pcDNA3.1 Myc-His B, and pEGFP-N1 Expression Vector

6.2.4 Transient transfection of Cos 7 cells with Myc tagged DIF1

To determine whether pcDNA3.1A/DIF1 expressed a polypeptide of the correct size, this construct was transfected into Cos7 cells. Western blot analysis of the soluble cell lysate using an anti-myc primary monoclonal antibody and an anti-mouse HRP-linked secondary antibody revealed a band of 43-44kDa. This confirmed that the pcDNA3.1A/DIF1 construct produced a protein of the correct size, as determined from the putative amino acid ORF of the clone. Although ExPASy protparam analysis reported a protein molecular mass for DIF1 of 33.8kDa, post-translational modifications as suggested by PROSITE analysis and the Myc-tag of approximately 2.5Kda resulted in a Myc-tagged protein of approximately 43-44kDa.

6.2.5 Transient transfection of 208F cell line with Myc tagged DIF1

The 208F, cell line was transiently transfected with the above described Myc tagged DIF1 expression vector and fixed twenty four hours post-transfection. The cells were stained by indirect immunofluorescence using an anti-myc primary mouse antibody, followed by anti-mouse FITC labeled secondary antibody that visualised the transfected cells and revealed the cellular localisation of DIF1. In conjunction, the cells were counter-stained with rhodamine-phalloidin (TRITC), to visualise F-actin. The methodology is described in Chapter 2, Section 2.23.

Control transfections were performed using the expression vector without a gene and also using the same expression vector with myc-tagged β-gal reporter gene. The empty expression vector did not stain for myc, whilst the myc tagged β-gal reporter localised to the cytoplasm. The F-actin staining for the control transfected cells and the untransfected cells determined that their was no difference in actin architecture (Data not shown).

Figure 6.8 details the localisation of the DIF1 in two transfected 208F cells. DIF1 is diffusely distributed throughout the cells. The expression is not excluded from the nucleus or cytoplasm. In the left panels, under Anti-Myc DIF1, the "holes" in expression may be the nucleolus of the nucleus and the golgi in the cytoplasm. The phalloidin staining for actin demonstrates that the 208F cells maintain their normal actin distribution. A normal fibroblastoid actin structure, as shown in Figure 6.8, is present in the DIF1-myc transfected cells. The merged pictures of DIF1 (green), and F-actin (red), staining in the transiently transfected 208F cells as shown by Figure 6.8 are shown in Panels (a), and (b), of Figure 6.13, and are characteristic of the transfected cell population.

Figure 6.7

Protein detection by Western blot analysis of pcDNA3.1(-)Myc-His B/DIF1 and pcDNA3.1(-)Myc-His B after transient transfection in Cos7 cells.

This figure details the protein detection of DIF1-myc by Western blotting. The cell extracts are from transient transfection in Cos7 cells with pcDNA3.1(-)Myc-His B/DIF1 and pcDNA3.1 (-)Myc-His B. The empty vector alone has no expression. The DIF1-myc cDNA in an expression vector results in a band around 43-44kDa.

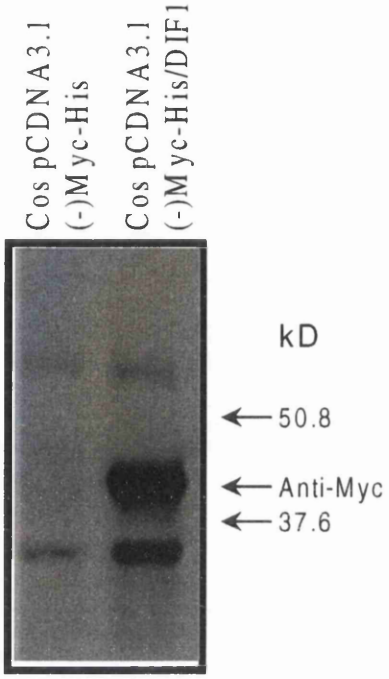


Figure 6.7
Cos 7 cells 72 hour Transient Transfection
3' Myc Tagged DIF1 cDNA

Figure 6.8

208F transiently transfected with myc tagged DIF1 expression vector and indirectly stained for FRP and F-actin

The confocal images are of transiently transfected 208F cells with myc-tagged DIF1. The DIF1 stained cells are pictured on the left, under Anti-Myc DIF1, and the same field stained for F-actin, under F-actin, is on the right. Other cultured but untransfected cells are also shown in the F-actin stained right half of the panel and the transiently transfected cell is marked by a yellow box.

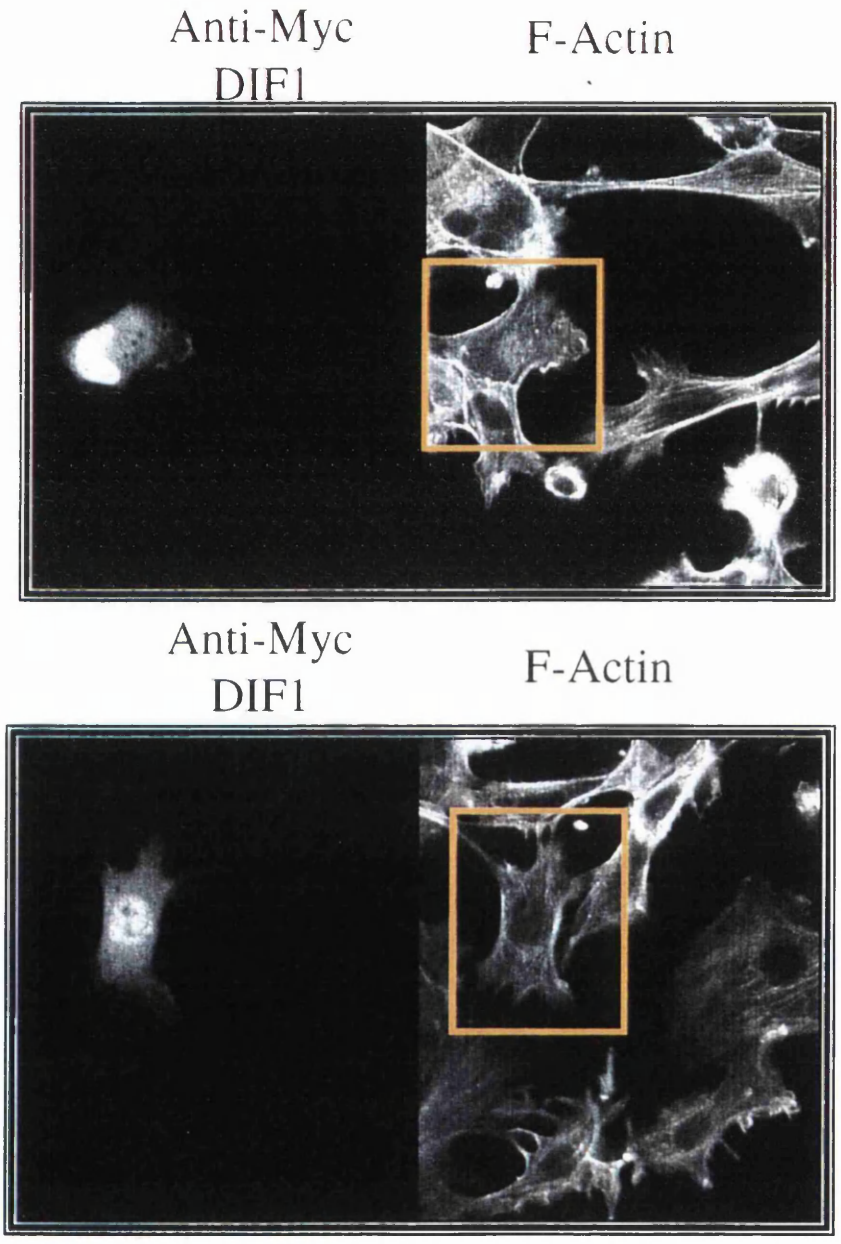


Figure 6.8
208F Transiently Transfected with DIF1-Myc

6.2.6 Transient transfection of FBR, and RAS Cell Lines with DIF1-GFP fusion protein

It proved extremely difficult to transfect FBR and RAS cell lines with DIF1 and numerous attempts to transiently and stably transfect FBR and RAS cell lines with the above described Myc tagged DIF1 expression vector were unsuccessful. The transient transfections were repeated at least ten times and no positively indirect myc stained cells were identified by confocal microscopy. Likewise, several attempts at generating stable clones proved unfruitful as all cells died during the neomycin selection procedure even when the concentration was reduced to 200μg/ml. In an attempt to overcome this DIF1 was cloned into the pEGFP-N1 expression vector so that DIF1 would be fused to the N-terminus of EGFP, a variant of wild-type GFP.

The GFP-DIF1 fusion transient transfections were also very difficult, although some positive cells were detected. Transiently transfected cells were fixed twenty four hours post-transfection and were stained with rhodamine-phalloidin (TRITC), to visualise F-actin. The methodology is described in Chapter 2, Section 2.23.

Control transfections were performed using empty pEGFP expression vector. Upon transfection, the empty expression vector localised in the nucleus. The F-actin staining for the control transfected cells and the untransfected cells determined that there was no difference in actin architecture (data not shown).

As previously described in chapter 5 the FBRs have a very different morphology to the 208Fs. The FBRs are bipolar and have no stress fibers. The F-actin runs as thick cables along the length of the bipolar cell and is concentrated in the cortex and pseudopodia, which are capped by actin rich bundles. The localisation of the GFP-DIF1 fusion protein in two representative fields of transfected FBR cells is shown by Figures 6.9 and 6.10. The expression of DIF1 in all of the transfected cells examined was of a complete cytoplasmic localisation with slight expression in the nucleus which is particularly obvious in the transfected cell as shown in Figure 6.9. In all of the FBR transfected cells examined, DIF1-GFP was also expressed throughout the pseudopod structures. The expression in FBRs was not only within the cell bodies but also in the pseudopods. Although the corresponding F-actin staining, as shown in the bottom panel of Figure 6.9, is difficult to determine due to the high population density of the cells. No significant change in morphology of the FBRs is apparent from the DIF1-GFP fusion expression pattern. The transfected cell, shown in the top panel in Figure 6.9, under DIF1-GFP Fusion, is of an unusual FBR appearance with a hint of cell flattening, and pseudopods with two to three spikes of various length and thickness as opposed to the characteristic bipolar phenotype. DIF1-GFP is also

expressed in these spike-like structures. The transfected cells shown in the top panel in Figure 6.10 are slightly flatter in appearance to a typical FBR and also have spike structures. The change in morphology and F-actin staining is difficult to assess due to the high density of the transiently transfected cells shown in the bottom panel of Figure 6.10. In addition, too few cells were successfully transfected to confirm that the morphological changes are of biological significance.

The merged pictures of DIF1-GFP and F-actin staining in the transiently transfected FBR cells shown by Figures 6.9 and 6.10 are shown in Panels (c), and (d), of Figure 6.13. These cells are characteristic of the transfected cell population.

The expression in RAS cell line was again distributed throughout the cell as the transfected cell in the top panel, under DIF1-GFP fusion shown by Figure 6.11 demonstrates. As discussed in the previous chapter the RAS cell line have a very different morphology to the fibroblastoid parental cells. They have multiple irregular shaped protrusions, often with an actin rich cap. The DIF1-GFP transiently transfected RAS cells are shown by Figures 6.11-6.12. These demonstrate that no apparent morphological difference exists between the transfected and untransfected cells. The cells shown by Figure 6.11 and 6.12, upper panel also have spike-like structures and the corresponding F-actin staining boxed in yellow in the lower panel, reveals an actin rich cap at the end of these spikes. The merged pictures of DIF1-GFP and F-actin staining in the transiently transfected RAS cells shown by Figures 6.11 and 6.12 are shown in Panels (e), and (f), of Figure 6.13. Panel (e), shows the merged picture of DIF1 GFP fusion protein and F-actin which not only highlights the expression of DIF1 in the cytoplasm, but also in the spike structures and at their outer edge. These cells are characteristic of the transfected cell population.

The difference in the localisation of DIF1 expression between the transformed cell lines, FBR and RAS, and untransformed 208F cell line is insignificant. However, transient transfections using the pEGFP-DIF1 expression vector should have been performed in 208F cell line. The great difficulty experienced in attempting to transiently transfect the transformed cell lines coupled with the phenotypic irregularities would indicate that DIF1 may be toxic to the transformed cells. However, no stables expressing DIF1 were produced and there is insufficient data to determine the toxicity of DIF1.

Figures 6.9 and 6.10

FBR transiently transfected with DIF1-GFP fusion expression vector and stained for F-actin

The upper panels are enlarged confocal pictures of the DIF1-GFP transiently expressed FBR cells under DIF1-GFP fusion. The lower panel are confocal pictures F-actin stained FBR cells, under F-Actin. The transfected cell field is shown by a yellow box.

DIF1-GFP Fusion

F-Actin

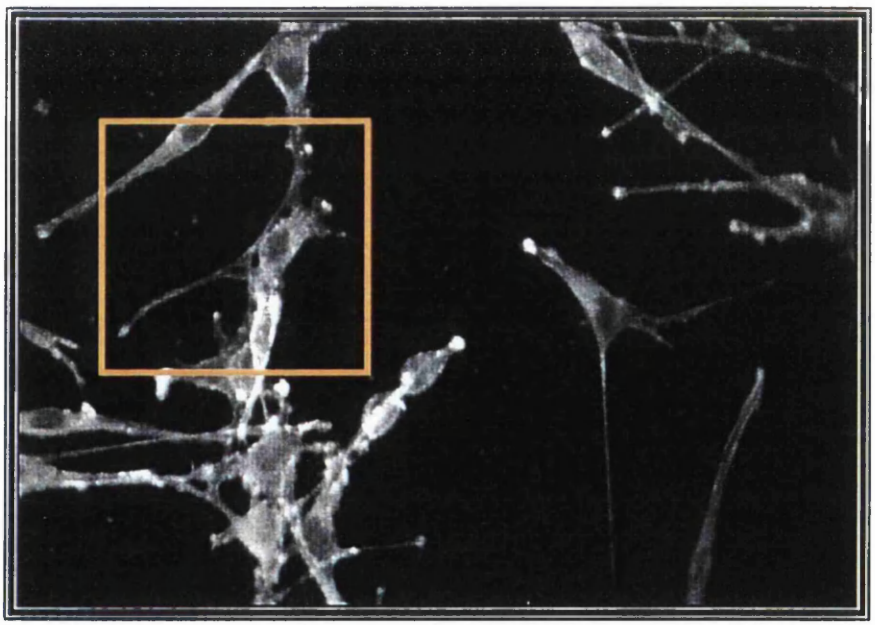
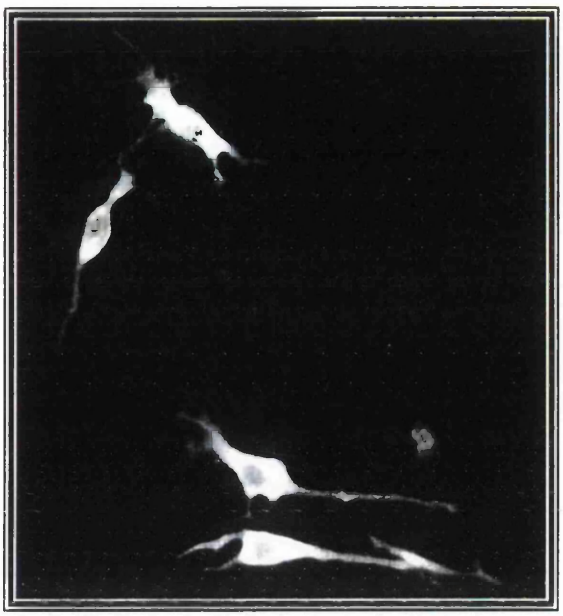


Figure 6.9
FBR Transiently Transfected with DIF1-GFP

DIF-GFP Fusion



F-Actin

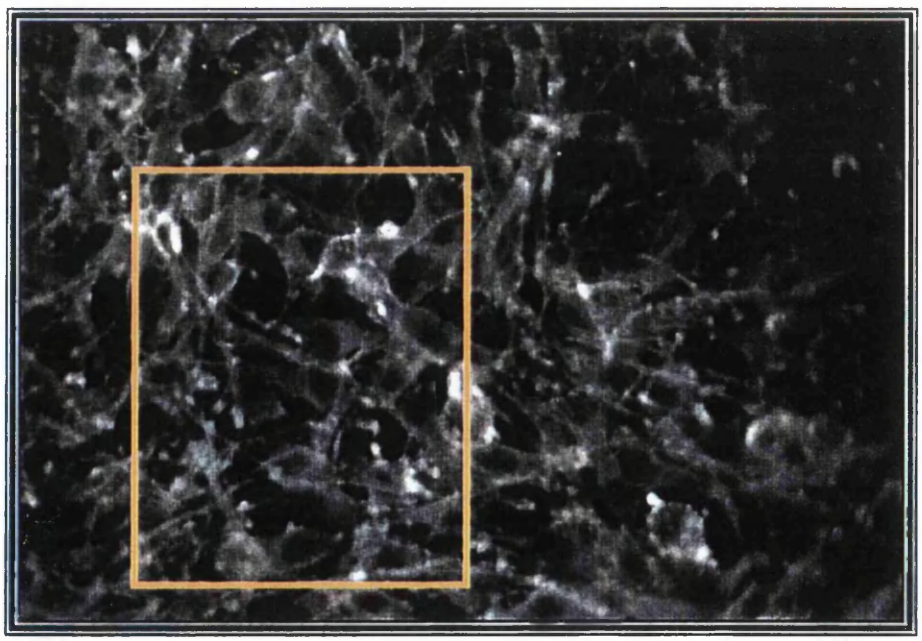


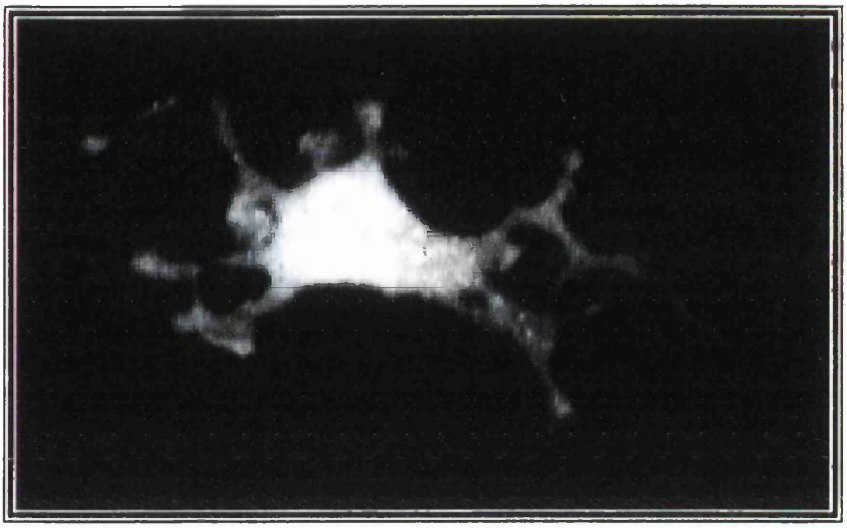
Figure 6.10
FBR Transiently Transfected with DIF1-GFP

Figures 6.11 and 6.12

RAS transiently transfected with DIF1-GFP fusion expression vector and stained for F-actin

The upper panels are enlarged confocal pictures of the DIF1-GFP transiently expressed RAS cells under DIF1-GFP fusion. The lower panel are confocal pictures F-actin stained RAS cells, under F-Actin. The transfected cell field is shown by a yellow box.

DIF1GFP Fusion



F-Actin

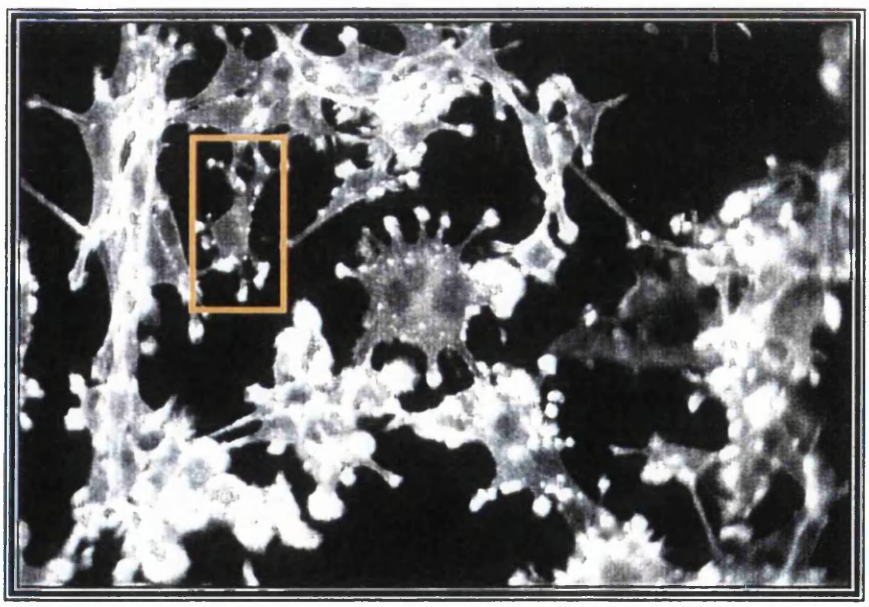
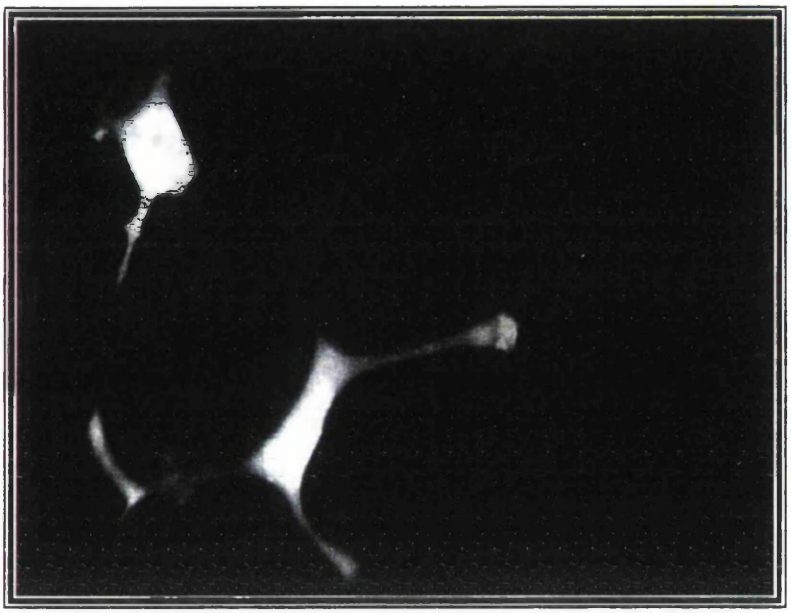


Figure 6.11
RAS Transiently Transfected with DIF1-GFP

DIF1-GFP Fusion



F-Actin

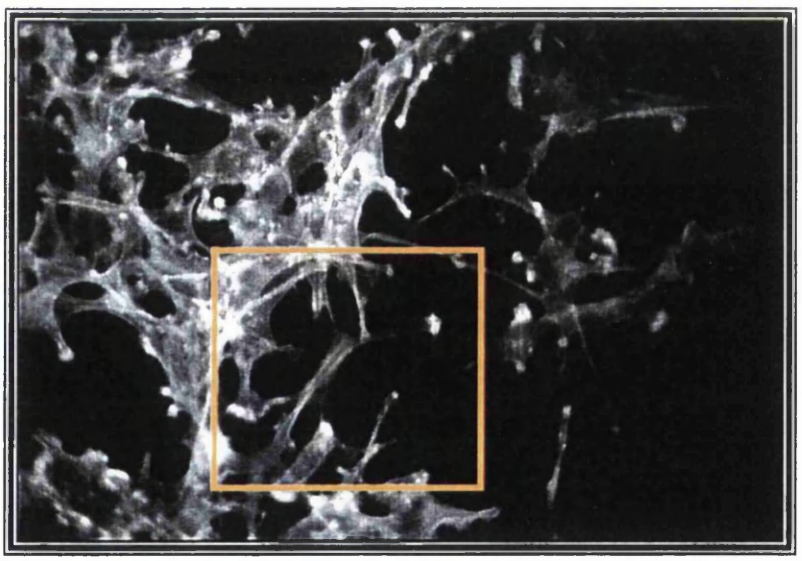


Figure 6.12
RAS Transiently Transfected with DIF1-GFP

Figure 6.13

Transient transfections in 208F, FBR and *RAS* cell lines: Merged pictures of DIF1-MYC and DIF-1-GFP and F-actin staining

Merged confocal images of transiently transfected cell lines. In 208F cells the indirect staining of DIF1-myc protein is shown in green, and for the FBR and RAS cells the DIF1-GFP fusion protein is also shown in green. The F-actin is shown in red. Panels (a), and (b), are two different 208F cells indirectly stained for DIF1, and F-actin, panels (c), and (d), are two different FBR fields expressing DIF1-GFP fusion protein and stained for F-actin, panels (e), and (f),are two different RAS fields expressing DIF1-GFP fusion protein and stained for F-actin.

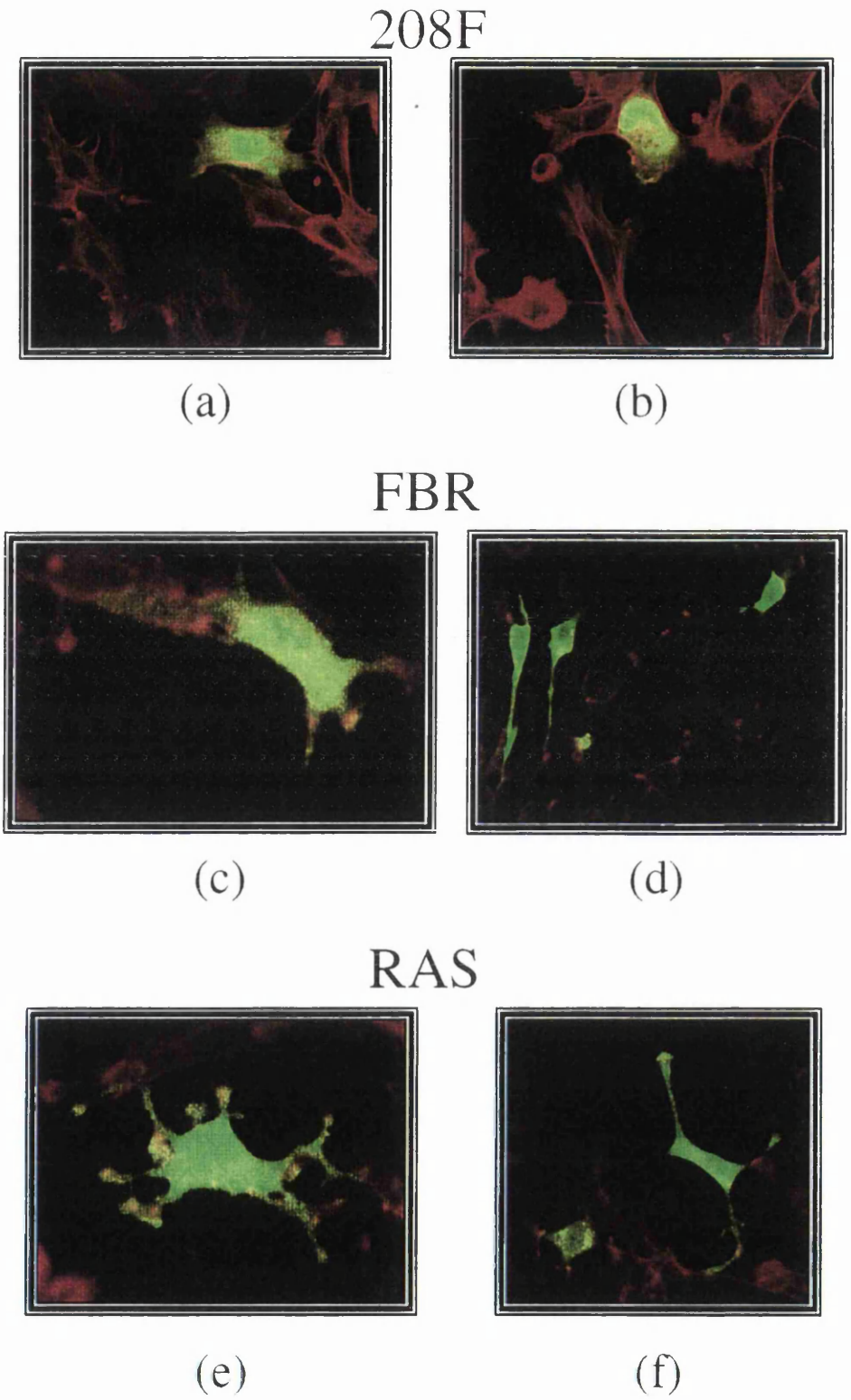


Figure 6.13
Transient Transfections in cell lines:
DIF1 and F-Actin staining

6.2.7 Sequence analysis

Clone CD10, was identified through the previously detailed database searches in chapter 3 as a clone with homology to rat FrpAP. Figure 6.14 details the homology at the nucleic acid level to FrpAP.

>gb|AF012891|AF012891 Rattus norvegicus frizzled related protein frpAP mRNA, complete cds Length = 1910
Plus Strand HSPs:
Score = 1947 (538.0 bits), Expect = 1.6e-151, P = 1.6e-151
Identities = 413/444 (93%), Positives = 413/444 (93%), Strand = Plus / Plus

Query: 1 TTATTCATGCCAAAATAAAAGCGGTCCAGAGGAGTGGTTGCAATGAGGTCACAACTGTGG 60
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Query: 61 TCGATGTAAAAGAGATCTTCAAGTCTTCATCACCTATCCCTCGAACGCAAGTCCCCCTCA 120
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Query: 121 TCACCAATTCCTCCTGCCAGTGTCCACACATCCTGCCCCATCAAGATGTCCTAATCATGT 180
Sbjct: 807 TCACCAATTCCTCCTGCCAGTGTCCACACATCCTGCCCCNTCAAGATGTCCTAATCATGT 866

Query: 181 GTTATGAGCGGCGTTCAAGGATGATGCTTTTTGAAAATTGTTTAGTTGAGAAATGGAGAG 240
Sbjct: 867 GTTATGAGCGGCGTTCAAGGATGATGCTTCTTGAAAATTGTTTAGTTGAGAAATGGAGAG 926

Query: 241 ATCAACTAAGCAGAAGGTCCACACAGTGGGAAGAGAGGCTTCAGGAACAGCAGAGAACAA 300
Sbjct: 927 ATCAACTAAGCAGAAGGTCCACACAGTGGGAAGAGAGGCTTCAGGAACAGCAGAGAACAA 986

Query: 301 CTCAGGACAAGAAGCAAATAGCCAGCCGCACCAGTCGCAGTAACCCCCCAAAGCCAAAAG 360
Sbjct: 987 CTCAGGACAAGAAGCAAATAGCCAGCCGCACCAGTCGCAGTAACCCCCCAAAGCCAAAGG 1046

Query: 361 GAAGGTCACCTGCTTCCAAACCTGCCAGTCCCTAAGAAGAACATCAAAGCTAGAATTGCA 420
Sbjct: 1047 GAAGGTCACCTGCTTCCAAACCTGCCAGTCCTAAGAAGAACATCAAAGCTAGAAGTGCAC 1106

Query: 421 CCCAAAAAANTCAACCCCAAGAAAA 444
Sbjct: 1107 CCAAAAAGTCAAACCCAAAGAAAA 1130

Figure 6.14 Homology of clone CD10 to FrpAP

6.2.8 Northern blot and tissue blot analysis

The expression pattern of clone CD10 was analysed by Northern blotting. The RNA was isolated from previously described cell lines, 208F and EGF, 208F, FBR, RAS and FBR TAM-67. The Northern blot was hybridised with ^{32}P labelled CD10 clone (refer to Figure 6.15A). Two CD10 RNA species of approximately 1.4Kb and 2.4Kb were detected in 208F mRNA, but no transcript was seen in the FBR mRNA. This data confirms that clone CD10 is down-regulated in FBR *v-fos* transformed cells. As shown no expression of the mRNA transcript was found in FBRs or in FBR Tam-67

cells. There is a marked down-regulation in K-*ras* transformed cell line. In EGF treated 208F cells the decrease in expression is partial.

To determine which tissues CD10 was expressed, a rat tissue blot was hybridised with ^{32}P labelled clone CD10. The two RNA species were detected in the tissue blot. The mRNA transcripts are abundantly expressed in skeletal muscle, but the transcripts are also expressed in brain, spleen and lung tissues but at a lower level.

6.2.9

6.2.9.1 Library screening and sequence analysis of full-length cDNA of CD10

The complete CD10 cDNA was isolated by screening a library derived from 208F mRNA with CD10. Approximately 1 x 10^6 plaques were screened (refer to Section 2.2.11). Three rounds of sequential screening of positive plaques resulted in the isolation of ten single positive plaques. The pBluescript cDNAs were excised from phage (refer to section 2.2.12), and analysed using restriction mapping and automatic DNA sequencing. Seven of the ten clones were false positives and the remaining three were not full-length. A further ten of the originally isolated clones from the first screen were subject to three separate platings, to isolate single positive plaques. Again, several of the clones were false positives and the remaining did not contain the full open reading frame. A further attempt to isolate the full length CD10 clone was performed by screening another 1.5 x 10^6 plaque forming units from the library. This resulted in the isolation of two clones which contained an identical open reading frame to FrpAP. The isolated clones were named FrpAP(FBR).

Two of the isolated FrpAP FBR clones contained the ORF for FrpAP but lacked the first nucleotide base as detailed in the published sequence, and the 3' end was truncated at 1583bp with a polyadenylation signal. The complete nucleic acid sequence and predicted conceptual protein translation of the open reading frame of, FrpAP (FBR) is shown by Figure 6.16. The nucleic acid sequence contained the ATG start site at position 94, located within a consensus translation initiation site (Kozak, 1987), and the stop site at position 1140. The coding region (1049bp), is flanked by a 5' untranslated region and a 3' untranslated region containing a polyadenylation signal as shown. The published FrpAP sequence contains an additional nucleotide at position one, with the ORF at positions 95-1141. The FrpAP(FBR) cDNA encodes a predicted polypeptide of 348 amino acids.

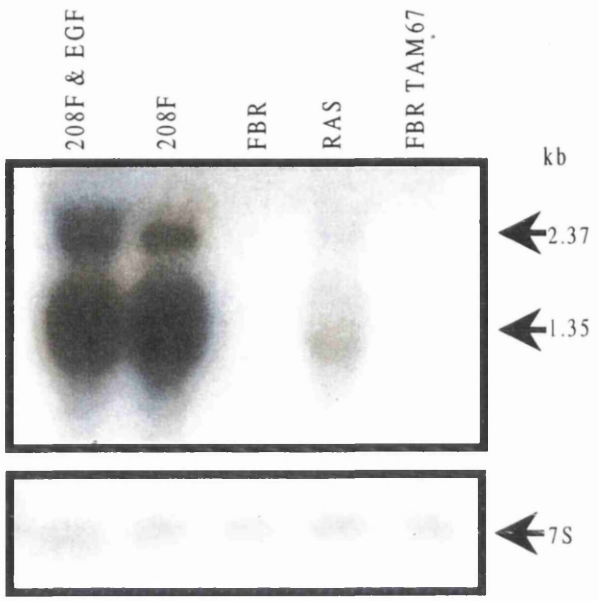
Figure 6.15

Northern blot and rat Tissue blot of clone CD10

In this figure, Panel A details the three day exposure X-ray film of Northern blot probed with Clone CD10, in the bottom panel the corresponding scanned X-ray film of the 7S Ribosomal loading control is shown. The Northern blot for CD10 was performed twice with the same result. B details the expression of CD10 in rat tissues. The actin loading control is shown on the right. The tissue blot was performed once using a Clontech rat tissue blot. The size of the mRNA transcripts is indicated by arrows with regards to the RNA ladder in kilobases and are approximately 2.0 and 2.8Kb.

A

NORTHERN BLOT



B

RAT TISSUE BLOT(CLONTECH)

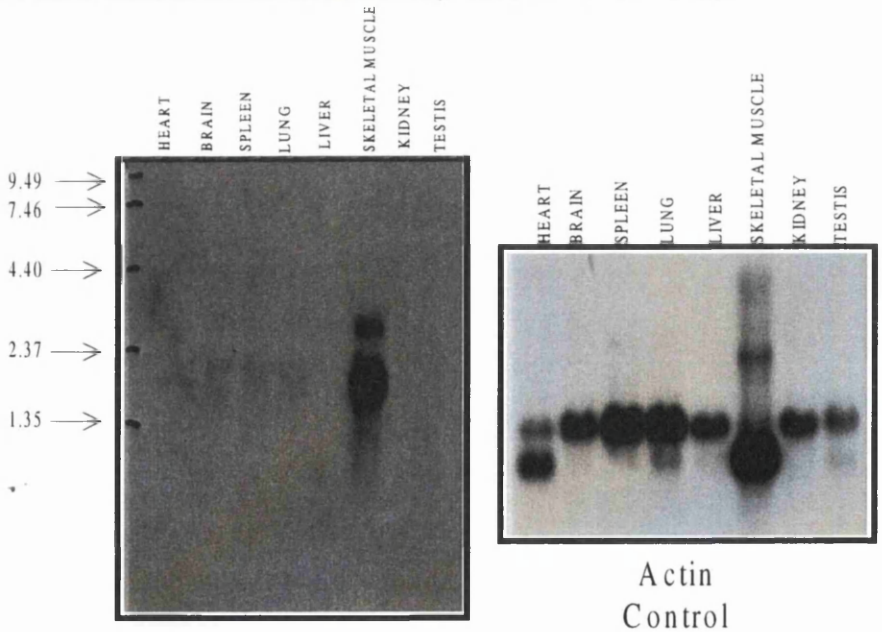


Figure 6.15
Northern Blot and Rat Tissue Blot of Clone CD10

6.2.9.2 Prosite analysis of ORF protein translation of FrpAP

FrpAP was identified as an apoptosis associated gene and was subsequently cloned (Wolf, *et al.*, unpublished), and contains a *frizzled* domain. Genes of the *frizzled* family have been found in many animal species and they have structural similarities. The ORF protein translation of FrpAP (FBR), was subject to Pfam, protein families database and HMMs alignments to identify the *frizzled* domain and also PROSITE analysis to identify theoretical protein pattern sites. These are detailed in Table 6.2 and their position in the amino acid sequence is highlighted in Panel A, Figure 6.17. The alignment of FrpAP(FBR) with the ten most closely related other *frizzled* family members in their domain and their amino acid sequence similarity is exemplified by Figure 6.18. Panel B in this figure exemplifies the very high homology that exists between FrpAP (FBR), sFRP4 and FrpHE. It would appear that the published sequence of FrpAP and the FrpAP (FBR), sequence herein described are rat homologues of sFRP4 and FrpHE.

ExPASy protparam analysis of FrpAP (FBR), predicts a molecular weight of 39.7kDa, theoretical pI of 9.04 with the amino acid composition containing 11% negatively charged residues (Asp & Glu), and 15% positively charged residues (Arg & Lys).

PROTEIN PATTERN	POSITION OF MATCH		
cAMP- and cGMP-dependent protein kinase phosphorylation site	1	282-285	RRST
	2	344-347	KKST
Protein kinase C phosphorylation site	1	281-283	SRR
	2	308-310	TST
	3	327-329	SPK
Casein kinase II phosphorylation site	1	49-52	STQE
	2	118-121	SWPE
	3	216-219	TVVD
	4	285-288	TQWE
	5	297-300	TTQE

Table 6.2

PROSITE protein patterns of FrpAP

The above table indicates the PROSITE protein pattern type, their location and motif.

Figure 6.16

Nucleotide-and predicted amino acid sequence of FrpAP cDNA

The nucleotides are numbered above the nucleic acid sequence. Amino acids are indicated in single letter code with the first predicted methionine at position 94. This is located with a translation initiation site GCG**ATG**G (Kozak, 1987). The termination codon is denoted by an asterisk at position 1140.

```
        10                  30                  50
gctcggagcgcacgccctaggtaaagacataaggagatcagagggacttgaagggaaggt

        70                  90                  110
ccccagtgggacgcagcccaggaggacagtgcgatgctcctctccatcctggtagcgtta
                                M  L  L  S  I  L  V  A  L

       130                 150                 170
tgcctgtgcgtgcgcctggctctgggagtgcgcggagcgccctgcgaggctgtgcgcatc
C  L  C  V  R  L  A  L  G  V  R  G  A  P  C  E  A  V  R  I

       190                 210                 230
cccatgtgtaggcacatgccctggaacatcacccggatgcccaaccacctgcaccacagc
P  M  C  R  H  M  P  W  N  I  T  R  M  P  N  H  L  H  H  S

       250                 270                 290
actcaggagaacgccatcctggccatcgagcagtacgaggagctggtagacgtgaactgc
T  Q  E  N  A  I  L  A  I  E  Q  Y  E  E  L  V  D  V  N  C

       310                 330                 350
agctctgtactgcgcttctttctctgtgccatgtatgcacccatctgtaccctggagttc
S  S  V  L  R  F  F  L  C  A  M  Y  A  P  I  C  T  L  E  F

       370                 390                 410
ctgcacgatcccatcaaaccctgcaaatctgtgtgccagcgcgcacgcgacgactgcgag
L  H  D  P  I  K  P  C  K  S  V  C  Q  R  A  R  D  D  C  E

       430                 450                 470
cccctcatgaagatgtataaccacagctggccagagagcctggcttgcgatgagctgcct
P  L  M  K  M  Y  N  H  S  W  P  E  S  L  A  C  D  E  L  P

       490                 510                 530
gtctatgaccgtggagtgtgcatctctccggaggcgatagtcactgaccttccggaagat
V  Y  D  R  G  V  C  I  S  P  E  A  I  V  T  D  L  P  E  D

       550                 570                 590
gtgaagtggatagacatcacaccagatatgatggttcaagaaaggtcctttgatgctgac
V  K  W  I  D  I  T  P  D  M  M  V  Q  E  R  S  F  D  A  D

       610                 630                 650
tgtaaacatctgagccctgatcggtgcaagtgcaaaaaggtgaagccaactttggcaacg
C  K  H  L  S  P  D  R  C  K  C  K  K  V  K  P  T  L  A  T

       670                 690                 710
tacctgagcaaaaactacagctatgttattcatgccaaaataaaagcggtccagaggagt
Y  L  S  K  N  Y  S  Y  V  I  H  A  K  I  K  A  V  Q  R  S

       730                 750                 770
ggttgcaatgaggtcacaactgtggtcgatgtaaaagagatcttcaagtcttcatcacct
G  C  N  E  V  T  T  V  V  D  V  K  E  I  F  K  S  S  S  P

       790                 810                 830
atccctcgaacgcaagtccccctcatcaccaattcctcctgccagtgtccacacatcctg
I  P  R  T  Q  V  P  L  I  T  N  S  S  C  Q  C  P  H  I  L

       850                 870                 890
ccccatcaagatgtcctaatcatgtgttatgagcggcgttcaaggatgatgcttcttgaa
P  H  Q  D  V  L  I  M  C  Y  E  R  R  S  R  M  M  L  L  E

       910                 930                 950
aattgtttagttgagaaatggagagatcaactaagcagaaggtccacacagtgggaagag
N  C  L  V  E  K  W  R  D  Q  L  S  R  R  S  T  Q  W  E  E

       970                 990                1010
aggcttcaggaacagcagagaacaactcaggacaagaagcaaatagccagccgcaccagt
R  L  Q  E  Q  Q  R  T  T  Q  D  K  K  Q  I  A  S  R  T  S

      1030                1050                1070
cgcagtaacccccccaaagccaaagggaaggtcacctgcttccaaacctgccagtcctaag
R  S  N  P  P  K  P  K  G  R  S  P  A  S  K  P  A  S  P  K

      1090                1110                1130
aagaacatcaaagctagaagtgcacccaaaaagtcaaacccaaagaaaagtacaagctaa
```

```
K  N  I  K  A  R  S  A  P  K  K  S  N  P  K  K  S  T  S  *

      1150                1170                1190
ttactttccaagttgaagccacccttacaggataaggtggtactgcctgggacagccttc

      1210                1230                1250
cgaaggccacacaccacttgccttaggacccactgtggttctctttatagacatgtcttg

      1270                1290                1310
cagcatttctcttaatgatagcttcagtatttctctttaaaccacagatccctgttattt

      1330                1350                1370
gtcagttttcttttatggaaaggtacctgtgttcagagtggcctgtgagcatactgtgtg

      1390                1410                1430
accttctgctttgagattttgacctaaaatctaccttataaaagttcacatactatgtcaa

      1450                1470                1490
gcaaaacttttttttgacaaaaatattggatatcctttgacttctgttaggaagttagtga

      1510                1530                1550
tatataaaatgttattgaaaaaaatgtttttgacaaaggcagaagaatgatacataaaag

      1570                1590
accttcatgtgtttgttgtctgcaaaaaaaaaaaaaaa
```

Figure 6.16
Nucleotide and predicted amino acid sequence of FrpAP cDNA

Figure 6.17

PROSITE and Pfam analysis of FrpAP amino acid sequence

A: Amino acids are numbered on the left beginning at the first methionine. The cAMP- and cGMP-dependent protein kinase phosphorylation sites are shown in red, Protein kinase C phosphorylation sites are shown in bold, and the Casein kinase II phosphorylation sites are shown underlined. The PSORT II signal sequence recognition for cleavable signal peptide is shown between positions 18 and 19 with a green forward slash.

B: Homology of FrpAP amino acid positions 24 to 137 to the *Frizzled* motif by pfam alignment program. The identical residues are shown by single amino acid code and similar residues are shown by + and the ten invariably spaced cysteine residues of the cysteine rich domain (CRD), are shown in red.

A

1 MLLSILVALC LCVRLALG/VR GAPCEAVRIP MCRHMPWNIT RMPNHLHHST

51 QENAILAIEQ YEELVDVNCS SVLRFFLCAM YAPICTLEFL HDPIKPCKSV

101 CQRARDDCEP LMKMYNHSWP ESLACDELPV YDRGVCISPE AIVTDLPEDV

151 KWIDITPDMM VQERSFDADC KHLSPDRCKC KKVKPTLATY LSKNYSYVIH

201 AKIKAVQRSG CNEVTTVVDV KEIFKSSSPI PRTQVPLITN SSCQCPHILP

251 HQDVLIMCYE RRSRMMLLEN CLVEKWRDQL SRRSTQWEER LQEQQRTTQD

301 KKQIASRTSR SNPPKPKGRS PASKPASPKK NIKARSAPKK SNPKKSTS

B

Alignment of Fz vs FrpAP positions 24-137

```
                 *->CepIpatipLCkdlgYNlTrmPNlLgHetqeEAgleveqFkPLvkvk
                    Ce+++  ip+C+++++N+TrmPN+L+H+tqe+A+l++eq+++Lv+v+
QuerySeq     24    CEAVR--IPMCRHMPWNITRMPNHLHHSTQENAILAIEQYEELVDVN 68

                 CSpdLkfFLCSlYAPvCtedlphkPIpPCRslCErakdgCaplMekfGFp
                 CS+ L+fFLC++YAP+Ct ++ h+PI+PC+s+C+ra+d+C+plM++++++
QuerySeq     69  CSSVLRFFLCAMYAPICTLEFLHDPIKPCKSVCQRARDDCEPLMKMYNHS 118

                 WPdsLnCdkFPvqnddpegLCm<-*
                 WP+sL+Cd++Pv+++   g+C+
QuerySeq    119  WPESLACDELPVYDR---GVCI    137
```

Figure 6.17
PROSITE and Pfam analysis of FrpAP amino acid sequence

Figure 6.18

Sequence similarity alignments of FrpAP with related *frizzled* family members

A: This figure indicates the conserved domains between FrpAP closely related secreted *Frizzled* sub-family members and the *frizzled* protein. The family member and their species origin is indicated on the left. They share a common N-terminal signal sequence as indicated with a blue box and the cysteine rich extracellular domain, CRD, boxed in pink. The *frizzled*-related proteins lack the seven transmembrane spanning regions found in *Frizzled* proteins as shown by a green box. The predicted number of amino acids for each protein is indicated to the right

B: The alignments of the *frizzled* related proteins and *frizzled* protein sequence is shown with the *Frizzled* sub-family member and species origin indicated in the left. The amino acid position is indicated above each row of alignment and the *frizzled* protein is truncated at position 376. Highly homologous regions, greater than 90% are shown in red with lower homology regions, 50%, are indicated in blue.

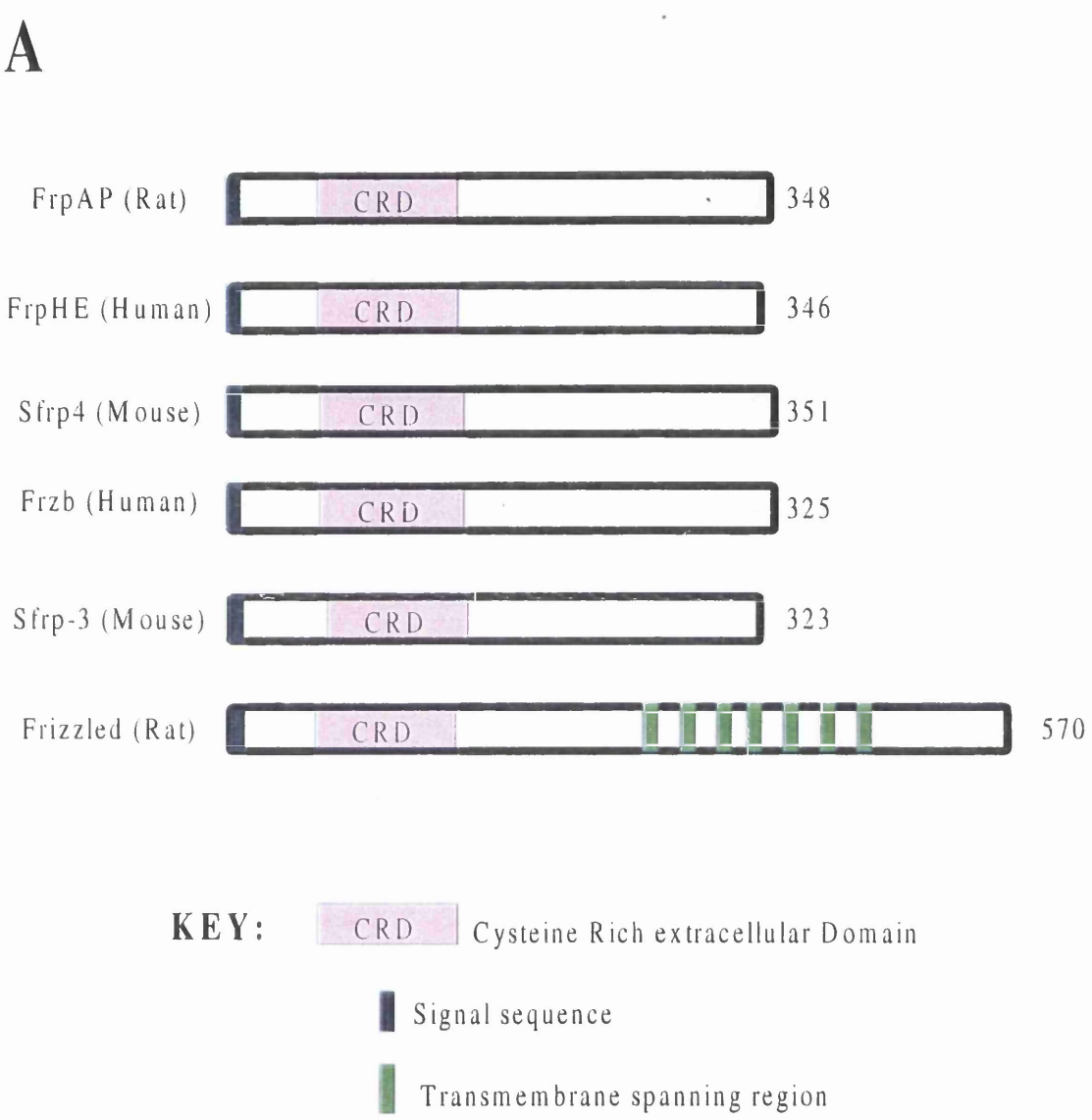


Figure 6.18A
Sequence similarity alignments of FrpAP with related *frizzled* family members

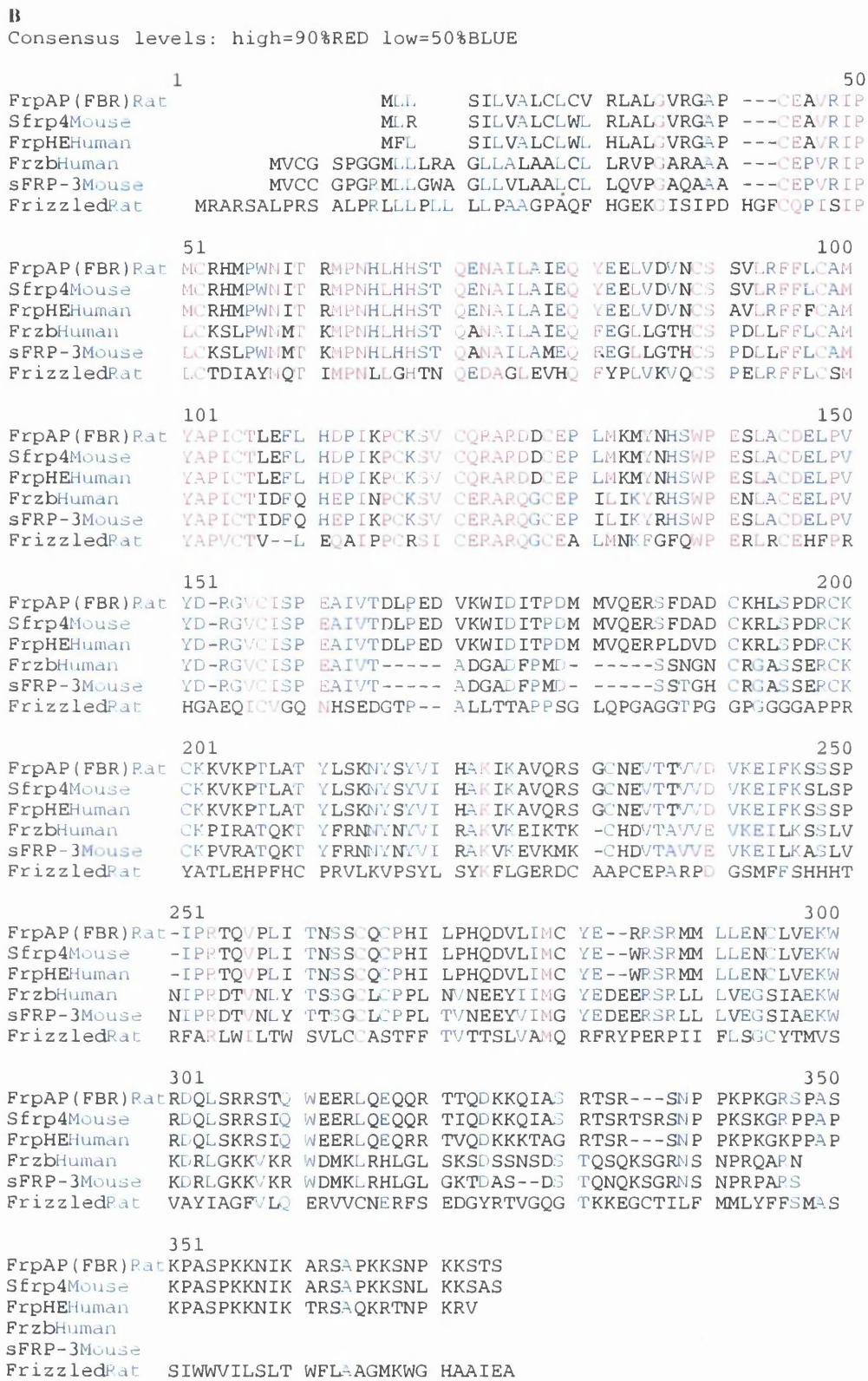


Figure 6.18B
Sequence similarity alignments of FrpAP with related *frizzled* family members

6.3 DISCUSSION

The clone KD1 was initially identified as a novel sequence with homology to the EST clone, mouse 7.5dpc ectoplacental clone, that exhibited a weak similarity to the helix-loop-helix transcription factor, Alf1. The characterisation of expression by Northern blotting revealed the expression patterns of KD1 in three model systems which activate AP-1 activity. In *v-fos* transformed 208F the transcript is undetectable. The KD1 transcript is down-regulated two-fold in K-*ras* transformed 208F fibroblasts. The KD1 transcript is not detectable in EGF transformed 208Fs. An approximate two-fold down-regulated transcript was detected in FBR-Tam67 cells. Collectively, these expression profiles indicate that KD1 is transrepressed by AP-1 transcription factor. The somewhat incomplete down-regulation by Tam-67 indicated that it may be an indirect target, although the quenching mechanism previously proposed for Tam-67 activity may be responsible.

As the KD1 transcript was significantly down-regulated in the three AP-1 models analysed by northern blotting, the tissue expression of KD1 in a rat tissue blot was examined. The transcript is testis specific. The sequence information coupled with the expression data indicated this clone to be an interesting down-regulated gene which may encode a novel transcription factor. Thus it was cloned for further analysis. The cDNA sequence of DIF1 predicts an open reading frame from the first Kozak ATG initiation signal resulting in a polypeptide of 300 amino acids. No nuclear localisation signal was detected. Therefore the DIF1 protein probably does not function as a helix-loop-helix transcription factor as initially believed.

Unfortunately, there was insufficient time to further study DIF1. The short term future work which would be interesting includes the cloning of DIF1 into an inducible expression system in attempt to generate stable DIF1 expressing clones. This would determine the exact morphological changes that DIF1 induces and would facilitate any change in proliferation or anchorage-independent growth to be studied. The characterisation of expression of DIF1 should be extended beyond the original tissue blot performed, to include more tissue types, as well as tumours from the tissues in which it is present versus normal. In addition, the myc-tagged DIF1 expression vector could be used to enable the production and purification of DIF1 in a bacterial system, to yield enough protein to raise an antibody to DIF1. This would permit histological studies to ascertain DIF1 cellular localisation in testis and in any other tissues in which it is expressed.

The rat *frizzled* related protein, FrpAP, was isolated as a *frizzled* related gene up-regulated in physiological apoptosis in corpus luteum of the rat (Wolf, *et al.*, 1997

unpublished). The characterisation of expression by Northern blotting revealed the expression patterns of FrpAP(FBR), in three model systems which activate AP-1 activity. In *v-fos* transformed 208F fibroblasts where AP-1 is consitutively expressed none of the two transcripts are detectable even when the blot is exposed for seven days. The K-*ras* oncogene transforms 208F fibroblasts and the concurrent cell signalling through the *ras* pathway activates AP-1, which markedly down-regulates the expression of both the FrpAP(FBR) transcripts. The other AP-1 model system, 208F treated with EGF which morphologically transforms 70-90% of the cell population through activation of cell signalling pathway via extracellular growth factor receptor, found the FrpAP(FBR) transcripts marginally down-regulated. No transcript was detected in FBR-Tam67 cells. Collectively, these expression profiles indicate that FrpAP(FBR) is indeed down-regulated in cells with activated AP-1.

The expression of FrpAp(FBR) in a rat tissue blot indicated a high functional activity in skeletal muscle, and low activity in brain, spleen and lung tissue. The tissue expression of sFRP family members is varied indicating that their antagonistic function is tightly controlled depending upon developmental stage and physiological state.

The cDNA sequence of rat FrpAP(FBR) predicts an open reading frame resulting in a polypeptide of 348 amino acids. The eukaryotic signal sequence indicating it's secretion is consistent with the typical signal sequence and secretion of other *frizzled* related family members. The protein contains the classical conserved CRD region of 120 amino acids which contains a motif of ten invariantly spaced cysteines. The extracellular domain, that binds *Wnt* gene products is clearly highlighted in Figure 6.18, Panel B.

The function of several cloned sFRP genes has been linked with various aspects of cellular proliferation, migration, differentiation and tissue morphogenesis during normal development (Hoang *et al.*, 1996; Wolf *et al.*, 1997; Mayr *et al.*, 1997; Leyns *et al.*, 1997; Rattner *et al.*, 1997; Finch *et al.*, 1997; Melkonyan *et al.*, 1997; Hu *et al.*, 1998; and Chang *et al.*, 1999). The FrpAp(FBR), presented here, is shown to be homologous to the mouse Sfrp4 and human FrpHE proteins. No current data is available of the function of Sfrp4 or FrpHE.

Two recent publications (Zhou *et al.*, 1998; Ugolini *et al.*, 1999) have linked the down-regulation of two sFRP family members in breast cancers, and one homologue, hsFRP was also up-regulated during apoptosis (Zhou *et al.*, 1998).

Cell growth and differentiation are tightly controlled by the apoptotic pathway and the cell cycle, and it is well documented that de-regulation of the genes involved in cell-cycle regulation can contribute to carcinogenesis (Hartwell and Kastan, 1994). The hsFRP gene, a new sFRP family member, was found to be up-regulated in

apoptotic and growth-arrested human immortalised breast epithelial cell line, HBL-100, which indicated a possible role in growth regulation through a cell-cycle/apoptotic mechanism or through the *Wnt* signalling pathway (Zhou *et al.*, 1998). An increase in hsFRP levels during apoptosis resulted in a subsequent decrease in β-catenin, which indicates that hsFRP can antagonise β-catenin levels through the *Wnt* signalling pathway. In addition, the down-regulation of hsFRP, up-regulates β-catenin which accumulates cytoplasmically, the same consequence as transforming *wnt*. The decrease in hsFRP level and subsequent up-regulation of β-catenin reduces apoptotic cell death, a characteristic feature of carcinogenesis. hsFRP may bind *wnt* and thereby antagonise binding to the cell-surface-signalling receptor, *Frizzled*, thus blocking transduction of the *wnt* signal. When expression of hsFRP is decreased, the *wnt* signal is unblocked thereby enabling signal transduction and can lead to abnormal, even transformed cell growth. These results indicated that hsFRP, a *frizzled* secreted sub-family member can block *wnt* signalling through cytosolic β-catenin, to regulate cell proliferation and perhaps transformation (Zhou *et al.*, 1998). The antagonistic mechanism of sFRP proteins, Frzb, on *Wnt* activity and subsequent signalling pathway is exemplified in Figure 6.19.

The expression of hsFRP as examined in cells derived from malignant breast carcinomas was found to be undetectable, whilst in three immortalized non-malignant breast epithelial cell lines it was expressed. Furthermore, lower expression was found in 5/5 of breast tumours, 2/4 of ovary tumours and 3/5 kidney tumours when expression was compared to adjacent normal tissue. This decreased hsFRP expression in breast tumours and carcinoma cell lines suggests a possible involvement in cell proliferation and breast tumourigenesis possibly through the *Wnt* signalling pathway.

The isolation of another breast tumour associated sFRP homologue, FRP1/FRZB was isolated in a differential expression assay on the short arm of chromosome 8p where there are frequent alterations through deletions and mutations associated with various types of cancers (Ugolini *et al.*, 1999).

Figure 6.19

sFRP(Frzb) can antagonise *Wnt* signalling

A: Frzb can compete and bind *Wnt* to prevent its interaction with a *Frizzled*-like *Wnt* receptor. In the absence of a *Wnt* signal through *Frizzled*-like receptor, the serine/threonine protein kinase GSK-3β phosphorylates cytoplasmic β-catenin, and in conjunction with the tumour suppressor protein APC, degrades β-catenin.

B: *Wnt* binds the *Frizzled*-like receptor, activating Dishevelled protein, Dsh, which inactivates GSK-3β. An increase in β-catenin level ensues, and β-catenin accumulates in the nucleus in association with the HMG-box DNA binding protein TCF(LEF). This β-catenin-TCF complex transcriptionally activates *Wnt* responsive genes which influence the fate and behaviour of cells.

(This figure was adapted from Zorn, 1997.)

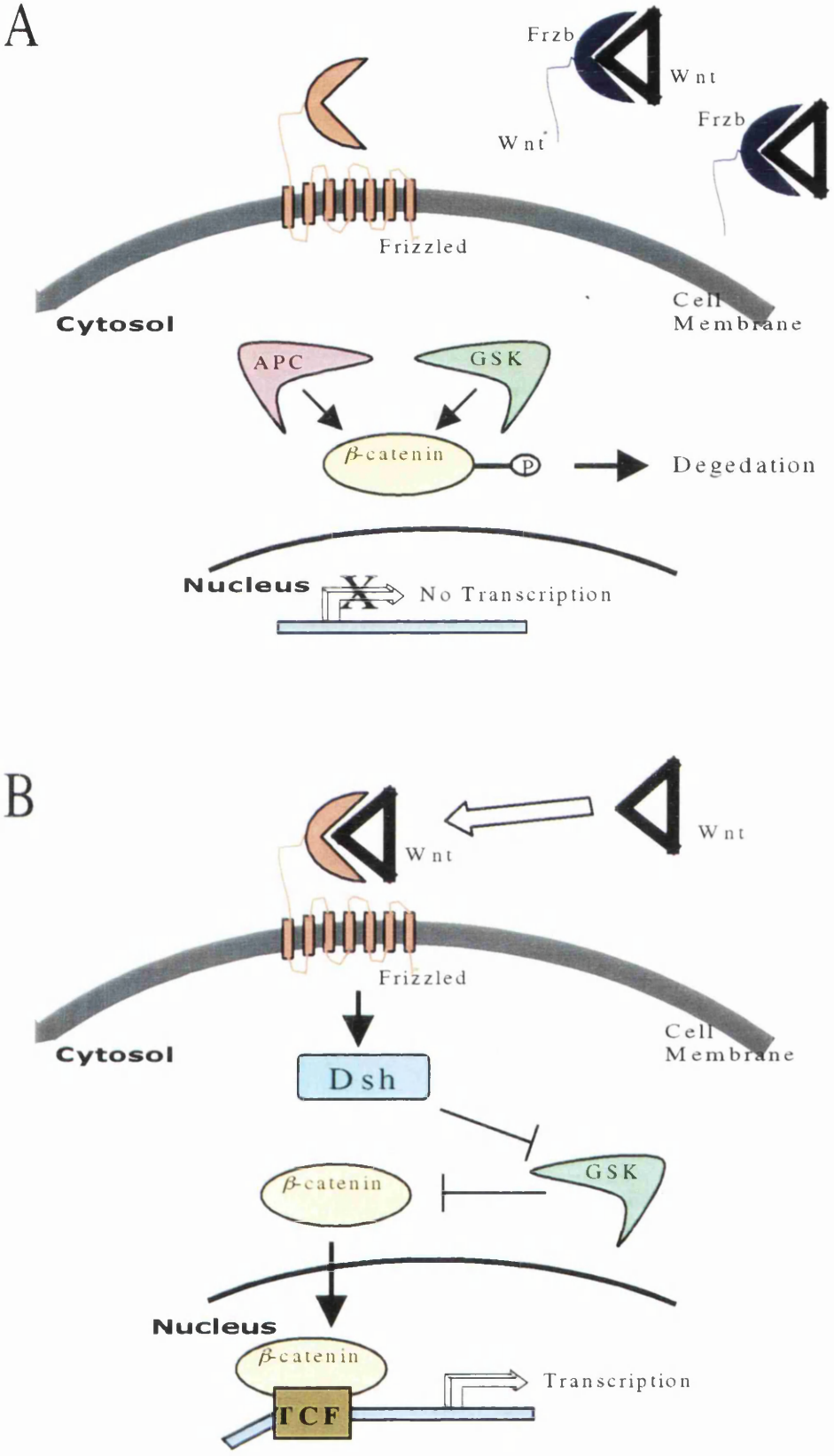


Figure 6.19
sFRP can antagonise Wnt signaling

Recent advances in using cDNA arrays, otherwise known as DNA chips can be used to detect and quantify differential gene expression of EST sequences on a large scale (for review Jordan 1998). This technology was used to examine the differential expression of a set of genes located in the p11-21 region of the short arm of chromosome 8.

The comparative expression analysis using MDA-MB-134 cells derived from ductal mammary carcinoma versus normal breast, and also between HBL-100, a cell line previously discussed, and breast carcinoma tumour 4885 and normal breast. One of the underexpressed transcripts in MDA-MB-134 and 4855 was clone y157a10, corresponding to the Frizbee-like protein (Zorn, 1997) called *Frizzled*-related protein-1, FRP1 (Finch *et al.*, 1997; Rattner *et al.*, 1997) or Secreted apoptosis related protein-2, SARP2 (Melkonyan *et al.*, 1997), was also undetected in tumour breast cells as compared to normal. The expression of FRP1 was examined in ninety breast carcinomas, eighty three of ductal origin, six lobular and one medullar; and sixteen benign tumours. The FRP1 transcripts was down-regulated in most of the malignant tumours and undetectable in 78%, however, in contrast only 19% of the benign tumours showed a reduction in expression as compared to normal breast. This demonstrated that FRP1 is expressed in normal breast and benign breast tumours, whereas it is severely repressed in the majority of breast tumours (Ugolini *et al.*, 1999). In agreement with the findings of hsFRP by Zou *et al.*, 1997; FRP1 has pro-apoptotic activity (Melkonyan *et al.*, 1997) and can antagonistically function to inhibit *Wnt* signalling (Finch *et al.*, 1997; Wang *et al.*, 1997).

The genomic localisation of FRP1 to the centromic region of chromosome 8 (p11-21) between microsatellite markers D8S532 and D8S268 maps it in a subregion of loss of heterozygosity, LOH V, in breast cancer. The up-regulation of FRP1 in apoptosis, the inactivation by deletion in the genome, combined with the loss of expression in breast tumours and breast carcinomas (Melkonyan *et al.*, 1997; Zhou *et al.*, 1998; Ugolini *et al.*, 1999) suggests a tumour suppressor role and inactivation is linked with breast malignancy.

Previous data pertaining to inappropriate activation of the *Wnt* pathway highlights its significance in human cancers whereby key components of the signalling cascade, such as APC, β-catenin or CBP can be altered resulting in abnormal proliferation (Pennisi, 1998; He *et al.*, 1998). In addition, the experimental evidence discussed above in relation to breast cancer strongly suggests that the inactivation of *Wnt* signalling pathway by the sFRP sub-family negative regulatory molecules may lead to carcinogenesis.

The data reported in this chapter with regards to ablation of FrpAp(FBR), sFRP signalling molecule in *v-fos* transformed fibroblasts and the down-regulation in K-*ras* and EGF transformed fibroblasts coupled with the above findings would suggest *Wnt* signalling is a key component of transformation. In FBR cell line, the down-regulation of FrpAP would therefore abolish *Wnt* negative control, thus allowing expression of *Wnt* signalling and responsive genes, many of which have been implicated in carcinogenesis, further supporting the transformed characteristics of these cells. Furthermore, the findings of Melkonyan *et al.*, 1997; Zhou *et al.*, 1998; Ugolini *et al.*, 1999 suggesting that sFRPs have a role as a tumour suppressor genes further supports the down-regulation of FrpAP(FBR), in FBRs. The FBR cell line has increased cell proliferation, migration, and adhesion, a characteristic of the transformed bipolar phenotype and invasive behaviour of these cells. It is fitting that the regulatory control mechanism with regards to these processes as previously reported for several cloned sFRP genes during normal development would be de-regulated in transformation by AP-1 transcription factor.

Chapter 7

Summary and Future Work

The FBR *v-fos* transformed fibroblasts were studied to provide a model of AP-1 transformation. The approach of SSH was used to identify AP-1 target genes. Two libraries containing a wealth of up-regulated and down-regulated genes were isolated. This approach isolated numerous down-regulated genes, an area of new territory. In total 206 clones were isolated from the SSH libraries that are predominantly differentially expressed in FBR *v-fos* transformants and are putative AP-1 direct and indirect target genes. Furthermore, the clones isolated represent a fraction of the libraries as Dr. J.Winnie has extended the isolation of new genes from the up-regulated library. The sequencing and Northern blotting has identified many previously undiscovered differentially expressed genes.

The characterisation of expression patterns of the sequenced clones by Northern blotting further confirmed their differential expression. In addition, most of the FBR *v-fos* up-regulated genes were also shown to be up-regulated in K-*ras* and EGF transformed 208F fibroblasts. Most of the FBR *v-fos* down-regulated genes were shown to be down-regulated in K-*ras* and EGF transformed 208F fibroblasts. Interestingly, the results of the Northern blot data using FBR v-fos transformants expressing Tam-67 revealed that the expression of both the up-regulated and down-regulated genes were somewhat reduced, with a few exceptions.

The mechanism of down-regulation by AP-1 is unknown, but has been hinted at by the recent association of dnmt1 DNA methylation, resulting in transcriptional repression (Bakin and Curran, 1999). The isolation of SAP18 and RbAp46 from the up-regulated library further strengthens the case for a transcriptional repression mechanism in AP-1 down-regulation.

The characterisation of the isolated genes did not extent to analysing promoter regions as it was beyond the scope of this project. However, some of the identified genes are known AP-1 targets such as, MMP-10 and CD44. Therefore, from the library controls and Northern data it is reasonable to assume that the identified genes are AP-1 targets, although many are probably not direct targets.

One of the down-regulated genes FRP when overexpressed in FBR *v-fos* transformants results in a 70% reduction of invasion in an *in vitro* invasion assay.

The down-regulated gene FRP, when overexpressed in FBR *v-fos* transformants resulted in a 70% reduction of invasion in an *in vitro* invasion assay. The future experiments to follow up this result would include the investigation into FRP expression by AP-1. Initially, a *fos*-inducible system could be used to determine if FRP is switched off during *fos* induction, and at what time point. The FRP promoter region could be analysed to identify the AP-1 consensus sequence. The endogenous cellular levels of FRP were not assayed in this study as no commercial antibody was available.

The raising of an antibody to FRP would facilitate the examination of endogenous levels and would also determine the localisation of the protein. The mechanism by which the secreted protein FRP blocks invasion is unknown. Therefore, interaction studies between FRP and ECM, Integrins would enable the identification of possible extracellular sites through which FRP functions.

The down-regulated gene DIF-1 was very difficult to transfect into FBR cell line. To overcome this, future experimentation should include a regulatable growth system to induce DIF-1 expression. This would enable the determination of the effects of DIF-1 on transfrormed cell morphology and actin structure.

In addition, the expression of FRP and DIF-1 in tumour and normal tissue should be examined.

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