Characterisation of a Novel Multi-tissue Tumour Suppressor Gene in Mouse

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Declaration

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

Abbreviations

ALL	acute lymphoblastic leukaemia
APC	adenomatous polyposis coli
A-T	ataxia telangiectasia
BICR	Beatson Institute for Cancer Research
c-	cellular
CDK	cyclin dependent kinase
cg1bp	putative cyclin G1 binding protein
CHLC	Cooperative Human Linkage Centre
CML	chronic myeloid leukaemia
CRC	Cancer Research Campaign
ER	oestrogen receptor
ES	embryonal stem cells
EST	expressed sequence tag
FAP	familial adenomatous polyposis
FISH	fluorescent in situ hybridisation
GAP	GTPase activating protein
HNPCC	hereditary non-polyposis colon cancer
HPV	human papilloma virus
HSP	heat shock protein
LOH	loss of heterozygosity
LTR	long-terminal repeat
MEF	mouse embryonic fibroblast
MEN	multiple endocrine neoplasia
mchr	mouse chromosome
MMCT	microcell-mediated monochromosome transfer
MMR	mismatch repair
NER	nucleotide excision repair
NIH	National Institute of Health
p	protein, plasmid
PAC	P1 artificial chromosome

PAGE	Polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PKC	protein kinase C
RB	retinoblastoma
RDA	representational difference analysis
RFLP	restriction fragment length polymorphism
RT	reverse transcriptase
SCC	squamous cell cancer
SCDR	smallest common deleted region
SSCP	single strand conformation polymorphism
TSG	tumour suppressor gene
Taq	*Thermus aquaticus*
TGF	transforming growth factor
TPA	12-*O*-tetradecanoylphorbol-13-acetate
UV	ultra violet
v-	viral
VHL	von Hippel-Lindau
wt	wild type
XP	xeroderma pigmentosum
YAC	yeast artificial chromosome

Reagents

APS	ammonium persulphate
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
DAB	diaminobenzidine
dH_2O	de-ionised water
DAPI	4',6-diamidino-2-phenylindole
DMBA	7,12-dimethylbenzanthracene
DMF	dimethylformamide
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid

dNTP	3′ deoxyribonucleoside 5′-triphosphate
EDTA	ethylenediaminetetra-acetic acid
FBS	foetal bovine serum
FITC	fluorescein isothiocyanate
IgG	immunoglobulin G
LB	Luria broth
MES	2-(N-morpholino) ethansulfonic acid
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
mRNA	messenger ribonucleic acid
PBS	phosphate buffered saline
PEG	polyethylene glycol
PI	potassium iodide
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
TAE	tris, acetic acid, ethylenediaminetetra-acetic acid
TBE	tris, boric acid, ethylenediaminetetra-acetic acid
TE	tris, ethylenediaminetetra-acetic acid
TEMED	tetramethylenediamine
Tris	2-amino-2-(hydroxymethyl)propane-1, 3-diol

Units

bp	base pair
Ci	Curie
cm	centimetre
Da	dalton
g	gram
g	gravity
hr	hour
k	kilo
l	litre
M	mega
μ	micro

m	milli
m	metre
M	molar
min	minute
n	nano
°C	degree Celsius
PD	population doublings
rpm	revolutions per minute
RT	room temperature
s	second
U	unit
v/v	volume for volume
w/v	weight for volume

Single letter amino-acid code

A	alanine
R	arginine
N	asparagine
D	aspartic acid
C	cystine
E	glutamic acid
Q	glutamine
G	glycine
H	histidine
I	isoleucine
L	leucine
K	lysine
M	methionine
F	phenylalanine
P	proline
S	serine
T	threonine

W	tryptophan
Y	tyrosine
V	valine

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Abstract

A considerable body of evidence from molecular, cytogenetic and functional studies points to the existence of a novel tumour suppressor gene on the long arm of human chromosome 7. This putative gene seems to be conserved between mouse and man, since a high frequency of allele loss is evident at mouse chromosome 6 band A2, the region syntenic to human chromosome 7q31, in the two-stage skin carcinogenesis system. In addition, introduction of human chromosome 7 into mouse tumour cell lines results in increased latency in tumorogenic assays, implying functional conservation. To date, this gene has not been isolated.

I undertook loss of heterozygosity studies in a series of mouse skin tumours produced by the two-stage carcinogenesis protocol. After saturating the area of interest on mouse chromosome 6A2 with all available published microsatellite markers, I defined a possible smallest common deleted region between the markers D6mit83 and D6mit50. Allelotyping analysis of a panel of mouse cell lines representative of the sequential stages in skin carcinogenesis demonstrated allelic imbalance of markers on chromosome 6. FISH analysis of a selection of these cell lines using whole chromosome paints confirmed aneuploidy with tri- or tertrasomy of chromosome 6 being common. The finding of trisomy of chromosome 6 was seen in both papilloma and carcinoma cell lines, suggesting that this is an early event in skin carcinogenesis.

Concurrent work in our laboratory on human chromosome 7 has generated a number of candidate tumour suppressor genes which map to chromosome 7q. Full-length cDNAs corresponding to three of these genes were cloned from a mouse foetal cDNA library, and analysed for mutational inactivation in the panel of mouse tumour cell lines. No mutations were evident. One of these genes was a novel homologue of *kelch*, an actin binding molecule first identified in *Drosophila*. This proved to possess both POZ and kelch repeat domains, commonly seen in the *kelch* family of genes, and showed a high degree of similarity to the human *kelch* homologue.

CHAPTER 1

INTRODUCTION

1. Introduction

1.1 Tumour Suppressor Genes and the Genetics of Cancer

Like much of biology, our understanding of cancer has undergone wholesale revision following the advent of the techniques of molecular biology in the 1970s. Prior to that time, tumour development was very much a black box, variously ascribed to viral infection, defective immunity and, nebulously, 'genetic alteration'. The unifying picture to emerge over the last two or three decades is that, put succinctly, cancer is a genetic disease of cells. Much of the research on carcinogenesis has focused on identification of those genes which, when altered, lead to the development of cancers. It has become clear that there is a finite number of commonly altered genes, whose normal role is as part of the signal transduction, cell cycle, cell differentiation and genome maintenance machinery. These genes are classifiable broadly as oncogenes or positive regulators of cell growth, and tumour suppressor genes (TSGs) or negative regulators. Epidemiological and other data point to there being a requirement for at least four or five genetic changes before development of the malignant phenotype. The emerging theory is that tumorigenesis is a multistage process requiring serial alterations in oncogenes and TSGs before the clinically recognised syndrome of cancer is seen. The sequence of these genetic changes is now becoming clear and is opening the way to novel therapeutic interventions.

1.1.1 Oncogenes

Oncogenes are genes which undergo 'gain of function' alterations in cancer cells. The study of oncogenes began with experiments on the Rous sarcoma virus (RSV), one of the acutely transforming retroviruses (reviewed in Weiss, 1984). These viruses were shown to possess, in addition to their normal genomic content, extraneous DNA sequences which were responsible for acute oncogenesis. In fact, RSV is unique in having all genes necessary for viral replication (*gag*, *pol* and *env*)

in addition to the oncogenic *v-src*; all other acute retroviruses lack one of the structural genes, rendering them replication incompetent. The entirely unexpected finding that the oncogenic DNA sequences hybridised to normal cellular DNA from a variety of vertebrates (Stehelin *et al.*, 1976) ushered in a new era in molecular oncology. Such proto-oncogenes transpired to be genes whose protein products have crucial roles to play in controlling the proliferation of normal cells. Their presence in viral RNA is thought to result from the process of transcription of proviral DNA, when they are 'captured' or transduced from the host genome (Bishop, 1982). In all, some twenty oncogenes were discovered in this fashion. Viral oncogenes have commonly undergone mutation in their coding region which alters the biochemical properties of the gene product. Others, however, harbour no detectable alterations and are oncogenic by virtue of the fact that they are under the transcriptional control of the constitutively active viral long terminal repeats. Indeed, retroviruses causing cancer after long incubation periods possess no oncogenes: they produce their oncogenic effect by proviral integration into or near host cellular proto-oncogenes (Hayward *et al.*, 1981).

There are in fact a number of mechanisms whereby proto-oncogenes can be activated aberrantly within normal cells, resulting in transformation. Cytogenetic alterations have long been associated with cancer cells, and were suspected to be causal of, or at least contributory to, the malignant phenotype. Translocations of the *myc* and *Ig* genes have been observed in B cell derived tumours in man and rodent species. In Burkitt's lymphoma and the B-cell leukaemias, the principle alteration is a juxtaposition of the *myc* oncogene and the *Ig* heavy chain gene (Leder *et al.*, 1983). By this mechanism, the *myc* oncogene is constitutively expressed, resulting in cellular transformation. Another means by which the action of proto-oncogenes can be altered is by the formation of fusion proteins. This is precisely the alteration occurring in the long recognised Philadelphia chromosome found in the vast majority of patients with chronic myeloid leukaemia (CML) and in a smaller proportion of those with ALL. Here, the classical translocation is between the long arms of chromosomes 9 and 22 (t(9;22)(q34;q11)), resulting in the fusion of the *abl*

proto-oncogene on chromosome 9 with the *bcr* gene on chromosome 22 and ultimately in a chimeric protein with constitutive tyrosine kinase activity (Heisterkamp *et al*, 1982). In over 90% of CML, *bcr* sequence is juxtaposed to exon 2 of *abl*, forming a 210kDa fusion protein, p210. That the bcr-abl protein is indeed oncogenic and causative of the disease can be proved by transgenic studies in mice: forced expression of the protein in haemopietic progenitor cells in mice results in a CGL-like syndrome (Elefanty *et al.*, 1990; Gishizky *et al.*, 1993).

Gene amplification is often visible cytogenetically as episomal double minute (DM) chromosomes (Schwab *et al.*, 1983), or as chromosomally integrated homologously staining regions (HSRs) (Alitalo *et al.*, 1983), and can again result in oncogenic activation by a gene dosage effect. Although long stretches (up to 2 megabases) of DNA can be so altered, sometimes making it uncertain which precise genes are involved, the majority of amplifications seen in human cancer involve the *myc* family of oncogenes, the *ras* family, and growth factor receptors such as *erbB-1* and *-2*. In small cell lung cancer, for example, all three members of the *myc* family are amplified with moderate frequency, whereas it is N-*myc* that is preferentially amplified in neuroblastoma cells (Schwab, 1993). These changes presumably confer some growth advantage, since they are not observed in normal cells. Many studies have attempted to correlate the presence of gene amplication with clinical outcome. The evidence would suggest that gene amplication is associated with a more invasive phenotype and thus a poorer prognosis. In the case of breast cancer, there has been huge interest in the presence of *erbB-2* overexpression, as detected by *in situ* hybridisation or immunocytochemistry, and its correlation with ER negative cancers and poor prognosis (Slamon *et al.*, 1987). The introduction of trastuzumab, a humanised monoclonal antibody against the extracellular domain of *erbB-2* (Carter *et al.*, 1992), as a therapy in metastatic breast cancer has refocused attention on the subject and may soon make *erbB-2* status as common a test and as relevant to treatment as ER status.

The extensive conservation of proto-oncogenes across all multicellular organisms points to a crucial biological role for these molecules. Invariably they form part of the signal transduction pathway relaying mitogenic signals from the cell surface to the nucleus. There are probably only three biochemical modes of action of oncogenes: phosphorylation of nascent proteins on tyrosine, serine or threonine residues; abnormal molecular switching by GTPases; and abnormal transcriptional activation by nuclear oncogenes. A classification of oncogenes and their cellular localisation is given in table 1.1. The cellular tyrosine kinases, both transmembrane and cytoplasmic, regulate key events controlling cell growth and morphology, and aberrant activation of these renders the cell mitogen-independent. In the case of membrane-associated tyrosine kinases, mutations that promote ligand independent dimerisation and subsequent kinase activity are seen as representing a general mechanism of activation for this class of oncogene. Similarly, non-receptor tyrosine kinases assume constitutive kinase activity after mutation, which activates downstream signalling molecules. The discovery of *ras*, the archetypal GTPase, and elucidation of its role as a binary switch (McCormick, 1989) was a crucial landmark in signal transduction research. The *ras* family (N- H- and K-*ras*) is one of the commonest class of oncogenes to undergo mutational activation in human cancer. In the GTP-bound state, *ras* activates complex signal transduction cascades including Raf and MAP kinase, which ultimately converge on nuclear oncogenes such as *myc* and *jun*. The nuclear protein family is implicated directly in the activation of gene expression (Lewin, 1991), and the majority have been shown to possess trans-activating activity. Figure 1.1 serves to give an overview of the role proto-oncogenes play in conducting mitogenic signals from cell surface to nucleus.

1.1.2 Tumour Suppressor Genes

One definition of a tumour suppressor gene, which relates historically to their mode of discovery, is any gene that is capable of suppression the malignant phenotype in *in vitro* assays or *in vivo* tumour models. In contradistinction to the oncogenes,

Table 1.1 A classification of oncogene

Growth Factors

FGF5, INT2, HST1, SHH, SIS/PDGFB, WNT1, WNT3

Growth Factor Receptors: Tyrosine Kinases

EPH, ERB-B/EGFR, FMS, KIT, MET, NEU/HER2/ERB-B-2, RET, ROS, SEA, TRK

Non-receptor Tyrosine Kinases

membrane-associated:
SRC, FGR, FYN, HCK, LCK, LYN, TKL, YES

cytoplasmic:
ABL, FPS/FES

Cytoplasmic Regulators of Protein Activity

SH2/SH3 containing adaptors:
CRK, NCK, SHC

guanine nucleotide exchange factors:
DBL, ECT2, LBC, TIAM1, VAV

other:
CBL

Membrane-associated G Proteins

HRAS, KRAS2, NRAS, 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

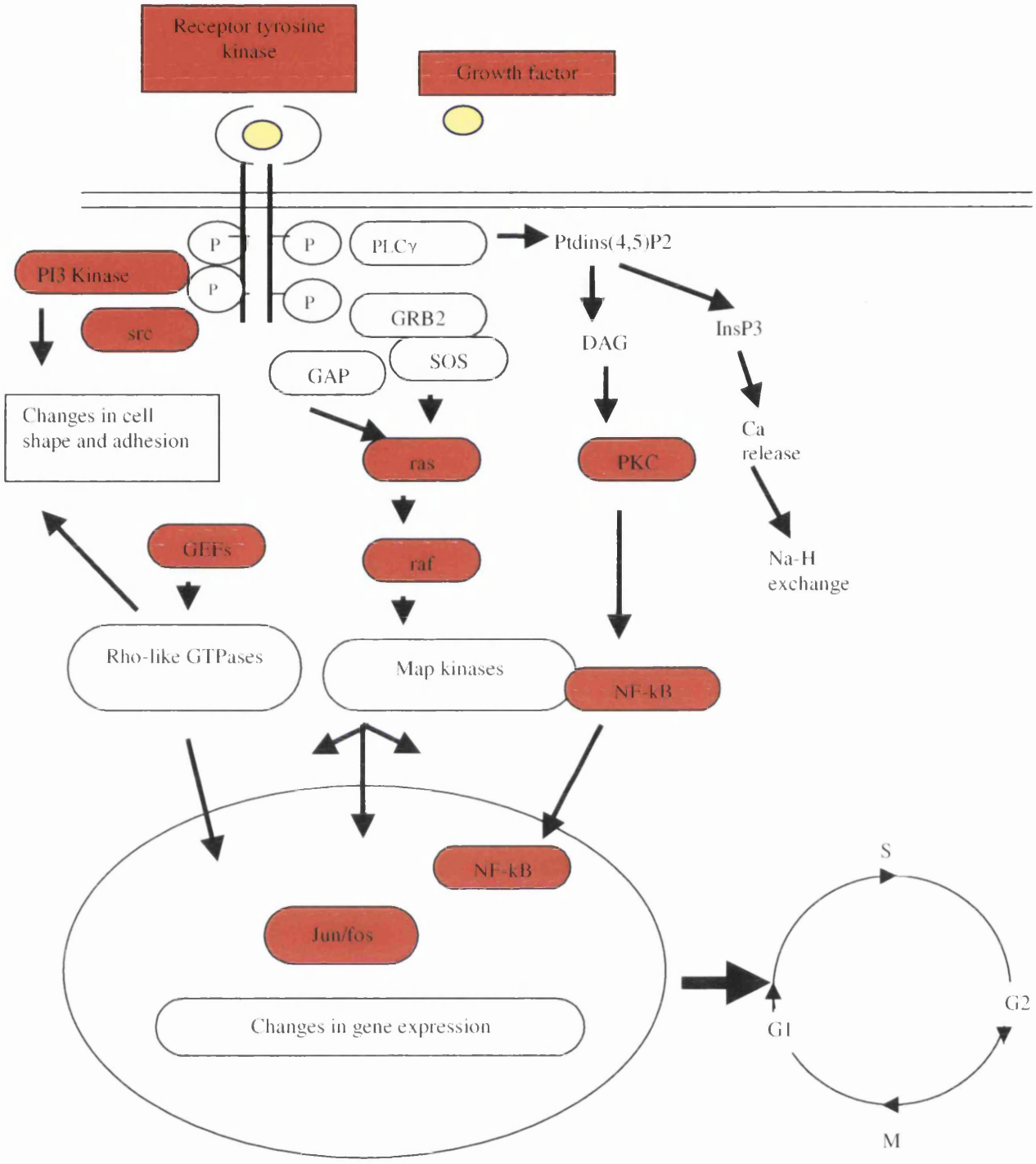


Figure 1.1 Simplified schema representing the flow of information in the mitogenic signalling path from cell surface to nucleus. Activation of receptor tyrosine kinase (RTK) is by binding of growth factor and phosphorylation of kinase domains. Phospholipase C (PLC), GTPase activating proteins (GAP) and *src* directly interact with the RTK, becoming phosphorylated and thus active. Those components of the pathway which are known oncogenes are shown in red. GEF, guanine nucleotide exchange factor; DAG, giacyl glycerol; InsP3, inositol triphosphate.

which act dominantly, TSGs are recessive at the cellular level, requiring deletion of both alleles before contributing to oncogenesis. As a direct consequence of being recessive, their identification has proved more demanding than has been the case with oncogenes since assays to detect non-transformed revertants in the context of proliferating, transformed cells are technically fraught. Nonetheless, about a dozen or so TSGs have now been identified and characterised. Their function is typically as regulators or impeders of cell cycle progression, or as promoters of differentiation.

The story of TSGs begins with observations made by Harris and colleagues (1969, 1988) that growth of murine cancer cells could be suppressed by fusion with non-tumorigenic cells. Such somatic cell hybrids tended to revert to the malignant phenotype over time, an event that coincided with the loss of chromosomes from the cell. It was soon observed that reversion was linked to loss of particular chromosomes, and not chromosome loss in itself. Together these observations supported the view that malignancy was a recessive trait, and that a particular chromosome or a small number of genes might be responsible for the tumour suppression. It required the advent of microcell medicated chromosome transfer techniques to test this hypothesis properly. Using this technique (figure 1.2), introduction of a tagged chromosome (Koi *et al.*, 1989) or subchromosomal region (Sanchez *et al.*, 1996) into a transformed cell can be accomplished.

Almost concurrently with these experiments, Knudson was conducting epidemiological investigations into the childhood cancer retinoblastoma (Knudson, 1971). Although usually a sporadic disease, it was recognised that retinoblastoma occurred in families, and that in these conditions bilateral disease was more prevalent. Such families also tended to develop the malignancy earlier compared to the sporadic form. Based only on a statistical analysis of his observations, Knudson framed one of the most influential ideas in the history of cancer genetics, the 'two hit' hypothesis. Here, he proposed that two events or mutations were necessary to the formation of retinoblastoma. In familial disease, the first mutation was present in

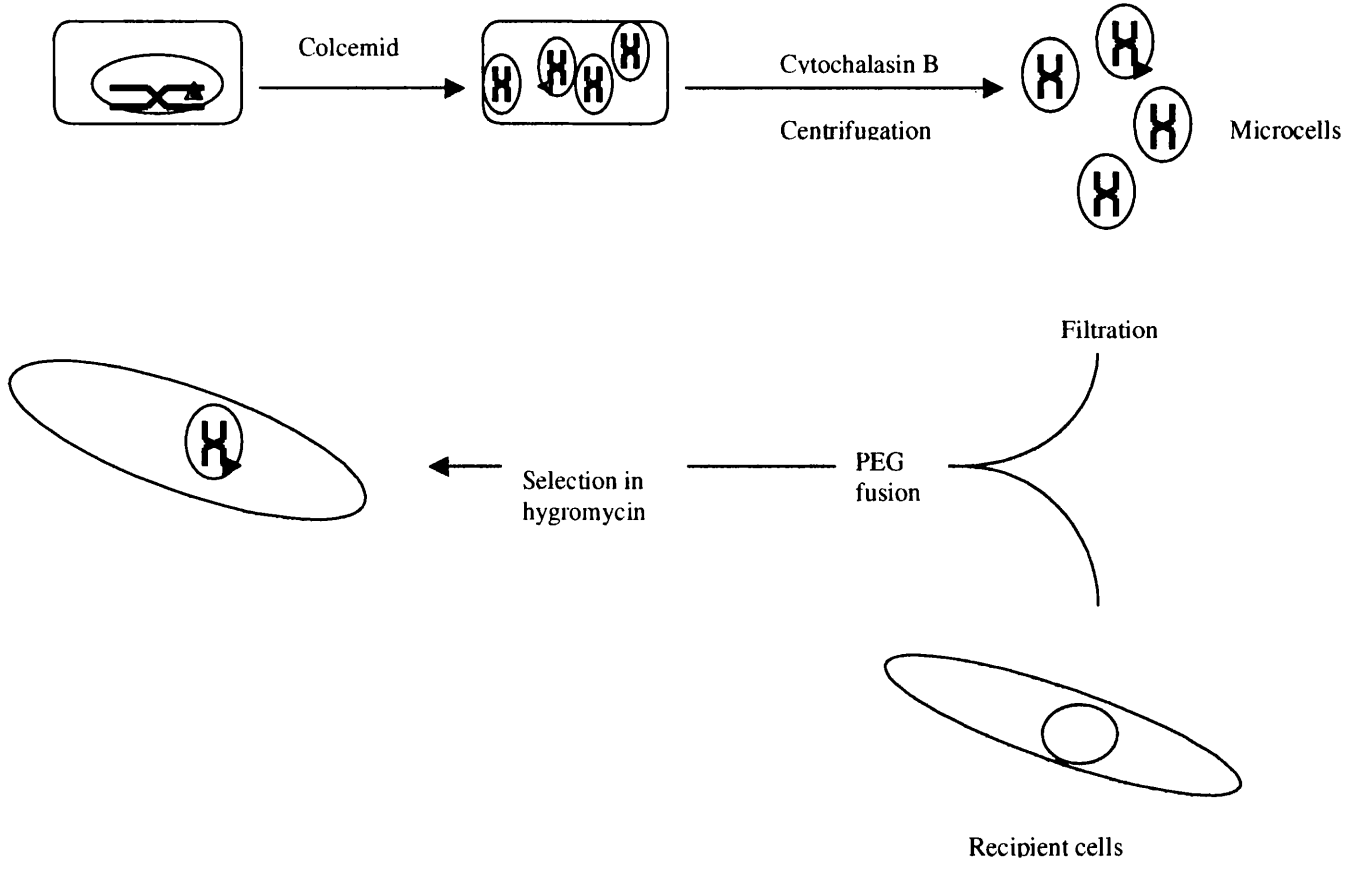


Figure 1.2. Schematic diagram of the steps involved in microcell-mediated chromosomal transfer (MMCT). Prolonged exposure to colchemid arrests the cells in metaphase. Micronuclei are recovered using a combination of cytochalasin and centrifugation. Filtration ensures large microcells are excluded from the PEG-facilitated fusion step. Finally, growth in hygromycin selects for those cells containing the tagged chromosome. The functional consequences of introduction of a single chromosome can therefore be observed. ▲ Represents the chromosomal tag.

the germ line of the patient and therefore in all somatic cells. One inactivating event was not, however, enough to result in disease, only to confer susceptibility. A second event was required and occurred by somatic mutation. Since the occurrence of two mutations is an unlikely event, the chance of sporadic cases developing bilateral disease is correspondingly low. Sporadic cases would also be expected to manifest later as is indeed the case. Experimental confirmation of Knudson's hypothesis came with cytogenetic and molecular studies of retinoblastoma cases (Cavenee *et al.*, 1983, 1985). A proportion of patients harbour a deletion in chromosome 13, providing the first mapping data for the retinoblastoms gene, RB1. The second 'hit' occurs as a microdeletion event, detectable by LOH analysis. A more detailed discussion of the techniques used to map and clone candidate tumour suppression genes follows.

Since its cloning in 1987 (Lee *et al.*, 1987), the retinoblastoma gene and its protein product (pRB) has become the focus of intense research. In many respects, it takes centre stage in the study of tumorigenesis since it functions as a key regulator of cell cycle progression. Arguably, it is the TSG about which most is known. Its importance was first hinted at when it became clear that transforming proteins derived from DNA tumour viruses (including adenovirus E1A and papillomavirus E7) bind to and inactivate Rb (Whyte *et al.*, 1988; Dyson *et al.*, 1989). It has since been suggested that the majority of (or perhaps all) cancer cells possess defects in this pathway, either upstream or downstream of pRB. To understand the importance of pRB it is necessary to understand more fully cell cycle biology.

In normal, physiological conditions, a cell is recruited into the cell cycle only when expansion of cell numbers is required, and this is effected by the balance of mitotic and antimitotic signals converging on the cell cycle regulatory machinery. Traditionally, the cell cycle has been divided into four phases (figure 1.3): S phase, DNA replication or synthesis; M phase, mitosis; and G1 and G2, gap phases when the cell is metabolically active and preparing for DNA replication and mitosis, respectively. Our understanding of the sequence of molecular events derives

primarily from work with yeast. Yeast genetics are such that it is easy to identify genes whose mutation results in impaired progression from one phase of the cell cycle to the next, and the seminal work of Hartwell and colleagues identified a number of genes whose products are obligatory to the progression of the cell cycle (reviewed in Hartwell, 1991). These proteins are known today as the cyclins and cyclin dependant kinases (cdks). Although identified in yeast, these molecules are remarkably well conserved in all eukaryote organisms. In man the situation is more complex, but the principle holds true: activity of certain cdks is specific to certain parts of the cell cycle which themselves require interaction with cyclins before becoming active kinases. It follows that the abundance of cyclins, which were again originally discovered through homology with yeast proteins, is extremely tightly regulated, both at the transcriptional level and by proteolytic degradation. Figure 1.3 demonstrates the pattern of cyclin-cdk activity at different phases of the cell cycle. As can be seen, a crucial watershed from the point of view of the cell is its

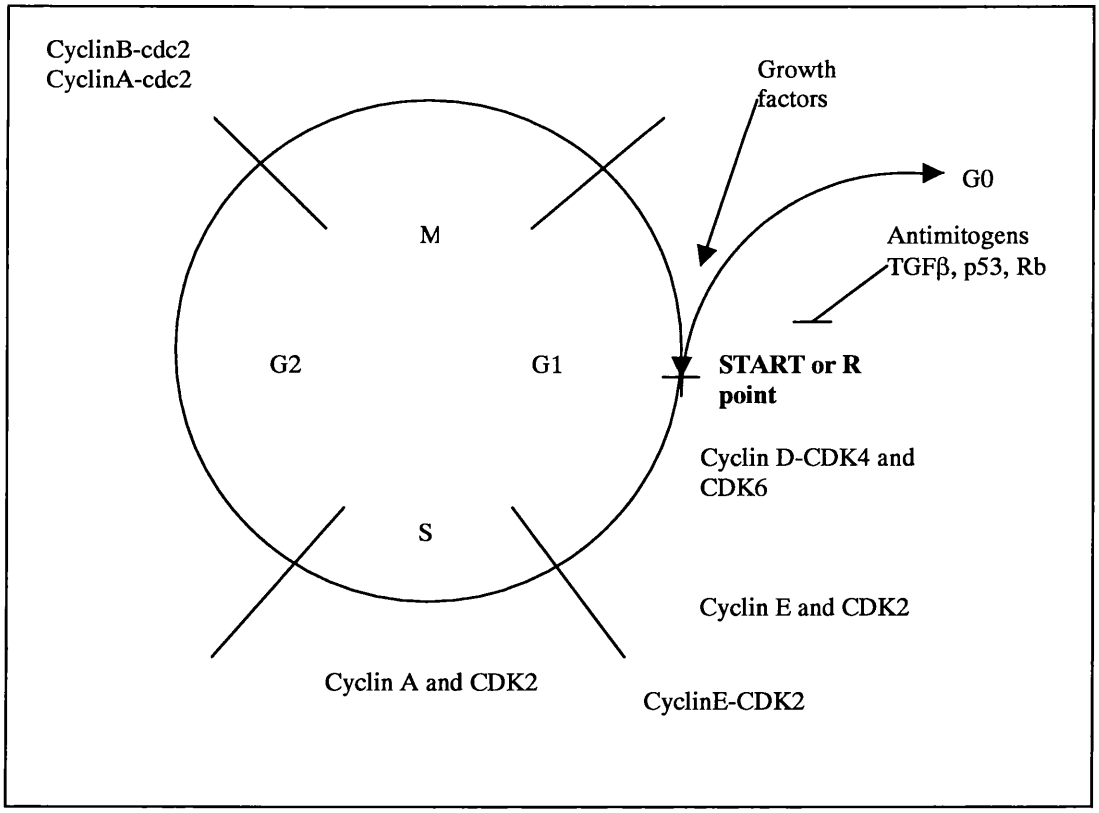


Figure 1.3 A simplified view of the cell cycle. See text for description.

(irreversible) commitment to a round of DNA synthesis and division. This control point is very clearly demarcated in yeast and is termed START (Cross, 1995). Although less purely defined, the corresponding decision for human cells is termed the R, or restriction, point (see Zetterberg *et al.*, 1995 for overview). It should be no surprise that this is the point at which the greatest number of signalling pathways converge and at which the control mechanisms are at their most stringent. The predominant G1 cyclin is the D-type cyclin group, whose specific role has been defined as the chief regulator of Rb. This it does through influencing Rb's phosphorylation status: hypophosphorylated pRb is capable of binding and sequestering large numbers of proteins such as E2F, a crucial transcription factor, whereas in the hyperphsphorylated state, E2F is released and able to function. In support of this, viral oncoproteins target only the hypophosphorylated form of pRB, thus permitting uncontrolled viral replication. Mutation or chromosomal deletion of pRB is moderately common in a range of cancer types, but alterations in upstream regulatory proteins are arguably more so. The cyclinD-cdk4 complex inactivates pRB thereby releasing E2F, and both these genes are mutated in a range of epithelial cancers. p15 and p16 both cdk inhibitors of the INK4 family, are also found to be altered not infrequently in tumour cell lines derived from common cancers such as lung, oesophagus and bladder (Kamb *et al.*, 1994; Nobori *et al.*, 1994). A number of pRB like proteins have now been described (Ewen *et al.*, 1991; Li *et al.*, 1993), and different proteins appear to bind different members of the E2F group of transcription factors. To date, however, no mutations have been identified in these related proteins, implying a distinct physiological role for pRB. Other targets for pRB are known. *c-abl* is ubiquitously expressed and has been shown to interact with pRB (Welch *et al.*, 1993). Its action may well be important in cell cycle regulation.

If pRb is the prototypical TSG, then *p53* has the distinction of being the most commonly mutated gene across the spectrum of human cancers. Originally thought to be an oncogene by virtue of its initial isolation in association with the SV40 large T antigen (Lane and Crawford, 1979), subsequent work showed that the wild type (wt) protein functions as a classical TSG. Reintroduction of wt *p53* into *p53*

deficient cells results in growth inhibition; cells exhibit growth arrest at or near the G1/S phase transition. A further observation, made early in *p53* research, is that exposure of cells to UV light, or other genotoxic agents, leads to accumulation of p53 and delayed cell cycle progression (Maltzman *et al.*, 1984; Kastan *et al.*, 1991). This response is particularly dramatic in response to double stranded DNA breaks, as might be produced by ionising radiation or alkylating agents. The evidence from numerous experiments is compelling that *p53* plays an important part in the cellular response to DNA damage. The current model is that *p53*, when activated, causes the cell to pause in G1 (and G2) while initiation of DNA damage repair takes place, thus maintaining the integrity of the genome (Shimamura and Fisher, 1996). The molecular events whereby *p53* acts have begun to be unraveled. p53 protein itself acts as a transcription factor with a number of downstream targets. Both *GADD45* and *p21* are upregulated in response to *p53* (Kastan *et al.*, 1992; El-Deiry *et al.*, 1994), and both proteins result in growth inhibition. *p21* acts directly to inhibit the G1 associated cdks, and is seen as a crucial downstream target of *p53*. Transfection studies also demonstrate that *p53* can lead to programmed cell death, or apoptosis, which it does through transcriptional control of *bax* and *bcl2* (Miyashita *et al.*, 1995). Although there appears to be a further, *p53*-independent pathway for apoptosis, an appealing proposal which has gained wide currency is that *p53* leads to the apoptotic destruction of cells which cannot be repaired following DNA damage. For these reasons, *p53* has earned the epithet 'guardian of the genome' (Lane, 1992).

To date, the majority of Mendelian cancer dispositions have been found to have as their aetiology a germline mutation in a TSG, with the exception of MEN2 where the *ret* proto-oncogene is altered (Ponder, 1995). This has been a crucial means of discovery of novel TSGs. The converse is, of course, not necessarily true. There may well be many TSGs not associated with heritable germline mutations and consequent cancer family syndromes. In addition to family linkage analysis, and as implied by the two hit model of Knudsen, a search for LOH is a common means of 'fishing' for tumour suppressors. Indeed, there are many chromosomal sites with moderate to high (20%+) levels of LOH where no novel TSG has yet been isolated. One such

site is the long arm of human chromosome 7 (reviewed in Zenklusen and Conti, 1996a) and the syntenic region of mouse, 6A2. It is the linkage method and the occasionally observed cytogenetic changes that have been most fruitful in identifying TSGs: WT1, BRCA1 and 2, APC, NF1, VHL, BLM and others (see table 1.2) were identified in this fashion. There remain several dozen cancer family syndromes where no germline mutation has as yet been discovered. These might in time be expected to yield information on novel TSGs.

Identification of the BRCA genes was the culmination of several years' work by a number of groups around the world, and the results were received with frenzied interest by both the public and scientific community. A family history of the disease is one of the strongest risk factors for breast cancer, implying a genetic component to its aetiology. Around 5% of all cases can be attributed to high penetrance genes and the two known breast cancer susceptibility genes, *BRCA-1* and *-2*, account for 90% of this number (Slattery and Kerber, 1993). As might be predicted from previous work, germline alterations in these genes are associated with an autosomal dominant breast cancer predisposition. *BRCA-1* was cloned following classical linkage analysis between marker D17S174 on chromosome 17 in women from large cancer families involving breast and ovary (Miki *et al.*, 1994). The existence of *BRCA-2* was implied from studies of families with a history of female and male breast cancer where linkage to the D17S174 marker was not seen. Evidence was subsequently forthcoming of linkage to 13q and, in 1995, the *BRCA-2* gene was cloned (Wooster *et al.*, 1995). The two genes have similar lifetime risk for breast cancer (60-85%), but the risk of ovarian cancer is higher in *BRCA-1* (~40%), whereas *BRCA-2* alone carries a risk of male breast cancer (Marcus *et al.*, 1996). Clearly, identification of these genes allows accurate quantification of risk within families with the mutation. The utility of a negative test in women who are at risk of carrying a breast cancer susceptibility gene is much less certain. In addition, the therapeutic options for proven carriers *viz.*, bilateral prophylactic mastectomies or chemoendocrine prevention, are not entirely satisfactory.

Table 1.2 Cancer predisposition syndromes and their genetic basis

Gene	Disease/Syndrome	Cancer Type	Chromosomal location
APC	FAP and Gardiner syndrome	Colon	5q21
AR		Male breast	Xq11-12
ATM	Ataxia telangiectasia	Multiple	11q21
BLM	Bloom's syndrome	Multiple	15q26
BRCA1		Breast and ovary	17q21
BRCA2		Breast	13q13
CDK4		Melanoma	12q13
CDKN2A/MTS2		Melanoma	9p21
DPC4	JPS	Colon	18q21.1
E-CADHERIN		Stomach	16q22
ER		Breast	6q23
IGFII	Beckwith-Wiedemann syndrome	Multiple	11p15
hMLH1	HNPCC	Colon	3p21
hMSH2	HNPCC	Colon	2p16
hPMS1	HNPCC	Colon	2q31
hPMS2	HNPCC	Colon	7p22
$p57^{KIP2}$	Beckwith-Wiedemann syndrome	Multiple	11p15
LKB1	PJS	Multiple hamartomata	19p13
MEN1	MEN type 1	Multiple endocrine	11q13
MET	HPRC	Renal	7q31
NF1	Neurofibromatosis type1 von Recklinghausen's disease	Neural crest	17q11
NF2	Neurofibromatosis type 2	Schwannoma	22q12
PTCH	Gorlin's syndrome/ BCNS	Skin	9q22
PTEN/MMAC1	BZS, CD, JPS, LDD	Multiple hamartomata	10q23
RB1		Retinoblastoma	13q14
RET	MEN type 2A and 2B	Multiple endocrine	10q11
TSC1	Tuberous sclerosis	Multiple	9q34
TSC2	Tuberous sclerosis	Multiple	16p13
TP53	Li-Fraumeni syndrome	Multiple	17p13
VHL	von Hippel-Lindau disease	Renal	3p25
WRN	Werner's syndrome	Multiple	8p11.1-21.1
WT1	Wilms' tumour/Denys-Drash syndrome/WAGR	Renal	11p13
XPA-G	Xeroderma Pigmentosum	Skin	

The DNA mismatch repair genes, germline mutations in which are responsible for the clinical syndrome of HNPCC, conform to the paradigm of other TSGs, yet nevertheless are felt to represent a distinct class or subclass of cancer genes. Observation of microsatellite instability in a proportion of bowel cancers (both inherited and sporadic) eventually resulted in the discovery of four genes that function to repair replication errors in DNA which are missed by the exonuclease activity of DNA polymerase (Liu *et al.*, 1995, 1996). Such a replication error (RER) positive phenotype is relevant to the treatment of these tumours: they appear intrinsically resistant to *cis*-platinum, and indeed treatment with alkylators may promote DNA instability. The concept of a hypermutable phenotype was first proposed by Loeb (Loeb, 1991), who felt that such a mechanism was required to account for the high mutation rate seen in cancer cells. One can imagine the result as being the acceleration of the whole process of tumorigenesis with fast emerging novel clones being selected on the basis of a growth advantage. This notion has both strengthened and refined the overarching hypothesis of cancer as a genetic disease of cells that requires a number of cumulative mutations before true invasion and metastasis is seen.

There are four other important genes whose disruption results in the Mutator phenotype. These are ATM, XPA, BLM and WRN. Each is associated with and was indeed discovered through the study of cancer disposing syndromes. Ataxia telangectasia (A-T), so named from two of the several conspicuous clinical signs observed in sufferers of the condition, confers a lifetime risk of cancer of around 40%, commonly leukaemia and lymphoma (Morell *et al.*, 1986). Cells from A-T patients examined microscopically are phenotypically abnormal, exhibiting macronucleus and demonstrating marked radiosensitivity. Indeed, for several years radiotherapists have known that exposing A-T patients to conventional doses of radiation has serious sequelae, seen only in normal subjects after very high doses. Cytogenetically, lymphocytes from homozygous mutant A-T patients show chromosomal abberations such as translocations and inversions (Kojis *et al.*, 1992) whose numbers increase as a consequence of irradiation. A provocative observation

is that these cells have defective DNA damage checkpoints, and lack a rise in p53 after irradiation, suggesting a role for the ATM protein in DNA damage response upstream of p53.

Whereas in some ways radiation sensitivity in A-T subjects is a laboratory artefact, since most will not be exposed to ionising radiation, exquisite photosensitivity is an integral part of the lives of xeroderma pigmentosum (XP) patients. XP is the prototype DNA repair defect syndrome. It results from defects in the nucleotide excision repair (NER) pathway which is only now becoming unravelled and understood. XP in fact comprises a group of genetic disorders (de Weerd-Kastelein *et al.*, 1972), and all seven genes have now been cloned (termed XPA-G). Two of these, XP B and D, encode DNA helicases. The genes BLM and WRN, defects in which cause the clinical syndromes of Bloom and Werner respectively, likewise encode helicases, although their physiological role is presently unclear. Certainly, these syndromes have cells which characteristically show genetic instability.

The discovery of genes which, though involved in cancer, do not themselves result directly in the transformed phenotype, has led to the proposal of the 'caretaker vs gatekeeper' hypothesis (Kinzler and Vogelstein, 1997). As elaborated by Kinzler this posits a caretaker class of TSGs whose role it is to maintain the integrity and stability of the genome and whose disruption leads to the mutator phenotype. Clearly, ATM, and the genes described above would belong to this group. On the other hand, gatekeepers are those genes which directly act to inhibit growth or promote cell death, and whose identity varies from tissue to tissue *e.g.* VHL in kidney cancer and APC in colon cancer. In some instances (such as the *BRCA* genes) assignment to one or other group is, on the basis of current evidence, not clearcut. Nevertheless, the description serves as a useful conceptual framework to facilitate understanding.

1.2 Animal Models in the Study of Cancer

The use of model organisms in the study of human disease is well established. Many disease phenotypes (both spontaneous and artificially produced) have been described in a wide variety of species, including fruitflies, zebrafish, pigs, rabbits, rodents and primates. Although studies in fruit flies and nematodes have yielded valuable and widely relevant information on developmental biology, the general utility of such evolutionary distant organisms to understanding human disease entities is limited. Mammalian models are clearly preferable by virtue of a more closely related physiology, and primates might be expected to fit the bill best of all. However, for reasons of expense, fecundity and significant ethical concerns over primate experimentation, the animal model *par excellence* has been and remains the humble housemouse, *Mus musculus*.

The *mus* genus has much to commend it as a model organism: ease of breeding, short lifespan and large numbers of offspring all facilitate laboratory handling and experimentation. Thanks to experience stretching back over several decades, mouse genetics are extremely well understood and, crucial to the mapping of novel genes, there exists a dense map of the mouse genome (Dietrich *et al.*, 1996). Of particular importance, large stretches of chromosomes show conserved gene and marker linkage (synteny) with human and other mammalian genomes. This is relevant since detailed mapping of mouse should aid investigation of homologous genes in humans. A high degree of conservation is often also evident at coding regions between mouse and man. Theoretically then, DNA probes and primers developed in one species would be expected to be useful and applicable to the other, and this is indeed true in practice.

Mice are beloved by geneticists because of the ease with which complex breeding protocols can be set up (table 1.3 and figure 1.4). This has been crucial to the placing of mutant phenotypes and clones on the mouse genetic map. Classically, mouse

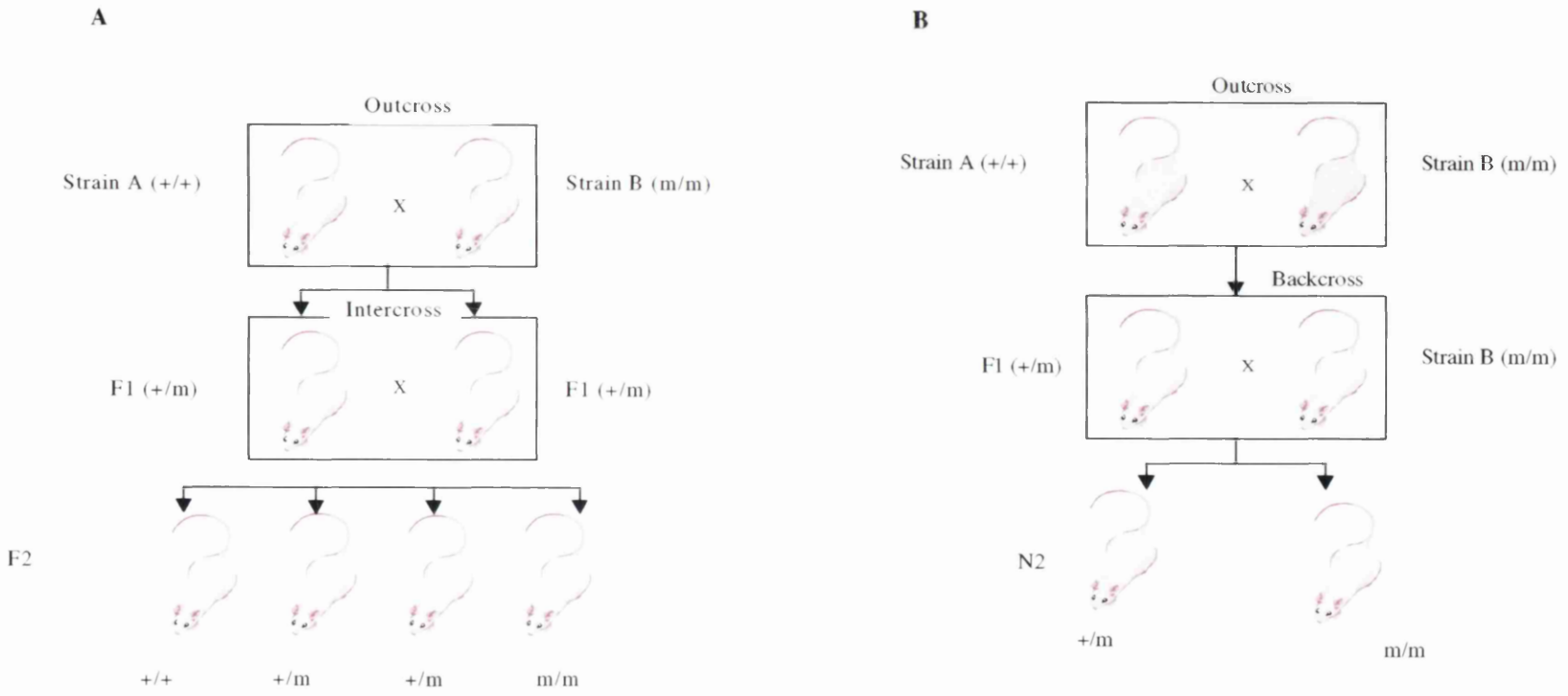


Figure 1.4. Two classic breeding schemes commonly used in mouse genetics. In both cases, two highly characterised inbred strains are crossed to each other. In the case of the outcross-intercross scheme, the F1 progeny are bred to each other . This is the scheme used in generation of recombinant inbred (RI) strains (see Silver, 1995 for comprehensive overview). In B, after outcrossing, an F1 generation mouse is crossed (backcrossed) to one of the parents. Note that these breeding protocols are used to generate linkage data. In the case of LOH studies, first generation (F1) mice are all that are required.

mapping had been done one locus at a time with the introduction of a mutation into a strain with another phenotypic marker, followed by breeding experiments to determine linkage. More recently, mapping panels have been employed. These are sets of DNA from mice with randomly recombined chromosomes produced by specific breeding protocols. Classic examples of such protocols are the interspecific backcross and recombinant inbred strains (Figure 1.4).

Table 1.3 Categories of Genetic Cross

	Types of Mating	Offspring Genotype	Uses
Outcross	A/A x a/a	A/a	Production of F1 hybrids
	a1a2 x a3a4	a1a3, a1a4, a2a3, a2a4	
Backcross	A/a x a/a	A/a, A/A	Linkage analysis
	A/a x A/A	A/a, a/a	
Incross	A/A x A/A	A/A	Maintenance of inbred strain
	a/a x a/a	A/a	
Intercross	A/a x A/a	A/a, A/A, a/a	Linkage analysis

The discovery of the microsatellite marker was hailed by geneticists as the advent of the magic bullet. These simple repeat sequences, consisting of di-, tri- or tetra-repeats, were discovered to be present at high density throughout mammalian genomes and, crucially, had unusually high polymorphic content. They therefore had distinct advantages over the basepair substitution type of polymorphism, as exemplified by restriction fragment length polymorphisms (RFLP) and, combined with the ease with which they could be assayed using PCR, quickly became the genetic tool of choice for mapping. Figure 1.5 illustrates the length polymorphism of microsatellites.

Interspecific crosses (crossbreeding between *M. musculus* and wild mouse strains) have led to major advances in mouse genetics (Bonhomme *et al.*, 1979; Copeland and Jenkins, 1991). Whereas use of intraspecies crosses between different musculus strains is hindered by relatively little genetic difference, interspecific F1 mice are 'superheterozygous' at virtually every marker. Aside from its implications for genetic mapping, this permits allelotyping to be performed and identification of loss of heterozygosity (Figure 1.5). Using simple microsatellite length polymorphisms

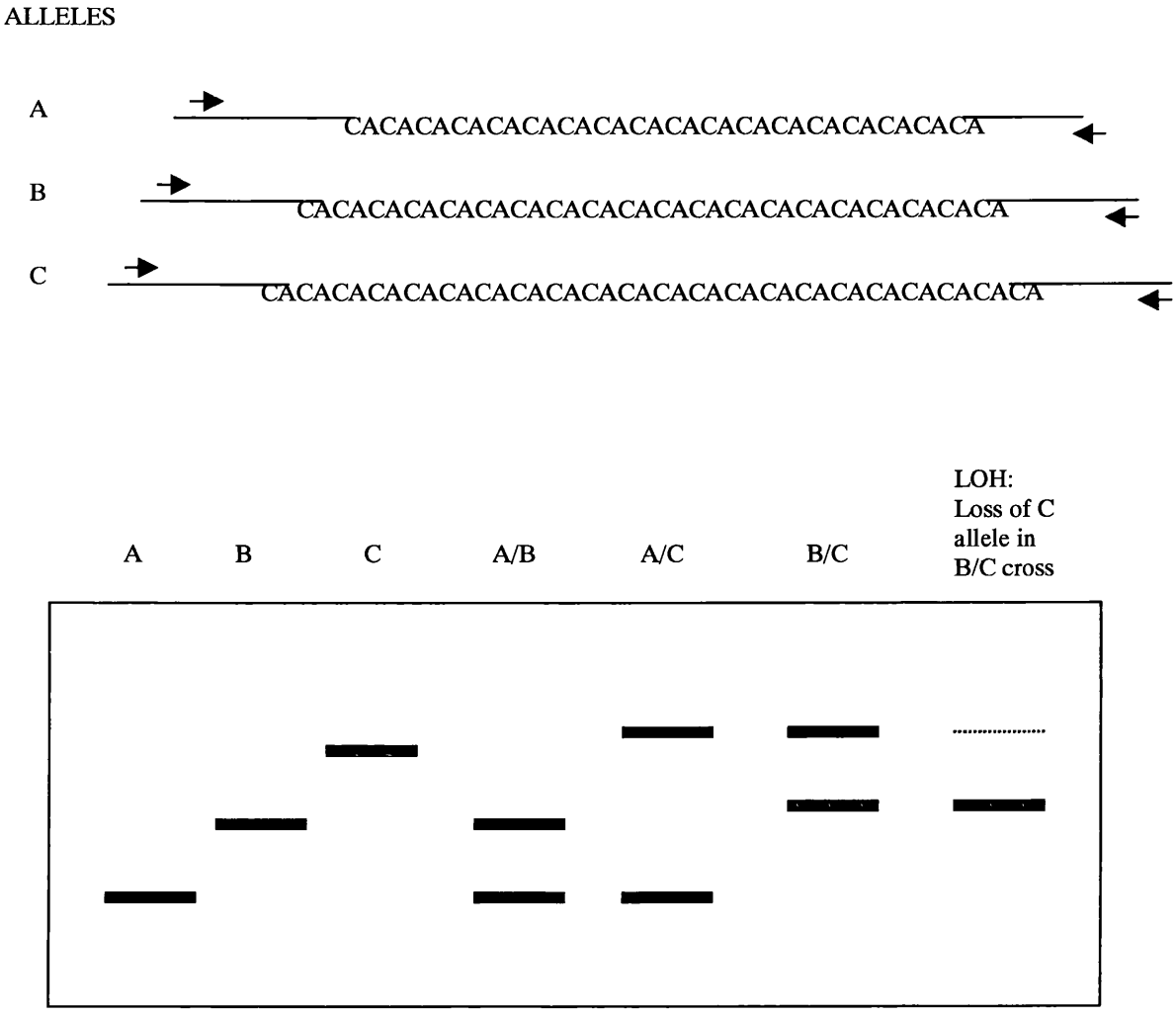


Figure 1.5. Depiction of microsatellite repeats of the $(CA)_n$ variety used as polymorphic markers in LOH studies. The polymorphism (e.g. between *M. musculus* strains or A, B and C in figure) resides in the number of CA repeats and therefore their length as assayed by PCR. Arrows represent PCR primers used in the amplification. Provided a mouse is heterozygous at a particular locus (*M. musculus x M. spretus* crosses virtually always are), LOH will be seen as an absence of a band on gel resolution (far right example).

assayed by PCR, parental alleles can be separately identified and followed. This is particularly applicable to allelotyping of chemically induced tumours in interspecific F1 mice, and is described below.

Most of the phenotypic variation between individuals is not of the 'all or none' variety that characterises Mendelian traits, but rather exists over a continuum. It is these traits which are of most interest to geneticists, and examples would include height, hair colour, longevity and, *apropos* cancer, tumour susceptibility. Although cancer families exhibit germline mutations in tumour suppressor genes which are transmitted in a classic Mendelian fashion, this is rare and accounts only for a small minority of human cancers. The likelihood is that cancer susceptibility is a polygenic phenomenon resulting from the combined effect of multiple low penetrance genes. The role of such genes in the development of cancer is arguably best studied in the mouse where a number of inbred strains show markedly differing susceptibility to cancer development, both spontaneous and induced. The use of mouse selective breeding protocols in combination with modern statistical techniques that allow modeling of quantitative trait loci has already resulted in the identification and mapping of a number of mouse tumour modifier loci, influencing tumour size, growth rate and other characteristics (Nagase *et al.*, 1995).

The mouse as model system has found renewed favour with the advent of germline manipulation technology. Pioneered in the 1980s, the technique of zygote pronuclear injection permitted the stable introduction of any gene into the germline of mice. ES cell technology has refined the technique further, permitting specific mutations to be engineered (Capecci, 1989; Melton, 1994). These experiments were crucial to exploring the role of oncogenes in carcinogenesis and in examining consequences of tissue specific oncogene expression. They also provided compelling evidence for cooperation between oncogenes in tumorigenesis by observing the offspring of crosses between two transgenic mice. For example, a synergistic action was observes between H-*ras* and *myc* when transgenes under the control of the MMTV promotor were expressed in breast epithelia (Sinn *et al.*,

1987). Application of the technique is clearly not confined to cancer research and has found particular use
commercially in generation of 'bioreactors' (Moffat, 1991) which produce large quantities of valuable proteins such as factor VIII.

Table 1.4 Knockout mice and their associated phenotype

Gene	Function	Tumour phenotype
Rb	Cell cycle regulator	Pituitary adenocarcinonias, MTC, phaeochromocytoma
p53	Transcription factor, 'guardian of the genome'	Lymphoma, sarcoma
Wt-1	Transcription factor	None
APc	Binds fl-catenin	Intestinal polyps
Nf-1	ras-GAP activity	Phaeochromocytoma, myeloid leukaemia
Nf-2	ERM cytoskeletal regulator	Sarcoma
Ink4a	Encodes p16 and $p19^{Arf}$	Lymphoma, sarcoma
p19	Binds mdm2, induces p53	Lymphoma, sarcoma
Brca1	Binds Brca-2 and RadSI; DNA repair?	None
Brca2	Binds Brca-1 and RadSI; DNA repair?	None
Smad4	TGF-β signal transducer	None
Dcc	Netrin-1 signal transducer	None
Msh2	Mismatch repair	Lymphoma, colon and skin carcinomas
Msh6	Mismatch repair	Lymphoma, intestinal adenocarcinoma
Pten	Lipid phosphatase	Thyroid and prostate hyperplasia, lymphoma

Investigation into the function and actions of tumour suppressor genes requires the ability to disrupt (knock out) both functioning copies of a gene. Such gene targeting is now routinely performed and the resultant knock-out mice are invaluable tools in the study of TSGs. Indeed these mice may serve as models for human cancer predisposition syndromes (table 1.4). One of the first was the *Rb* knockout mouse (Jacks *et al.*, 1992; Clark *et al.*, 1992). Interestingly, loss of *Rb* does not in the mouse result in retinoblastoma; these mice die of pituitary adenocarcinomas, medullary thyroid carcinaoma and phaeochromocytomas without any evidence of retinal pathology (Harlow, 1992). One suggestion, supported by the observation that transgenic expression of SV40 T antigen induces retinoblastomas, is that Rb related proteins, p107 and p130, require to be inactivated. Rb -/+; p107 -/- chimaeric mice

do indeed develop retinoblastoma (Robanus-Maandag, 1998), but tumours seem to arise from a different cell layer to those related to T antigen overexpression. As can be seen from the *Brca-1* and *–2* knockouts, this technology does have its limitations: no detectable tumour phenotype can be correlated with knockouts in these genes.

1.2.1 The Mouse Carcinogenesis Model

The observation over fifty years ago that skin cancers in mice can be induced consistently and reproducibly by topical application of a number of organic chemicals paved the way for the development of a valuable and powerful tool in the field of cancer research. Although not the only animal chemical carcinogenesis system available (table 1.5), for reasons of ease of application and observation, the mouse skin two stage carcinogenesis model has become one of the most widely used and best understood. Indeed, much of the early evidence in support of the multistage development of cancer came from observations in this system (Peraino *et al.*, 1975; Becker, 1975; Burns *et al.*, 1983).

Table 1.5 Animal chemical carcinogenesis models of human cancer

Target	Species	Agents	Tumour Type
Skin	Mouse, rat	Nitrosamines, alkylating agents, aromatic amines, PAH	Squamous cell cancer, basal cell cancer
Liver	Hamster, guinea pig	DMBA	Melanoma
Lung	Mouse, rat, hamster, dog	Nitrosamines, asbestos, PAH	Adeno- and squamous cell carcinoma, mesothelioma
Breast	Mouse, cat, dog	DMBA, aromatic amines, NMU	Adenocarcinoma
Colon	Mouse, rat	Nitrosamines	Adenocarcinoma
Pancreas	Rat, hamster	Nitrosamines	Adenocarcinoma
Bladder	Mouse, rat, hamster	Nitrosamines, aromatic amines	Urothelial cancer

Observations made using mouse and other systems allow the carcinogenesis process to be divided into three operational steps: initiation, promotion and progression (Yuspa and Dludosz, 1991). A single application of an initiator results in an irreversible, heritable change in the cells which is not phenotypically evident.

Multiple applications of a promoter will produce numerous benign (but preneoplastic) papillomas, 5-10% of which will spontaneous progress to carcinomas. Both squamous carcinomas and the more aggressive and less well differentiated spindle cell carcinomas are formed. The genetic events underlying each of these steps have begun to be unraveled.

1.2.1.1 The initiation process

That most potent initiators are mutagens, that the process is to some degree dose-dependent and that the initiated state persists for some time (and is often irreversible), all points to a genetic basis for initiation. The classic approach to experimental induction of skin tumours was painting with coal tar but this has now been superceded by application of the pure chemical carcinogen. Presently, commonly used compounds in the process of initiation are 7,12-dimethylbenzanthracene (DMBA) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). Although their use results in no immediately obvious phenotypic change in the context of intact skin, initiated keratinocytes *in vitro* display an altered response to signals for terminal differentiation (Yuspa and Morgan, 1981). This fact has allowed isolation and detailed molecular analysis of initiated cells in culture, and has led to some remarkable observations. It became apparent that over 90% of DMBA initiated cells from papillomas carried an identical A-T transversion in codon 61 of H-*ras* and that the same mutation was present in pretumorous but initiated cells (Quintanilla *et al.*, 1986). It is of particular note that many spontaneous human skin tumours, both benign and malignant, exhibit the same mutation. Proof that this mutation was causal came from crucial observations made by Balmain and colleagues, who found that introduction of mutant *ras* could substitute for chemical initiation (Balmain and Pragnell, 1983).

1.2.1.2 Promotion

In contrast to initiation, the process of promotion is reversible and requires repeated application to be effective. In addition, most tumour promoters are not detectably genotoxic. This would suggest that the process is an epigenetic phenomenon. Tumour promotors can be identified by their ability to reduce the latency period to the development of preneoplastic and neoplastic lesions after application of initiators. They do not require metabolic activation and are to some degree tissue specific (table 1.6). In general terms most promoters act to induce cellular proliferation (Burns *et al*, 1983); indeed 'wounding' has been known for some time to take the place of a promoting agent. The explanation advanced to account for their general mode of action, now widely accepted, is that they cause selective clonal expansion of an initiated cell population which in turn provides an increased number of cells that are targets for further changes in the neoplastic process. The phorbol ester TPA is one of the most potent promoters known, and is thought to exert its effects on cell proliferation partly by interacting with Protein kinase C (PKC) (Castagna *et al.*, 1982). In normal cells, exposure to TPA results in accelerated differentiation whereas initiated cells are resistant to PKC mediated differentiation. It seems likely that by allowing continued proliferation, further genetic events are permitted to take place.

Table 1.6 Site specificity of promoting agent

Initiation	Promotion	Tissue Site
BHBN	Phenobarbital	Liver
	Saccharin	Bladder
NMU	Phenobarbital	Liver, thyroid
	Saccharin	Bladder
4-Acetylaminostilbene	-	Sebaceous glands
	Phenobarbital	Liver
	DDT	Liver
	DES	Liver, breast

Other molecular events are clearly involved at this stage. Tumour promoters are recognised as causing aneuploidy. Trisomies of chromosomes 6 and 7 are a common observation in papillomas and carcinomas (Aldaz *et al.*, 1989), implying

that they harbour genes important to the neoplastic process. Trisomy of chromosome 7 is thought to duplicate the mutant *ras* allele, conferring selective advantage to an initiated cell. Loss of all or part of chromosome 6 has also been seen. The gene(s) involved here have however yet to be isolated.

1.2.1.3 Malignant Progression and the Squamous-Spindle Transition

Papillomas that appear following initiation and promotion may be divided into high risk and low risk groups on the basis of the likelihood of progression to malignant tumours. Papillomas of the high risk class tend to erupt early, grow to a large size and fail to regress following withdrawal of promoter (Hennings *et al.*, 1990a). Phenotypic markers can also help distinguish them: they express cytokeratin K1 rather than the K13 antigen commoner on low risk tumours (Nischt *et al.*, 1988), and they lack TGFβ1 and 2 (Glick *et al.*, 1993). The TGFβ group of growth factors interacts with the cognate receptor thereby inhibiting growth in normal keratinocytes and other epithelial systems, including colon. Although the genetic basis for these different characteristics is not known, that this stage of progression is genetically controlled is suggested by the ability to produce a higher rate of malignant progression with the application of carcinogens. In the future these genetic differences may be identified by novel molecular techniques, such as genomic array comparative genome hybridisation (CGH) (Kashiwagi and Uchida, 2000) or SNP (single nucleotide polymorphism) analysis (Lai, 2001).

Spindle cell carcinomas, so-called from their fibroblast like morphology, are poorly differentiated tumours thought to arise from squamous cells. They invariably lack a range of cell surface differentiated markers, such as integrins and cell adhesion molecules. The genetic basis for the transition again remains to be elucidated, but a high proportion of spindle cell lines have undergone homozygous deletion at the *p16/CDKN2A* locus. It has been suggested that metastasis may occur *via* a spindle cell intermediate, though firm evidence for this is lacking.

1.3 Multistep Carcinogenesis

The question arises as to whether inferences can be made about human epithelial cancer from data derived from the mouse skin chemical carcinogenesis system. Thankfully all the indications are that they can: the range of genes altered in the mouse skin system is the same as is found in spontaneously occurring human cancers; treatment with carcinogens, rather than forcing cells to transform, merely accelerates changes that take place spontaneously, albeit at a much slower rate; and, similar sequential genetic changes are observed in cancers of other epithelia, particularly the urogenital, digestive and aerorespiratory tracts.

The three commonest genes altered in the mouse skin system are H-*ras*, *p53* and *p16*. As discussed above, mutation of H-*ras* is seen as an early, initiating event in skin tumorigenesis. *p53*, residing on mouse chromosome 11, appears to be altered at a later stage of tumour development, often in high grade squamous cancers. Interestingly, *p53* mutation may be more common in the smaller percentage of squamous carcinomas that retain TGFβ expression, perhaps conferring resistance to this growth inhibitor. In any case, *p53* alterations appear to occur as a late event. Likewise, *p16*, which appears homozygously deleted in a high proportion of spindle cell carcinomas, is altered as a late event.

Useful parallels can be drawn between the mouse skin model and the human multistep process, as paradigmed in the genetic model of colon cancer. The acquisition of sequential mutations in the dysplasia-benign papilloma-invasive carcinoma procession, first described and elaborated by Kinzler and Vogelstein (1996), is the best known and most detailed carcinogenesis model in man. The theory serves to unify much of what is known about oncogenes, tumour supressor genes and mutator genes. The initiating event is a mutation in the APC gene which is seen as a gatekeeper to subsequent events. The obvious parallel in the mouse system would be H-*ras*. The *ras* family of oncogenes seems also to be involved in human colon cancer, but at a later stage, with 50% of large adenomas possessing

mutations in N- or K-*ras*. Interestingly, these changes are also seen occasionally in hyperplastic polyps and collections of normal cells. This serves to reinforce the notion that the critical event is an alteration in APC and that cooperation between genes is crucial: in the presence of an APC mutation, *ras* contributes to transformation, which can be reversed by reintroduction of normal *ras* gene. *p53* is involved in human colon cancer as a late event, in common with the changes in mouse skin. A schematic of these changes, highlighting similarities, is shown in figure 1.6.

1.4 Gene Cloning

The possession of biochemical data, or even purified protein from which sequence information can be inferred, is not usually a luxury enjoyed when attempting to identify a cancer-associated gene. The typical starting point is an indication of the chromosomal or perhaps subchromosomal location of the gene. This can be derived from the discovery of cytogenetically visible chromosome abnormalities in individuals with the disease, or by formal (and laborious) linkage analysis. Linkage is a function of genetic recombination at meiosis, and simply implies that two DNA sequences are inherited together because of physical proximity. Linkage data as generated by computers are expressed as LOD scores (logarithm of the odds of linkage, Z). These methods of generating positional data apply to any disease-related gene. The technique of LOH, and the related homozygous deletion, is specific to TSGs and is particularly relevant to the study of sporadic cancers. Invoking once again the 'two hit' model, the first hit is commonly a mutation or other relatively subtle change. The hit on the second allele, however, is often much less discreet and may involve loss of a whole chromosome, or a large deletion (as might be detected cytogenetically). Cavanee and colleagues (1986) gave a comprehensive description of the means whereby this inactivating event might occur (figure 1.7). Closely linked genetic markers would also be expected to be lost in this deletion and, if the patient is heterozygous for a marker at a particular locus, the event can be observed

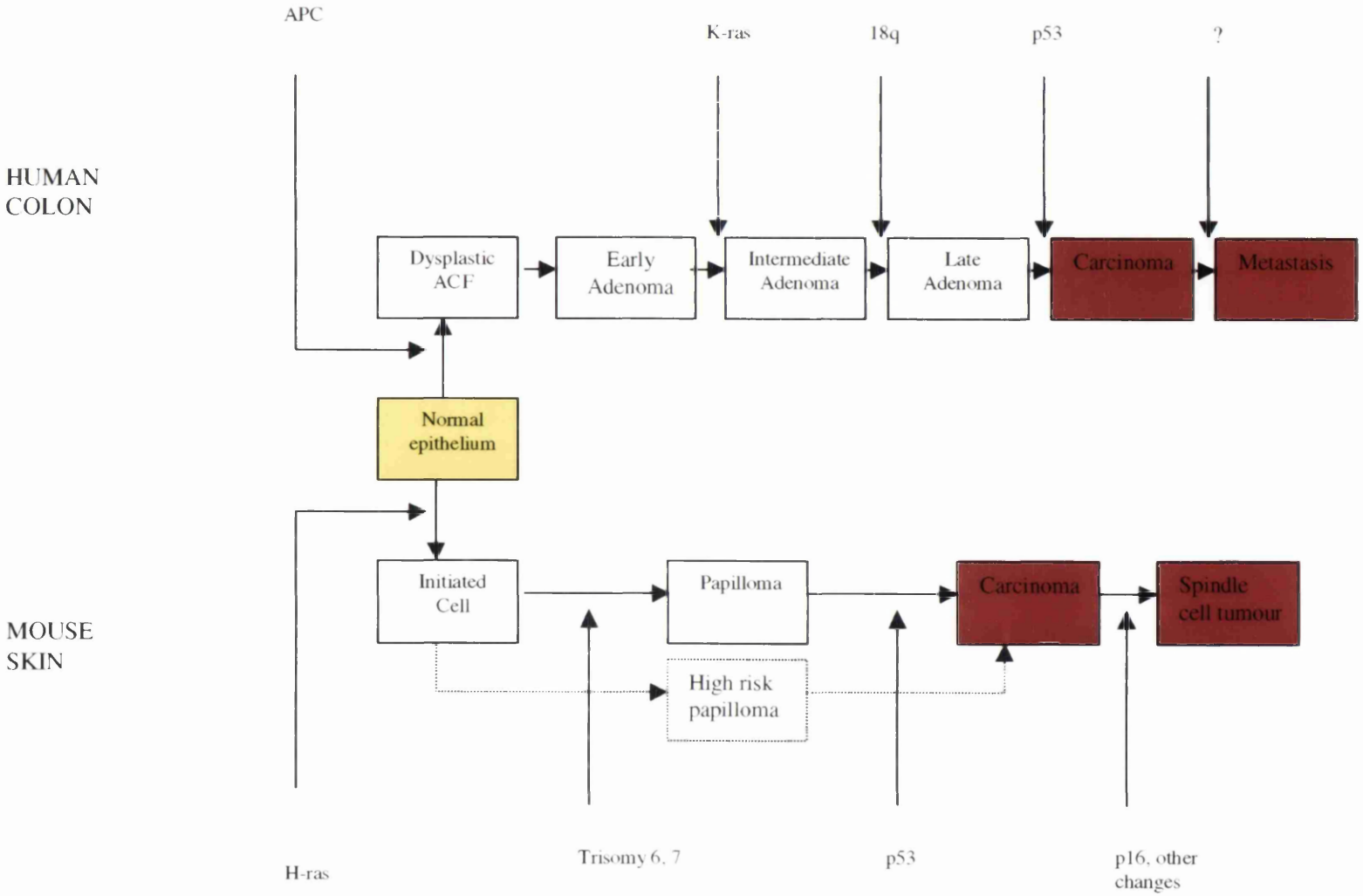


Figure 1.6 Stepwise genetic changes observed in human colorectal cancer and mouse skin. See text for description. ACF, aberrant crypt foci.

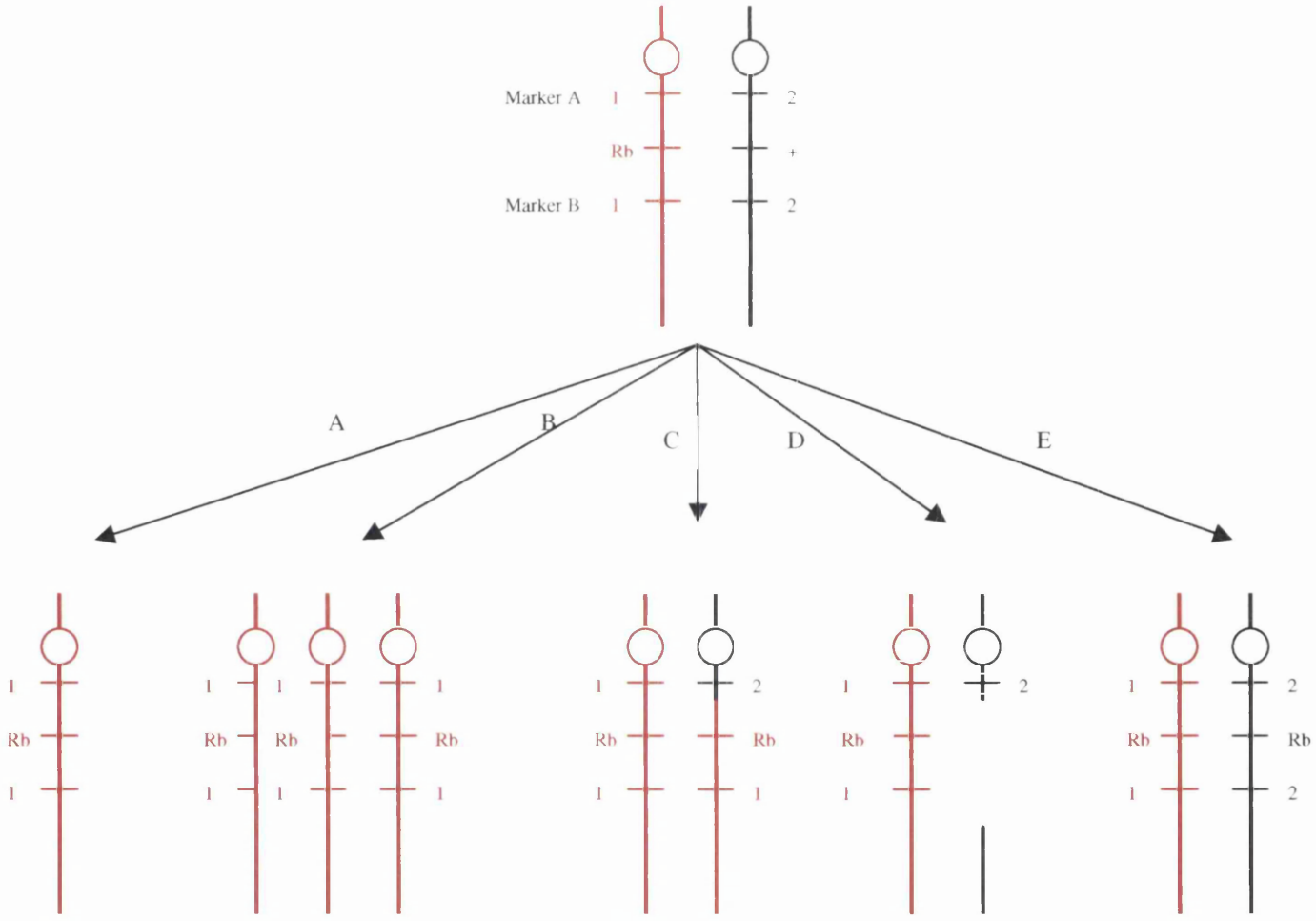


Figure 1.7. Diagram depicting the various molecular mechanisms whereby a wild-type can be lost. In the example, the subject is heterozygous for a mutant retinoblastoma gene (red type). A, mitotic nondisjunction. B, loss of chromosome as in A, followed by reduplication. C, mitotic recombination at a site proximal to the mutant Rb locus. D, loss of wild type allele by deletion. E, point mutation in wild type allele. All of these mechanisms result in loss of adjacent markers and can be detected as loss of heterozygosity, apart from in E, where no loss of genetic information has occurred. After Cavenee *et al.*, 1986

as LOH. Following markers for which a patient is homozygous is of course noninformative. Employing highly polymorphic markers such as microsatellites greatly facilitates the search for LOH since most patients will be heterozygous for any given marker chosen. As already described, interspecific crosses between mice results in a 'superheterozygous' state in the offspring, making LOH analysis simple to perform in F1 progeny.

The use of positional data to identify a gene is known as positional cloning. Often, and particularly in the context of the human genome project, genes are known to exist in the area of interest, and these candidates can then be investigated for evidence of disease association. It is expected that on completion of the human genome project, the positional candidate gene approach to identifying human cancer (and other) genes will be the most efficient and therefore the preferred method.

Generation of high resolution mapping data for the gene of interest is commonly the rate limiting step in the process. A *sine qua non* of positional cloning is possession of a large number of polymorphic markers which can be used to generate such a map. Clones are required which span the region of interest, from which physical maps can be derived. If contigs (contiguous clones) have not already been generated, they require to be constructed - a formidable and time-consuming task. Again, due mainly to human genome project, much of this work has now been performed: large insert clones such as YACs or PACs exist for much of the human genome which can be subcloned into smaller, more manageable fragments *e.g.* cosmids for further analysis. Sequence data in public databases may also point to the presence of genes within the cloned region, facilitating the positional candidate gene approach.

Once achieved, however, the candidate genes so identified must be rigorously examined for evidence of involvement in the disease. Good circumstantial evidence of involvement might be expression of the gene in the tissue of interest. For example, failure to find corresponding mRNA in breast in a candidate breast cancer gene would not bode well. A more specific means is to screen for mutational

alteration of the gene in the cancer of interest from a number of affected cases. This can be done rapidly and with acceptable sensitivity by the technique of single strand conformational polymorphism (SSCP) which aims to identify any difference from the normal sequence (see 4.2 for a fuller description of this). Functional assays provide a compelling argument that the gene of interest has been found. Reintroduction of a TSG into a cell with subsequent suppression of the malignant phenotype would provide this supporting evidence, and subsequent reversion upon loss of the gene would be stronger evidence still. Animal models might provide further positive data. Creation of a transgenic mouse with a phenotype resembling the disease would obviously be supportive of the case.

The technique of subtraction cloning, and its modern corollary RDA (Lisitsyn *et al.*, 1993; Lisitsyn and Wigler, 1995), provides a method of scanning an entire genome for differences from normal DNA. Although technically difficult to perform, it has had a number of notable successes: *dystrophin* and *PTEN* (Li *et al.*, 1997) were both identified in this way. It is expected that this technique, combined with microarray technology, will accelerate our understanding of the genetics of cancer.

1.5 Aims

A large body of evidence points to the existence of a TSG on the long arm of human chromosome 7. Frequent loss of heterozygosity has been reported in a variety of human cancers, including breast, ovarian, gastric, pancreatic and haematological malignancies, and these data have been supported by cytogenetic studies (reviewed in Zenklussen and Conti, 1996). In common with other TSGs, the putative gene seems to be conserved in other species such as mouse, since a high frequency of allele loss has been seen in mouse squamous tumours in the region of mouse chromosome 6 syntenic with human 7q31 (Zenklussen *et al.*, 1994). Furthermore, inhibition of tumorigenesis has been demonstrated by chromosome transfer of human chromosome 7 into murine squamous carcinoma cell lines (Zenklussen *et al.*,

1994), indicating that the encoded protein is highly conserved between these two species.

The aim of this work was to identify and clone the putative multi-tissue TSG on mouse chromosome 6. Firstly, I chose to confirm and extend the published LOH data from the mouse skin system and define a region of interest. This was achieved by saturating the area of interest with all published microsatellite markers. Next, utilising data from concurrent research on human chromosome 7 within our laboratory, I aimed to identify and examine mouse homologues of human candidate tumour suppressor genes. Finally, having cloned a number of such genes, I examined their potential as candidate tumour suppressor genes.

CHAPTER 2

MATERIALS AND METHODS

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals

All chemicals not listed below were obtained (AnalaR grade) from BDH Chemicals Ltd., Poole, Dorset, UK. Solutions and buffers were prepared using de-ionized water (dH_2O) obtained from a Millipore MilliRO 15 system.

Chemical	Source
Redivue [α-^{32}P] dCTP~3000Ci/mmol	Amersham International plc., Amersham, Buckinghamshire, UK
CsCl Hepes Mops	Boehringer Mannheim UK, Lewes, East Sussex, UK
Butan-1-ol Chloroform 38% (w/v) Formaldehyde Propan-1-ol	Fisher Scientific UK. Ltd., Loughborough, Leicestershire, UK
Dimethyl formamide	Fluka Chemika-Biochemika AG, Buchs, Switzerland
Ethanol	James Burrough Ltd., Witham, Essex, UK
Tris Trizol	Life Technologies Ltd., Paisley, UK
Deoxyribonucleotides	Promega, Southampton, UK
Water-saturated phenol	Rathburn Chemicals Ltd., Walkerburn, UK

Chemical	Source
Bovine serum albumin (BSA)	Sigma Chemical Co.
Bromophenol blue	Ltd.,
$CuSO_4$	Poole, Dorset, UK
DEPC	
Dithiothreitol	
Ethidium bromide	
MES	
NP40	
TEMED	
Tween 20	

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
Klenow polymerase Proteinase K	Boehringer Mannheim UK, Lewes, East Sussex, UK
NovoZyme	Novo BioLabs, Bagsvaerd, Denmark.
Dnase-free RNase A	Sigma Chemical Co. Ltd., Poole, Dorset, UK
Taq polymerase	Bioline, London, UK
T4 DNA ligase	Northumbria Biologicals Ltd., Cramlington, Northumberland, UK

2.1.3 Kits

Kit	Source
HighPrime random-priming labelling mixture	Boehringer Mannheim UK, Lewes, East Sussex, UK
TA-cloning kit	Invitrogen, NV Leek, Netherlands.
First strand cDNA synthesis kit	Life Technologies, Paisley, UK
Geneclean II	BIO 101 Inc., Vista, CA, USA
ABIPRISM DNA sequencing kit	PE Applied Biosystems, Warrington, UK

2.1.4 General plasticware

	Source
Filter pipette tips	Greiner Labortechnik Ltd., Gloucestershire, UK
Falcon tubes	Becton-Dickinson Labware, Plymouth, UK
5 ml bijous 20 ml universals	Bibby-Sterilin Ltd., Staffordshire, UK
microcentrifuge tubes pipette tips	Elkay, Galway, Eire

2.1.5 Miscellany

	Source
MicroSpin S-200 and S-400 HR columns	Pharmacia Biotech. Inc., Herts., UK
Sonicated, denatured genomic DNA from human placenta Torula yeast RNA type VI	Sigma Chemical Co. Ltd., Poole, Dorset, UK
FITC labelled STAR FISH whole mouse chromosome paints	Cambio Ltd., Cambridge, UK

2.1.6 Electrophoresis gels

	Source
Agarose, ultrapure, electrophoresis grade	Life Technologies, Paisley, UK
Polyacrylamide	Severn Biotech Ltd., Kidderminter, UK
Sequagel	BS+S Edinburgh, UK

2.1.7 Molecular weight markers

Marker	Source
ϕX174 DNA/ Hae III fragments λ DNA/ Hind III fragments	Life Technologies Ltd., Paisley, UK

2.1.8 Paper, membranes, and X-ray film.

	Source
Hybond nylon membranes	Amersham International plc., Amersham, Buckinghamshire, UK
3MM filter paper	Whatman International Ltd., Maidstone, Kent, UK
X-ray film (X-OMAT-AR)	Eastman Kodak Co., Rochester, New York, USA

2.1.9 Microbial host, media, and supplies.

Sterile glassware and Luria (L)-broth (Maniatis *et al.*, 1989) were prepared by Beatson Institute for Cancer Research (BICR) central services.

	Source
Petri dishes	Bibby-Sterilin Ltd., Staffordshire, UK
Bacto-agar Bacto-peptone Bacto-yeast extract Tryptone Yeast nitrogen base without amino acids	Difco, Detroit, MI, USA
DH5α competent E. Coli NZY broth	Life Technologies, Paisley, UK
Ampicillin Kanamycin	Sigma Chemical Co. Ltd., Poole, Dorset, UK

2.1.10 Plasmid vectors

Vector	Source
pBluescript SK (+/-)	Stratagene Ltd., Cambridge, UK.

2.1.11 Libraries

Library	Source
Mouse fetal brain cDNA library Mouse B6 genomic library	Stratagene Ltd., Cambridge, UK

2.1.12 Cell culture media and supplies

Sterile glassware, PE, PBS and water were prepared by the Beatson Institute for Cancer Research central services.

	Source
Freezing vials	A/S Nunc, Roskilde, Denmark
Cell culture plastic dishes	Becton-Dickinson Labware, Plymouth, UK
FCS	Bioclear UK Ltd. Devizes, Wilts, UK
DMSO	Fisher Scientific UK. Ltd., Loughborough, Leicestershire, UK
L-glutamine	Life Technologies, Paisley, UK

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

2.1.13 Cell lines

The cell lines listed below were kindly provided by Professor A. Balmain, CRC Beatson laboratories, Medical Oncology, Glasgow, UK.

Cell Line	**Description**	**Origin**
C5N C50	non-tumorigenic, immortalized cell line	isolated by single cell cloning of MCA3D cells derived from a Balb/c mouse
MSCP1	derived from papillomas	*M. spretus* / *Mus musculus* Fl hybrid
B9	squamous explant from the MSC11 carcinoma	*Mus spretus* / *Mus musculus* Fl hybrid mouse (single cell clone)
A5, D3	spindle explants from the MSC11 carcinoma	*Mus spretus* / *Mus musculus* Fl hybrid mouse (single cell clones)
CarB, carC	highly aggressive spindle cell lines isolated from carcinomas	NIH mouse
SN161	squamous / spindle mixed morphology derived from a lymph node metastasis	129 / NIH mouse
E4	squamous cell line	single cell cloning of SN161
Hll	spindle cell line	single cell cloning of SN161
PDV	squamous cell line	keratinocyte isolated from C57BL mouse and transformed *in vitro* by DMBA

2.1.14 Mouse skin tumour samples

Mouse skin tumours were gifted to me by Professor A. Balmain, CRC Beatson laboratories, Medical Oncology, Glasgow, UK. Tumours were induced and harvested as follows: all mice were bred in-house and given food and water *ad libitum*. The dorsal area of 7 to 10 week old mice was shaved and tumours induced by twice-weekly application of DMBA, or alternatively a single application of DMBA followed by twice-weekly applications of TPA. Occasionally, animals treated with DMBA and TPA were also treated with MNNG. Carcinomas were removed as they appeared and frozen, and any papillomas present were also removed. DNA was isolated from the tumours as described in 2.2.2.1.

2.1.15 Websites

Genome Data Base:

http://gdbwww.gdb.org

Jackson Laboratory:

http://www.informatics.jax.org/

National Center for Biotechnology Information (NCBI):

http://www.ncbi.nlm.nih.gov

Whitehead Institute/MIT Center for Genome Research:

http://www-genome.wi.mit.edu/

2.2 Methods

2.2.1 Cell culture

Cell lines derived from mouse skin or from epithelial tumours were routinely maintained at 37°C in sealed plastic flasks containing Special Liquid Medium (SLM) supplemented with 10% foetal calf serum (FCS) and 2mM glutamine in an incubator adjusted to 5% CO_2. Cells were routinely passaged when subconfluent by aspirating the medium, washing once with PBS then adding a small volume of trypsin (10% v/v trypsin, 0.01% EDTA) in PBS. They were incubated at 37°C until the cells detached. The cells were then resuspended in fresh serum-containing medium to inactivate the trypsin, and reseeded at the appropriate dilution.

Cell cultures were routinely monitored for *Mycoplasma* infection by DAPI staining, and were found to be negative.

2.2.2 Nucleic acid preparation and quantitation

2.2.2.1 Extraction and purification of mouse genomic DNA

Genomic DNA was prepared from mammalian cell lines according to Laird *et al.* (1991). Cells were first harvested by trypsinisation and then pelleted by brief centrifugation in a microcentrifuge tube. Cells were resuspended and simultaneously lysed by trituration in 1 ml of lysis buffer (100 mM Tris.HCl pH 8.5, 5 mM EDTA, 0.2% (w/v) SDS, 200 mM NaCl, 100 μg Proteinase K/ml), followed by incubation for several hours at 37 °C with constant agitation. DNA was precipitated by addition of an equivalent volume of propan-2-ol with gentle mixing until viscosity was gone. The aggregated precipitate was then removed by lifting from the solution with a sterile plastic inoculation loop. Excess liquid was dabbed off and DNA was rinsed in 70% (v/v) ethanol. After being allowed briefly to air-dry, DNA was dispersed into 0.5 ml TE (10 mM Tris.HCl, 1 mM EDTA pH 8.0).

2.2.2.2 Bacterial clone DNA preparation

Cosmid clones were supplied as agar stab cultures. These were first streaked out on an L-broth plate (1% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract, 170 mM NaCl, 1.5% (w/v) agar) supplemented with kanamycin (50 ug/ml). A single colony was then used to inoculate a flask containing L-broth and kanamycin. For the preservation of bacterial stocks, a 0.5 ml aliquot of over-night culture in liquid medium was mixed with an equivalent volume of 50% (w/v) glycerol, chilled on ice and then stored at -70 °C in plastic cryotubes. Cultures were subsequently re-established by inoculation of 5ml of L-broth medium, containing the appropriate antibiotic, with 20 µl of the glycerol stock.

Plasmid and cosmid DNA were isolated from over-night cultures of transformed bacteria by alkaline lysis. Cultures were first refrigerated for 20 minutes before being pelleted by centrifugation, and resuspended in the appropriate volume of solution I (100 mM Tris.HCl pH 8.0, 100 mM EDTA) (table below). Bacteria were then lysed by addition of solution II (0.2 M NaOH, 1% (w/v) SDS) and gentle mixing by inversion. After a 5 minutes incubation on wet ice, detergent and protein were precipitated by addition of ice-cold solution III (3M KOAc pH 4.8) and momentary vigorous shaking. After incubating on ice for a further 15 minutes, the flocculate was removed by centrifugation at 10,000 g. Supernatant containing plasmid or cosmid DNA was decanted into a fresh polypropylene tube. (For small to medium scale preparations of bacterial clone DNA, RNase was added at this stage at a final concentration of 10 µg/ml and the lysate incubated for 15 minutes at 37°C.) 1/10 volume of chloroform was then added to the lysate to remove residual protein, the two phases mixed by shaking, and separated by centrifugation. The upper aqueous phase was decanted and DNA was precipitated by addition of 0.6 volumes of propan-2-ol, followed by washing in 70% (v/v) ethanol. DNA was resuspended in TE (pH 8.0).

Table 2.1 Solution volumes in bacterial clone DNA preparation

	Culture volume		
	1 ml	50 ml	500 ml
Solution I	100 µl	1 ml	20 ml
Solution II	200 µl	2 ml	40 ml
Solution III	150 µl	1.5 ml	30 ml

2.2.2.3 Total RNA extraction from mouse cell lines

Extraction of total RNA from cell lines was carried out following the manufacturer's protocol for Trizol. Sub-confluent cells grown in 10 cm dishes were washed twice in ice-cold PBS which was completely removed by aspiration. Cells were subsequently lysed directly in the dish by the addition of 1 ml Trizol. A disposable cell scraper was employed to homogenise the cells, and the RNA was solubilised by passing the lysate a few times through a pipette tip before being transferred to a fresh microcentrifuge tube. Chloroform was added (0.2 ml per 1 ml lysate) and the microcentrifuge tube vortexed for 15 s, then incubated on ice for 5 minutes. The samples were centrifuged at 12,000 g at 4°C for 15 minutes after which the upper colourless aqueous phase was transferred to a fresh microcentrifuge tube and an equivalent volume of propan-2-ol added. The samples were stored overnight at -20°C. The RNA precipitate was pelleted by centrifugation at 12,000g at 4 °C for 25 minutes. The RNA pellet was washed once with 1.5 ml ice-cold 75% (v/v) ethanol by vortexing and centrifugation at 7,500 g at 4°C for 8 minutes. The RNA was air-dried and re-dissolved in 50 µl (DEPC-treated) RNase-free water.

2.2.2.4 First strand cDNA synthesis

First strand cDNA, prepared for RT-PCR, was synthesised using a kit, according to the manufacturer's instructions. Approximately 1 µg of total RNA in DEPC-treated H_20 was combined with 1 x first strand buffer (50 mM Tris.HCl pH 8.3,

75 mM KCl, 3 mM $MgCl_2$), 10 mM DTT, and 0.5 mM of each of the four dNTPs (dATP, dCTP, dGTP, dTTP), and incubated for 5 minutes at 65°C. The reaction mixture was chilled on ice and 1 μl (200 units) of M-MLV reverse transcriptase added, followed by incubation at 37°C for 1 hr.

2.2.2.5 Synthesis and Purification of oligonucleotides

Oligonucleotides were synthesised at the BICR as a core service on an Applied Biosystems model 392 or 394 RNA/DNA synthesiser using phosphoramidite chemistry according to the manufacturer's instructions. As part of the synthesis 5′ trityl groups were removed and the oligonucleotides eluted into a solution of 29% (v/v) ammonia. This eluate was then incubated at 55°C overnight in order to de-protect the oligonucleotides. Vials were then chilled on ice and the DNA-ammonia solutions transferred to 15 ml Falcon tubes. Oligonucleotides were precipitated by the addition of 0.1 volumes of 7.5 M ammonium acetate and 3 volumes of ethanol, followed by incubation on dry ice for 30 minutes. The DNA was then pelleted by centrifugation in a Sorvall HB-6 rotor at 10,000 g for 15 minutes. The pellet was washed in 70% (v/v) ethanol, air-dried and dissolved in 0.5 ml of de-ionised water. DNA concentrations were calculated as described below (2.2.2.6) and the oligonucleotides were then stored at -20°C until required.

2.2.2.6 Quantitation of nucleic acid concentrations

Nucleic acids were quantified by spectrophotometric determination of their UV light absorbency. Sample aliquots of 5 μl were added to 495 μl of de-ionised water and the absorbency of the solution measured at 260 nm and 280 nm in a quartz cuvette, using de-ionised water as a blank. The concentration of the solution was calculated on the basis that an optical density of 1.0 at 260 nm corresponds to a concentration of 50 μg/ml for double-stranded DNA, 40 μg/ml for RNA, and 33 μg/ml for single-stranded oligonucleotides. Pure preparations of DNA and RNA have a ratio of A_{260}/A_{280} readings between 1.8 and 2.0.

2.2.3 Polymerase chain reaction (PCR) protocol and product analysis

2.2.3.1 PCR

The following conditions were used to perform the majority of PCR amplifications both for allele loss studies and SSCP analysis, but occasional slight alterations (to the annealing temperature or concentration of magnesium ions) were required to optimise results. 100 ng of human or mouse genomic DNA or 10 pg bacterial clone DNA was subjected to PCR amplification using 1μM oligonucleotide primers, 1.5 mM $MgCl_2$, 50μM dNTPs, 1 x reaction buffer (50 mM KCl, 10 mM Tris.HCl pH 8.0) and 1 unit *Taq* polymerase in a total reaction volume of 25 μl. 1 μCi [α^{32}P]-dCTP per reaction was included for *in situ* radiolabelling of amplification products when desired. The thermal cycling parameters consisted of 30 rounds of 1 minutes denaturation at 94°C, 30 s annealing at 55°C, and 30 s extension at 72°C, using an MJC Research PTC-200 thermal cycler (Genetic Research Instrumentation Ltd., Dunmow, Essex, UK). Following PCR, amplification products were digested where appropriate by direct addition of 10 units of restriction enzyme without change or modification of the buffer and incubated for several hours at the appropriate temperature.

2.2.3.2 Denaturing polyaclylamide gel electrophoresis

Radiolabelled PCR products from amplified polymorphic markers were resolved on 6% (w/v) polyacrylamide gels under denaturing conditions. A gel solution was prepared from Sequagel stock solutions which contained 6% (w/v) acrylamide, 0.2% (w/v) bisacrylamide, 1 x TBE, and 8 M urea. The solution was polymerised by the addition of 150 μl of 20% (w/v) ammonium persulphate and 75 μl of TEMED per 60 ml of gel. This solution was then poured between glass plates separated by 0.4 mm spacers and was allowed to set at room temperature. Gels were pre-run at 100 W for 45 minutes prior to the loading of samples to warm the gels to approximately 50°C. 5μl PCR product was mixed with 5 μl of STOP buffer (95% formamide, 200mM EDTA pH 8.0, 0.01% (w/v) xylene

cyanol and bromophenol blue) and denatured by heating at 94°C for 10 minutes, followed by quenching on ice. 3 μl was then subjected to electrophoresis at 100 W for 2-3 hr depending upon the size of the PCR product. The plates were then separated and the gel transferred to a sheet of Whatman 3MM paper, followed by drying under vacuum at 80°C for 45 minutes, prior to detection of PCR products by autoradiography using x-ray film in a cassette with intensifying screens at -70 °C.

2.2.3.3 Tumour allele loss studies

100 ng template DNA was subjected to PCR using the standard reaction conditions above (2.2.3.1); amplification products were radiolabelled *in situ*. 28 cycles of amplification was taken to be in the linear part of the amplification, permitting the assumption that the ratio of the optical densities arising from two alleles would be the same for both normal and tumour DNA samples if no LOH occurred. PCR products were resolved on 6% (w/v) polyacrylamide denaturing gels and visualised by autoradiography (section 2.2.3.2). Allele loss was determined by visual examination of autoradiographs.

2.2.3.4 Non-denaturing polyacrylamide gel electrophoresis

For non-denaturing polyacrylamide gels, a 30 % (w/v) stock solution of acrylamide, comprising 29 parts acrylamide to 1 part N,N′methyl-bisacrylamide was diluted to give a 6% (w/v) gel forming solution containing 1 x TBE and 3% (v/v) glycerol. This solution was polymerised by the addition of 150 μl of 20% (w/v) ammonium persulphate and 75 μl of TEMED per 60 ml of gel. After mixing, the solution was poured into vertical plates with 0.4 mm spacing and allowed to set at room temperature. 4 μl PCR product was mixed with 8 μl of STOP buffer and denatured by heating at 94°C for 10 minutes, followed by quenching on ice. 3 μl was then subjected to electrophoresis at a constant 400 V for run lengths (of several hours) determined by the migration of the xylene cyanol dye front and the size of the PCR product. Gels were fixed by heating at

80°C under vacuum, and exposed directly to X-ray film for detection by autoradiography.

2.2.3.5. SSCP analysis

For SSCP-heteroduplex analysis, 100 ng DNA prepared from tumours or tumour-derived cell lines were subjected to PCR using the standard reaction conditions above (2.2.3.1). Radiolabelled amplification products were then resolved on 6% (w/v) non-denaturing polyacrylamide gels (section 2.2.3.4) and visualised by autoradiography. Electrophoretic mobility shifts in either single or double stranded DNA products were visually determined from the autoradiographs.

2.2.3.6 Automated chain terminator 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. 0.3–0.5μg of plasmid or 50–100ng of a PCR product was mixed with 20 ng of sequencing primer, 8 μl of Dyedeoxy reaction mix in a total reaction volume made up to 20 μl with water. DNA was subjected to cycle sequencing in a DNA thermal cycler (Perkin Elmer Cetus) for 25 cycles (each cycle comprises 15 s at 96°C to denature DNA, 1 s at 50°C for annealing, and 4 minutes at 60°C to extend), and the products were then ethanol precipitated, washed with 70% (v/v) ethanol, and air dried prior to being re-suspended in loading buffer (95% (v/v) formamide, 25 mM EDTA pH 8.0, 1.5 mg/ml dextran blue). Samples were then denatured by heating at 94°C, chilled on ice, and subjected to electrophoresis as advised by the manufacturer. Sequence was analysed using the Sequencing Analysis program v3.0.

2.2.4 Recombinant DNA techniques

2.2.4.1 Restriction digestion of bacterial clone or genomic DNA

Approximately 1 μg of plasmid DNA, 5 μg of cosmid DNA, or 10–20 μg of mouse genomic DNA was digested in a final volume sufficient to dilute the volume of restriction enzyme added 10-fold. Bacterial clone digests were routinely performed using 10 units of enzyme per μg of DNA in each reaction and were incubated at the recommended temperature for at least 1 hr. Genomic DNA was digested overnight with at least 10 units of enzyme being added for each μg of DNA to be digested. A fresh aliquot of enzyme (10 units/μg DNA) was added to genomic digests the following morning and the digest continued for a further 3 hrs. Digest reactions were stopped by the addition of EDTA to a final concentration of 20 mM. Genomic DNA was ethanol precipitated as previously described and then air dried prior to being resuspended in an appropriate volume of TE for subsequent loading on an agarose gel. 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 in sequential reactions separated by ethanol precipitation.

2.2.4.2 Ligation of DNA/PCR fragments into plasmid DNA

Plasmid DNA was digested as described above. The DNA fragment to be inserted was also digested as above to produce blunt ends or complementary sticky ends with the vector, and then isolated by gel electrophoresis and purified as described in Section 2.2.5.3. 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, followed by a chloroform extraction, and plasmid DNA was then ethanol precipitated. For ligation, 100 ng of vector and two 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 $MgCl_2$, 1 mM

ATP, 1 mM DTT) and T4 DNA ligase (5-10 units), and incubated at room temperature for a minimum of 3 hours. PCR products were cloned using a TA-cloning kit according to the manufacturer's instructions.

2.2.4.3 Transformation of bacterial cells with recombinant plasmid DNA

Competent *E. coli* (DH5α [*supE*44 Δ*lac*U169(φ80 *lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1], INVαF' [F' *supE*44 Δ*lac*U169(φ80 *lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1]) were thawed on ice, and aliquots transferred to pre-chilled 1.5 ml screw-cap microcentrifuge tubes. Approximately 10 ng 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 °C for 45 s and then placed on ice for a further 2 minutes. 400 µl of SOC medium (2% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract, 20 mM glucose, 10 mM NaCl, 10 mM $MgCl_2$) was then added to the mixture and the cells were incubated at 37°C for 1 hr in an orbital shaker at 240 rpm. After this time cells were pelleted by briefly pulsing in a microcentrifuge, 450 µl of supernatant was discarded and the cells resuspended in the remaining 50 µl. Cells were then spread on L-broth plates containing 1.5% (w/v) agar supplemented with the appropriate antibiotic. Plates were incubated in an inverted position overnight at 37 °C.

2.2.4.4 Colony lifts

Hybond N membranes were layered onto plates containing recombinant *E. coli* and left for 1 minute. Five orientation marks were made with a needle through the membrane into the agar. After transfer, the filter was carefully lifted ensuring not to drag the membrane across the plate, and inverted over 3MM filter paper wetted with denaturation solution (1.5 M NaCl, 0.5M NaOH) for 5 minutes, and then transferred to 3MM filter paper wetted with neutralisation solution (1.5 M NaCl, 0.5M Tris.HCl pH 8.0) for 5 minutes. Finally, the filter was rinsed in 2 x SSC, briefly blotted with 3MM filter paper, and UV-crosslinked using a UV

Stratalinker 1800 (Stratagene). Filters were probed as described below (Section 2.2.5.5) and exposed overnight to Kodak X-OMAT AR film with intensifying screens at -70°C. The films were orientated against the filters using the needle marks, and positive clones determined using the strongest signals on the film. Plasmid DNA was prepared from positively hybridising colonies as described in Section 2.2.2.2.

2.2.5 Blotting and hybridisation protocols

2.2.5.1 Agarose gel electrophoresis of restriction digested bacterial clone or genomic DNA and unlabelled PCR products

DNA restriction fragments from plasmids or genomic DNA or unlabelled (cold) PCR products were separated on non-denaturing agarose gels and visualised by staining with ethidium bromide and UV transillumination. Typically, gels were prepared by dissolving between 1 and 2% electrophoresis grade agarose in 1 x TAE (40 mM Tris.HCl pH 8.0, 20 mM sodium acetate, 2 mM EDTA). After being heated in a microwave to dissolve the agarose, molten gels were cooled to approximately 60°C and ethidium bromide added to a final concentration of 5 μg/ml before being poured into an appropriate gel former. Once solid, gels were placed into electrophoresis tanks containing 1 x TAE. Samples were mixed with one-fifth volume of gel-loading buffer (40% (w/v) sucrose, 0.05% (w/v) bromophenol blue and 0.05% (w/v) xylene cyanol) prior to being loaded into the wells of the gel. Electrophoresis was performed at 5 V/cm. 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.

2.2.5.2 Purfication of DNA fragments from agarose gels

DNA bands of interest were excised from agarose gels using a scalpel with the aid of UV transillumination. The excised DNA was extracted from the agarose using a Geneclean kit according to the manufacturer's instructions. Excised agarose blocks were chopped into small pieces to aid extraction. 2.5 volumes of 6M NaI was added and the agarose incubated at 55°C for 5 minutes to melt the gel. Following this, 5 µl of glassmilk was added for every 5 µg, or less, of DNA. This was mixed and left at room temperature for 5 minutes to allow binding. The glassmilk was pelleted by brief centrifugation and washed 3 times by mixing with 300 µl NEW wash (a Tris base and EDTA-buffered solution of NaCl, ethanol, and H_20), pelleting the glassmilk and removing the supernatant. Finally, half the desired volume of TE was added to the cleaned pellet and heated to 55°C for 5 minutes. The glassmilk was pelleted and the TE containing DNA removed to a fresh microcentrifuge tube. This was repeated to elute all the DNA. Typically for most applications DNA was eluted in a final volume of 20 µl.

2.2.5.3 Preparation of random-primed radiolabelled probes

The probes used in this study for the analysis of Southern blots are listed in appendix 1.

All probes were radiolabelled with [α^{32}P]-dCTP via the method of random priming using a HighPrime kit. Following each labelling reaction, unincorporated nucleotides were removed by spin-column chromatography using MicroSpin S-200 HR columns according to the manufacturer's instructions. Before being used in reactions, all double-stranded probes were denatured by addition of an equivalent volume of 0.4 M NaOH.

2.2.5.4 Southern analysis

Following electrophoresis of DNA samples, DNA was depurinated by soaking the gel in freshly prepared 0.25M HCl for 15 minutes at room temperature with constant shaking. The gel was rinsed in de-ionised H_20 and then soaked for a further 15 minutes in 0.4 M NaOH to denature the DNA strands. DNA was transferred overnight onto Hybond N+ membrane by capillary blotting using 0.4 M NaOH, essentially as described in Maniatis *et al.* (1989).

Membranes were pre-hybridised at 65°C for four hours in hybridisation solution (5 X SSPE, 5 X Denhardt's, 0.5% (w/v) SDS, 1 mg/ml torula yeast RNA type VI), and then hybridised overnight at 65°C in a minimal volume of fresh hybridisation solution with added radiolabelled probe (5 x 10^6 cpm/ml). Following hybridisation, probe bound non-specifically to the membrane was removed by washing the membranes twice in 0.2 x SSC/0.1% (w/v) SDS for 20 minutes at 65°C, and then once in 0.1 x SSC/0.1% (w/v) SDS for a further 20 minutes at 65°C. Excess liquid was removed and membranes wrapped in Saranwrap before being exposed to X-ray film (X-OMAT-AR, Eastman Kodak Co., Rochester, New York, USA) in a cassette with an intensifying screen at -70°C for detection of hybridisation by autoradiography.

For successive hybridisations to the same membranes, radiolabelled probe was removed from the membranes by washing in a boiling 0.1% (w/v) SDS solution. Membranes were then agitated at room temperature until the SDS solution had cooled to room temperature. Blots were then wrapped in Saranwrap and stored at 4°C until further required.

2.2.6 cDNA library screening

A fetal mouse brain cDNA library constructed in the ZAP II vector was purchased from Stratagene. Plating and screening of the library was carried out essentially as described in the Stratagene instruction manual, summarised below.

2.2.6.1 Titering phage

The Lambda ZAP II phage was diluted in 4 serial 10-fold dilutions in SM buffer (l0 mM $MgSO_4$, 50 mM Tris.HCl pH 7.5, 10 mM NaCl, 0.01% (w/v) gelatin). In a 15 ml sterile tube, 600 µl of XL1-blue cells diluted to OD_{600} = 0.5 were mixed with 1 µl of each Lambda phage dilution and incubated at 37°C for 15 minutes to allow the phage to attach to the cells. 8 ml molten top agar (0.7% (w/v) agarose in L-broth cooled to 50°C) was added to the culture-phage mix and poured immediately onto warmed (37 °C) 140 mm L-broth plates. The plates were left to set at room temperature and then incubated at 37°C overnight. The number of plaques per plate were counted and the titre calculated using the formula:

titre = (no. plaques x 1000 x dilution factor) pfu/ml.

2.2.6.2 Screening cDNA library

Approximately 150,000 pfu were plated on each of four large 245mm square L-broth plates with 2 ml of OD_{600} = 0.5 XL 1-Blue cells/plate and 30m1 top agar/plate. These were incubated overnight at 37°C. The plates were refrigerated for 2 hours at 4°C prior to taking lifts, as this prevents the top agar from sticking to the nitrocellulose filter. Filters were prehybridised, hybridised and washed as described in section 2.2.5.4. They were then wrapped in Saranwrap and exposed overnight to Kodak X-OMAT AR film with intensifying screens at -70°C. The films were orientated against the filters using the needle marks, and positive clones determined using the strongest signals on the film. The end of an inverted pasteur pipette was used to core the putative clones from the stock agar plates, and the agar plug placed into an microcentrifuge tube with 500 µl SM buffer and 20 µl chloroform. The tube was briefly vortexed and incubated overnight at 4°C to elute the phage. The phage stock is stable for 1 year at 4°C.

For each positive clone, l µl of a 200-fold dilution of eluted phage in SM buffer was added to 600µl XLl-Blue cells at OD_{600} = 0.5, incubated at 37°C for 15 minutes, mixed with 8 ml molten top agar, and poured onto l40 mm L-broth

plates. Transfer to nitrocellulose filters and screening was carried out as described above. This procedure was repeated for a maximum of three platings, such that single positive plaques were isolated.

2.2.6.3 *In vivo* excision of the pBluescript phagemid

In vivo excision of the cloned insert is dependent upon the simultaneous infection of XLl-blue cells with both the lambda vector (containing cloned insert) and the M13 helper phage (ExAssist). For a full description of this, see the Stratagene manual.

Single plaques, isolated as described above, were placed in 500 µl SM buffer with 20 µl chloroform and incubated overnight at 4 °C. In a 15 ml conical tube 200 µl of OD_{600} = 1.0 XLl-Blue cells, prepared as in section 2.2.6.1, were combined with 100 µl eluted phage stock and 1ul ExAssist helper phage, and incubated at 37°C for 15 minutes. After which, 3 ml of terrific broth (for 1 litre: 12g bacto tryptone, 24g bacto yeast extract, 4m1 glycerol. After autoclaving add 100 ml solution B {0.17 M KH_2PO_4, 0.72 M K_2HPO_4 }) was added and incubated at 37°C with shaking for 2½ hours. The tube was heated at 70°C for 15 minutes, and centrifuged for 5 minutes at 4000g. The supernatant containing the pBluescript phagemid, packaged as phage particles, was stored at 4°C.

To rescue the phagemid, 1 µl supernatant from above was added to 200 µl OD_{600} = 1.0 SOLR host cells, and incubated at 37°C for 15 minutes. Following incubation, 10 µl and l00 µl of the phage/SOLR mix was spread onto L-broth plates containing 50 µg/ml ampicillin, and incubated overnight at 37 °C. Minipreparations of plasmid DNA were prepared as described in section 2.2.2.2.

2.2.7 Fluorescent *in situ* hybridisation

2.2.7.1 Preparation of metaphase spreads

Established cell lines were cultured until subconfluent, at which time they were treated with colcemid (0.0l μl /ml) for 2-3 hours at 37°C, and then harvested. The cells were centrifuged at 1500rpm for 5 minutes. Most of the supernatant was removed and the cells were resuspended in the residual medium. Pre-warmed hypotonic buffer (0.075M KCI, 37°C) was added dropwise. The cells were incubated at 37°C for 5-10 minutes, then centrifuged at 1500 rpm for 5 minutes. Most of the supernatant was removed and the cells again resuspended in the remaining buffer. The swollen cells were then fixed in 3:1 methanol/acetic acid and left at room temperature for 15 minutes, followed by centrifugation at 1500rpm for 5 minutes, to remove the supernatant. Fixation was repeated twice. The cells were dropped from a height on to clean, moist slides and immediately washed in 3:1 methanol/acetic acid.

2.2.7.2 Preparation of probes

FITC-labelled whole mouse chromosome paints were purchased from Cambio. For each slide, l0μl of probe was denatured at 75°C for 5 minutes, and incubated at 37°C for 30 minutes to allow for pre-annealing of C_{ot}l repetitive DNA prior to applying to metaphase chromosomes.

2.2.7.3 *In situ* hybridisation

Metaphase spreads were fixed in 3:1 methanol / acetic acid for 1 hour, rinsed in 2 x SSC, and then treated with RNase (100g/ml) in 2 x SSC at 37°C for 1 hour. Slides were rinsed in 2 x SSC, then treated with 0.01% pepsin in 0.01M HCI at 37°C for 10 minutes. After rinsing in phosphate buffered saline (PBS) and fixing in Streck Tissue Fixative for 10 minutes at room temperature, the chromosomes were dehydrated in 70% and 100% ethanol.

Chromosomes were denatured prior to thin hybridisation in 70% formamide in 2 x SSC at 80°C for 3 minutes. Slides were quenched in ice-cold 70% ethanol, then dehydrated. The probe was applied to the denatured chromosomes, and hybridisation was carried out under a sealed coverslip at 37°C overnight in a humidified chamber.

After hybridisation, coverslips were removed in 2 x SSC and slides were washed in 50% formamide/1 x SSC and then 2 x SSC, both at 42°C for 20 minutes each. Metaphase spreads were washed for 10 minutes in 4 x SSC-T and then dehydrated in 70% and 100% ethanol, and mounted in anti-fade medium (Vectashield, Vector Labs) containing 0.1μg/ml DAPI and 0.34g/ml PI. The mouse whole chromosome paints were directly labelled with FITC. Therefore, following the wash steps in 50% formamide and 2xSSC, slides were dehydrated in 70% and 100% ethanol, and mounted in anti-fade medium containing DAPI and PI as before. Fluorescence was analysed using a BioRad MRC-600 laser scanning confocal microscope equipped with a krypton argon ion laser, using 488/568nm line excitation, and dual channel 522nm and 585nm filters.

Chromosome copy numbers were obtained by analysing a minimum of 20 metaphase spreads per cell line. Total numbers of chromosomes were derived from the mean of 10 individual metaphase spreads.

CHAPTER 3

RESULTS

LOSS OF HETEROZYGOSITY STUDIES

3. Results

3.1 Loss of Heterozygosity Analysis in Mouse Skin Tumours

Previous work by Zenklusen and colleagues had demonstrated a high freqency of allele loss on mouse chromosome 6, band A2 in both the murine skin and hepatoma carcinogenesis models (Zenklusen *et al.* 1996b, 1997), suggesting the presence of one or more tumour suppressor genes in this area. I chose initially to confirm these observations and to extend them by saturating the area of interest with microsatellite markers obtained from the public databases. In so doing I aimed to define a smallest common deleted region (SCDR).

The use of interstrain and interspecific F1 hybrid mice greatly facilitates polymorphic microsatellite analysis since the likelihood of length polymorphism and therefore informativeness is high. This is particularly so in the case of interspecific crosses. With this in mind, over thirty microsatellite repeats of the simple $(CA)_n$ variety spanning the first 8 cM of mch6 (the area of highest loss in previous studies) were examined for length polymorphisms in the following strains of mice: *M. spretus*, NIH/Swiss, SENCAR, BALB/c, B6, FVB and 129. As expected, the highest degree of informativeness was between *M. musculus* strains and *M. spretus*. Figure 3.1 demonstrates simple sequence length ploymorphisms (SSLP) between strains. Using the 21 informative markers identified in this way, I then searched for allele loss in papillomas and carcinomas induced in NIH x *Spretus* F1 (10 papillomas, 17 carcinomas), 129 x NIH F1 (15 carcinoma, 6 papilloma) and SENCAR x Balb (5 carcinoma, 2 papilloma) F1 mice. A total of 55 cases were examined, consisting of 18 papillomas and 37 carcinomas. Tumours had been induced and harvested in house, and had been gifted to me by Professor A. Balmain. Naturally not all markers were informative for all hybrids. Figure 3.2 shows representative autoradiographs of PCR amplified microsatellite repeats demonstrating LOH and tables 1, 2 and 3 show the pattern of losses seen within each of the three crosses.

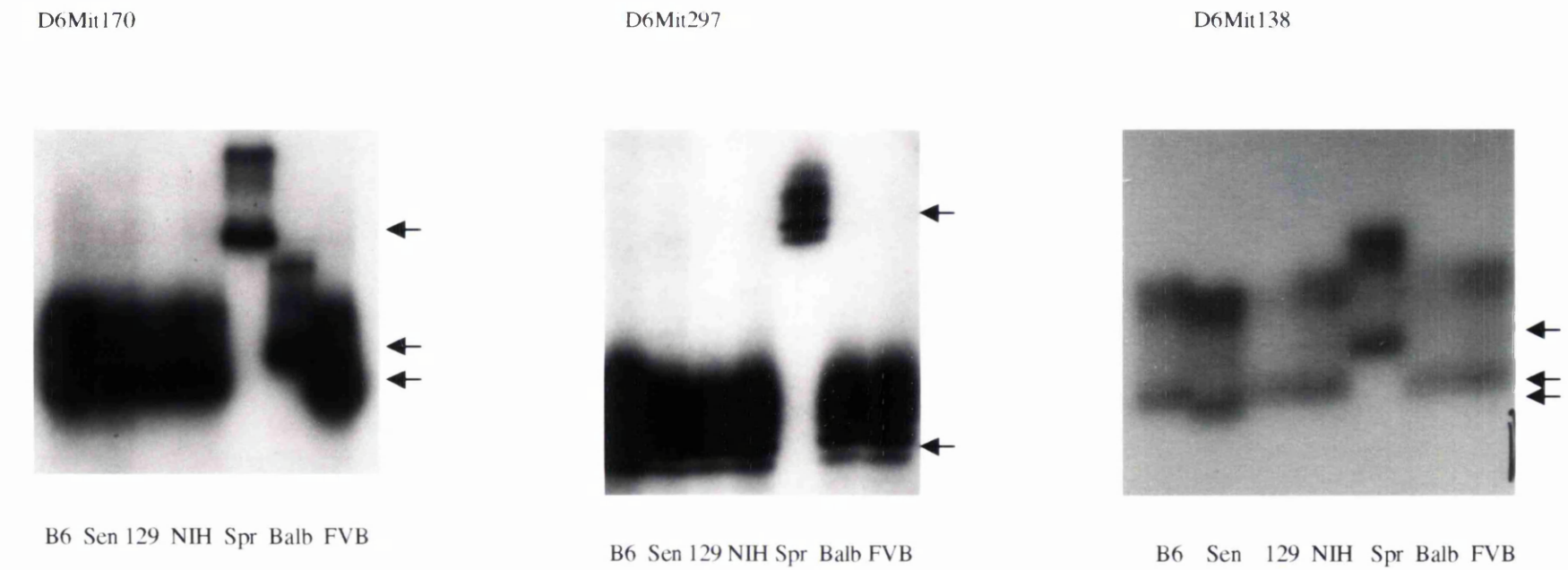


Figure 3.1. Examples of sequence length polymorphisms between different mouse strains. Different alleles are arrowed. Note the marked length polymorphism between *M. Spretus* and the other strains, all of which are derived from *M. musculus*. From the first autoradiograph it can be seen that marker D6mit170 would not be informative in a 129 x NIH F1 cross.

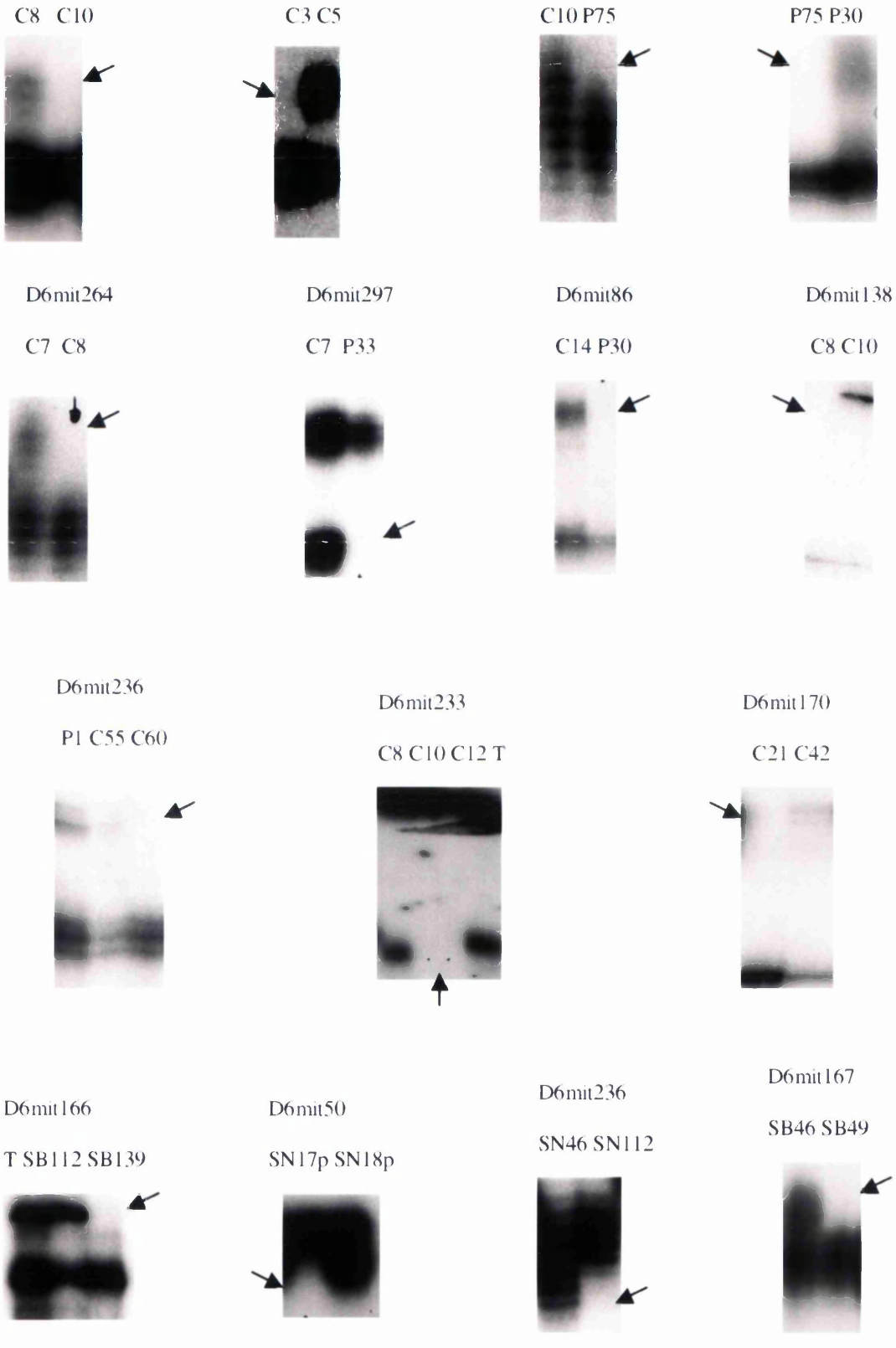


Figure 3.2 Examples of loss of heterozygosity in carcinoma (C) and papilloma (P) samples as seen by radiolabelled PCR analysis of microsatellite markers resolved on acrylamide gels. The arrow demonstrates the lost allele. SN, 129 x NIH F1 cross; SB, SENCAR x Balb F1 cross; C and P, *Spretus* x NIH F1 cross. In some cases tail (T) samples are also shown.

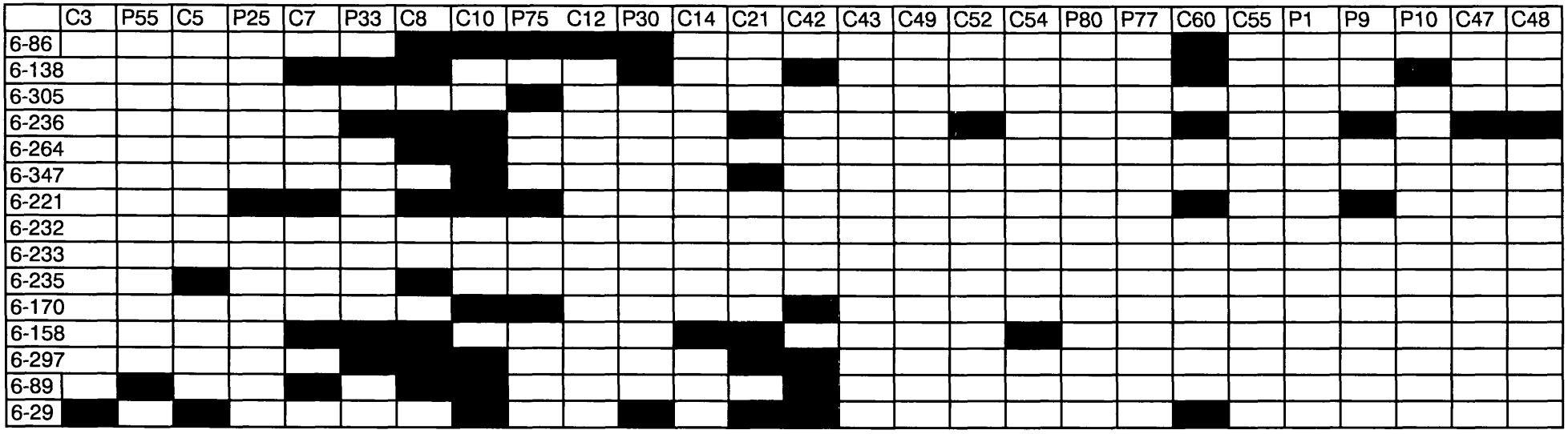


Table 3.1. Loss of heterozygosity in carcinoma (C) and papilloma (P) tumours derived from *Spretus* x NIH F1 mice. Tumour cases are shown on the x-axis while microsatellite markers, ordered acromeric to telomeric, are shown on the y-axis. Loss of heterozygosity is denoted by filled boxes.

	SN17p	SN18p	SN37c	SN39c	SN42c	SN44c	SN48c	SN51c	SN53p	SN61c	SN62c	SN63c	SN64c	SN65c	SN87p	SN91c	SN95c	SN119c	SN120c	SN122p	SN156p
6-83																					
6-236	■	■													■	■					
6-50		■																			
6-167	■	■																			
6-89																					

Table 3.2. Loss of heterozygosity in carcinoma (c) and papilloma (p) tumours derived from129 x NIH mice. Tumour cases are shown on the x-axis while microsatellite markers, ordered acromeric to telomeric, are shown on the y-axis. Loss of heterozygosity is denoted by filled boxes.

	SB46c	SB49c	SB111p	SB112p	SB125c	SB126c	SB139c
6-166				■			■
6-296							
6-83							
6-236			■	■	■	■	
6-167		■				■	
6-170							
6-158							
6-89							

Table 3.3. Loss of heterozgosity in papilloma (p) and carcinoma (c) tumours derived from SENCAR x Balb/c mice. Tumour cases are shown on the x-axis while microsatellite markers, ordered acromeric to telomeric, are shown on the y-axis. Loss of heterozygosity is denoted by filled boxes.

A moderate frequency of allele loss seems to be common in the region examined (summarised in table 3.4). The highest frequency of loss is seen at markers D6mit236 (3.1cM from the acromeric tip of mchr6) and D6mit166 (0.6cM), although in the latter case the total number of tumours examined is small. These markers are found on mchr6 region A2, a region syntenic with human chromosome 7q31.1-2. The most distant marker, D6mit29 on mchr 6A3, also showed moderately high incidence of loss (26%), although to date synteny has not been established to any human chromosome.

Table 3.4 Summary of tumour loss of heterozygosity data. Microsatellite markers are ordered from acromeric to telomeric

Marker	cM Position	Loss in papillomas	Loss in carcinomas	total (%)
D6Mit86	0.5	2/10	4/17	22
D6Mt166	0.6	1/2	1/5	29
D6Mit296	0.625	0/2	0/5	0
D6Mit138	0.65	3/10	4/17	26
D6Mit83	2.7	0/8	0/28	0
D6Mit305	2.8	1/10	0/17	4
D6Mit236	3.1	7/18	10/37	33
D6Mit264	3.2	0/10	2/17	7
D6Mit347	3.2	0/10	2/17	7
D6Mit50	3.3	1/6	0/15	5
D6Mit167	3.4	2/8	2/20	14
D6Mit221	3.5	3/10	4/17	26
D6Mit232	3.5	0/10	0/17	0
D6Mit233	3.5	0/10	2/17	7
D6Mit235	3.5	0/10	2/17	7
D6Mit170	4.0	1/12	2/22	9
D6Mit158	6.0	1/12	5/22	18
D6Mit297	6.0	1/10	4/17	18
D6Mit89	7.0	1/18	4/37	9
D6Mit29	30.0	1/10	6/17	26

Looking at the pattern of losses in different crosses, it is difficult to draw firm conclusions as to the existence of a SCDR. This is particularly so in the case of the *Spretus* x NIH cross. Three out of 27 cases show isolated loss of heterozygosity at marker D6Mit236, but the same could be said of other markers at different areas. The *Spretus* x 129 and Sencar x Balb F1 tumours are more helpful: losses do seem to be centred around marker D6Mit236 and this could potentially represent a SCDR. Loss of heterozygosity throughout the region

examined was not confined to carcinomas; both papillomas and carcinomas were involved in roughly equal proportions.

3.2 Analysis of Cell Lines

3.2.1 Molecular analysis of cell lines

I examined the panel of cell lines in my possession for allele loss. These cell lines are representative of the various sequential steps in mouse skin carcinogenesis (see Materials and Methods 2.1.13 for a full description of origin). Some of these lines were derived from interspecific F1 crosses, and therefore lent themselves to classical LOH analysis. Others were from individual mouse strains which, although not amenable to LOH analysis, would still demonstrate homozygous deletion at a locus if present. Clearly this would be very helpful in attempting to localise any putative tumour suppressor gene, since it implies a small and specific deletion.

No homozygous loss was apparent at any marker locus. Indeed no absolute loss of heterozygosity was detectable in any cell line. As demonstrated by previous investigators (Kemp *et al.*, 1993b), there was evidence of allelic imbalance (figure 3.3). This was likely to be indicative of aneuploidy, since it has been established that a number of the cell lines are tri- or tetrasomic for mouse chromosome 6. The cell lines I had examined and which showed allelic imbalance had not hitherto been analysed for ploidy. I therefore decided to establish the copy number of chromosome 6 using whole chromosome painting.

3.2.2 FISH analysis of cell lines

Chromosome painting is a specific application of fluorescent *in-situ* hybridisation (FISH) technology which allows direct visualisation of specific chromosomes in metaphase spreads and interphase cells. The paints themselves represent a large collection of DNA probes generated by fluorescently-labelled PCR from a single chromosome which hybridise to the entire length of the corresponding

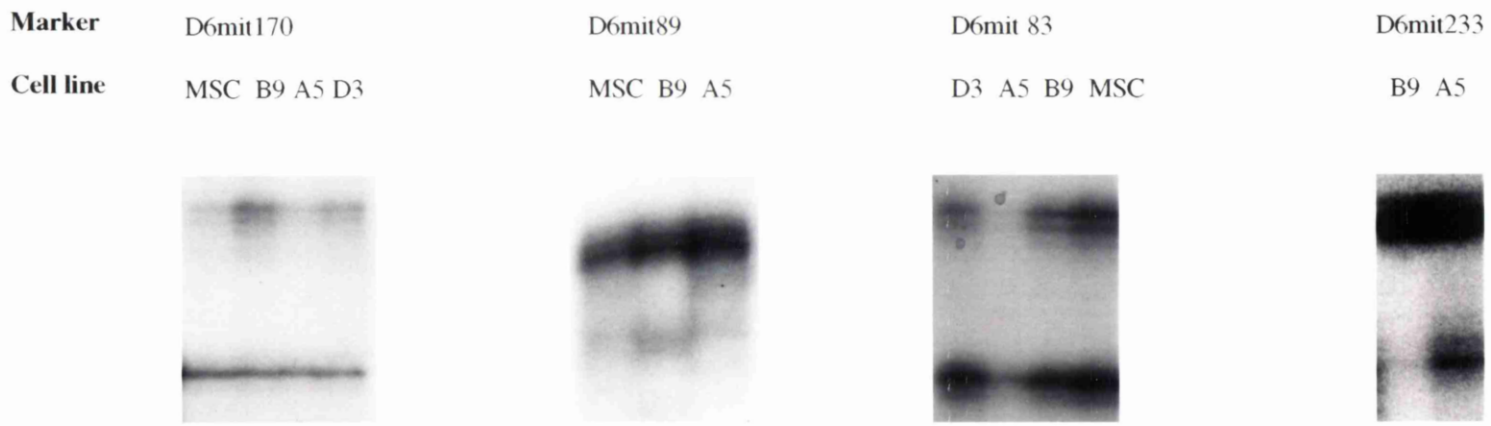


Figure 3.3. Examples of allelic imbalance in mouse cell lines as assayed by PCR amplified microsatellites. MSC, MSCP. See 2.1.13 for a description of cell lines.

chromosome (Rabbitts *et al.*, 1995). In addition to allowing determination of chromosome copy number, paints have found widespread application in defining *de novo* chromosome rearrangements in clinical genetics and cancer cytogenetics (Trask, 1992; Ried *et al.*, 1998).

I examined the copy number of mouse chromosome 6 (and, in a smaller number of cases chromosome 15), in cell lines representing the stages in mouse skin cancer progression. Described briefly, C50 is an immortal, non-tumorogenic line; MSCP is derived from a papilloma; E4 is from a squamous explant; and carB and H11 are derived from highly aggressive spindle cell cancers. Chromosome 15 was chosen as a control because, in contrast to chromosome 6, it is not known to harbour any genes involved in skin cancer progression. Table 3.5 shows representative numbers from the FISH analyses, and figures 3.4 and 3.5 representative chromosome spreads.

Table 3.5 Summary of FISH analysis on mouse cell lines using whole chromosome paints specific to mouse chromosomes 6 and 15

Cell Line	Chromosome 6	Chromosome 15	Total chromosome number
Macrophage*	2	-	40
C50	4	4	65
MSCP	3	-	62
E4	3	2	63
Car B	3	-	62
H11	3	4	69

* Data not shown

All the cell lines examined possessed more than two copies of chromosome 6, helping to explain the finding of allelic imbalance. The cell line C50, an immortal, non-tumorogenic line often taken as representative of the normal keratinocyte, would appear from the point of view of chromosome number to be anything but this. In fact the cells are near-tetraploid, an observation also made of a related non-tumorigenic line, C5N (S. Frame, unpublished).

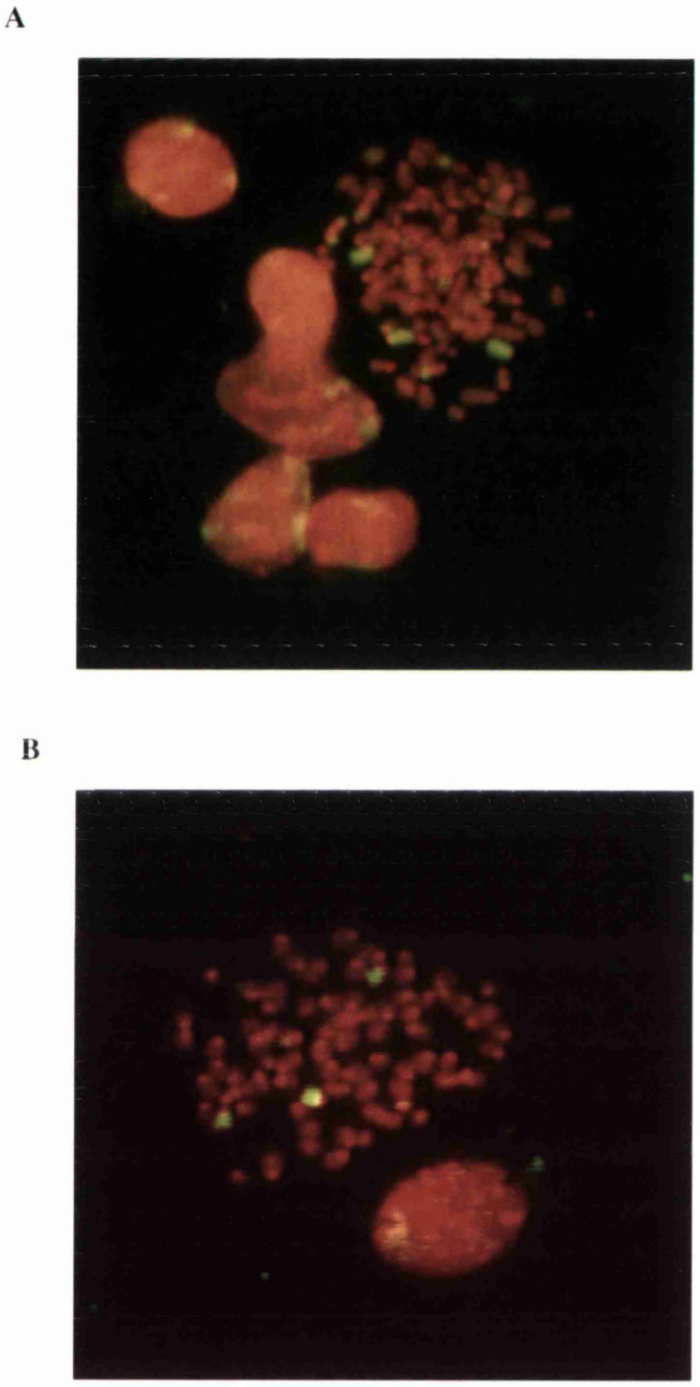
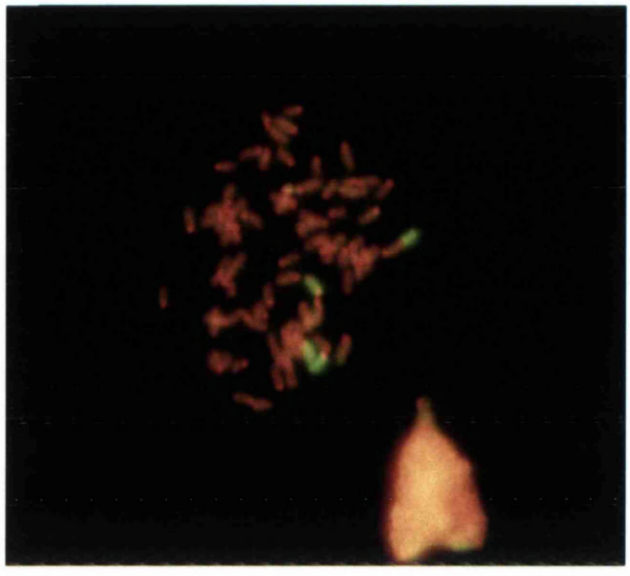


Figure 3.4. Examples of chromosome painting using fluorescently-labelled whole chromosome paints. A, E4 cells using chromosome 6 paint; B, MSCP cells using chromosome 6 paint.



B

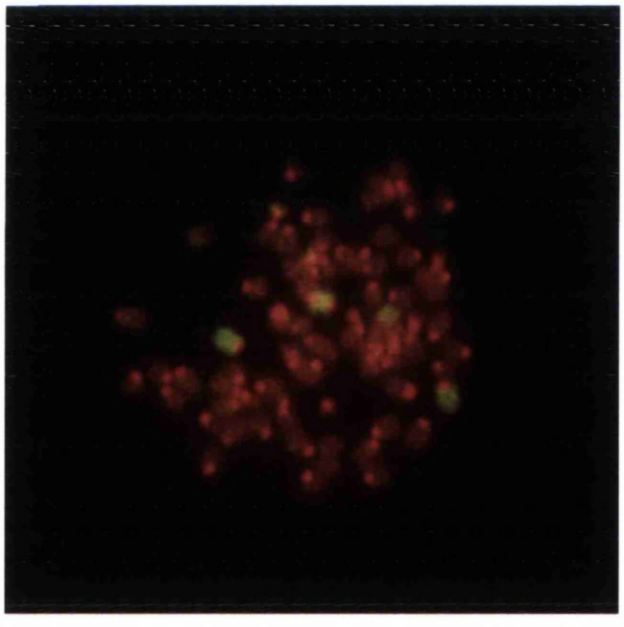


Figure 3.5. Examples of chromosome painting using fluorescently-labelled whole chromosome paints. A, C50 cells using chromosome 6 paint; B, H11 cells using chromosome 15 paint.

3.2.3 Southern blot analysis of candidate TSGs in cell lines

The ability to use information on a potentially homologous gene in human epithelial cancer is a boon of this project: mapping information from work on human chromosome 7 should facilitate the search for the putative TSG in mouse, and *vice versa*. Data generated within our laboratory on human candidate genes were used to look for homozygous deletions at the homologous loci in the mouse cell lines. The two human candidate genes which showed the most promise as tumour suppressor genes were *caveolin-1* and a novel member of the *kelch* gene family, as yet unnamed.

In the case of *caveolin-1*, a 700bp probe derived from the coding region of the human sequence was hybridised directly to Southern blots prepared from mouse cell lines, since homology was expected to be high between the two species. With the novel kelch gene, an 800bp probe was generated from a mouse foetal brain cDNA library using primers derived from mouse ESTs that matched the cloned human kelch cDNA sequence. As a positive control, the Southern blots were probed with a mouse H-*ras* probe. The blots and corresponding gel are shown in figures 3.6 and 3.7. As can be seen, no homozygous deletions were discovered.

3.3 Summary of Allelotyping Data

A moderate degree of LOH is present at mouse chromosome 6A2 and the SCDR may be centred on marker D6mit236. Figure 3.8 shows the position of marker D6mit236 in the context of a gene map of the region, and also demonstrates established synteny with human chromosome 7. The marker D6mit236 has now been mapped to an exonic region of the murine *cftr* gene. Interestingly, the SCDR in LOH studies examining human breast tumours maps to intron 17b of the *CFTR* gene (A. Hurlstone, unpublished).

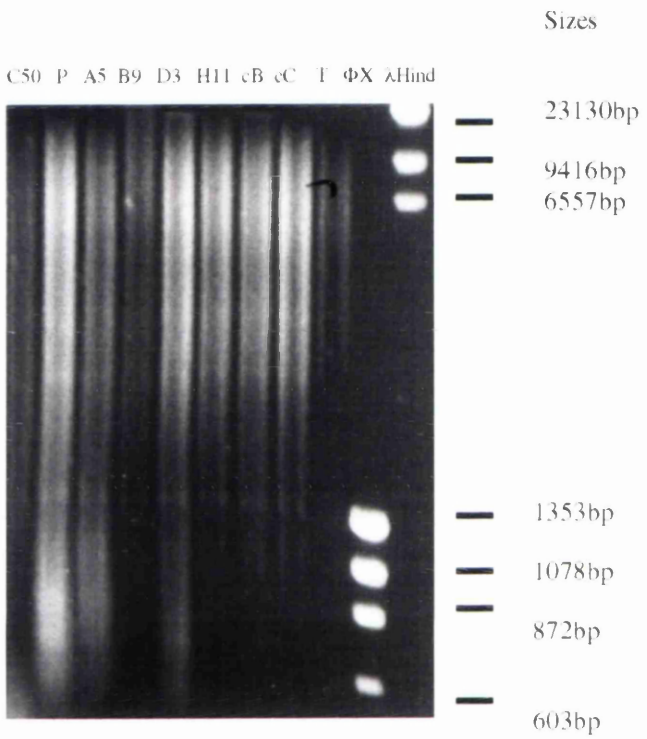


Figure 3.6. Photograph of mouse cell line DNAs (5µg per lane) digested with Bam H1/EcoR1 and run on an agarose gel. Size markers are identified to the right.

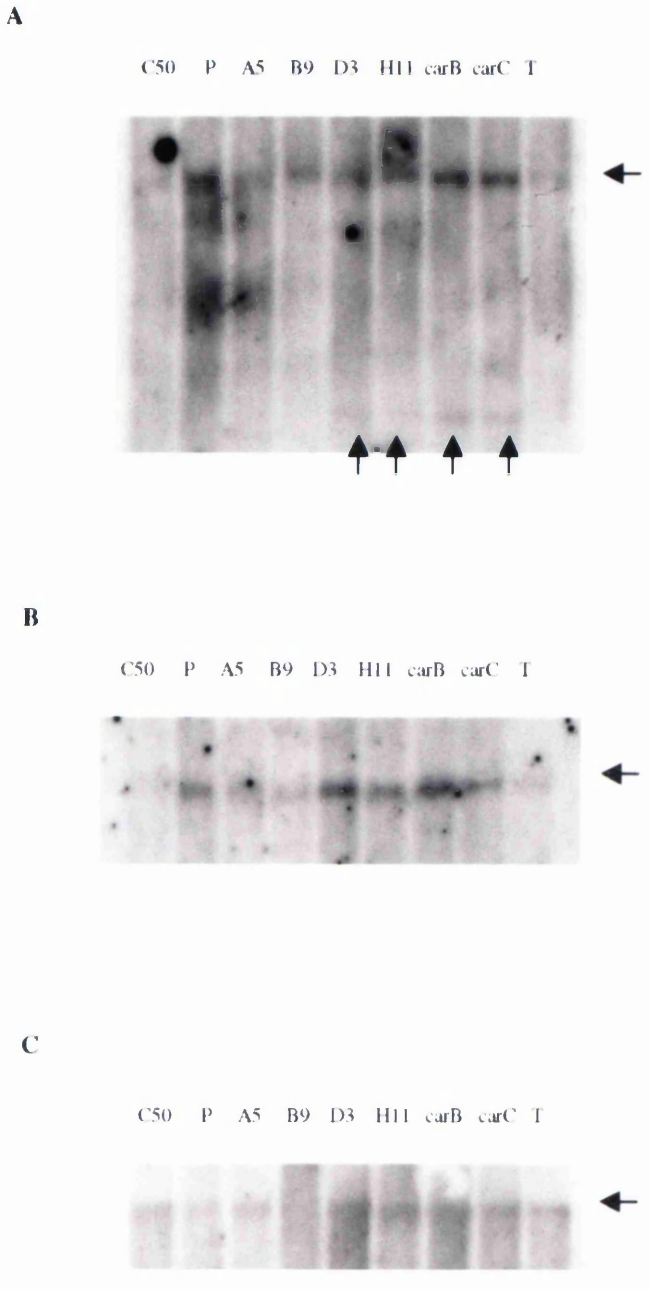


Figure 3.7. Southern blot analysis of cell lines. The blot corresponds to the gel shown in figure 3.6. A is probed with H-*ras* as a positive control. Note the (expected) presence of mutant H-*ras* in some cell lines (vertical arrows). B is probed with *kelch* related gene, and C is probed with *caveolin-1*.

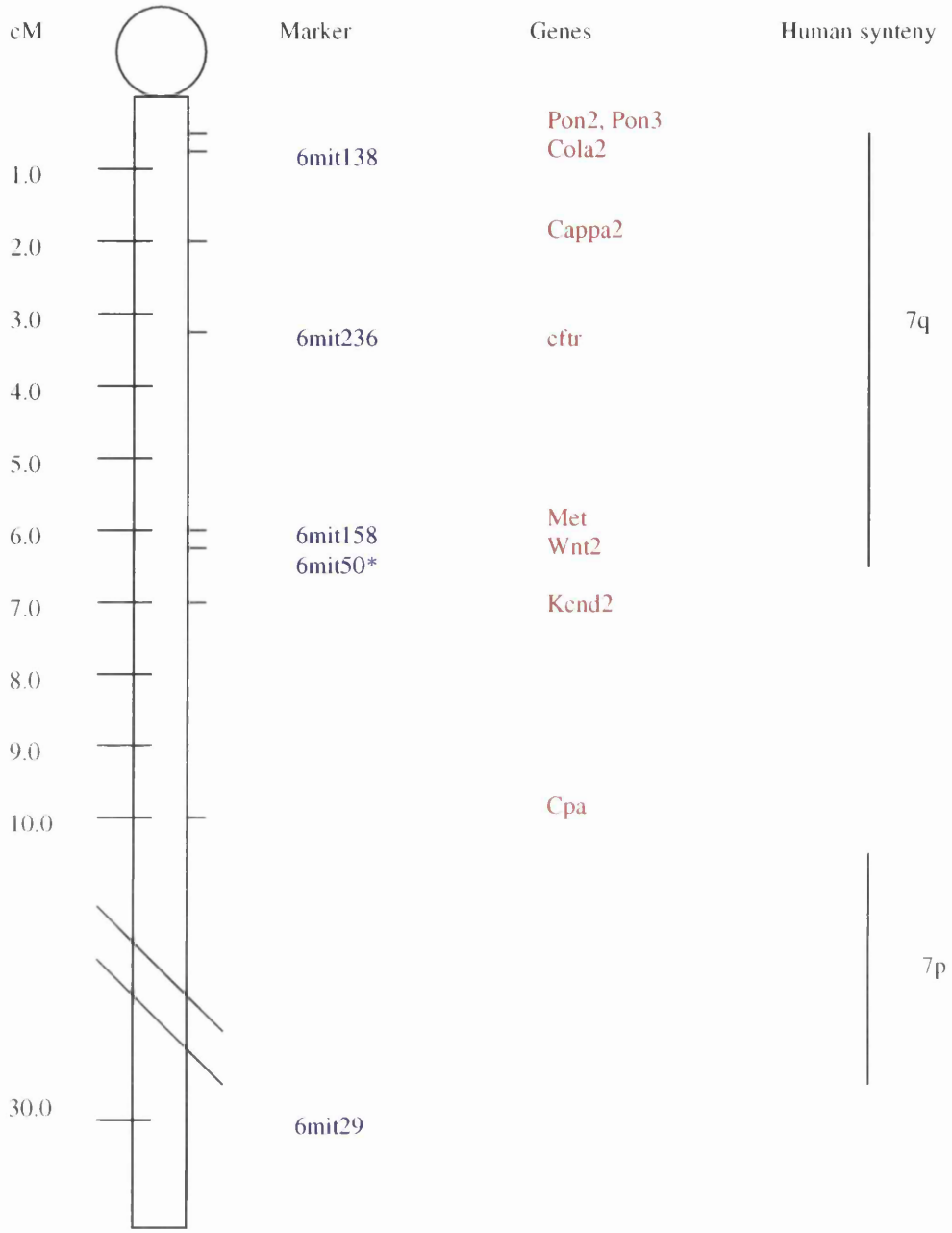


Figure 3.8. Representation of mouse chromosome 6 showing positions of a number of microsatellite markers and genes. Also shown are approximate stretches of known synteny with human chromosome 7.

* D6mit50 (~6cM) represents the SCDR as defined by Zenklusen *et al.*, 1996. This marker has recently been re-ordered, and is now situated at 3.3cM.

CHAPTER 4

RESULTS

ANALYSIS OF CANDIDATE GENES

4. Results

4.1 Cloning of Candidate Genes

Generation of candidate genes that can be examined for mutations in tissues of interest is an integral part of the positional candidate gene approach. In an effort to identify the human putative tumour suppressor gene from human chromosome 7q31, a number of genes were identified from a human senescent fibroblast library which mapped to the area of interest. As already stated, an advantage of this project is that data generated from human studies can expedite a search for the mouse ortholog, and *vice versa*. Full use has therefore been made of information on potential human candidates, and several of these were cloned in the mouse and subsequently screened for mutations in the mouse skin carcinogenesis model. The cloning of three such genes is described.

An overall schema showing the steps involved in isolating genes of interest from a cDNA phagemid library is shown in figure 4.1. Bacteriophage infected host bacteria are poured over agar plates and incubated for several hours, allowing plaques to appear on a bacterial 'lawn'. Plaques are lifted onto a nitrocellulose filter, denatured, washed and dried. The DNA is fixed to the membrane by exposure to UV light. A radiolabelled probe is then applied to the membrane lifts in hybridisation buffer, the filters are washed and exposed to photographic film to obtain an autoradiograph. From the Xray film, positive colonies are identified and isolated. The process is repeated twice to obtain positive secondary and tertiary clones. The isolated positive tertiary clones are then grown in liquid culture, the DNA isolated and finally analysed.

4.2 Putative cyclin G1 binding protein

One of the genes isolated from a human senescent fibroblast cDNA library (by virtue of its being upregulated) and which mapped to the human region of interest

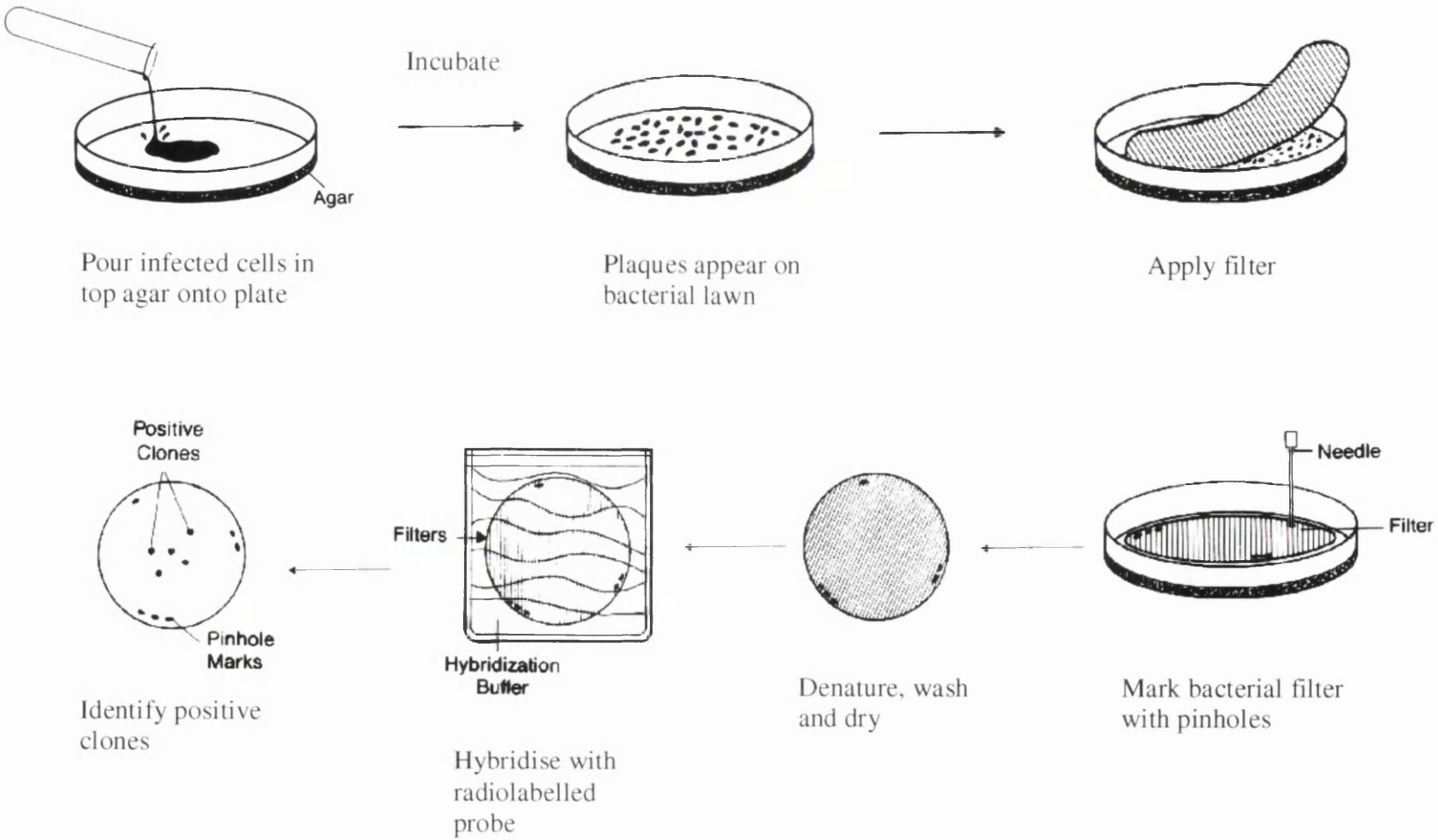


Figure 4.1. Stages involved in screening of a library (in this case a mouse full length cDNA library) for a sequence of interest. The plaques formed represent infection of bacteria by phage particles, with subsequent lysis of cells. See text for details.

was a putative cyclin G1 binding protein. It is known from information in public databases to map to the human region 7q22-31 and, since a high degree of synteny is observed between 7q and mouse 6A, it is expected to map to the area of high frequency LOH in the mouse skin carcinogenesis system. It therefore qualifies tentatively as a potential candidate gene.

A search of the mouse EST databases was undertaken using the full-length human cDNA sequence and, from homologous hits, primers were designed to amplify a ~300bp fragment to be used as a probe. Figure 4.2A shows the PCR product using these primers with cDNA prepared from a mouse keratinocyte cell line (C5N) and genomic (tail) DNA as template. As can be seen the PCR product from genomic DNA is significantly larger than that from the cDNA template, implying the presence of an intron. The 300bp fragment was excised, radiolabelled and used to probe a mouse fetal cDNA library. Figure 4.2B shows the autoradiograph of the primary filter. Figure 4.3A shows an autoradiograph of the secondary filter obtained after plating one of the chosen clones arrowed in figure 4.2B. Two clones were then isolated, and figure 4.3B shows the insert size of both after excision from vector and resolution on an agarose gel. Both clones were sequenced and the resulting sequence used to search the mouse EST database for homologies using the blast protocol (Altschul *et al.*, 1997). Both sequences in fact corresponded to the mouse putative cyclin G1 bindng protein. Clone 2 was however more complete, spanning the start and stop codons.

Figures 4.4 and 4.5 show the complete DNA coding region of the mouse putative cyclin G1 binding protein and its degree of homology to the human sequence at the DNA and protein levels. As might be expected the level of homology between man and mouse is high: 88% at the nucleic acid level, and 78% at the amino acid level.

I next looked for evidence of mutations by single strand conformational polymorphism (SSCP) in the cloned gene in the panel of mouse cell lines representative of the multiple stages in skin carcinogenesis. SSCP is a relatively

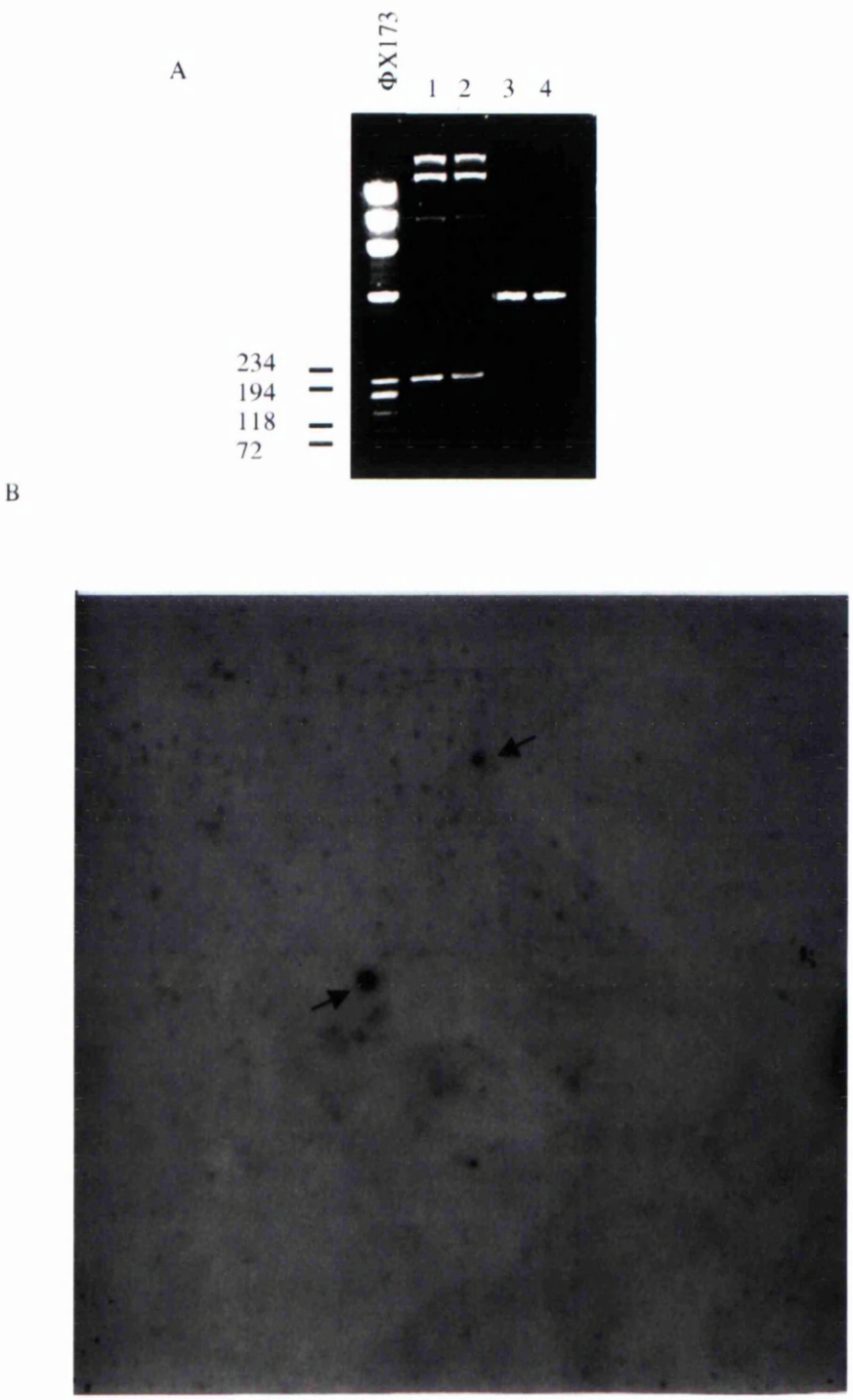


Figure 4.2. A, PCR product used to probe the primary filter of a mouse foetal brain cDNA library. Lanes are numbered 1-4: 1 and 2 are identical, as are 3 and 4. This was to ensure high yield of DNA for radiolabelling. Template for PCRs 1 and 2 is C5N cDNA, and for 3 and 4, genomic (tail) DNA. Marker sizes are shown in bp to the left. The ~300bp product was excised from the agarose gel and genecleaned before use. B, picture (60% lifesize) of autoradiograph from primary filter probed with radiolabelled probe shown in A. The clones chosen to be plated onto secondary plates are arrowed.

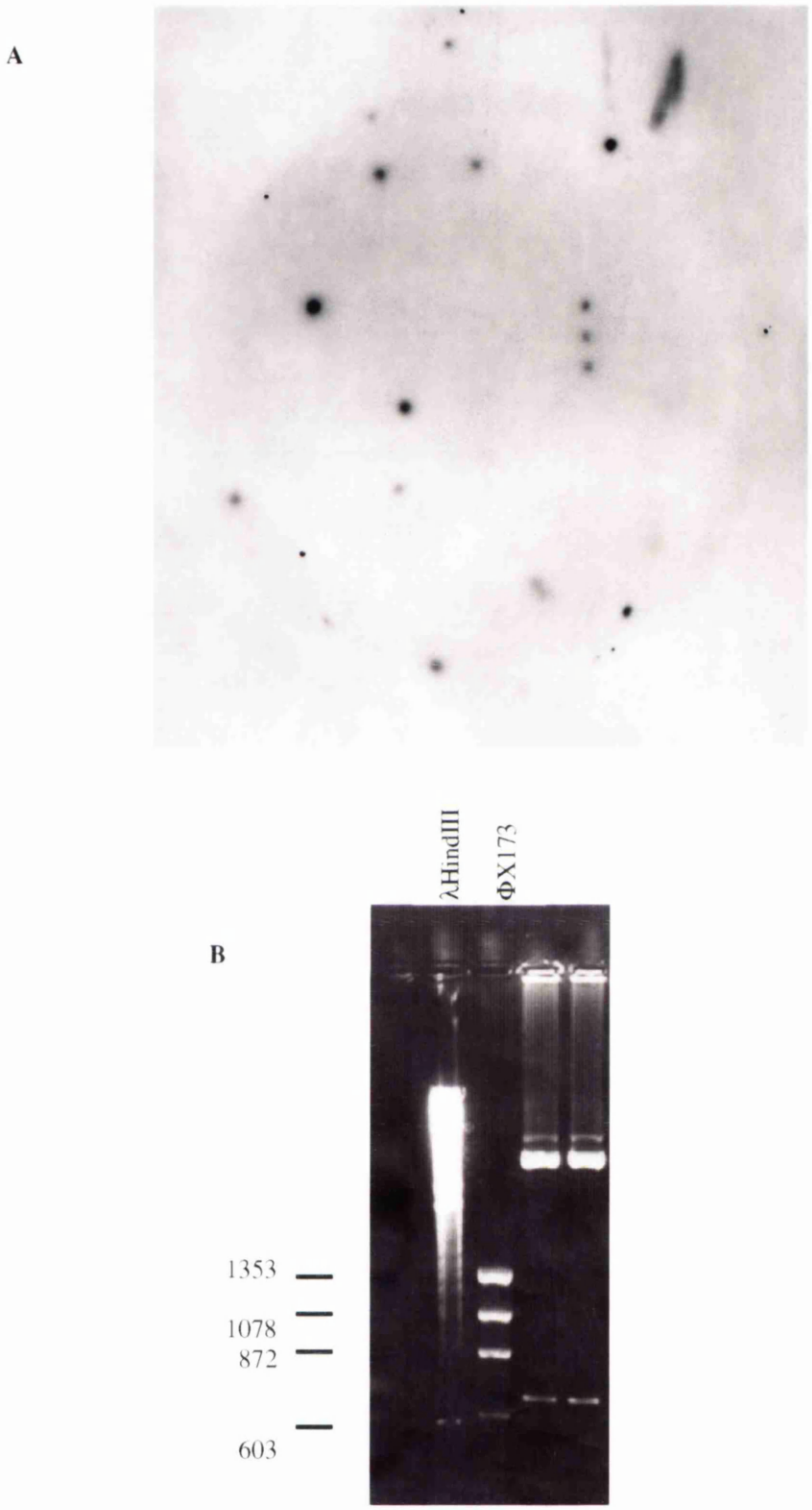


Figure 4.3. A, autoradiograph of secondary filter of cg1bp clones reprobed with the original PCR product shown in figure 1. B, excised inserts of two clones shown in A, after resolution on an agarose gel. Size markers are on the left.

A

```
ccaatggtggagaagaaacccgccgttcgctcacaggaccctggacagcggcgggtgctg
    M  V  E  K  K  P  A  V  R  S  Q  D  P  G  Q  R  R  V  L
gacagggcagccaggcagcggcggatcaaccgacagctggaggcattggagaatgacaac
 D  R  A  A  R  Q  R  R  I  N  R  Q  L  E  A  L  E  N  D  N
ttccaggatgacccccatgccggcctcccccagctgggcaagaggctacctcagtttgat
 F  Q  D  D  P  H  A  G  L  P  Q  L  G  K  R  L  P  Q  F  D
gatgatgcagacacaggaaagaaaaagaagaaaactcgaggtgaccactttaaacttcgc
 D  D  A  D  T  G  K  K  K  K  K  T  R  G  D  H  F  K  L  R
ttccggaaaaacttccaggctttgctggaggagcagaacctgagtgcatctgagggcccc
 F  R  K  N  F  Q  A  L  L  E  E  Q  N  L  S  A  S  E  G  P
aactacttgacagcctgtgccggcccccatctcggccccagcgtcccttctgtgctgtg
 N  Y  L  T  A  C  A  G  P  P  S  R  P  Q  R  P  F  C  A  V
tgtggcttcccatccccgtatacctgtgtgagctgtggtgcccggtactgcaccgtgcgc
 C  G  F  P  S  P  Y  T  C  V  S  C  G  A  R  Y  C  T  V  R
tgcctgggtacccatcaggagaccaggtgtctgaagtggaccgtgtaaacctggcatcag
 C  L  G  T  H  Q  E  T  R  C  L  K  W  T  V STOP
ggagagaaagtacccccaccttcccaacccaccccagctcat
```

B

```
Mouse: 1   MVEKKPAVRSQDPGXXXXXXXXXXXXXXINRQLEALENDNFQDDPHAGLPQLGKRLPQFXX 60
           MVEKK +VRSQDPG               INRQLEALENDNFQDDPHAGLPQLGKRLPQF
Human: 1   MVEKKTSVRSQDPGQRRVLDRAARQRRINRQLEALENDNFQDDPHAGLPQLGKRLPQFDD 60

Mouse: 61  XXXXXXXXXXXXXXXHFKLRFRKNFQALLEEQNLSASEGPNYLTACAGPPSRPQRPFCAVC 120
                          HFKLRFRKNFQALLEEQNLS +EGPNYLTACAGPPSRPQRPFCAVC
Human: 61  DADTGKKKKKTRGDHFKLRFRKNFQALLEEQNLSVAEGPNYLTACAGPPSRPQRPFCAVC 120

Mouse: 121 GFPSPYTCVSCGARYCTVRCLGTHQETRCLKWTV 154
           GFPSPYTCVSCGARYCTVRCLGTHQETRCLKWTV
Human: 121 GFPSPYTCVSCGARYCTVRCLGTHQETRCLKWTV 154
```

Figure 4.4. A, complete coding sequence and translation of the mouse putative cyclin G1 binding protein. B, homology at the protein level between mouse and human sequences. The consensus is highlighted.

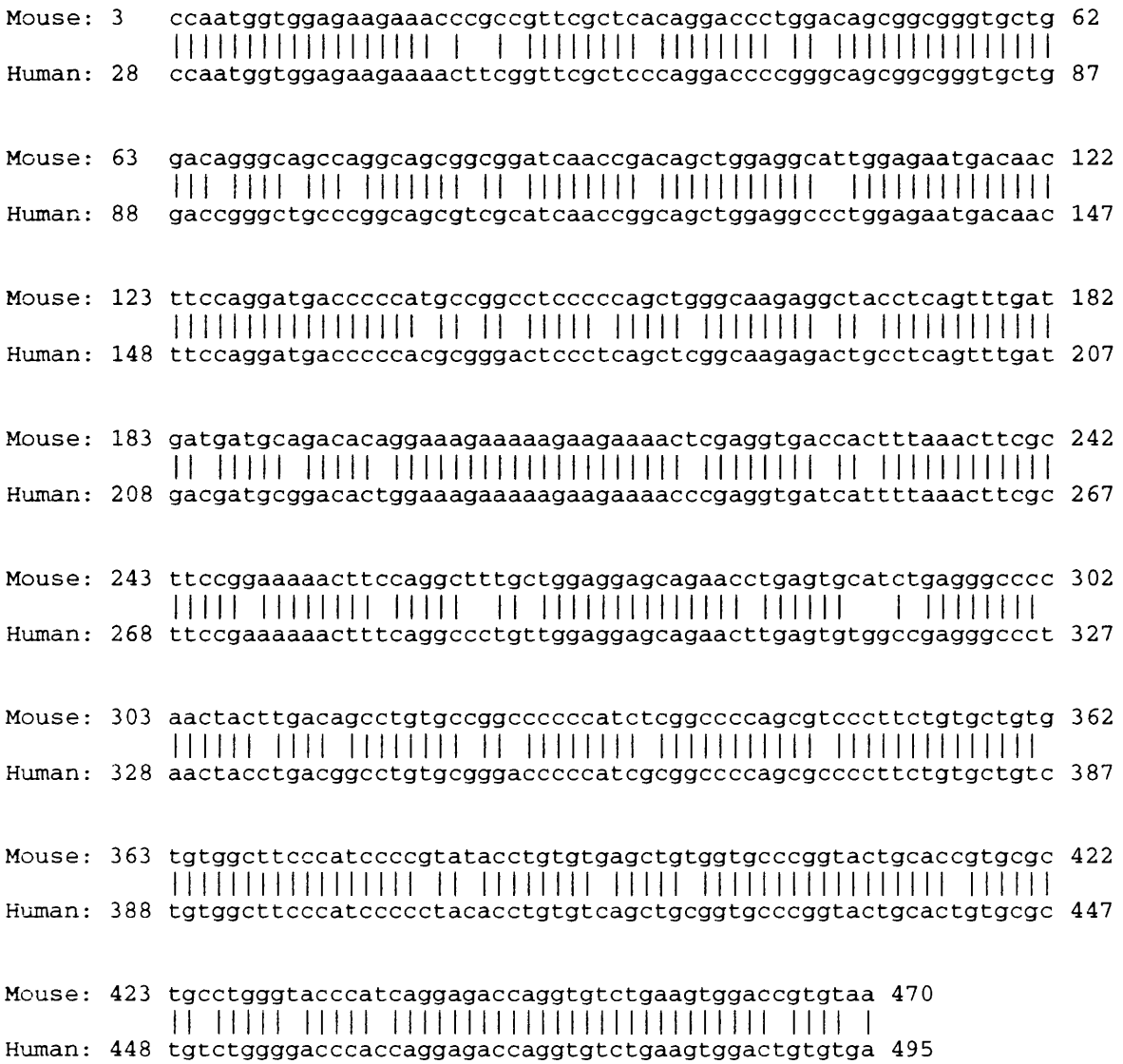


Figure 4.5. cDNA sequence comparison between the mouse and human putative cyclin G1 binding protein

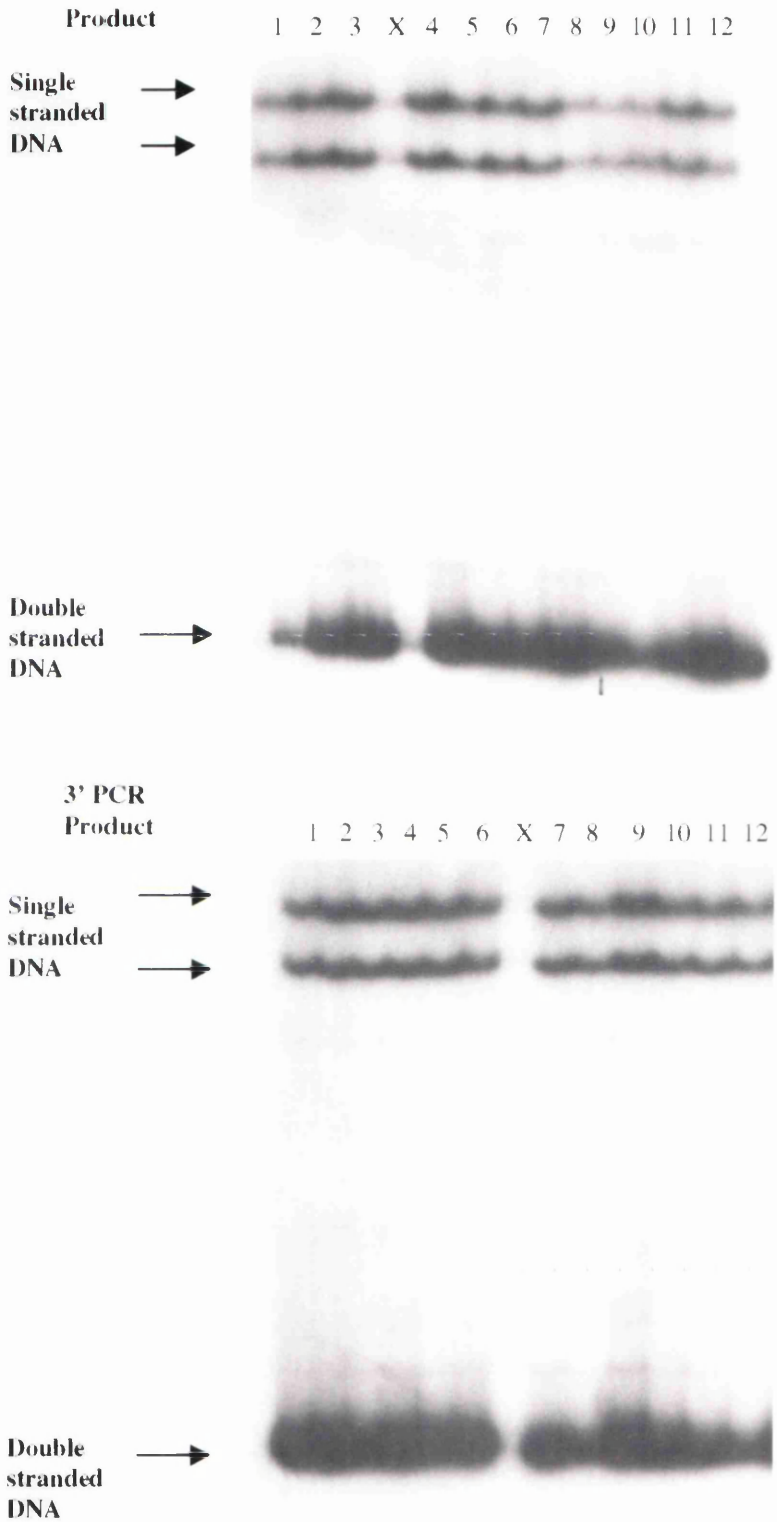


Figure 4.6. SSCP analysis of putative cyclin G1 binding protein in mouse cell lines. The gene was analysed in two regions, 5' and 3' products. Lanes are marked 1-12, corresponding to the following cell lines: 1, C5N; 2, C50; 3, MSCP1; 4, B9; 5, A5; 6, D3; 7, CarB; 8, CarC; 9, SN161; 10, E4; 11, H11; 12, PDV

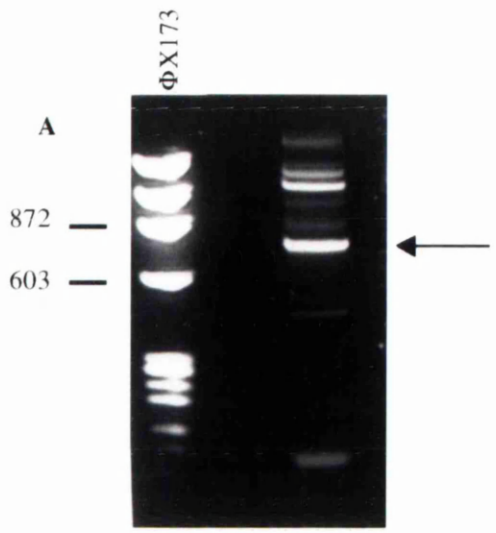
simple technique which allows rapid screening of DNA samples for mutations or deletions with an acceptable level (~80%) of sensitivity. The technique is predicated on the fact that electrophoretic mobility of single stranded DNA is dependent not only on length but also on conformation, which is in turn dictated by sequence. As originally described, the technique involved digestion of genomic DNA prior to running on a neutral polyacrylamide gel. However, the technique can be applied equally well to PCR amplified genomic DNA and, as in this study, cDNAs created by the RT-PCR method. The optimum length of sequence for analysis is ~200bp, but 100-300bp fragments are acceptable.

The SSCP gels are shown in figure 4.6. As can be seen, no sequence variants were detected by SSCP.

4.3 Novel *kelch* Gene

The second human candidate gene, once more identified from a human senescence library by virtue of its upregulation, appeared to be a novel homologue of the *Drosophila kelch* gene (Xue and Cooley, 1993). Again, it mapped to the long arm of human chromosome 7.

Human cDNA sequence was used to identify mouse ESTs from the public database, from which primers were designed to generate an 800bp mouse probe. Figure 4.7 shows the PCR product which was radiolabelled and used to probe the mouse cDNA library, along with the autoradiograph of the primary filter. Figure 4.8 shows examples of the corresponding secondary and tertiary filters. A total of five clones were isolated and sequenced to ensure complete coverage of the mouse *kelch* coding region. PCRs using a variety of primers designed from mouse ESTs and compared to the known human sequence are shown in figure 4.9. This provided a rapid method of "sequencing" the clones. The entire cDNA and amino acid sequences are shown in figure 4.10, and figure 4.11 depicts the positions of the conserved protein domains as predicted by the blastp search protocol. Homology to the human *kelch* related



B

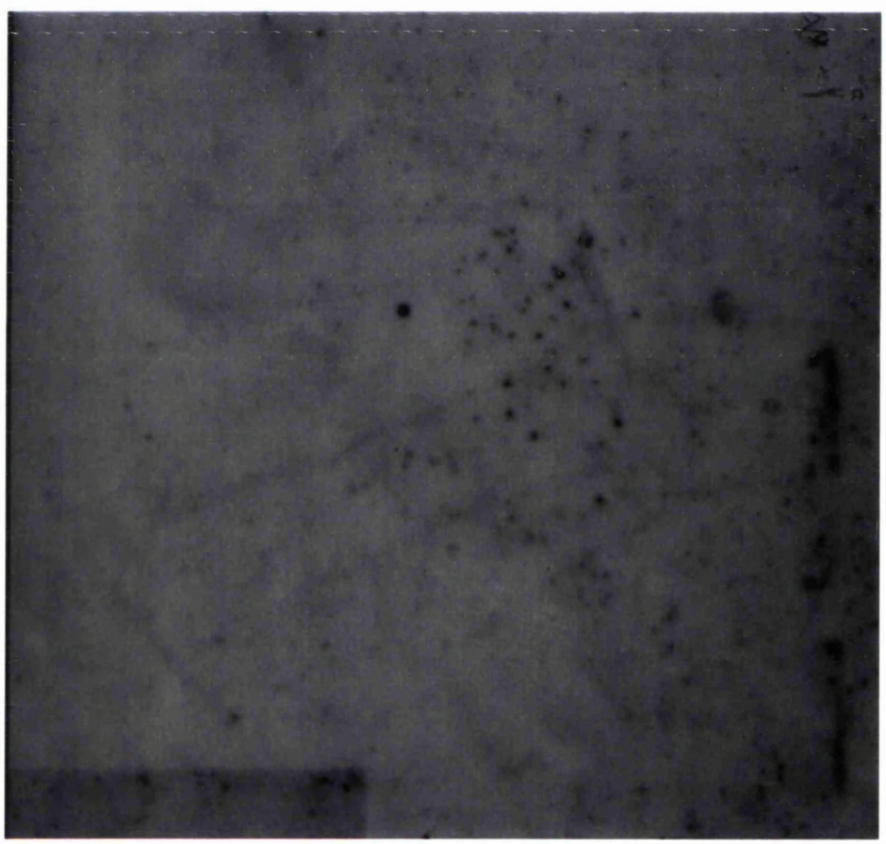


Figure 4.7. A, Kelch probe (arrowed) generated using PCR primers designed from mouse ESTs found to be homologous to the human *kelch* sequence. The probe was excised and genecleaned before use. Size markers are identified on the left. B, autoradiograph of primary filter probed with the radiolabelled PCR product shown in A.

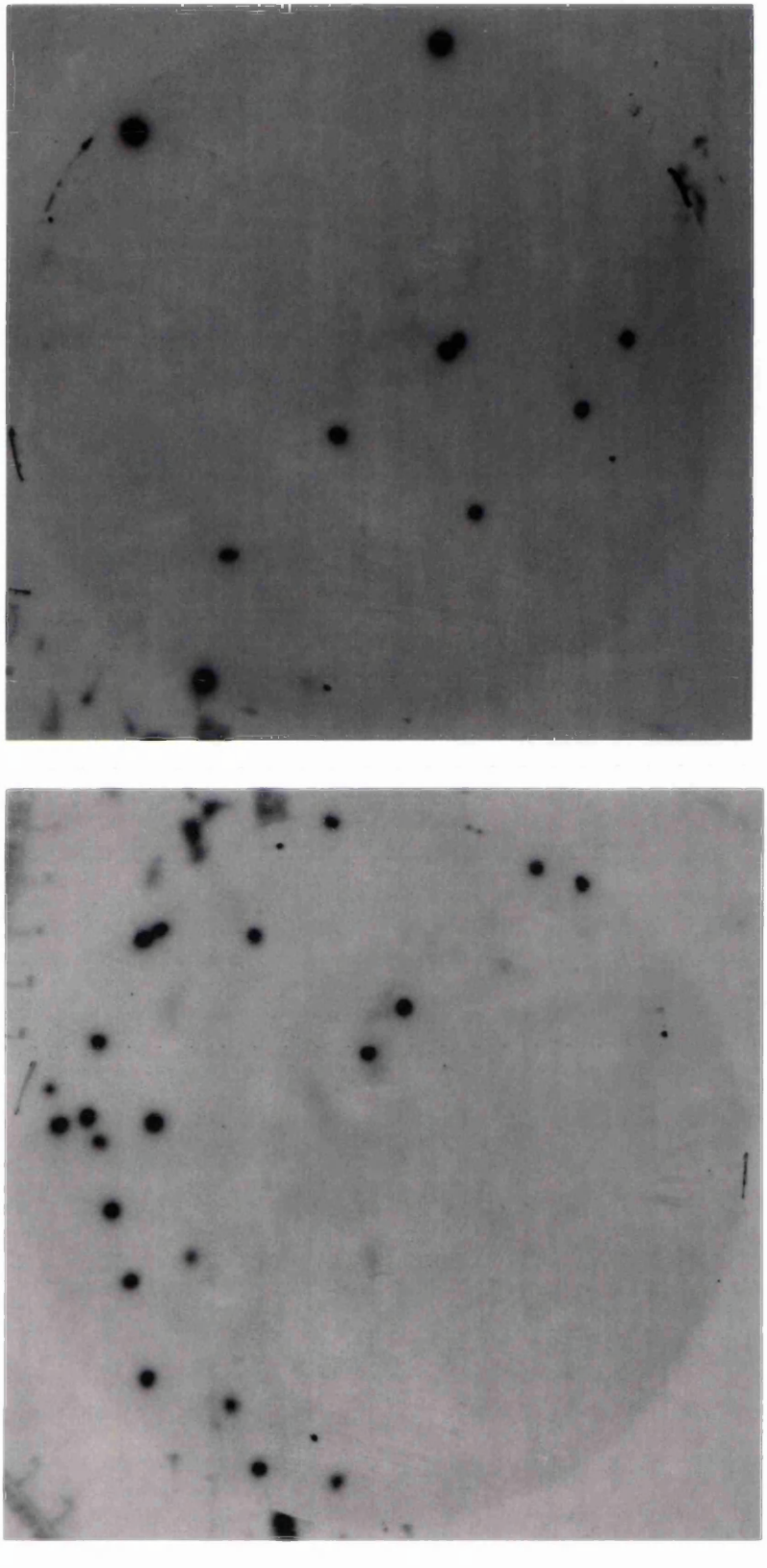


Figure 4.8. A and B, autoradiographs of a secondary and tertiary filter, respectively, of mouse *kelch* clones isolated from the primary filter shown in figure 4.7. Several secondary and related tertiary plates were produced, from which a total of five clones were isolated

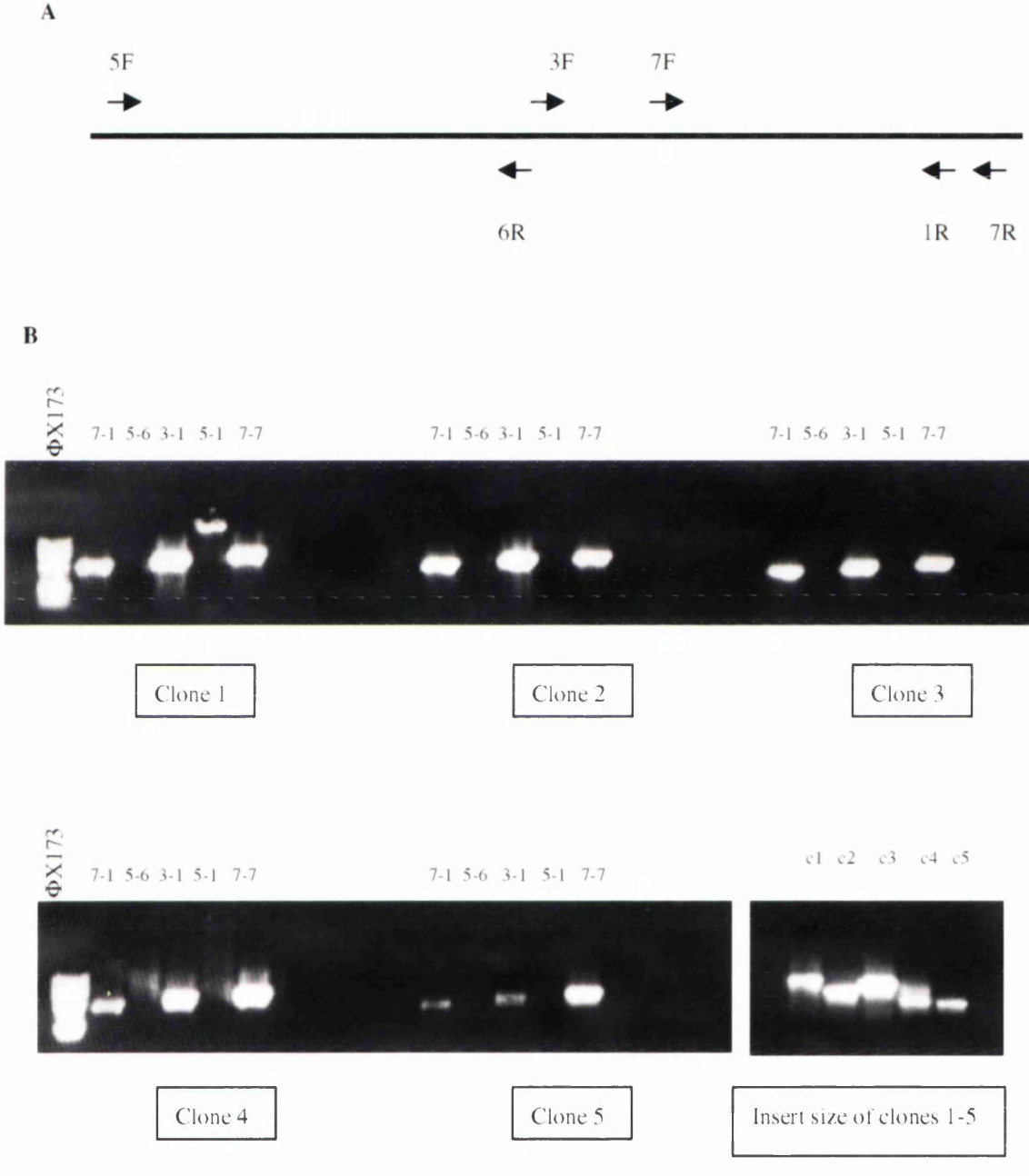


Figure 4.9. A, primers were designed from mouse ESTs corresponding to the human *kelch* related gene, and used to amplify the mouse clones. Their approximate relative positions are shown. B, PCR amplification products corresponding to the five mouse clones. Failure to amplify implies absence of related sequence. Also shown is the insert size of each clone using SK and KS primers which bound the polylinker site. The apparent band corresponding to product 5-6 from clone 4 is artefactual. The 5F-6R primer combination consistently failed to amplify.

```
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                                        M  I  P  N  G  Y  L  M
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 F  E  D  E  N  F  I  E  S  S  V  A  K  L  N  A  L  R  K  S
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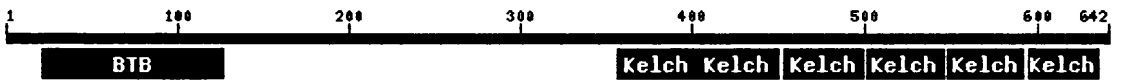
Figure 4.10. Complete coding sequence of the novel *kelch* gene, with protein tranalation.

A



B

repeat	from	to	fragment
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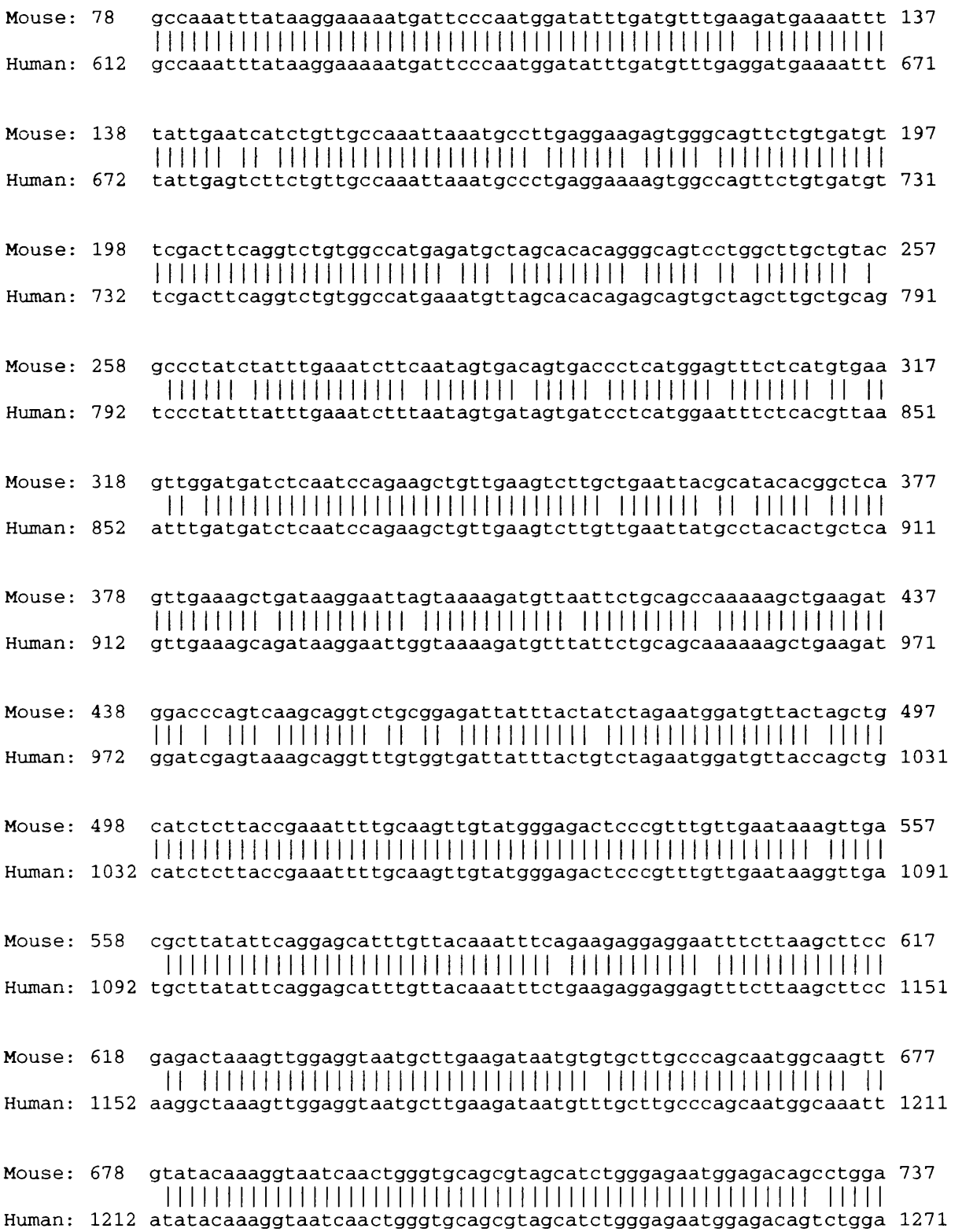
KELCH	416	463	QLYVVGGSNGHSDDLSCGEMYDPNIDDWTPVPELRTNRCNAGVCALNG
KELCH	465	512	LYIVGGSDPYGQKGLKNCDVFDPVTKSWTSCAPLNIRRHQSAVCELGG
KELCH	514	559	LYIIEGAESWNCLNTVERYNPENNTWTLIAPMNVARRGAGVAVLDG
KELCH	560	606	KLFVGGGFDGSHAISCVEMYDPTRNEWKMMGNMTSPRSNAGITTVGN
KELCH	608	642	IYAVGGFDGNEFLNTVEVYNPQSNEWSPYTKIFQF

Figure 4.11. A, Pictogram of the primary amino-acid sequence of *kelch* related protein showing the approximate positions of the POZ/BTB domain and kelch repeats. B, amino-acid number position and composition of the kelch repeats, as identified using the blastp protocol.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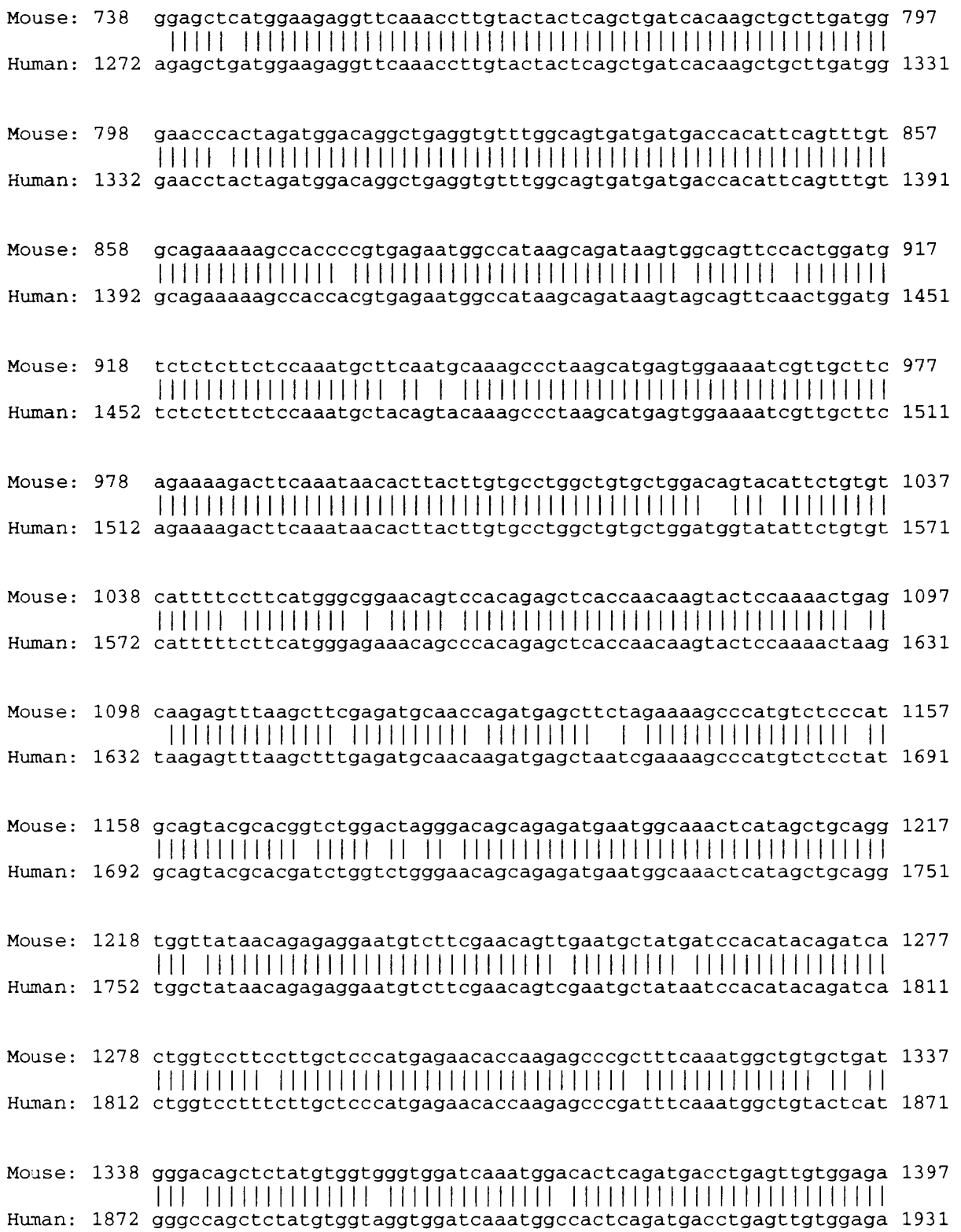
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```

Figure 4.12. Demonstration of homology between mouse and human novel *kelch* cDNA sequences. Start and stop codons are highlighted in red.

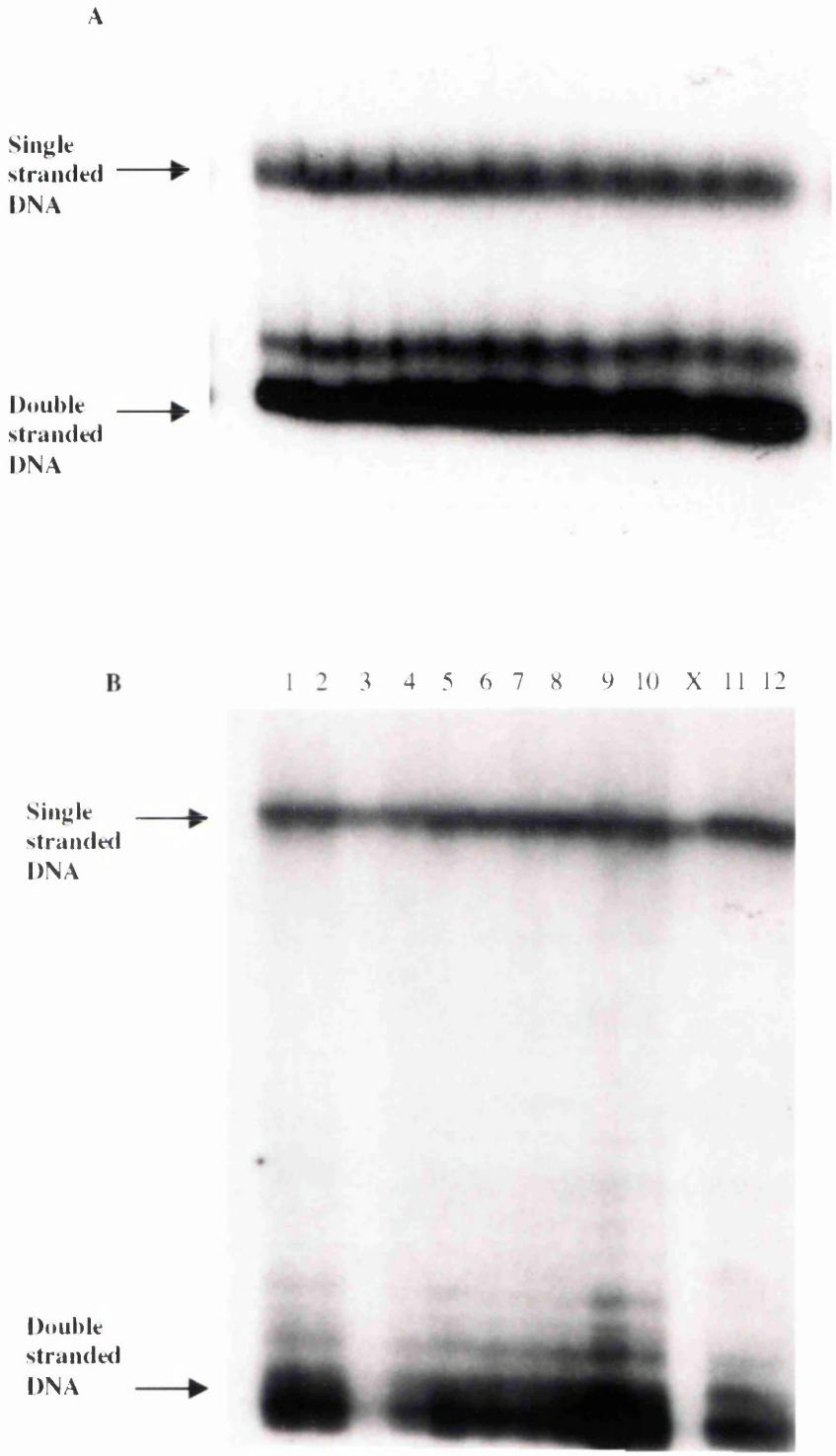


Figure 4.13. Example of SSCP analysis of cDNAs corresponding to mouse *kelch*-related gene. A, 250b segment from the 5' end. B, 200b segment from the centre portion of the gene. Lanes are marked 1-12, corresponding to the following cell lines: 1, C5N; 2, C50; 3, MSCP1; 4, B9; 5, A5; 6, D3; 7, CarB; 8, CarC; 9, SN161; 10, E4; 11, H11; 12, PDV

gene cloned in our laboratory is shown in figure 4.12. Again a high degree of homology was evident between man and mouse: 93% overall homology at the DNA level and 94% at the protein level.

Again, mutations were looked for in the cloned gene in mouse cell lines using SSCP. Examples of the SSCP gels are shown in figure 4.13. No sequence variations and therefore mutations were evident

4.4 Novel Member of *l(2)gl* Gene Family

The third gene examined was a novel member of the lethal in giant larvae (*l(2)gl*) gene family. This gene is known to be a tumour suppressor in *Drosophila*, where it was first characterised (Strand *et al.*, 1994). It was isolated from the human senescent fibroblast library. Its chromosomal position was not known. However, given its known role in embryogenesis and its tumour suppressor function in *Drosophila,* it was thought worthy of further study.

A 900bp human probe was used directly to probe the mouse cDNA library. The corresponding positive primary and secondary lifts are shown in figure 4.14. Despite a total of eight clones being isolated, I was unable to find any that were representative of the 3' and of the gene. I therefore designed forward primers from the 3'most sequence to be used in combination with the KS primer of the vector, yielding a ~600bp probe. This too failed to identify any clones representing the 3' end of the gene. Primers designed directly from the 3' region of the known human sequence were not helpful. I was therefore unable to clone the entire coding region of the novel *l(2)gl* gene. The obtained sequence is shown in figure 4.15.

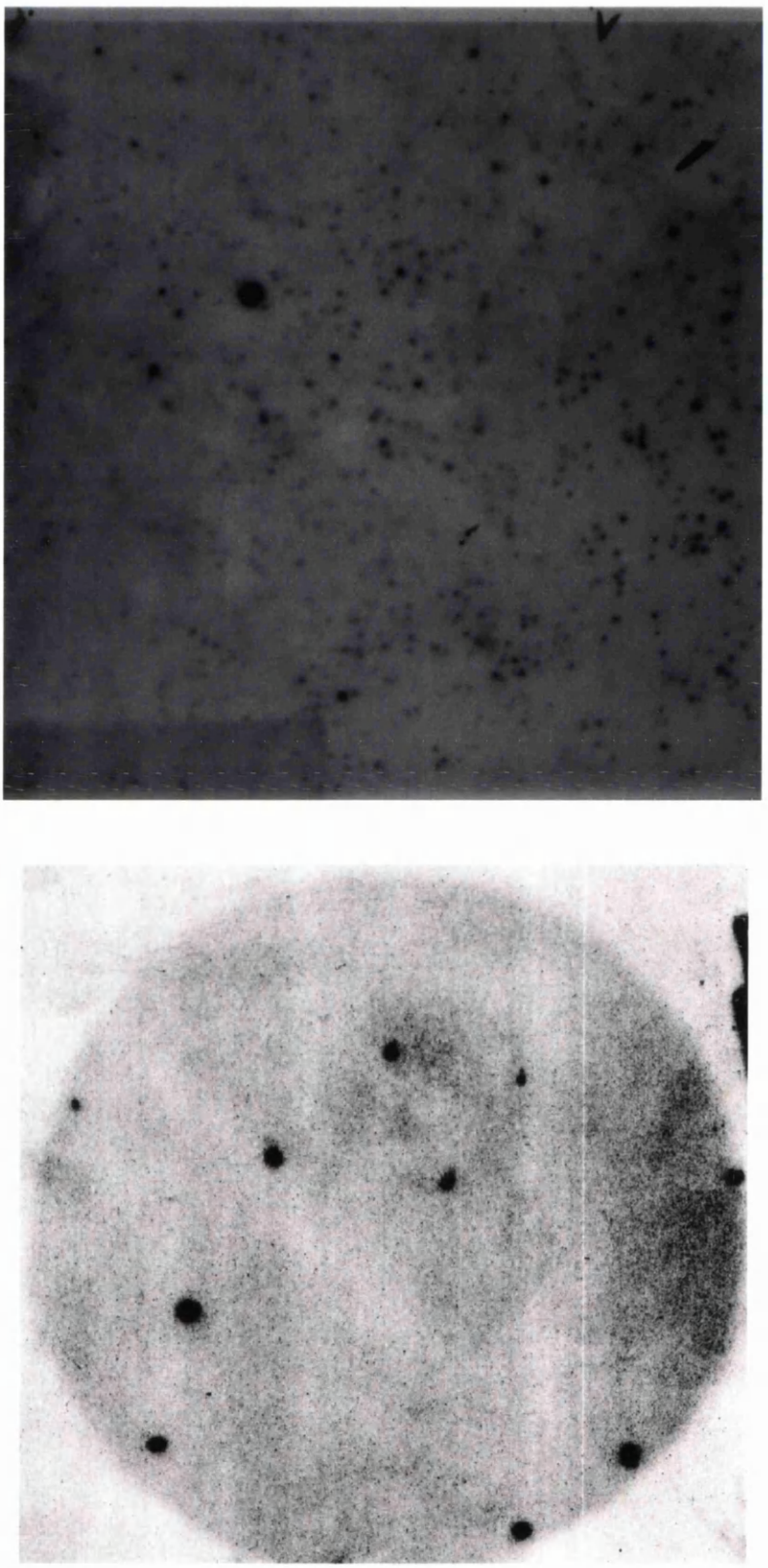
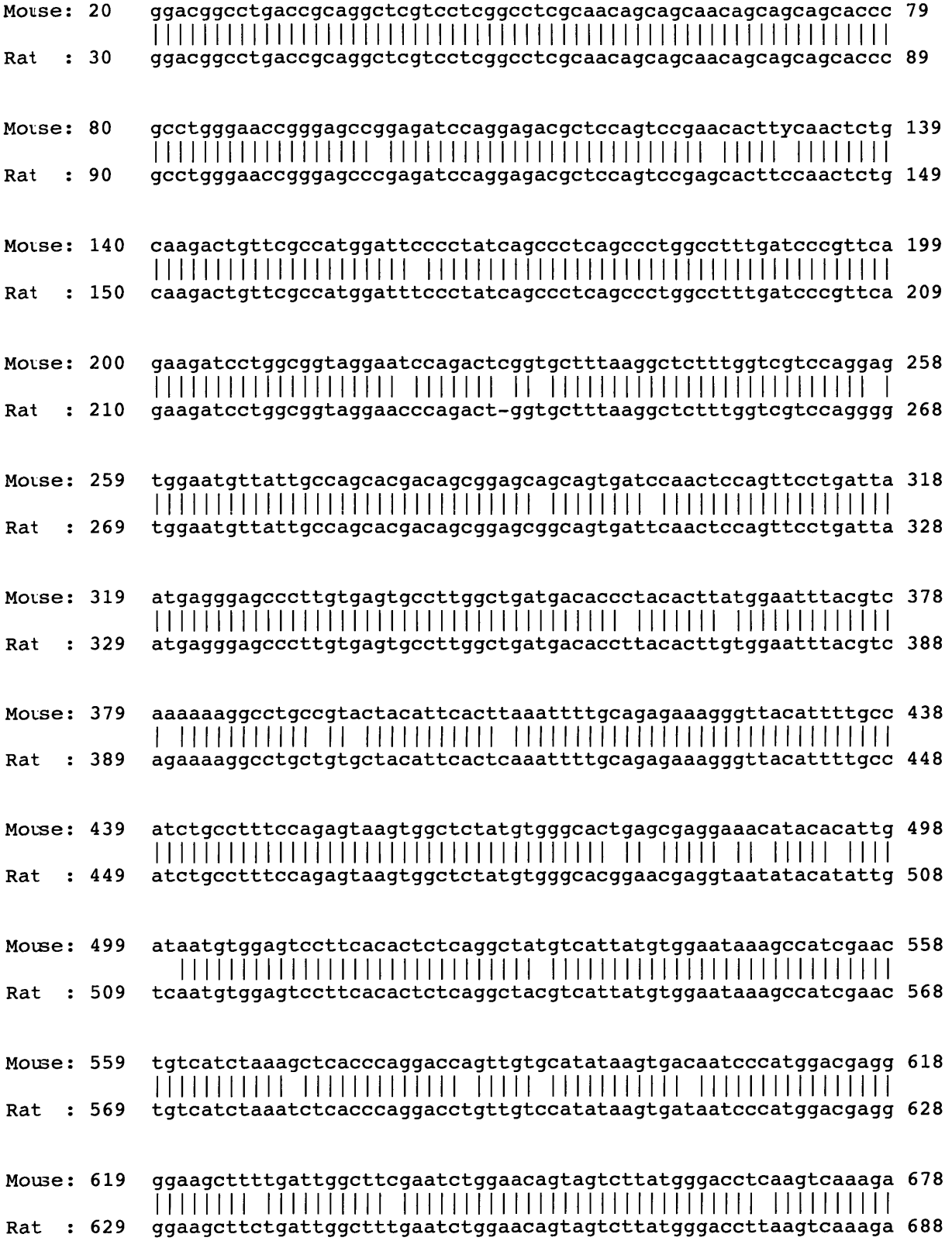


Figure 4.14. Autoradiographs of primary (A) and secondary (B) filters corresponding to mouse *l(2)gl* clones.


Mouse: 20 ggacggcctgaccgcaggctcgtcctcggcctcgcaacagcagcaacagcagcagcaccc 79
Rat : 30 ggacggcctgaccgcaggctcgtcctcggcctcgcaacagcagcaacagcagcagcaccc 89
Mouse: 80 gcctgggaaccgggagccggagatccaggagacgctccagtccgaacacttycaactctg 139
Rat : 90 gcctgggaaccgggagcccgagatccaggagacgctccagtccgagcacttccaactctg 149
Mouse: 140 caagactgttcgccatggattcccctatcagccctcagccctggcctttgatcccgttca 199
Rat : 150 caagactgttcgccatggatttccctatcagccctcagccctggcctttgatcccgttca 209
Mouse: 200 gaagatcctggcggtaggaatccagactcggtgctttaaggctctttggtcgtccaggag 258
Rat : 210 gaagatcctggcggtaggaacccagact-ggtgctttaaggctctttggtcgtccagggg 268
Mouse: 259 tggaatgttattgccagcacgacagcggagcagcagtgatccaactccagttcctgatta 318
Rat : 269 tggaatgttattgccagcacgacagcggagcggcagtgattcaactccagttcctgatta 328
Mouse: 319 atgagggagcccttgtgagtgccttggctgatgacaccctacacttatggaatttacgtc 378
Rat : 329 atgagggagcccttgtgagtgccttggctgatgacaccttacacttgtggaatttacgtc 388
Mouse: 379 aaaaaaggcctgccgtactacattcacttaaattttgcagagaaagggttacattttgcc 438
Rat : 389 agaaaaggcctgctgtgctacattcactcaaattttgcagagaaagggttacattttgcc 448
Mouse: 439 atctgcctttccagagtaagtggctctatgtgggcactgagcgaggaaacatacacattg 498
Rat : 449 atctgcctttccagagtaagtggctctatgtgggcacggaacgaggtaatatacatattg 508
Mouse: 499 ataatgtggagtccttcacactctcaggctatgtcattatgtggaataaagccatcgaac 558
Rat : 509 tcaatgtggagtccttcacactctcaggctacgtcattatgtggaataaagccatcgaac 568
Mouse: 559 tgtcatctaaagctcacccaggaccagttgtgcatataagtgacaatcccatggacgagg 618
Rat : 569 tgtcatctaaatctcacccaggacctgttgtccatataagtgataatcccatggacgagg 628
Mouse: 619 ggaagctttgattggcttcgaatctggaacagtagtcttatgggacctcaagtcaaaga 678
Rat : 629 ggaagcttctgattggctttgaatctggaacagtagtcttatgggaccttaagtcaaaga 688

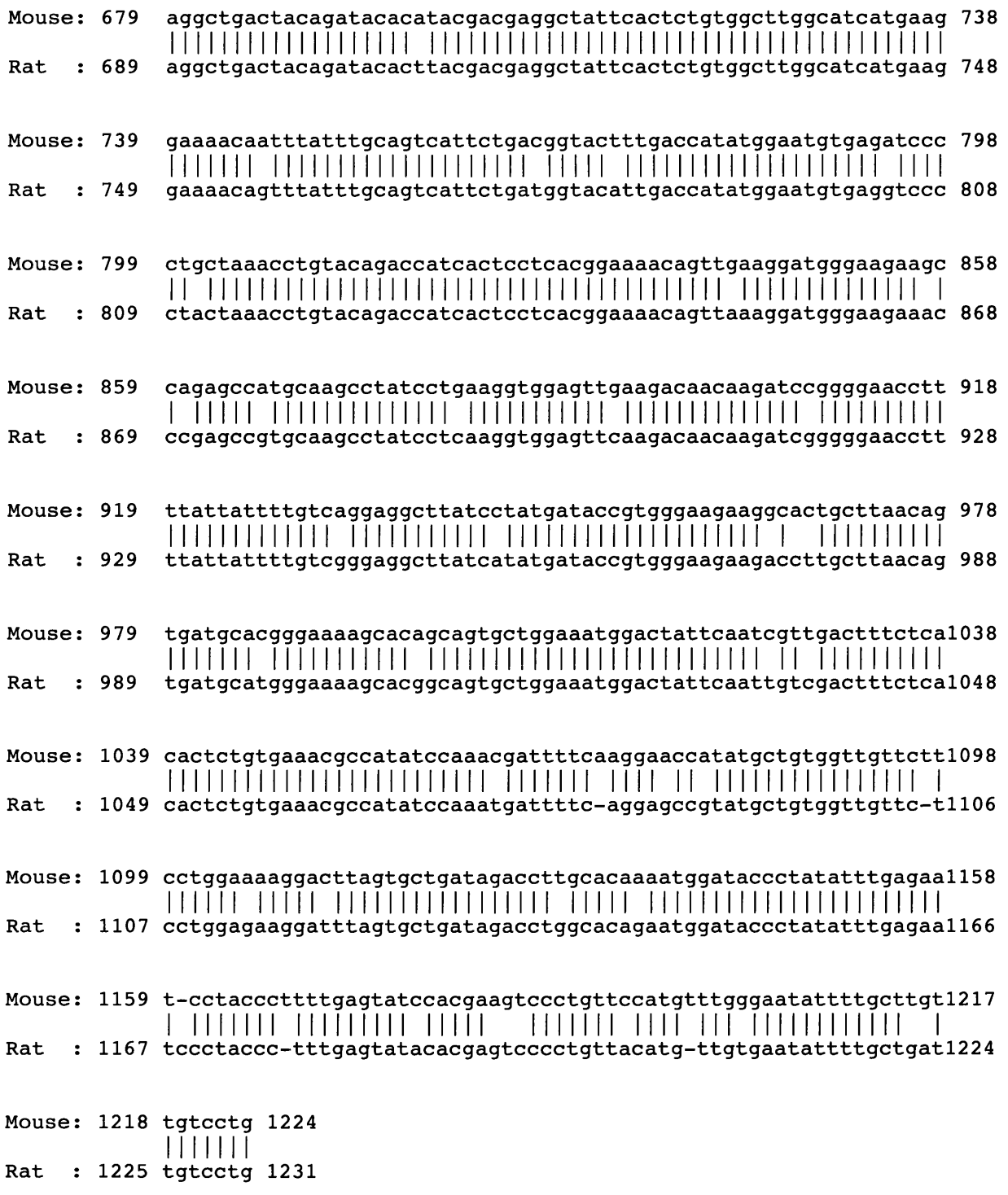


Figure 4.15. Demonstration of cDNA sequence homology between the 5' end of the cloned *l(2)gl* related gene and rat *tomosyn*

CHAPTER 5

DISCUSSION

5. Discussion

Based on my observations, there is a moderate frequency of loss of heterozygosity on the acromeric region of mouse chromosome 6 in the two stage skin carcinogenesis model. The highest frequency of loss (33%) is seen at marker D6Mit236, situated 3.1cM from the acromere and corresponding cytogenetically to band A2. The data are suggestive, particularly from analysis of the *Spretus* x NIH and Sencar x Balb tumours, that loss of this marker and the region between D5mit83 and D6mit50 represents a smallest common deleted region (SCDR), implying the presence of a tumour suppressor gene.

The significance of loss of heterozygosity data is however sometimes difficult to judge, and should be seen at most as suggestive of the presence of novel tumour suppressor genes. Certainly it should be borne in mind that there is an expected background level of LOH in sporadic tumours, consistent with general genetic instability, and not necessarily implying the presence of a TSG. Conventional wisdom is that a background frequency of 10% is to be expected, and that a frequency of over 20% is significant. Relative to this, a rate of 33% at the D6Mit236 locus in a series of 55 tumour cases is likely to be of significance. It may be however that particular parts of the mouse genome are prone to greater instability than others. This is known to be the case in humans. In particular, the 7q31 region is recognised as harbouring a recombinational hotspot as well as an aphidicolin –inducible fragile site, identified during attempts to clone the cystic fibrosis gene (Karem *et al.*, 1989; Huang *et al.*, 1998a, b). This may provide an alternative explanation of the high frequency LOH in the region, *viz.*, intrinsic genomic instability rather than targeted inactivation of a TSG. Recent work however suggests otherwise: introduction of chromosome 7 into a highly aggressive prostate carcinoma cell line resulted in increased tumour latency by at least twofold, with introduction of chromosome 12 having no effect (Zenklusen *et al.*, 2000). This does lend weight to the argument that chromosome 7 harbours a TSG.

The large discrepancy between the frequency of LOH in my studies and that in published series (Zenklusen *et al.*, 1996b, 1997), even when using the same markers, may be explained in a number of ways. Firstly, in deciding to score for LOH only those tumours with unequivocal allele loss as determined by visual examination, I may have underestimated the true frequency. I chose this line of action in order to minimise false positive results from contaminating stromal tissue. Secondly, the strains of mice used to create F1 hybrids in my experiments are different from those chosen by Zenklusen in his investigations, and would have different genetic backgrounds that might influence genetic changes during tumorigenesis. It is certainly recognised that strains of mice differ in their propensity to develop tumours secondary to carcinogen exposure, a phenomenon that undoubtedly has a genetic cause. Clearly also the grade or stage of tumours may have been different between the two studies. Lastly, it is worth mentioning that the number of tumour cases examined by Zenklusen and coworkers was considerably smaller than that examined here. Small series may well overestimate the frequency of losses.

This is the third report of consistent LOH on mouse chromosome 6A2 in tumours induced by chemical carcinogenesis. Zenklusen and colleagues have reported high frequencies of allele loss in tumours derived from mouse skin and also rat liver, another well defined chemical carcinogenesis protocol ((Zenklusen *et al.*, 1996b, 1997). In addition, several sources have confirmed consistent LOH in a variety of human epithelial cancers at 7q31, the region syntenic to mouse 6A2. Allele loss distal to the *met* oncogene at 7q31-33 is reported to be as high as 81% in breast cancer (Zenklusen *et al.*, 1994b). These same authors found a similar order of loss (73%) in ovarian tumours at the same locus DS522 (Zenklusen *et al.*, 1995), confirming earlier reports (Cliby *et al.*, 1993). Allelotyping data from SCC of the head and neck (Nawroz *et al.*, 1994) again suggest that LOH in this region is common in this tumour type (30-50%). Lastly, in colon and prostate cancers, LOH on chromosome 7 at frequencies in excess of 80% have been documented (Zenklusen *et al.*, 1995b; Zenklusen *et al.*, 1994). These molecular studies confirm earlier cytogenetic observations of common deletions in the long arm of chromosome 7 (reviewed in Zenklusen and Conti, 1996). Together, these

data argue for the presence of a novel TSG at these loci which is conserved across species. Functional studies have added to the strength of this argument. Tumorigenicity can be repressed by the introduction of human chromosome 7 into a murine carcinoma cell line (CH72) using microcell mediated transfer techniques, an effect specific to chromosome 7 since introduction of chromosome 15 has no discernible effect (Zenklusen *et al.*, 1994a). Interestingly, two of the seven microcell fusion clones obtained from these experiments were at least as aggressive as the original CH72 cell line. One of these clones was shown on allelotyping to lack markers in the 7q31 region, implying a deletion. These observations are entirely consistent with previous findings of LOH at this locus in solid tumours.

Using somatic cell genetics, this work has been taken further in our laboratory. Systematic deletion mapping has been undertaken of immortal segregants arising from human fibroblast/chromosome 7 fusion clones. These segregants (or revertants) are clones that, having lost the immortal phenotype subsequent to introduction of chromosome 7, then revert to the original phenotype as a consequence of loss of genetic material. Although losses occurred at three loci spanning 10 Mb (D7S522, D7S821 and D7S633-17TA-5/17B-RE3), one of these coincided almost exactly with solid tumour LOH studies. A recent analysis of 7q loss in ovarian cancer undertaken by a collaborator at Queen's University, Belfast found the highest loss at marker 17TA-5/17B-RE3, in agreement with our studies (H. Russell, unpublished). Interestingly, several tumours were found to have partial deletions spanning *CFTR* and the region just proximal to this (A Hurlstone, PhD Thesis). The highest losses found in my experiments in mouse occurred at marker D6Mit236, contained within an intron of mouse *cftr*. Further, the deletion found in one of the two revertants in Zenklusen's mouse cell/chromosome 7 fusion experiments discussed above was located at the *cftr* locus.

Attempts to define further a SCDR might take the form of large scale tumorigenesis studies with deletion analysis of mouse carcinoma/chromosome 7 derived tumours whose growth kinetics have reverted to wild type, implying inactivation of a TSG on chromosome 7. The introduction of fragments of chromosome 7, and indeed the

smallest possible region required to induce tumour suppression, might facilitate mapping efforts. Employment of the RDA technique might be an applicable strategy, particularly in the analysis of possible microdeletions in those segregants with no obvious allele loss.

The finding of aneuploidy in chemically induced papillomas, as well as carcinomas and spindle cell tumours, in chromosome painting studies supports the notion that this is an early event in the mouse skin system. This is likely to reflect general chromosomal instability, and may be due to an event that occurred during initiation or specifically to the action of the promotor. One should of course exercise caution in interpreting karyotype changes in cultured cell lines, since continuously passaged cells are doubtless in a state of genetic flux that may not reflect the situation *in vivo*. This is underlined by the chromosome painting analysis of C5N, a 'normal' keratinocyte line which karytypically is highly abnormal. However, these findings *in vitro* do seem to be borne out from both cytogenetic and molecular studies *in vivo*. Aldaz has suggested (Aldaz *et al.*, 1996) that, following TPA treatment, an initiated population of cells undergoes random chromosomal alteration. Subsequently, clones of cells develop which harbour trisomies of chromosome 6 (and 7) as a result of the growth advantage imparted by them. The finding of LOH in papillomas at a rate as frequent as in carcinomas reinforces that these genetic changes are an early event in carcinogenesis.

Trisomy is not necessarily associated with tumour suppressor genes, and in many cases implies selection for abundance of a growth promoting gene product. This is likely to explain the selection for multiple copies of chromosome 7, where the mutant H-*ras* gene resides. The role played by H-*ras* in the initiation of mouse skin tumours has been firmly established. However, trisomy is also entirely compatible with TSG inactivation. For example, once a chromosome carrying a mutated TSG is duplicated (through non-disjunction) to produce a trisomy, complete inactivation of the gene can occur by mitotic recombination with the defective chromatid, which is then detectable at the molecular level as LOH.

Having found a significant level of LOH on mouse chromosome 6 A2 and defined, tentatively, a smallest common deleted region centred on marker D6mit236, the task of narrowing down on a potential TSG could take several forms. The known genes in the area should be screened for mutational inactivation in the panel of cell lines from the mouse carcinogenesis system, by examination for SSCP in cDNAs and exonic genomic DNA sequences. Examples of these genes would be *wnt2*, *met* and *cappa2*. Although there is no obvious biological explanation nor any prior evidence supporting the notion that *cftr* is a TSG, it should be included within the list of candidates. Assuming that the area will also harbour genes not yet discovered and sequenced, the area encompassing the SCDR would have to be spanned with clone contigs, and these sequences screened for genes. Again, the candidate genes identified would require to be examined for mutations. The recent publication of the first complete sequence of the human genome will provide further potential candidates. Indeed, positional cloning as previously understood will be more and more superseded by the positional candidate gene approach as a consequence of the human genome project. There are, of course, other methods of gene silencing than deletion or mutational activation of TSGs: loss of expression may result from mutation of transcription controlling elements, or from methylation changes (Merlo *et al.*, 1995; Herman *et al.*, 1995). These can be looked for by expression studies, and Southern blot analysis using methylation specific restriction enzymes. Once found, a good candidate gene would be expected to induce tumour suppression when introduced into a tumour cell line.

The candidate genes I cloned in the mouse system were chosen for their upregulation in a human senescent fibroblast library relative to non-senescent cells. Senescence describes the state of irreversible growth arrest reached by a cell after a finite number of doublings (eponymously termed the Hayflick number) (Hayflick, 1965). Although first described in *in vitro* studies of primary epithelial cultures, there is evidence that senescence pertains *in vivo* (thoroughly reviewed by Campisi, 1996). Induction of replicative senescence is thought to be one method of tumour suppression, by acting to limit the doubling potential of cells. Although loss of the senescent phenotype may not be crucial at the early stages of carcinogenesis, it may well be a requirement for the

development of invasion and metastases. There is independent evidence in man that there exists a senescence gene on the long arm of chromosome 7 (Otaga *et al.*, 1993). The exciting hypothesis being examined in our laboratory is that this gene is one and the same as the putative tumour suppressor gene at 7q31. Genes which are upregulated in senescing fibroblasts relative to pre-senescent cells, and which map to human chromosome 7, are therefore potential candidate tumour suppressor genes.

The first gene I examined, a putative cyclin G1 binding protein, had already been cloned in mouse, but the exercise allowed me to establish proof of principle and gain experience of the techniques involved. The gene is small and therefore recoverable in a single clone. Its putative function is based on observations that its protein product interacts with cyclin G1 in the yeast-2-hybrid system, an observation that may or may not hold true *in vivo.*

Arguably the most interesting gene examined was a novel *kelch*-related gene, again identified first in the human senescence library. This gene has not hitherto been cloned in mouse in its entirety. First identified in 1993 from a female sterile *Drosophila* mutant, kelch was shown to be a constituent of oocyte ring canal structures which permit cytoplasmic transport between the oocyte and nurse cells (Xue and Cooley, 1993). The repeating structural motif, now termed the kelch motif, first described in the *Drosophila* kelch ORF1 protein, comprises approximately 50 amino acids, is repeated three to six times and has been shown to interact with actin. The kelch motif in *Drosophila* is required for the correct localisation of the protein to ring canals.

The use of bioinformatics has shown that this motif is ancient and widely dispersed during evolution. It is present, for example, in the A55R and C2L ORFs of vaccinia virus, spe-26 of *Caenorhabditis elegans* (Varkey *et al.*, 1995), the predicted IPP protein of mouse (Kim *et al.*, 1999), α- and β-scruin of the *Limulus* crab (Way *et al.*, 1995a,b) and the NRP/B and NS1 binding proteins in humans (Kim *et al.*, 1998; Wolff *et al.*, 1998). Direct evidence of actin association has been demonstrated in α-scruin of *Limulus* as well as colocalisation of actin filaments with *Drosophila* kelch in oocyte ring

canals. Fortuitously, the kelch repeat has also been identified in fungal galactose oxidase, for which the crystal structure has been solved (Ito *et al.*, 1994). In this enzyme, the seven kelch motifs together form a conserved tertiary structure, the β-propellor. Each kelch unit forms a four stranded β-sheet, corresponding structurally to one blade of the propellor. The series of blades is arranged around a single axis. Although galactose oxidase is the only kelch related protein for which the crystal structure has been determined, the highly conserved primary sequence and theoretical predictions make it very likely that other members of this family also form β-propellors.

A subset of the kelch family, which includes *Drosophila* kelch and my novel kelch related protein, can be defined by the presence of an additional motif, the POZ (**Po**xvirus and **Z**inc finger) domain, also known as the BTB (**B**road complex **T**ramtrack and **B**ric a Brac) domain. This 120 amino acid motif is thought to permit homo- and heterodimerisation, and is particularly important in the group of zinc finger transcription factors. In the human promyelocytic leukaemia zinc finger (PLZF) protein, the POZ domain has transcriptional repressor activity and interacts with components of the histone deacetylase complex (Ahmad *et al.*, 1998). The crystal structure of this protein has also been solved and shows a tightly intertwined dimer with an extensive hydrophobic interface (Ahmad *et al.*, 1998). The amino acid sequence at the formed groove between the two protins appears to be highly conserved and is suggestive of a peptide binding site, for example with other nuclear proteins.

The physiological and biochemical functions of the known kelch family members, of which there are about twenty, remain largely to be determined. Despite sharing common structural features, their known functions seem to be quite diverse. Certainly their cellular localisation includes the nucleus, cytoplasm , cell surface and even extracellular (reviewed in Adams *et al.*, 2000). Although the kelch repeat domain in a number of proteins is known to interact with actin, not all kelch containing proteins are actin binding molecules. *Mayven* (*KLHL2*), the human homlogue of *Drosophila kelch*, and IPP seem to be intimately involved in cytoskeletal modulation *via* actin association. Calicin, on the other hand, though still involved in cytoskeletal and organelle structure,

is located in mammals in actin-negative structures termed calyces which are important in spermatocyte morphogenesis (von Bulow *et al.*, 1995). The recently characterised NS1-BP (Wolff *et al.*, 1998) is likely to have a role in mRNA splicing. This molecule was discovered by virtue of its interaction with a virally encoded protein, NS1. Upon infection with influenza virus, NS1-BP relocates throughout the nucleoplasm. Lastly, attractin, present in many mammals including man, is found extracellularly in serum and promotes monocyte spreading (Duke-Cohan *et al.*, 1998).

Evidence that members of the *kelch* family may be involved in oncogenesis has come from a couple of sources. Recent cloning and molecular characterisation of *KLHL3*, a new human homologue of the *Drosophila kelch* gene, showed that it maps to human chromosome 5, corresponding to a smallest common deleted region in myeloid leukaemias (Lai *et al.*, 2000). However, a search for inactivating mutations by SSCP in malignant myeloid cells was negative. In addition, recent evidence has suggested that overexpression of *krp1*, a novel rat *kelch* family member thought to be a transcriptional target of AP1, results in dramatically elongated pseudopodia in transformed rat fibroblasts, which may facilitate cell motility and invasion (Spence *et al.*, 2000).

The novel *kelch* molecule cloned here has a single open reading frame of 1929 bp, resulting in a derived protein sequence of 642 amino acids and a predicted molecular weight of 71kDa. It contains both a POZ domain and a kelch repeat domain. The kelch motif is tandemly repeated six times, at amino acids 369 to 415, 416 to 463, 465 to 512, 514 to 559, 560 to 606 and 608 to 642. From my experiments, there appeared to be no inactivating mutations in the series of cell lines representing various stages in the mouse carcinogenesis model. It is unlikely, based on these experiments, that kelch is a tumour suppressor gene involved in skin carcinogenesis.

Nevertheless much work remains to be done on this interesting molecule. Despite several attempts, I was unable to clone the novel mouse *kelch* from a genomic library. This needs to be achieved before the intron-exon boundaries can be identified. Patterns of expression need to be determined by Northern examination of tissue specific RNAs.

In addition, chromosomal localisation should be sought using FISH or PCR of chromosome specific DNA. Expression of the protein in an expression system may allow antibodies to be raised to the molecule, which would allow cellular localisation studies by immunocytochemistry. Binding partners can also be sought, either by immunoprecipitation techniques or by employing the yeast-2-hybrid system. Going a stage further, and making full use of the mouse as model system, a knock out mouse could be constructed to explore further the role of this novel *kelch*.

The last gene cloned, albeit partially, in the mouse system was a novel homologue of the *l(2)gl* gene, first identified as a tumour suppresser in *Drosophila*. Over fifty genes have been described in *Drosophila* whose mutation produce tissue specific tumours, the best known of which is **l**ethal **(2) g**iant **l**arvae (Strand *et al.*, 1994a). Homozygous disruption of this locus results in malignant transformation of the neuroblasts of the presumptive adult optic centres in the larvae. The protein product has been shown to participate in a cytoskeletal network and undercoat the cell membrane in areas of cell-cell contact. Sequence comparisons with mammalian proteins show similarities with known mammalian junctional proteins. It is tempting to speculate that neoplastic transformation is a result of disruption of the cytoskeletal network.

Cloning of the mouse homologue of this gene confirmed the presence of related molecules in higher vertebrates (Tomotsune *et al.*, 1993). Expression of *mgl-1*, the mouse homologue of *Drosophila l(2)gl*, was found to be subordinate to *Hox-C8*, a mouse homeobox gene crucial to pattern formation in the embryo, and was found in highest abundance during embryogenesis in brain and spinal cord.

Despite numerous attempts, I was able only to clone the first 1200bp of the 5' end of the cDNA. Interestingly, Jacob and colleagues state that in their attempts to clone the cDNA of the *Drosophila* gene, and despite extensively screening three cDNA libraries, they found only two cDNAs out of 32 characterised cDNAs (Jacob *et al.* 1987). This may be due to secondary structure in the mRNA precluding reverse transcription.

Unfortunately, while I was working on this novel *l(2)gl* related gene, the rat sequence of *tomosyn*, a syntaxin-1 binding protein, was published which demonstrated very close homology to the 5' sequence in my possession (Fujita *et al.* 1998). It is highly likely that I had cloned the mouse homologue of this new gene. Tomosyn (**tomo** (friend in Japanese) of **syn**taxin) forms a novel complex with syntaxin in nerve terminals and is probably one of the many components of the neurotransmitter release process. It is found specifically in the outer plexiform area of the retina and the post synapse of cerebellum. Presently, it is unclear how or whether this role as a likely synapse molecule, and its homology to a known *Drosophila* tumour suppressor gene, are related. It seems unlikely that it has a role in oncogenesis.

Our understanding of cancer has advanced dramatically since the discovery of oncogenes and tumour suppressor genes in the 1980s. This knowledge has permitted us to devise novel therapeutic approaches to the disease, based on a molecular understanding of the underlying cellular defects. Although drugs targeted at molecular pathways are currently few, the number is rising. Identification of new oncogenes and tumour suppressors clearly provides us with a broader range of targets which are expected to be more tumour specific and therefore less toxic. Arguably the most mature of the novel therapeutic approaches is targeting of the *ras* mitogen-activated protein kinase pathway. This strategy is particularly promising because the frequency of *ras* mutation is among the highest of any gene in human cancer (Bos, 1988; Kiaris and Spandidos, 1995). Ras proteins are among a small group of compounds undergoing a post-translational modification known as farnesylation, a process crucial to membrane binding and physiological function (Lerner *et al.*, 1997; Sebti and Hamilton, 1997). Blockage of this process results in severe impairment of ras protein function. Farnesyl transferase inhibitors are remarkably specific and have been demonstrated to cause no gross systemic toxicity in animals. Dose limiting toxicities in humans when administered orally include myelosuppression, diarrhoea and lethargy (Eskens *et al.*, 1999). These compounds are currently in Phase I and II clinical trials. Interestingly, it is becoming increasingly clear that inhibition of ras farnesylation may not be the most important antitumour effect of this molecule. Activity does not seem to be dependent on

ras mutation status, and recent evidence points to rhoB as a more likely target (Lebowitz and Prendergast, 1998). An alternative strategy to the inhibition of this pathway would be the use of an antisense oligonucleotide, also the subject of a Phase I trial (Gordon *et al.*, 1999).

The tumour suppressor gene *p53* is also commonly mutated in human cancers, particularly at the time of relapse following therapy, and has been evaluated as a potential therapeutic target. Expression of wild type *p53* can result in apoptosis in human cancer cells (Takahashi *et al.*, 1992). Replacement of functional *p53* using a variety of delivery systems (adenoviral, retroviral, liposomal) has been examined, both alone and in combination with standard cytotoxic chemotherapy (Wills *et al.*, 1994; Fujiwara *et al.*, 1994; Hupp *et al.*, 2000). A particularly elegant approach has been to exploit the molecular difference between malignant and normal cells *vis-à-vis p53* status. The best known of these is the ONYX015 adenovirus, which harbours an E1B deletion allowing replication and cytolysis only in *p53* deficient cells (Biscoff *et al.*, 1996), although recent work has questioned the molecular basis for this specificity (Rothmann *et al.*, 1998). This concept has been explored in a variety of tumour types, including ovary (Vasey *et al.*, 2000) and, more promisingly, head and neck (Khuri *et al.*, 2000).

On the available evidence, the putative novel tumour suppressor, which formed the subject of this thesis, is inactivated in a very broad spectrum of solid tumours and at high frequency. This would imply an important and common role in tumorigenesis, and potentially a useful novel therapeutic target. The search for this putative multi-tissue tumour suppressor gene on the long arm of human chromosome 7, and the A2 region of mouse chromosome 6, should therefore continue.

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APPENDIX

Table A1 List of mouse ESTs used in designing PCR primers

Gene	Mouse EST Accession Number
Putative cyclin G1 binding protein	AA764288
Kelch	AA109625
	AA140498
	AA553216
	AA673812
	AA619726
	AA690294
	AA516929
l(2)gl homologue	AA437465

Table A2 Description of probes used for Southern analysis

Probe	Description
H-*ras*	300bp ECOR1 insert of BS9 (Ellis *et al.*, 1980)
Caveolin-1	700bp *Sma1* fragment encompassing exon 1
Kelch homologue	800bp product spanning central coding region and first kelch repeat

Table A3 List of PCR primers used for SSCP analysis of mouse putative cyclin G1 binding protein

Primer	Sequence
1F	5'TGCTCGGGACTCCGCCCTGGA3'
1R	5'TCCTCCAGCAAAACCTGGAAG3'
2F	5'ACCACTTTAAACTTCGCTTC3'
2R	5'TCTGTCCTTTGTGAATGAGCT3'

Table A4 List of PCR primers used to amplify mouse *kelch* homologue

Primer	Sequence
5F	5'GCGTTCTCAGTGACACATTTC3'
5R	5'CTTCTTGGCTGCAGAATAAACATC3'
6R	5'CATTCTTTTAGATGTACGCTGC3'
1F	5'GATCCAACTAGAAATGAATGGAAG3'
1R	5'AGTTGGATCATACATCTCCACAC3'
2F	5'CTAGAAAAGCCCATGTCTCCC3'
2R	5'AGTTGGATCATACATCTCCACAC3'
3F	5'GATGCAACCAGATGAGCTTCTAG3'
3R	5'TCTCGAAGCTTAAACTCTTGCTC3'
4F	5'GTTACAAATTTCAGAAGAGGAGGA3'
4R	5'CCATTAAATCATCATCGCTCTC3'
7F	5'GCTATGATCCACATACAGATCAC3'
7R	5'CATCACTAAGCCTGTTAGTTT3'

Table A5 List of PCR primers used to amplify mouse *l(2)gl* homologue

Primer	Sequence
1F	5'TCCGAACACTTCCAACTCTGC3'
1R	5'CTTTGACTTGAGGTCCCATAAG3'
2F	5'ACCCTCAGTTCTCCGTTC3'
2R	5'TACTGCACCTCAGCACC3'
3F	5'AAGCTTTTGATTGGCTTCGAATC3'
4F	5'AGTTATTGCAGAAGCGTCCCAG3'
4F	5'TAATTTGTCAGGCTTATCC3'

Table A6 Primers used in sequencing

Primer	Sequence
KS	TCGAGGTCGACGGTATC
SK	CGCTCTAGAACTAGTGGATC
T7	GTAATACGACTCACTATAGGGC
T3	AATTAACCCTCACTAAAGGG