The role of Harvey-ras in mouse skin tumorigenesis

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To Mum and Dad and to Declan a good friend and University colleague who lost his fight against cancer on August 27th 1990.

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Abbreviations

DNA	deoxyribonucleic acid
cDNA	complementary DNA
RNA	ribonucleic acid
mRNA	messenger RNA
A	adenine
C	cytosine
G	guanine
T	thymine
N	A, C, G or T
dNTP	deoxyribonucleoside triphosphate
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dTTP	deoxythymidine triphosphate
Arg	arginine
Asn	asparagine
Cys	cysteine
Ile	isoleucine
Phe	phenylalanine
Leu	leucine
DNase	deoxyribonuclease
RNase	ribonuclease
bp	base pairs
kb	kilobase pairs
mm	millimetres
ml	millilitres
mM	millimolar
uM	micromolar
pM	picomolar
ug	microgrammes
ng	nanogrammes
pg	picogrammes
W	watts
mA	milliamps
kV	kilovolts
^{o}C	degrees centigrade
f/c	final concentration
TM	trademark
dH_2O	distilled water

BSA	bovine serum albumin
EDTA	ethylenediamine tetra-acetic acid
EtBr	ethidium bromide
Tris	tris(hydroxymethyl)methylamine
PEG	polyethylene glycoll
SDS	sodium dodecyl sulphate
FCS	foetal calf serum
FBS	foetal bovine serum
PBS	phosphate buffered saline
PMSF	phenylmethylsulfonyl fluoride
G418	geneticin
GANC	gancyclovir
LIF	leukemia inhibitory factor
SLM	special liquid medium
DMBA	7,12-dimethyl benz(a)anthracene
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
HaMSV	Harvey murine sarcoma virus
TPA	12-O-tetradecanoylphorbol-13-acetate
PCR	polymerase chain reaction
LOH	loss of heterozygosity
RFLP	restriction length polymorphism
H-ras	Harvey-ras
K-ras	Kirsten-ras
% w/v	weight in grammes per 100ml water
% v/v	volume in ml per 100ml water

Abstract

This thesis outlines two alternative approaches to investigating the role of H-ras in mouse skin tumour development. The first, the *in vitro* approach utilises a number of unique cell lines representative of a late stage of tumorigenesis, the conversion from a well differentiated squamous carcinoma to a undifferentiated spindle form. Through manipulation of the gene dosage ratio of mutant:normal H-ras in these cell lines, by plasmid transfection and virus infection, it was hoped to provide some clues as to the function of H-ras in this late stage transition event. The second approach involves the technique of gene targeting. The aim of this approach was to create a mouse hemizygous or completely deficient in H-ras and to examine the effects in the context of both chemical carcinogenesis studies and normal mouse development.

Initial observations of the squamous/spindle transition event showed that the cell lines representative of both phenotypes were characteristically very different. The squamous cell line B9 produced a well differentiated tumour when injected into nude mice whereas the spindle cell lines A5 and D3 gave rise to aggressive, disorganised tumours with a much reduced latency. Genetic analysis of the cell lines showed that the most dramatic difference between the squamous and spindle derivatives was in the copy number and expression levels of H-ras. Both spindle cell lines A5 and D3 showed an approximate 5-10 fold increase in mutant H-ras, best explained by a genetic amplification mechanism. Experimentally overexpressing mutant H-ras in the squamous cells, and vice versa the levels of normal H-ras in the spindle variants, was unable to mimic the conversion event. However it was found that several A5 and D3 transfectant clones showed a dramatic reduction in tumorigenicity. This correlated not with the levels of the introduced normal H-ras but the absolute levels of endogenous mutant H-ras. Spontaneous loss of mutant copies of H-ras resulted in clones that morphologically

resembled spindle cells yet produced tumours with a vastly increased latency period. It seems therefore that in this system mutant H-ras is the critical determinant of tumorigenicity, and relates perhaps to the growth of the tumour cell *in vivo*, possibly providing the driving force for the conversion. However, alteration of the H-ras gene dosage ratio was not in itself sufficient for the transition of a squamous to a spindle cell carcinoma, implying that another locus as yet unknown must play a role in controlling the epithelial phenotype.

Finally, the gene targeting approach, provides a means to examining the earlier stages of mouse skin tumorigenesis, in particular the proposed role of normal H-ras in suppressing the development of papillomas from an initiated cell. For instance, if the consistent duplication of the chromosome carrying the mutant H-ras gene, seen in the development of papillomas, is related to overcoming a suppressive effect of normal H-ras, then carcinogen treatment of a mouse deficient in H-ras may result in a mouse more susceptible to tumorigenesis. Experiments with the gene targeting technique are on going and as yet no targeted ES cell clone has been isolated, but hope remains that this approach will provide new insights into the functions of H-ras both in early tumorigenesis and normal development.

Chapter 1
Introduction

1.1 The history of oncogenes

1.1.1 Lessons from viruses

The identification of oncogenes as genes with the potential to cause cancer, initially arose from the study of a class of retroviruses called acute transforming viruses. Many of these acute transforming viruses, like the Rous sarcoma virus (RSV) isolated over 80 years ago, were identified in crude extracts of spontaneously arising tumours, as agents that could reproducibly induce the growth of tumours in previously healthy animals, after a very short latency period (reviewed by Bishop, 1983).

Compelling evidence that RSV encoded a protein that induced transformation came in 1970 from the isolation of mutants that were temperature-sensitive for transformation (Martin, 1970). Spontaneous revertants that were unable to transform at any temperature arose by deletions of the RNA genome that occurred during RSV replication.

The gene or oncogene defined by the deletion, which gives RSV its ability to transform cells and induce tumours was called src (Stehelin et al., 1976a). cDNA probes from the region of the src oncogene were shown to anneal to normal genomic DNAs from several avian species (Stehelin et al., 1976b). Sequences homologous to src were shown to be widespread among vertebrate species, and to be expressed in normal cells (Spector et al., 1978)

The concept that the viral oncogene, v-src, had a cellular counter-part or "proto-oncogene", c-src, that had been in some way transduced by the virus, indicated for the first time the existence of cellular genes with the potential to cause cancer (Bishop, 1981).

Perhaps 40-50 oncogenes have now been identified as components of retroviruses, isolated from a number of different species. The astounding conservation of these oncogenes can be appreciated when one estimates that the nucleotide sequence of the

chicken and human c-src genes have diverged by only 10-15 percent (Shilo and Weinberg, 1981; Shibuya et al., 1980). This high degree of genetic stability suggests a critical role for the proto-oncogenes in cellular metabolism.

Many other oncogenes have been identified not as a component of a retrovirus, but rather as a cellular gene disrupted by the integration of a retrovirus within or close to the coding region of the gene. In many cases, this brings the gene under the powerful transcriptional control of the viral LTRs. This class of oncogenes will be discussed in the context of oncogene activation, Section 1.2.2.

1.1.2 Lessons from gene-transfer studies

The establishment of mouse 3T3 cells that can be transformed *in vitro* (Shih et al., 1981), and the technique of calcium phosphate transfection (Graham and van der Eb, 1973), together formed the basis for the 3T3 transformation assay. This assay has played a prominent role in the study of cellular oncogenes, by enabling the introduction of single copy genes into mammalian cells inorder to determine their transforming abilities (Shih et al., 1979b).

Transfection of DNA from chemically transformed mouse fibroblasts into monolayer cultures of NIH3T3 cells resulted in the development of transformed foci (Shih et al., 1979a). This suggested that the original mutagenic event which resulted in chemical transformation of the cells was dominant and transmissible through several rounds of transfection.

It could be argued that despite the presence of cellular counterparts, oncogenes were a peculiarity of RNA tumour viruses, and played no role in human cancer. However this appeared less likely when high molecular weight DNA from the human bladder carcinoma cell line, T24 (Perucho et al., 1981) or EJ (Tabin et al., 1982; Pulciani et al., 1982), and lung carcinoma lines A2182 and LX-1 (McCoy et al., 1983; Der et al., 1982) were shown to transform recipient NIH3T3 cells. Through the use of human specific repetitive Alu sequences, it was possible to follow the cosegregation of the human fragments with the phenotype of malignant transformation, leading to the eventual isolation and cloning of the candidate genes (Perucho and Wigler, 1981).

The transforming genes of the bladder and lung cell lines were shown to hybridise to the Harvey and Kirsten sarcoma viruses. These were not new oncogenes but instead cellular homologues of the transforming retroviruses, themselves able to induce cellular transformation. This suggested a causal role in the development of certain human tumours, by an alteration event that did not change the gross structure of the genes.

Thus from both the study of acute transforming viruses, and the study of human tumours, the same genes were recognised as being causal and were subsequently cloned. The obvious question that now arose was what were the qualitative or quantitative changes that enabled a cellular gene, present and expressed in normal cells, to become a cancer causing gene ?

1.2 Mechanisms of activation of cellular oncogenes:

It had been clear for some time that many different agents could induce cancer in animals. For instance, the early work on the carcinogenic properties of X-rays (Muller, 1927), coal tar, and nitrogen mustards (Auerbach et al., 1947), as well as the abundant data on viral oncogenesis, Section 1.1.1. It appeared there was the same diversity at the genetic level. Viral transduction of a cellular gene could induce rapid tumorigenesis in a healthy host, whereas the Avian leukosis virus (ALV) induced

lymphomas only after a long latency period, as a result of viral integration at a unique genomic site (Hayward et al., 1981). This led to the notion that proto-oncogenes could be activated by proviral insertions as well as by viral transduction. In addition, early observations by Rowley using chromosome banding suggested a gross chromosomal mechanism for oncogene activation (Rowley, 1973). The Philadelphia chromosome associated with chronic myelogenous leukemia, was shown to be a result of a balanced translocation between human chromosomes 9 and 22. The observations of Barbacid and Weinberg, indicated that single point mutations were capable of activating cellular proto-oncogenes (Pulciani et al., 1982; Tabin et al., 1982). Clearly there were a number of different mechanisms for the generation of genetic alterations associated with neoplasia.

1.2.1 Point mutation

The first demonstration of a lesion in cellular DNA, whose occurrence was directly related to the carcinogenic process, came from analysis of the human bladder cell line EJ (Tabin et al., 1982) or T24 (Reddy et al., 1982; Taparowsky et al., 1982). Differences were detected in the migration of the p21 proteins of the H-ras gene. The alternative species were the result of a single point mutation leading to an amino acid substitution, glycine to valine at codon 12 of the p21 product. It was also shown that the Harvey sarcoma virus deviated from its cellular counterpart at the same position with a glycine to arginine substitution (Tabin et al., 1982). It thus appeared that a single point mutation in a cellular gene was sufficient to alter its normal function.

In agreement, a single point mutation in the neu proto-oncogene (Bargmann et al., 1986a; Schechter et al., 1984), leading to an amino acid substitution in the transmembrane domain of the protein occurs in the development of rat neuro- and glioblastomas (Bargmann et al., 1986b). However, as yet no point mutations in the c-neu gene have been identified in human tumours.

The pp60 product of the c-src gene is also modified by several point mutations (Levy et al., 1986), leading to amino acid changes in the tyrosine kinase domain of the protein. The result is an elevation of the tyrosine kinase activity of pp60src, which is thought to directly induce cellular transformation. As with the c-neu oncogene, there are no examples of c-src activation by point mutation in spontaneously arising human tumours. However, the FMS proto-oncogene which encodes the receptor for colony-stimulating factor 1 and the gsp oncogenic G protein, gsp, have been reported to be activated in human tumours by the mechanism of point mutation (Sherr et al., 1985). Mutations in codons 301 and 969 of the FMS gene were seen in several patients with myelodysplasia and acute myeloblastic leukemia (Ridge et al., 1990), whereas gsp has been shown to be activated both in tumours of the thyroid and pituitary (Clementi et al., 1990; Suarez et al., 1991; O'Sullivan et al., 1991).

Clearly substitution of amino acids as a result of a point mutation, is sufficient to alter the activity of a protein product, and constitutes a common mechanism for activation of a proto-oncogene.

1.2.2 Pro-viral insertion

Replicating retroviruses can cause tumours in a host animal after long incubation periods, in contrast to the acute transforming retroviruses, which with the exception of v-src are replication incompetent (Temin, 1963). The tumours show common pro-viral insertion sites, implying the dominant cis-activation of cellular genes by viral sequences (for a review see Nusse, 1986).

The first oncogene recognised as being activated in this manner was the myc oncogene, which had previously been identified as the transduced component of myelocytomatosis virus 29, or MC29. Levels of myc-specific RNA were 30-100 fold higher in ALV-induced lymphomas as a direct result of viral integration at several sites upstream of the c-myc gene (Hayward et al., 1981; Robinson and Gagnon, 1986). This was the first finding to suggest that over-expression of a normal cellular gene could result in neoplastic transformation, and led to the unmasking of the c-myc and c-abl genes at chromosomal breakpoints in non-viral tumours (Section 1.2.4).

Transcriptional enhancement is also seen in the activation of the int-1 and int-2 genes by MMTV (Nusse et al., 1984). The protein encoding domains of the int genes remain intact, indicating tumorigenesis is caused by the over-expression of an unaltered gene product (Nusse, 1988). Insertions of MLV near Pim-1 follow the same principle, most frequently found in the 3' untranslated region of the gene, perhaps indicating stabilisation of the Pim-1 mRNA as a contributing factor (Selten et al., 1986).

Proviral insertions are also found in the c-erb-B gene in chicken erythroleukemias (Nilsen et al., 1985), and in the c-myb oncogene in mouse lymphosarcomas (Sheng-Ong et al., 1986). In both cases, the protein product is disrupted by the provirus insertion, resulting in a novel spliced viral-oncogene transcript.

Thus insertional activation of cellular genes by the transcriptional enhancement or generation of novel chimeric proteins represents an additional mechanism responsible for oncogenesis.

1.2.3 Amplification

Another mechanism which results in increased dosage of cellular oncogenes, occurs through genetic amplification of a region harbouring the proto-oncogene. This represents a frequent event in tumour cells, and is thought to provide an indicator to the clinical prognosis of some malignancies (Nau et al., 1985).

Amplification of the cellular myc oncogene was originally seen in the human cell line HL-60 (Favera et al., 1982b; Collins and Groudine, 1982). The list of amplified oncogenes presently includes the myc and ras genes as well as abl, myb, EGFR, ERBB2(neu) (Semba et al., 1985), and HST. Amplification is more common in solid tumours than with haematopoietic malignancies which only rarely show signs of amplification. In addition, certain solid tumour types display genetic amplification more readily, such as neuroblastomas, small cell lung cancers, breast, ovarian and colon cancers (for a recent review see Schwab and Amler, 1990).

The copy number of the amplified gene ranges from 5 to 700. The c-Ki-ras gene is amplified 30-60 fold in mouse adrenocortical tumours, often residing on double minutes or homogeneously staining regions (Schwab et al., 1983) (reviewed by Cowell, 1982).

Amplification has been shown to correlate with the status and clinical prognosis of malignancy, for example, the most aggressive forms of neuroblastoma, stage III and IV, display genetic amplification of the N-myc oncogene (Schwab et al., 1984; Brodeur et al., 1984). Also the large cell variants of small-cell lung carcinomas have amplified c-, N- ,and L-myc genes, and show a poorer prognosis accordingly (Nau et al., 1985).

The structure of the amplified cellular oncogene appears unaltered with the exception of a single colon carcinoma line, COLO 320 (Schwab et al., 1986), and a single neuroblastoma cell line, NMB (Amler and Schwab, 1989).

The neu oncogene reported in an earlier section as being activated by point mutation has also been shown to be amplified in breast carcinomas (Slamon et al., 1987). The amplified gene is normal and shows no point mutations (Slamon et al., 1989). It thus appears, that at least in some cancers, overexpression of a normal oncogene product constitutes an important factor in the progression to a malignant phenotype.

1.2.4 Chromosomal translocation

Chromosomal translocations have been shown to be associated with a variety of haematopoietic malignancies (reviewed by Haluska et al., 1987). In 1973, Rowley with the use of chromosome banding, showed that the Philadelphia chromosome was as a result of a balanced translocation between human chromosomes 9 and 22. In 1982 molecular analysis of the chromosome translocation involved in Burkitts lymphoma, provided an explanation for the occurrence of translocations in neoplasia. The distal end of the long arm of chromosome 8 where the c-myc oncogene resides is translocated to chromosomes 14, 22, or 2. The outcome is that the c-myc gene is juxtaposed to an immunoglobulin gene on the respective chromosome, resulting in elevated constitutive expression of c-myc (Favera et al., 1982a; DeKlein et al., 1982; Taub et al., 1982).

Other oncogenes that are activated by translocation include bcl-1, bcl-2, and c-abl (Tsujimoto et al., 1984b; Tsujimoto et al., 1984a; Groffen et al., 1984; Tsujimoto and Croce, 1986).

Activation of the ret proto-oncogene in human thyroid papillary carcinomas occurs not by rearangement between different chromosomes but by an intra-chromosomal alteration between two genes that reside close to each other at position q11.2 of chromosome 10. The result is a fusion between the novel PTC (for papillary thyroid carcinoma) gene and the tyrosine kinase domain of the ret oncogene (Grieco et al., 1990).

Table 1. PROTO-ONCOGENES AND HUMAN CANCER

Proto-oncogene	Neoplasm	Mechanism of activation
ABL	Chronic myelogenous leukemia	Translocation
ERBB-1	Squamous cell carcinoma; astrocytoma	Amplification
ERBB-2(NEU)	Adenocarcinoma of breast, ovary and stomach	Amplification
GSP	Adenoma of pituatary gland; carcinoma of thyroid	Point mutations
MYC	Burkitt's lymphoma	Translocation
	Carcinoma of lung, breast and cervix	Amplification
L-MYC	Carcinoma of lung	Amplification
N-MYC	Neuroblastoma; small cell lung carcinoma	Amplification
H-RAS	Carcinoma of colon, pancreas, lung, melanoma and squamous carcinoma of head and neck	Point mutations
K-RAS	Acute myelogenous and lymphoblastic leukemia; carcinoma of thyroid; melanoma	Point mutations
N-RAS	Carcinoma of genitourinary tract and thyroid; melanoma	Point mutations
RET	Carcinoma of thyroid	Rearrangement
TRK	Carcinoma of thyroid	Rearrangement

Table from J.M.Bishop Cell64:235-248

In summary, the activation of cellular oncogenes may be acheived by a number of alternative mechanisms. In addition, the same oncogene may be activated in certain malignancies by different means (see Table 1). For example, in human breast carcinomas the c-neu proto-oncogene is amplified several-fold (Slamon et al., 1987), whereas in rat neuro- and glioblastomas a single point mutation represents the activating event (Bargmann et al., 1986b).

1.3 Evidence for oncogene cooperation

A number of oncogenes have now been identified from the analysis of consistent genetic changes associated with specific tumour types, and from *in vitro* assays of cell transformation. These can be grouped into a small number of classes, on the basis of shared functional properties and on the basis of the nuclear or cytoplasmic localisation of the gene products (reviewed by Weinberg, 1985). Among the nuclear oncogenes are variants that exhibit some structural homology such as c-myc, N-myc, myb, and adenovirus E1A. These along with other nuclear oncogenes, such as p53, polyoma large T (Rassoulzadegan et al., 1983), and SV40 large T, display some common functions, namely the ability to immortalise primary rat embryo fibroblasts (REFs) (Mougneau et al., 1984).

In contrast, the cytoplasmic oncogenes such as H-ras, K-ras, N-ras, src, and polyomavirus middle T antigen, are generally weak in their ability to immortalise cells but strong in their ability to promote the phenotypes of anchorage independence, alterations in cell shape and reduction in growth factor requirements.

Transfection of polyoma middle T, E1A, or ras oncogenes alone, are unable to transform cultures of primary cells. However, combinations of oncogenes, such as E1A and E1B, or polyoma middle T with large T, were shown to fully convert primary cells to tumorigenic cells (Land et al., 1983; Ruley, 1983). The concept of activated oncogenes collaborating to fully transform cells in culture, provided credance to the nuclear/cytoplasmic classification, since the oncogene products that act in the nucleus cooperate best with those that act in the cytoplasm (Weinberg, 1985).

Most of the nuclear oncoproteins can cooperate with activated ras to elicit transformation of primary REFs (Land et al., 1986; Parada et al., 1984)(for review a see Ruley, 1990).

However, there are exceptions to the rule, in that certain adenovirus E1A mutants collaborate with ras but not with the cytoplasmic product of polyoma middle T (Ruley, 1987). There are also examples of two nuclear oncoproteins (GLI and E1A) (Ruppert et al., 1991), or two cytoplasmic oncoproteins (bcl-2 and c-H-ras) that cooperate to transform REFs (Reed et al., 1990).

Additional evidence for oncogene cooperation has come from studies with transgenic mice. F1 offspring from a cross between MMTV-myc and MMTV-ras transgenic mice showed considerable acceleration in the appearance of tumours compared to the parental strains (Sinn et al., 1987). The concept of functional complementation between oncogenes, suggests a limited number of inter-related regulatory pathways governing cell proliferation. In addition, the necessity for two oncogenes to cooperate to fully transform a primary cell, provides perhaps the first indication that multiple oncogenic events are responsible for the development of a tumour.

To understand the molecular basis of oncogene cooperation, it is necessary to examine the functions of the various proto-oncogenes.

1.4 The functions of cellular oncogenes

It is now clear that proto-oncogenes cover the full array of cellular interactions, from growth factors and growth factor receptors, through cytoplasmic protein kinases and GTP binding proteins, to nuclear transcription factors.

Growth factors such as the platelet-derived growth factor (PDGF) and epidermal growth factor (EGF), and the inhibitory factor, transforming growth factor β, TGFβ, activate a variety of cellular signaling devices through transmembrane receptors (reviewed by Cross and Dexter, 1991). The end point in the signaling pathways that govern cell proliferation appears to be at the level of transcription.

Activation of receptor tyrosine kinases transduce the signals across the plasma membrane. Stably linked transmembrane homodimers (Insulin receptor), or monomers induced to form homo- or hetero-oligomers (PDGF receptor and NGF receptor), autophosphorylate on tyrosine residues upon activation. Receptor autophosphorylation is thought to act as a switch to induce the binding of cytoplasmic signaling proteins to the receptor. The SH-2 domains found in a variety of cytoplasmic molecules including GAP, PLC-γ, and src (Sadowski et al., 1986), are thought to complex with the autophosphorylated receptors to enable the catalytic domains of the cytoplasmic molecules to come into close proximity with the receptor kinase domain. The subsequent phosphorylation of the cytoplasmic signaling molecules stimulates downstream pathways, such as the ras pathway (for a recent review see Pawson, 1992).

Mutant forms of growth factor receptor have been identified such as the EGFR encoded by the v-erb-B gene (Ullrich et al., 1984), or the CSF1R, a product of the v-fms oncogene (Coussens et al., 1986).

Other oncogenes can be grouped as mutant proteins associated with the inner surface of the plasma membrane such as the src non-receptor protein tyrosine kinase or the ras gene products. Whereas, Mos, Pim-1 and Raf represent an additional group of cytoplasmic oncogenes whose products are protein serine-threonine kinases, involved in signal transduction and cell cycle regulation (Sagata et al., 1989; Roy et al., 1990)(reviewed by Cantley et al., 1991).

The transcription factor oncogenes represent the final group of oncogenes which can release a cell from the constraints of normal signal transduction processes (reviewed by Lewin, 1991). They can be divided into three classes. The first are the nuclear receptors for ligands such as steroid and thyroid hormones, an example of this class is the c-erbA gene product. The second class represented by the c-fos and c-jun proteins, are inducible by extracellular signals and are nuclear in location. The final class is also inducible by upstream signals but cytoplasmic in location (c-rel).

Mutant forms of fos and jun are thought to elicit their oncogenic response by increasing the transcription of genes required for growth, whereas mutant forms of rel and erb A are thought to promote growth by repressing the transcription of genes that suppress growth (Lewin, 1991).

It thus appears that the cellular function of oncogenes fall into a number of classes, which cover the entire spectrum of cellular responses, from mitogenic stimulation to transcriptional regulation.

In recent years it has been recognised that the cooperation of genes to elicit a transformed phenotype not only involves the dominant interaction of oncogenes, but also recessive alterations in negative growth controlling genes, losely termed "tumour suppressor genes". The inactivation of such genes whose function may counteract the stimulatory effect of dominantly acting oncogenes, offers an additional array of genes now believed to play a critical role in tumorigenesis.

1.5 Tumour suppressor genes

1.5.1 Evidence from cell fusion studies

The idea that loss-of-function mutations participate in the development of tumours first came from studies on the tumorgenicity of cell hybrids (reviewed by Harris, 1988).

The fusion of malignant cells to non-malignant cells can produce non-tumorigenic hybrid cell lines, which can subsequently segregate to become tumorigenic once again (Harris et al., 1969; Ephrussi et al., 1969; Engel et al., 1969). Analysis revealed the tumorigenic segregants had lost several chromosomes present in the non-tumorigenic hybrids (Stanbridge et al., 1981). Stanbridge and colleagues noted the loss of chromosomes 11 and 14 in HeLa x fibroblast tumorigenic hybrids. Klinger also noted the loss of chromosome 11 as well as other chromosomes such as 2, 13, 17, and 20, in similar HeLa x fibroblast hybrids (Klinger, 1982).

Hybrid studies between tumorigenic fibrosarcoma x fibroblast cell lines implicated the loss of chromosomes 1 and 4, suggesting perhaps that for different malignancies there was a unique set of tumour suppressor genes involved (Benedict et al., 1984).

Experiments with the technique of microcell transfer enabled the introduction of a single chromosome from a donor to a recipient cell (Saxon and Stanbridge, 1987; Fournier and Ruddle, 1977). The transfer of chromosome 11 derived from a normal human fibroblast cell, into a Wilms' tumour cell line (Weissman et al., 1987), HeLa cells, and tumorigenic HeLa x fibroblast hybrids, resulted in the suppression of tumorigenicity (Saxon et al., 1986). This provided direct evidence for the existence of a tumour suppressor gene on chromosome 11.

Viral and oncogene induced, as well as spontaneous tumorigenicity, has been shown to be suppressed in hybrid studies. For example, hybrids between SV40 transformed and non-tumorigenic BALB/c3T3 cells produced some non-tumorigenic clones. Analysis of these clones showed the presence of integrated SV40 DNA and the

expression of T antigen (Howell and Sager, 1981). Similarly a Chinese hamster embryo fibroblast cell line when fused to a tumorigenic transfectant carrying the EJ c-H-ras oncogene showed suppression in most hybrids despite the continued presence and expression of the H-ras oncogene (Craig and Sager, 1985). This data indicates that suppression proceeds independent of the presence of the oncogene encoded product.

1.5.2 Evidence from familial studies

The concept that loss of tumour suppressor genes could assist in the development of neoplasia was further endorsed by the study of familial cancers (reviewed by Knudson, 1986). Knudson originally suggested that retinoblastomas resulted from two sequential mutations (Knudson, 1971). This became more specific from the finding that the two target loci are the two copies of the Rb gene, and that critical alterations serve to inactivate Rb function (Friend et al., 1986; Bookstein et al., 1988; Horowitz et al., 1989). In the familial case, a mutant allele is inherited, whereas in the sporadic forms of the disease both mutant alleles develop from somatic mutations. Loss of heterozygosity studies have been used to identify candidate suppressor genes both in familial and sporadic forms of tumours. To date, there are six known, or presumptive human tumour suppressor genes that have been isolated as molecular clones. These include p53 (Baker et al., 1989), retinoblastoma (Rb)(Friend et al., 1986), von Recklinghausen neurofibromatosis type 1 (NF1)(Cawthon et al., 1990), Wilm's tumour (WT-1) (Call et al., 1990), familial adenomatosis polyposis (FAP) (Solomon et al., 1987; Bodmer et al., 1987), and deleted in colon carcinoma (DCC) (Fearon et al., 1990; Marshall, 1991b). All with the exception of DCC have been shown to be passed through the germline causing a congenital predisposition to a variety of tumours. However, there is circumstantial evidence to suggest a role for DCC in a syndrome, with autosomal dominant inheritance, which predisposes individuals to carcinomas of the colon, and other organs (Lynch et al., 1985).

1.5.3 The functions of tumour suppressor genes

The biochemical functions of most of the tumour suppressors are still poorly understood. However, the fact that it is loss of gene function which contributes to tumorigenesis, infers that the gene products may act as repressors of cellular proliferation or inducers of differentiation.

An initial clue to the function of the Rb gene product came from the discovery of its' association with the E1A protein (Whyte et al., 1988). Loss of normal function of pRB by mutation or sequestration by a viral oncoprotein, in some way contributed to the neoplastic process. It now appears, there is a role for pRB in the regulation of transcription during the cell cycle. The Rb product has been shown to be an inhibitory component of the E2F transcription complex (Chellappan et al., 1991; Hiebert et al., 1992). In addition, pRB is phosphorylated just before the S-phase of the cell cycle (Buchkovich et al., 1989; Chen et al., 1989). Binding to the E2F complex is inhibited by pRB phosphorylation. In this way, pRB may control the transcription of genes governing the cell cycle (DeCaprio et al., 1989). This may in turn be controlled by the phosphorylation status of pRB. An additional factor which may play a role in this particular scheme, is the inhibitory growth factor TGF-β, which can prevent the cell cycle dependent phosphorylation of pRB (Laiho et al., 1990). One consequence of loss of pRB function may therefore be a loss of responsiveness to TGF-β (Roberts et al., 1991).

There is some evidence to suggest a link with the cell cycle in the function of the p53 protein. Over-expression of the wild-type p53 gene was shown to block the growth of osteosarcoma and colorectal carcinoma cells, by preventing entry into S phase (Diller et al., 1990; Baker et al., 1990).

Although the specific function of cellular p53 is still unknown, it is thought to inhibit DNA synthesis following DNA damage, by blocking the cells at the G1 phase of the cell cycle. Increased levels of p53 protein were detected in proliferating normal human bone marrow progenitor cells after DNA damage (Kastan et al., 1991). The

increase in p53 protein occurred via a post-transcriptional mechanism and was temporally correlated with a transient arrest in the G1 phase of the cell cycle. However, cells with mutant p53 or no p53, continued through the cell cycle after DNA damage. The exact role for this increase in p53 protein is unclear, although it may provide a mechanism allowing surveillance of the genome for genetic damage. The interaction of the normal p53 protein with DNA appears to be direct. A concensus binding sequence has been identified in several genomic clones consisting of two copies of a 10 base pair motif. Mutants of the p53 protein failed to bind to the concensus dimer (El-Deiry et al., 1992). Co-transfection studies have shown that wild-type p53, but not oncogenic forms of the protein, are able to activate expression of genes adjacent to the p53 DNA binding sites (Kern et al., 1992). Furthermore, co-transfection of mutant and normal constructs indicated that mutant p53 was capable of inhibiting the inducing activity co-ordinated by the normal gene. This may be explained by complexes forming between the wild-type and mutant proteins which are unable to bind DNA and thus unable to control the transcription of genes necessary for the proper maintenance and regulation of the cell cycle.

In contrast to the nuclear localised normal products of the p53 and RB genes, the DCC gene, a candidate suppressor gene located on chromosome 18q, commonly inactivated in colon carcinomas (Fearon et al., 1990), shows considerable homology to the cell adhesion molecule N-CAM. It is therefore presumed to act at the cell surface. The reduction in expression of a putative cell adhesion molecule in colorectal epithelial cells may suggest a mechanism of clonal expansion by loss of growth-restraining signals associated with cell adhesion (Fearon and Vogelstein, 1990). A recent paper by Narayanan and co-workers provides the first biological evidence for DCC as a tumour suppressor. High levels of DCC antisense RNA resulted in inhibition of cell adhesion in Rat-1 fibroblasts. The Rat-1 fibroblasts were capable of anchorage-independent growth and showed an increased tumorigenicity *in vivo* (Narayanan et al., 1992).

The functions of tumour suppressor genes appear as diverse as the functions of dominantly acting oncogenes. In very few cases, are two oncogenes activated in the same tumour, but there are several instances where two or more tumour suppressor genes display alterations. For example in lung and osteosarcomas both p53 and RB show genetic alterations (Harbour et al., 1988; Takahashi et al., 1989), and in colorectal tumorigenesis possibly three putative suppressor genes, FAP, DCC, and p53, are implicated at various stages of progression (Fearon and Vogelstein, 1990). Thus tumorigenesis occurs as a result of the inactivation of multiple tumour suppressor genes, and the concerted activation of dominantly acting oncogenes. No doubt additional tumour suppressor genes will be uncovered, which may allow further elucidation of the synergistic pathways by which tumour suppressors and oncogenes control cell growth.

1.6 The mammalian ras proto-oncogenes are members of a ras superfamily

The products of the mammalian ras proto-oncogenes, Harvey-ras (H-ras), Kirsten-ras (K-ras), and N-ras, are members of a family that includes at least 40 ras-related small GTP-binding proteins, which share the ability to interconvert between an inactive (GDP-bound) and an active (GTP-bound) state (for a review Barbacid, 1987). They are involved in a wide spectrum of biological functions, such as growth, differentiation, organisation of the actin cytoskeleton and intracellular vesicle transport and secretion (reviewed by Hall, 1990).

The ras gene super-family can be divided into 3 classes, the ras-related, the rho-related (Drivas et al., 1990) and the rab-like proteins (Zahraoui et al., 1989) In the context of this thesis, we will concentrate on the ras-related family.

The various ras-related family members are about 50-60% identical, in terms of amino acid homology, and their molecular masses vary from 21kDa (for ras) to 27kDa (for ral). In addition to the ras proto-oncogenes, mammamlian members include Ral(A and B), Rap1(A and B), Rap2, and R-ras. Many other homologues have been identified in lower eukaryotes such as in the fruit fly *Drosophila melanogaster* (Neuman-Silberberg et al., 1984), the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Powers et al., 1984; DeFeo-Jones et al., 1985), and in the slime mould *Dictyostelium discoideum* (Reymond et al., 1984).

Several members of the ras gene superfamily have been isolated by screening with highly conserved oligonucleotide probes, for example spanning the GTP binding site (Touchot et al., 1987). Others such as the rap1 and rap2 genes were isolated by low stringency screens of human cDNA libraries using the Drosophila Dras3 probe (Pizon et al., 1988), or in the case of the human R-ras gene with the v-H-ras probe (Lowe et al., 1987).

1.7 The structure of the mammalian ras proto-oncogenes

The ras proto-oncogenes H-ras, K-ras, and N-ras are expressed in most, if not all mammalian cell types and have remarkable sequence conservation. The first 85 amino acids encoded by the three genes are identical between mouse and human. The product of the genes is a 21kDa protein, encoded by four exons, except for K-ras-2 which possesses two alternative fourth coding exons (exons IVA and IVB) encoding two isomorphic p21 proteins of 188 and 189 residues (McGrath et al., 1983; Capon et al., 1983).

The promoter regions of the cellular ras genes resemble so-called housekeeping genes, in that they lack a TATA box but possess multiple copies of the sequence GGGCGG, the SP1 concensus binding site (Ishii et al., 1986; Ishii et al., 1985). The promoter structure together with the degree of sequence conservation, lends support to the belief that the ras proteins perform essential cellular functions.

As in the case of the distantly related G proteins, normal p21ras binds to GTP and catalyses its hydrolysis to GDP (Scolnick et al., 1979; Shih et al., 1980). The ras protein cycles between an active GTP-bound and an inactive GDP-bound state. The regions important for GTP binding have been identified by mutagenesis (Sigal et al., 1986; Lacal et al., 1986) and X-ray crystallography studies (Krengel et al., 1990). Oncogenic mutations of p21ras occur at or near the sites of interaction with the β- and γ-phosphates of the nucleotide (residues 12, 13, 59, 61, and 63)(Colby et al., 1986; Der et al., 1986; Fasano et al., 1984), or sites of interaction with the guanine base (residues 116, 117, 119 and 146)(Walter et al., 1986; Der et al., 1988; de Vos et al., 1988). The former reduce the GTPase activity of the protein while the latter decrease its' affinity for nucleotide, hence increasing the rate of exchange of bound GDP for cytosolic GTP. The overall effect is to cause an accumulation of the GTP-bound active form of p21, thus eliciting an oncogenic signal (Barbacid, 1987).

While some substitution mutations reduce the biological effect of ras proteins (in yeast complementation and mammalian NIH3T3 focus forming assays), they do not disrupt their nucleotide binding, cellular localisation or stability. In this respect, residues 32-40 have been associated with the effector activity of the ras proteins (Sigal et al., 1986). This region is hydrophilic and presumably lies on the external surface of the ras molecule (Willumsen et al., 1986). X-ray crystallography studies have shown that this region in particular shows dramatic conformational changes after GTP hydrolysis (Jurnak et al., 1990; Milburn et al., 1990). The trigger for these alterations appears to be a change in the coordination of the active-site Mg^{2+} ion as a result of loss of the γ-phosphate of GTP (Schlichting et al., 1990).

1.8 Processing of the ras protein

To function properly the ras protein must be localised to the plasma membrane. This localisation involves a complex series of post-translational modifications, centering on the carboxy-terminal motif CAAX (where C is cysteine, A any aliphatic amino acid and X any uncharged amino acid) found in almost every ras superfamily protein. Processing begins with the proteolytic removal of the three C-terminal amino acids to leave Cys186 at the carboxyl terminus. Carboxyl methylation of Cys186 and the attachment of a polyisoprenyl group then proceeds (Gutierrez et al., 1989; Hancock et al., 1991b). Further processing occurs for H-ras, N-ras and K-rasA with palmitoylation of the two upstream cysteines (181 and 184). However, the predominant form of K-ras, K-rasB lacks these upstream cysteines and is consequently not palmitoylated (Hancock et al., 1989). Instead a string of basic residues close to Cys186 appear to be important in its' membrane localisation (Hancock et al., 1990; Hancock et al., 1991a).

Mutation of the Cys186 residue has been shown to block both membrane localisation and transformation (Willumsen et al., 1984), thus providing proof that membrane attachment is essential for cellular transformation.

1.9 Biochemical aspects of ras regulation

The ratio of GTP:GDP on p21ras can in theory be regulated by either altering the rate of GTP hyrolysis or by altering the rate of nucleotide exchange. There is evidence that both mechanisms operate in the regulation of the ras pathway, indeed perhaps acting synergistically to contribute to ras activation (Feig and Cooper, 1988b).

1.9.1 Isolation of GAP - a GTPase-activating protein

In 1987 Trahey and McCormick reported that a cytoplasmic factor present in *Xenopus* oocytes was capable of stimulating the rate of GTP hydrolysis of purified injected p21N-ras (Trahey and McCormick, 1987). The factor stimulated GTP hydrolysis more than 200-fold compared to the intrinsic *in vitro* rate of purified p21ras, although it was shown to have no stimulatory effect on two codon 12 ras mutants (Trahey and McCormick, 1987). The factor, GTPase-activating protein (GAP), was shown to interact with p21ras at a site previously identified as the "effector" site (Cales et al., 1988). It was subsequently purified and cloned from bovine brain and human placenta (Trahey et al., 1988; Vogel et al., 1988). The transforming ability of these mutants most probably results from the accumulation of the active p21 GTP-bound state.

GAP is a 120kDa protein that is mainly cytosolic, although the amino terminal which is dispensable for GTPase activation is extremely hydrophobic, and is thought to play a role in membrane attachment. The region of GAP carboxy to this is highly conserved, 98% identical between bovine and human species (McCormick, 1989). The protein contains two SH2 and one SH3 motifs, regions of homology to non-catalytic portions of pp60src (Sadowski et al., 1986), thought to bind phosphotyrosine residues of other proteins.

From the evidence to date, it appears that GAP operates as an upstream negative regulator of ras, but it may also function as a downstream effector. Data to support this, has come from ras proteins with mutations in the "effector" region, residues 32-40 (Adari et al., 1988; Cales et al., 1988). Mutations of this domain prevent GAP interaction and delete the transforming potential, but do not effect the nucleotide binding, membrane localisation, and stability of the ras proteins. Additional data to support GAP having an effector function has come from studies on the effects of ras and GAP on the opening of receptor-coupled potassium channels in atrial cells (Yatani et al., 1990). The blocking of the channels was shown to occur through the

interaction of p21ras with GAP, by the uncoupling of the heterotrimeric G protein, G_k, from its receptor. These experiments propose a role for GAP as a downstream effector. Further corrobarative data, comes from mutation studies such as the double yeast mutant of ras, Leu68/RAS1(term.), which is truncated at residue 185 and thus lacks the membrane localisation site. The mutant protein is kept in the GTP bound form and is cytosolic in location. The resultant block in ras action induced by the double mutant can be overcome by simply increasing the amount of GAP in the cell (Gibbs et al., 1989).

However, evidence against GAP having a major effector role has emerged from studies where GAP overexpression in cells transformed by v-src and high levels of normal ras, caused reversion to a normal phenotype, indicating a solely negative role (Zhang et al., 1990; DeClue et al., 1991; Nori et al., 1991).

1.9.2 Regulation of GAP

At the very least, GAP functions as a negative regulator of p21ras. It thus seems important to be able to, in turn, regulate GAP. A possible mechanism for such regulation, has been proposed by Tsai and colleagues, who have shown that GAP activity is blocked by the interaction of lipids and breakdown products such as phosphatidic acid, phosphatidylinositol phosphates and arachidonic acid (Tsai et al., 1989b). The model proposes that activation of tyrosine kinases by mitogenic stimuli, which involves phospholipid metabolism, can in turn activate the ras pathway by inhibiting GAP activity (Tsai et al., 1989a). A potential problem with this theory, is that the concentration of lipids necessary to inhibit GAP are unlikely to represent the levels found *in vivo*. However, another protein which may compete with GAP has been shown to interact with certain lipids (Tsai et al., 1990). This so called GTPase inhibitory protein, is activated by phospholipids which normally inhibit GAP. Phospholipids produced as a result of mitogen stimulation may thus inhibit GAP and

stimulate the GTPase inhibitory protein, with the overall effect of increasing the proportion of ras bound to GTP, and promoting the biological activity of ras.

A number of growth stimulatory receptor and non-receptor tyrosine kinases have been shown to phosphorylate GAP (Molloy et al., 1989; Ellis et al., 1990). However, tyrosine phosphorylation of GAP does not alter its activity. Instead, the more likely outcome of phosphorylation appears to be the formation of heteromeric complexes either directly with the tyrosine kinases (for example the activated PDGF and CSF-1 receptors) or with other cytoplasmic proteins. Two such proteins p62 and p190 have been shown to associate with GAP (Ellis et al., 1990; Moran et al., 1991). In v-src or EGF stimulated fibroblasts, up to half of the cytosolic GAP is found complexed with p190 compared to very little in normal proliferating fibroblasts. Complexes of GAP with p62 and p190 may reduce the capacity of GAP to interact with p21ras, thus promoting accumulation of p21-GTP and activation of the pathway. However, the alternative explanation cannot be ruled out, that complex formation stabilises an active conformation of GAP that functions as an effector for ras signalling.

An additional factor in ras/GAP regulation has come from the isolation of a cDNA encoding a 21kDa protein with 50% amino acid homology with p21ras. Rap1A or K-rev1, was isolated on the basis of its ability to revert a Kirsten sarcoma virus transformed NIH3T3 cell (Kitayama et al., 1989; Pizon et al., 1988). The product binds to GAP in a strictly GTP-dependent manner, but does not undergo GAP-mediated hydolysis (Frech et al., 1990). If GAP bound to oncogenic p21 elicits a response, then K-rev-1 may act to block this effect by sequestering the available GAP molecules.

1.9.3 Isolation of another GAP-like molecule, product of the neurofibromatosis gene

The story is further complicated by the isolation of an alternative GAP-like molecule the product of the neurofibromatosis 1 (NF1) gene. As previously discussed (Section 1.5.2: Tumour Suppressor Genes), the NF1 locus represents a tumour suppressor gene. The NF1 protein shows strong homology to GAP and to the yeast equivalent proteins, IRA1 and IRA2 (Buchberg et al., 1990). Furthermore, expression of the NF1 homology domain is able to complement the loss of the yeast IRA genes, and is able to stimulate GTPase activity of yeast RAS2 and human H-ras proteins. However, as in the case of GAP, it is unable to stimulate the GTPase activity of oncogenic H-ras$^{val\text{-}12}$ and yeast RAS2$^{val\text{-}19}$ mutants (Xu et al., 1990), and is also unable to bind to the ras effector mutants. Interestingly, it has a much higher affinity for p21ras than GAP (more than 20-fold), but has a slower turnover (30-fold lower specific activity) (Martin et al., 1990). The exact significance of NF1 to ras regulation remains uncertain, but the kinetics suggest that NF1 may play a significant role particularly at low p21ras concentrations, whereas at high p21ras concentrations GAP may be the more critical regulator (Martin et al., 1990).

1.9.4 Guanine nucleotide exchange factors

The rate of exchange of guanine nucleotides from the ras proteins is very slow in the presence of physiological concentrations of Mg^{2+}. It therefore seemed necessary for the presence of a factor able to accelerate the release of GDP. Such a factor was discovered in yeast encoded by the CDC25 gene (Jones et al., 1991), and also in the *Drosophila* system of eye development, encoded by the Sos gene (Simon et al., 1991). Similar exchange factors have now been identified in eukaryotes. A 60kDa protein has been isolated in cytoplasmic extracts of human placenta (REP for Ras Exchange-Promoting) (Downward et al., 1990) and a 100-160kDa factor isolated from rat brain

cytosol (RAS-GRF) (Wolfman and Macara, 1990). Both have been shown to act reversibly, and to promote exchange of GDP for GTP even on ras mutants insensitive to the action of GAP. These factors identify what appears to be a common mechanism for the control of the ras pathway, acting in opposition to the GTPase enhancing activity of GAP and NF1. In the case of the rho proteins, there are two types of GDP/GTP exchange proteins, the GDP dissociation stimulator (GDS), similar in function to the exchange factors already described (Isomura et al., 1991). In addition, there is a GDP dissociation inhibitor (GDI) factor, which inhibits the GDP/GTP exchange reaction (Ueda et al., 1990; Fukumoto et al., 1990). The interaction of these factors with the rho proteins, may present an additional means of regulation, at the level of post-translational modification and membrane localisation. GDI forms a cytosolic complex with rho-GDP. An unknown signal may induce complex dissociation, and allow the GDS factor to form a rho-GTP membrane localised complex. Rho GAP may then catalyse the hydrolysis reaction to a rho-GDP bound form, which would then reunite with GDI to stimulate its' removal from the membrane (Isomura et al., 1991). Post-translational modifications of the C-terminus of rho have been shown to be important for the membrane localisation and interaction with the GDP/GTP exchange proteins, but not with GAP (Hori et al., 1991). Whether a similar mechanism exists for the regulation of the mammalian ras proteins remains to be seen.

It therefore appears, that regulation of the ras signaling pathway is complex, involving the interaction of multiple factors, both stimulatory and inhibitory. These themselves, may in turn be regulated by an additional set of molecules.

1.10 Studies of lower eukaryotes

A comprehensive understanding of the complexities of the mammalian ras pathway may come from studies of lower eukaryotes such as yeast, the nematode worm (*Caenorhabditis elegans* or *C. elegans*), and the fruit fly (*Drosophila melanogaster*). In the yeast, *Saccharomyces cerevisiae*, the ras proteins, RAS1 and RAS2 regulate adenylate cyclase activity and cAMP concentrations (reviewed by Broach and Deschenes, 1990). The concentration of cAMP controls the growth and cycling of the yeast cells. Nutritional starvation results in a decrease in cAMP and exit from the cell cycle. The link between the nutritional state of the cell and cAMP concentrations, is thought to occur through the action of the product of the yeast CDC25 gene. Evidence suggests, that the CDC25 product is a guanine nucleotide exchange factor for RAS, and is itself regulated by nutrients and metabolites. The yeast RAS genes are also negatively regulated by two GAP/NF1 homologues, products of the IRA1 and IRA2 genes. The biochemical similarities between the yeast and mammalian pathways are marked, considering the evolutionary distance. However, there is no evidence that mammalian ras controls cellular levels of cyclic AMP.

Similar biochemical analogies have surfaced from the studies of *C. elegans* . The product of the let60 gene of *C.elegans* has been shown to be 84% identical in its first 164 amino acids to other vertebrate and invertebrate ras gene products. The let60 gene product is necessary for specifying the fate of vulval precursor cells, through a signal transduction pathway, during inductive processes in normal development (Han and Sternberg, 1990).

Mutations that elevate the expression of let60 activity or extrachromosomal arrays containing about 30 copies of wild-type let60 cause a multivulva (Muv) phenotype. Whereas recessive partial loss-of-function mutations, that lead to the reduction of let60 activity result in an opposite vulvaless (Vul) phenotype. Thus the level of expression of the let60 gene is critical for the outcome of cell fate. Dominant activating mutations of let60 which result in a Vul phenotype are caused by point

mutations of codon13, which results in an amino acid substitution, glycine to glutamic acid (Beitel et al., 1990).

The discovery of another *C.elegans* gene let23 necessary for vulval induction may shed some light on the mammalian ras pathway. The product of let23 is a tyrosine kinase of the EGF receptor family (Aroian et al., 1990). Let23 functions upstream of let60 ras, indicating a link between growth factor receptors and ras.

A similar link between a tyrosine kinase and ras has emerged from studies of a *Drosophila* system. The product of the *Drosophila* sevenless (sev) gene encodes a transmembrane protein tyrosine kinase which has been implicated in the development of the *Drosophila* compound eye (reviwed by Ridley and Hall, 1992b). In the absence of functional sev each ommatidia of the eye lacks a particular photoreceptor, the R7 cell. It seems that sev functions as a receptor for a signal (most likely Boss, Bride of sevenless) that determines whether R7 develops into a photoreceptor or non-neuronal cell. By screening for mutations in genes which decreased the effectiveness of signalling by Sev, the gene Sos (Son of sevenless), was isolated (Simon et al., 1991). Sos is homologous to the yeast CDC25 product and is thought to act as a guanine nucleotide exchange factor for Ras1 (Peters, 1990). In addition, a *Drosophila* GTPase activating protein, Gap1 has been isolated by Gaul and co-workers. Their genetic data from loss-of-function mutants, indicates a solely negative role for Gap1 in the Sev signaling pathway (Gaul et al., 1992). Recently, Rubin and colleagues have shown that Boss and Sev null mutants can be rescued by transgenic activated $Ras1^{val12}$ mutants, providing proof that ras activation is a direct result of signal transduction from the sev receptor (Fortini et al., 1992).

Hence, from the studies of lower eukaryotes, it may be possible to decipher something of the complexities of the mammalian ras signal transduction pathway. In particular, it would be useful to learn more about the downstream effectors of ras-mediated signal transduction.

1.11 Ras signalling pathways and cellular transformation

The biological effects of ras on cell growth have been well documented. Both proliferative and differentiative cellular responses have been accredited to the effects of ras.

Microinjection of ras proteins induces fibroblasts to proliferate, in particular the oncogenic form of ras, which elicits dramatic effects on cell morphology, causing plasma membrane ruffling, enhancing fluid-phase pinocytosis, and stimulation of phospholipase A (Bar Sagi and Feramisco, 1986). However, in rat pheochromocytoma (PC12) cells ras induces NGF-like differentiation, altering the adrenal chromaffin-like cells into sympathetic neuron-like cells (Bar Sagi and Feramisco, 1985).

The biological response to ras-mediated signals, therefore appears to reflect intrinsic properties of a specific cell type, which are as yet not fully understood. However, the roles for normal ras as an intermediate in signal transduction, and oncogenic ras in cellular transformation, are gradually becoming clearer.

The introduction of oncogenic ras into a cell activates gene transcription via AP-1 transcription factors, of which Fos and Jun are members (Bos et al., 1988; Rauscher et al., 1988; Sassone-Corsi et al., 1988; Chui et al., 1988). In addition, it has been shown that insulin-induced expression of Fos can be blocked by the introduction of ras inhibitory mutants, H-ras(Asn-17) and H-ras(Leu61,Ser-186) (Medema et al., 1991). Similarly the dominant negative H-ras(Asn-17) mutant can inhibit the effects of both v-src and H-ras on c-Jun activity and phosphorylation (Smeal et al., 1992). The mechanism for c-Jun induction by Src or Ras is thought to involve disruption of the c-Jun:inhibitor interaction which is postulated to operate through the δ negative regulatory region (Baichwal et al., 1991).

Together these data advocate a role for ras as an intermediate in a growth factor-induced signalling cascade. Fos and Jun appear likely candidate transription factors, able to convert signals from growth factors and transforming oncoproteins into changes in gene expression.

Differentiation of neuronal PC12 cells can be induced by the treatment with nerve growth factor (NGF), which results in the tyrosine phosphorylation of multiple cellular proteins. These proteins have now been identified as serine/threonine or tyrosine kinases which function as part of a mitogen stimulated protein phosphorylation cascade (Thomas et al., 1992; Wood et al., 1992).

In a recent paper, the serine/threonine kinase encoded by the c-Raf-1 gene has been shown to be an upstream activator of the serine/threonine kinase MAP kinase-kinase, MAPK-K (Kyriakis et al., 1992). This protein kinase in turn tyrosine phosphorylates and activates MAP kinase, which is thought to exert its effect through stimulation of c-Jun activity. While oncogenic H-ras is a constitutive stimulator of c-Jun activity and phosphorylation, normal H-ras is a serum dependent modulator of Jun activity (Smeal et al., 1992).

In addition to the kinase-cascade pathway, there appears to be a protein kinase C (PKC)-dependent pathway. The end-point of which, once again appears to be through the modulation of transcription factors such as Fos and Jun. The exact mechanism through which ras activates PKC is still unclear, but may be a result of enhanced phospholipid turnover (reviewed by Marshall, 1991a).

A further component of signalling pathways from activated receptors has been recently reported by Pelicci and colleagues (Pelicci et al., 1992). SHC proteins bind to activated EGF receptors with high affinity *in vitro*. The highly conserved SHC gene encodes at least two different protein products. Both contain a single SH-2 domain but differ in their N-termini. They are hypothesised to act as intermediates, possibly binding to downstream effectors with their alternative N-terminal domains. Interestingly, overexpression of the SHC gene in NIH3T3 cells leads to morphological transformation and tumour formation in nude mice, suggesting an oncogenic role in signal transduction.

In conclusion, cellular transformation by activated ras appears to act through PKC-dependent and PKC-independent pathways, which terminate in an alteration of the cellular transcription program.

1.12 Prevalence of ras mutations

The products of the H-ras, K-ras and N-ras genes are expressed in almost every human fetal and adult tissue (Furth et al., 1987). In some cell lineages, immature cells express more p21ras than mature cells. However, in some fully differentiated cells, such as neurons and epithelial cells of the endocrine glands, p21ras is expressed in abundant amounts.

In human tumours, the ras mutations observed include those of codon 12, 13 and 61 (Parada et al., 1982; Bos et al., 1985) (for a review Bos, 1989). The overall incidence of ras mutations in human tumours is estimated to be about 10-15% (Barbacid, 1987). The incidence of K-ras mutations is high in colon adenocarcinomas 40-50%, and in lung adenocarcinomas 20-30%, while almost all pancreatic adenocarcinomas harbour K-ras mutations (Bos et al., 1987). In some cases, the frequency of ras mutations in a particular tissue, correlates with the level of expression of the ras gene, for example in the colon and pancreas. There are exceptions, such as in lung and liver where the

level of expression of the ras genes is very weak yet the incidence of mutations is relatively high (Leon et al., 1987).

1.13 Timing of ras activation

The causal nature of ras activation in tumorigenesis is best demonstrated by studies of transgenic animals. Mice carrying the ras oncogene under the control of tissue specific promoters show a correlation in tissue expression and tumour formation (Sinn et al., 1987). However, in many cases, oncogene expression precedes tumour formation by several months, for example MMTV LTR-ras showed a latency period of 1-6 months (Sinn et al., 1987; Groner et al., 1987). These observations suggest that oncogene expression is necessary, but not in itself sufficient to generate tumours.

The incidence of ras mutations at different stages of human malignancy has been investigated by Lemoine and co-workers. They found that the frequency of ras activation in human thyroid tumours was similar at all stages: benign microfollicular tumours (50%), differentiated follicular carcinomas (53%), and undifferentiated follicular carcinomas (60%), with all three ras genes displaying mutations (Lemoine et al., 1989). This data demonstrates the presence of ras mutations in benign lesions, establishing ras activation as an early step in tumorigenesis.

Similarly, ras mutations have been identified in benign lesions such as keratocanthomas of the skin (Leon et al., 1988) and adenomatous polyps of the colon (Bos et al., 1987; Forrester et al., 1987).

However, tumour heterogeneity in human seminoma implys that at least in this system ras activation is not the initiating event (Mulder et al., 1989). In addition, in the case of acute myeloid leukemia (AML), N-ras mutations are considered to occur at a late stage of progression (Farr et al., 1988).

1.14 Animal models

Tumours can be induced in animals either by the inbreeding of laboratory strains susceptible to spontaneous tumour development, or more commonly through the treatment of animals with carcinogenic agents. The development of animal model systems for the study of tumorigenesis, offers the experimentor a more controlled and reproducible environment, than for instance, the analysis of human tumours.

Such model systems for the study of tumour induction and progression, include the rodent mammary model system. Mammary carcinomas develop in 90% of the animals, with a latency period of about 6-12 months after a single dose with the alkylating agent N-nitrosomethylurea (NMU) (Derynck et al., 1986).

In contrast, lymphomas of the thymus can be induced with intraperitoneal injections of NMU after a latency period of about 3 months. Gamma irradiation of mice also results in thymic lymphomas but with a longer latency period, of about 6 months (Guerrero et al., 1986). The spectrum of ras mutations are different depending on the treatment used, with activation of N-ras occurring after NMU exposure (Guerrero et al., 1985), whereas K-ras mutations are more prevalent after radiation treatment (Guerrero et al., 1984; Guerrero and Pellicer, 1987). Additional genetic factors are clearly important, with differences in tumour yield occurring between different mouse strains. For example, the mouse strain 129/J develops a high incidence of tumours after NMU treatment but a low yield after whole body radiation (Meruelo et al., 1981). The precise reason for these differences remain unclear.

Studies of tumours of the central and peripheral nervous system have been aided by the observation that a single dose of the alkylating agent ethylnitrosourea (ENU), between 15 days of gestation and 10 days post-partum, develop neuroectodermal tumours with a high incidence 4-10 months later (Rajewski et al., 1979).

However, the model system that shall be discussed in the context of this thesis is the mouse-skin model system. It provides an organ which is accessible to treatment, and easily monitored for the development of tumours.

1.15 The mouse-skin carcinogenesis model

1.15.1 Introduction

The two-stage protocol for the mouse-skin model system, involves the treatment of mouse skin with initiating and promoting agents. The initiation step is considered irreversible because initiated cells can remain dormant in the skin, capable of activation by promoter application for up to one year (Van Duuren et al., 1975). This is followed by a reversible promotion stage, involving multiple applications of a tumour-promoting compound. After several weeks the formation of benign papillomas develop, some of which (10-20%) progress to form carcinomas.

The complete carcinogenesis protocol on the other hand, involves a large single dose of carcinogen, or multiple small doses of an initiator such as DMBA. This results in fewer papillomas but more carcinomas.

1.15.2 Mechanisms of initiation

The observation that the initiating chemical used correlated with the type of mutation formed, supported the proposal by Barbacid and co-workers (Zarbl et al., 1985b; Zarbl et al., 1985a), that the carcinogen interacts directly with the DNA to create a genetic mutation.

In studies of skin carcinogenesis with DMBA as the initiator, over 90% of papillomas that developed after promotion with TPA displayed the same A -> T transversion mutation at codon 61 of the H-ras gene (Quintanilla et al., 1986). The same mutation was not observed when MNNG was used as the initiating chemical (Quintanilla et al., 1986).

The notion that a genetic mutation of the H-ras gene could act as an initiating event in mouse skin tumorigenesis was substantiated by experiments in our laboratory. It was shown that viral ras genes could substitute for the chemical initiation stage of mouse skin carcinogenesis. When Harvey- or Balb-murine sarcoma viruses were applied to mouse skin, followed by treatment with TPA, papillomas developed after 4-5 weeks, some of which progressed to form carcinomas (Brown et al., 1986).

In addition, transgenic mice expressing mutant H-ras from a suprabasal-specific keratin 10 promoter, develop generalised hyperkeratosis of the skin and forestomach. Animals that survived to the age of 7-8 weeks developed highly differentiated papillomas at sites of wounding (Bailleul et al., 1990). This data has since been corroborated by Leder and colleagues using a similar transgenic model (Leder et al., 1990). A transgenic line carrying a fusion gene, consisting of an embryonic globin promoter linked to the activated v-H-ras oncogene, developed papillomas at sites of skin abrasion. In addition, more than 90% of the mice developed papillomas within 6 weeks of application of phorbol 12-myristate 13-acetate (PMA).

1.15.3 Mechanisms Of Tumour Promotion

1.15.3.1 The Role Of TGF-β

In its most simplistic form, promotion can be viewed as selection for an initiated cell amongst the background of normal cells. This "clonal selection" hypothesis has gained credibility from the study of growth factors TGF-α and in particular TGF-β. These factors are released from platelets (Wakefield et al., 1988), produced by keratinocytes upon wounding (seen as a promotional stimulus), and after treatment of the skin with tumour promoters such as TPA (Akhurst et al., 1988; Pittelkow et al., 1989). TGF-α is thought to provide a mitogenic stimulus to epithelial cells, while TGF-β has been shown to inhibit keratinocyte proliferation *in vitro* (Furstenberger et al., 1989a; Coffey et al., 1988).

We (Haddow et al., 1991) and others (Glick et al., 1991), have shown that epidermal cells which harbour mutant H-ras genes can still respond to the negative effects of TGF-β. These data present an apparent paradox, in that the growth of "initiated cells" can still be down-regulated *in vitro* by TGF-β. A possible explanation has come from *in situ* hybridisation data, and immunocytochemical staining of skin tumours. While both benign and malignant tumours produced high levels of TGF-β mRNA, only low levels of protein could be detected. Early tumours may therefore evade the negative effects of TGF-β by altered translational or post-translational processing of the newly-synthesised factor (Haddow et al., 1991).

There is evidence to suggest that TGF-β mediates its inhibitory effect on cell proliferation through c-myc expression (Pietenpol et al., 1990a). The block in c-myc expression occurs at the level of transcriptional initiation and is thought to be mediated by the product of the retinoblastoma gene, pRB (Pietenpol et al., 1990b). Intrestingly, a retinoblastoma cell line that was resistant to the negative effects of TGF-β was shown to have lost TGF-β receptors from the cell surface (Kimchi et al., 1988).

1.15.3.2 TPA and PKC

Phorbol esters such as TPA, may also exert an effect on gene expression through the modulation of the PKC subspecies.

The functions of the various PKC proteins are diverse. They appear to have dual functions, both positive, in the control of gene expression such as induction of the interleukin-2 receptor and some proto-oncogenes, and negative such as in the control of cell proliferation (Nishizuka, 1986).

TPA stimulates PKC activity it is thought by masquerading as a diacylglycerol-like molecule, the normal activator of PKC. TPA, as well as activating PKC, also degrades the protein, and it has been suggested that removal of PKC from the cell may relieve the negative control on cell proliferation, leading to uncontrolled cell growth (Nishizuka, 1988).

TPA has been shown to induce c-jun expression (Lamph et al., 1988), and most recently it has been reported that phorbol esters can enhance the trans-activation potential of c-Jun by phosphorylation of residues 63 and 73 (Franklin et al., 1992).

Indirect evidence that TPA may stimulate cell proliferation through the ras kinase signalling cascade, comes from the observation that antigen stimulation of the T cell receptor results in the hyperphosphorylation of c-raf via the serine/threonine kinase PKC (Siegel et al., 1990).

1.15.3.3 TPA and genetic damage

An alternative aspect to tumour promotion, is the role of TPA in the creation of DNA damage (Furstenberger et al., 1989b). TPA can induce sister chromatid exchanges in hamster fibroblasts (Kinsella and Radman, 1978). It is also capable of creating DNA single strand breaks in human leukocytes (Birnboim, 1982), and in mouse keratinocytes (Hartley et al., 1985).

TPA, but not the second stage promoter RPA, has also been shown to contribute to the aneuploid state of the cell, with the appearance of double minutes being a common feature. These structural aberrations are described as non-random with mouse chromosomes 1, 2, 5, 6, and 18 displaying a preference for genetic alterations (Petrusevska et al., 1988).

In summary, it appears that the mechanisms of tumour promotion with chemicals such as TPA appear multi-faceted. Clonal selection of an initiated cell from the inhibitory environment of normal cells may be fulfilled through the action of growth factors such as TGF-β or alterations in cellular signalling through a PKC-dependent pathway. Genetic damage may also contribute to the outgrowth of papillomas possibly by the loss of tumour suppressor genes which control cell growth or genes which control genomic stability.

1.16 Genetic changes associated with mouse-skin tumour progression

1.16.1 Allelic imbalance of mouse chromosome 7 is a frequent event in mouse-skin tumorigenesis

Although animal model systems have been used to study oncogene activation in chemical carcinogenesis (reviewed by Barbacid, 1987; Balmain and Brown, 1988; Guerrero and Pellicer, 1987), loss of heterozygosity studies have been hindered by the lack of informative polymorphisms in inbred mouse strains. Loss of heterozygosity (LOH), at a particular chromosomal locus in a tumour suggests the presence of a tumour suppressor gene. However, it may also be that the primary lesion is an activating mutation which is unmasked by loss of the corresponding normal allele. LOH studies in human tumours have been successfully used to map and clone several tumour supprressor genes.

We have previously described an experimental approach to overcome the lack of informative restriction length polymorphisms (RFLPs) present in the inbred laboratory mouse strain. By crossing different inbred strains, and generating tumours in F1 hybrids, it has been possible to study genetic events, other than activation of oncogenes, that take place in mouse skin tumour progression.

Through the study of such tumours it has been possible to provide the first demonstration of mitotic recombination and non-disjunction in mouse tumours (Bremner and Balmain, 1990). Initial data indicated that imbalances between parental alleles on mouse chromosome 7 arose at high frequency only in tumours carrying activated H-ras. Most frequently this occurred by non-disjunction, but in certain cases the mechanism was best explained by a mitotic recombination event. Since H-ras resides on chromosome 7, it was thought that these gross-chromosomal events provided a mechanism by which mutant H-ras was duplicated and/or the normal allele lost. Further experiments, using the more informative hybrids between outbred *Mus*

spretus and inbred *Mus musculus*, have provided more conclusive data on these mouse chromosome 7 imbalances. An XbaI RFLP enables the parental *spretus* and *musculus*, normal and mutant alleles to be distinguished.

Even in early papillomas it has been shown that the chromosome carrying the mutant allele was over-represented in the form of a trisomy of chromosome 7. However, under no circumstances was the normal H-ras allele duplicated (Bremner and Balmain, 1990). These results, together with the expression data on the levels of the normal and mutant alleles (Jandrig, Haddow, Bremner and Balmain, in preparation), indicate that over-representation of the mutant H-ras is a frequent and apparently crucial event in mouse skin tumorigenesis.

The underlying impetus for loss of normal and/or duplication of mutant alleles implied that there may be some form of tumour suppressor role for the normal H-ras gene. Only by loss or under-representation of the normal allele would the malignant phenotype induced by the mutant gene manifest itself.

1.16.2 A role for normal ras as a tumour suppressor gene

The question of whether mutant ras acts in a dominant or a recessive fashion in tumorigenesis has been considered in the past (Guerrero et al., 1985). Examples of loss of the normal allele have been reported by other groups. Human mammary carcinoma cell lines that possess the H-ras codon 12 mutation in some cases also show the loss or under-representation of the normal allele (Kraus et al., 1984).

This has been demonstrated not only in cell lines but also in primary tumours. In murine thymic lymphomas, reduction or loss of normal K-ras and N-ras alleles have been reported (Diamond et al., 1988; Guerrero et al., 1985).

This suggests that the mutant gene is co-dominant or even recessive in nature. However, what of the *in vitro* models of ras fibroblast transformation which indicated a dominant role for ras ? These experiments may be misleading since they either required a complementing oncogene (Land et al., 1983), or very high levels of

expression (Spandidos and Wilkie, 1984). In addition, there may be inherent differences between fibroblasts and epithelial keratinocytes in their response to alterations in ras gene dosage.

However, despite the quantity of data on loss of heterozygosity on mouse chromosome 7, the co-incident loss of a putative gene closely linked to H-ras cannot be eliminated.

1.16.3 Evidence for another locus on mouse chromosome 7

Data from our group that may support the involvement of another locus on mouse chromosome 7, has come from the analysis of a primary carcinoma, SN158 (and a lymph node metastasis from the same mouse). Tumour SN158 showed loss of heterozygosity for markers distal to H-ras while maintaining heterozygosity at the ras locus and for proximal markers. The most likely explanation for the observed allele ratios was a mitotic recombination of a region distal to H-ras (Bremner and Balmain, 1990). This region may therefore harbour a putative tumour suppressor gene. In agreement with this concept, are studies of human chromosome 11 which contains the syntenic region of mouse chromosome 7, including the H-ras locus. Loss of alleles at 11p15 is a common genetic feature in many human tumour types (Mannens et al., 1988; Koufos et al., 1984; Riou et al., 1988; Mackay et al., 1988).

As well as loss of syntenic regions on human 11, the 11q13 region has been shown to be amplified in a number of different human cancers, including breast, squamous cell and bladder carcinomas (Ali et al., 1987; Lammie et al., 1991; Berenson et al., 1989)(reviewed by Lammie and Peters, 1991). The candidate genes for this region include INT2, HSTF1 (hst-1), BCL1 and PRAD1 (D11S287). Both HSTF1 and INT2 have been implicated in tumorigenesis. INT2 is one of the most frequent targets for transcriptional activation by MMTV (Nusse, 1986), whilst HSTF1 originally identified in assays of human tumour cell DNA can also be activated by the integration of MMTV (Peters et al., 1989). The co-amplification of HSTF1 and INT2 in about 15% of breast cancers and about 50% of esophageal carcinomas (Tsuda et al., 1989) clearly indicates the pathogenic importance of this region in a number of different human cancers. However, whereas amplification of these genes does not result in detectable transcripts, the closely linked PRAD1 gene shows elevated expression in neoplastic mammmary tissue as compared to normal.

Interestingly, there is also a candidate suppressor gene, MEN1 that has been mapped to 11q13. This gene has been implicated in the rare autosomal dominant condition, familial MEN1 or Wermer syndrome (Larsson et al., 1988).

Thus several candidate genes residing on regions syntenic to the H-ras locus of mouse chromosome 7, have been identified in human malignancies. Whether these genes play a role in mouse skin tumorigenesis remains to be seen.

1.16.4 Other genetic changes associated with tumour progression

Through the use of hybrid mice and highly polymorphic microsatellite sequences, which can be distinguished by the polymerase chain reaction (PCR), we have accumulated LOH data on several mouse chromosomes. These studies have confirmed the previously reported chromosome 7 changes (Bremner and Balmain, 1990), and in addition have shown LOH on mouse chromosomes 11, 6 and at lower frequencies on 1 and 4 (C.Kemp and F.Fee, unpublished results). Alterations on 6

and 7 have been seen in both benign and malignant tumours, whereas changes on chromosomes 11, 4, and 1 are only detected in carcinomas. The important gene on chromosome 11 is thought to be the p53 tumour suppressor gene. Candidate genes on the other chromosomes are as yet unknown.

1.17 Progression of squamous to spindle cell carcinomas

1.17.1 Spindle cell carcinomas represent a distinct entity

Mouse skin tumorigenesis thus appears to occur through a series of discrete steps associated with specific genetic alterations, from an initiated cell, to a benign papilloma, and subsequently to a fully malignant carcinoma. It now seems that this final carcinoma stage can be categorised into two distinct histological entities. This sub-division is between a well-differentiated form of carcinoma, termed the squamous cell carcinoma, and a much less differentiated form, the spindle cell carcinoma.

Spindle cell carcinomas have been characterised in human tumours for many years (Evans and Smith, 1980). However, the origin of these tumour cells have been the subject of much controversy. Some pathologists have classified the tumours as epidermal in origin, whereas others believe they are derived from fibroblasts (Battifora, 1976).

In humans, spindle cell carcinomas are found as rare components of cancers mostly of the oral cavity and upper aero-digestive tract. Clinicians define the tumours as biphasic, with a carcinomatous and a sarcomatous component. Spindle metaplasia has also been identified in neoplastic tissue of the lung (Matsui and Kitagawa, 1991), breast (Kaufman et al., 1983) and the uterine cervix (Steeper et al., 1983).

1.17.2 Spindle cells show loss of differentiation markers

Intermediate filaments are cytoskeletal components of cells expressed in a tissue specific fashion. Epithelial cells normally express keratins, whereas mesenchymal cells express vimentin. In epithelial cells, it has been suggested that vimentin expression correlates with reduced cell-to-cell contact, which may reflect a reversion to a more embryonic pattern. It has been shown that during embryogenesis cells that express vimentin intermediate filaments can be derived from keratin-positive cells which apparently switch off keratin intermediate filament expression (Franke et al., 1981).

Studies on tumours induced *in vivo* have demonstrated changes in the cytokeratin specific differentiation markers (Klein-Szanto et al., 1983; Roop et al., 1988). Cytokeratins (CK) 8 and 18 are the pair of keratins associated with simple epithelia, and early stages of embryogenesis. CK 8 and 18 are not found in adult epidermis but have been shown to be reexpressed in human skin carcinomas, SV40-transformed human epidermal keratinocytes (Hronis et al., 1984), and in mouse keratinocytes transfected with oncogenic H-ras (Diaz-Guerra et al., 1992).

Studies by our group have shown a similar loss of differentiation markers in a number of mouse spindle cell lines. The highly malignant carB spindle cell line fails to express any keratins and the cell adhesion protein, E-cadherin. However, other spindle cell lines A5 and D3, still express keratins but stain negatively for E-cadherin (Stoler, Steinback and Balmain, submitted). This suggests that A5 and D3 represent an earlier stage of the spindle phenotype, and that E-cadherin may be lost before the keratin filament network is disrupted.

Although vimentin and keratin expression provide markers for the spindle cell phenotype, they in themselves do not give rise to the spindle phenotype (Sommers et al., 1992), with reexpression of E-cadherin in the spindle cell line, carB, producing no obvious morphological change (A.Cano, unpublished data).

1.17.3 A role for H-ras in the squamous to spindle conversion

Analysis of mouse skin tumours containing both differentiated and spindle cell parts, both components carried the same H-ras codon 61 mutation. However, the spindle component had a much higher mutant:normal H-ras gene dosage ratio. This correlation was obtained from PCR amplification using H-ras amplimers of material derived from paraffin sections of carcinomas of defined histological types (Buchmann et al., 1991). In addition, over the past few years we have isolated several cell lines from tumours at differrent stages of progression. Recently, we have been fortunate enough to obtain cell lines, representing both the squamous and spindle components from a single primary tumour, MSC11. Preliminary analysis of these cell lines, provided further correlative data for a role for H-ras in the squamous to spindle conversion (Data presented in Chapter 3 of the Results Section). Figure 1 provides a schematic overview of the genetic changes associated with the various stages of mouse-skin tumorigenesis, from H-ras activation in the initiated cell, through trisomy of chromosomes 6 and 7 in papillomas to mutational inactivation of p53 in the well differentiated squamous form of carcinomas. Finally as discussed, there is the transition to a undifferentiated spindle cell carcinoma, with the as indicated involvement of H-ras and possibly other loci. It is the investigation of this late stage of tumour progression that forms a large body of the work covered in this thesis. However, as will become clearer, we are also interested in the involvement of H-ras in the earlier stages of tumour development, symbolised by the consistent trisomy of mouse chromosome 7.

MULTI - STEP TUMORIGENESIS IN THE MOUSE SKIN SYSTEM

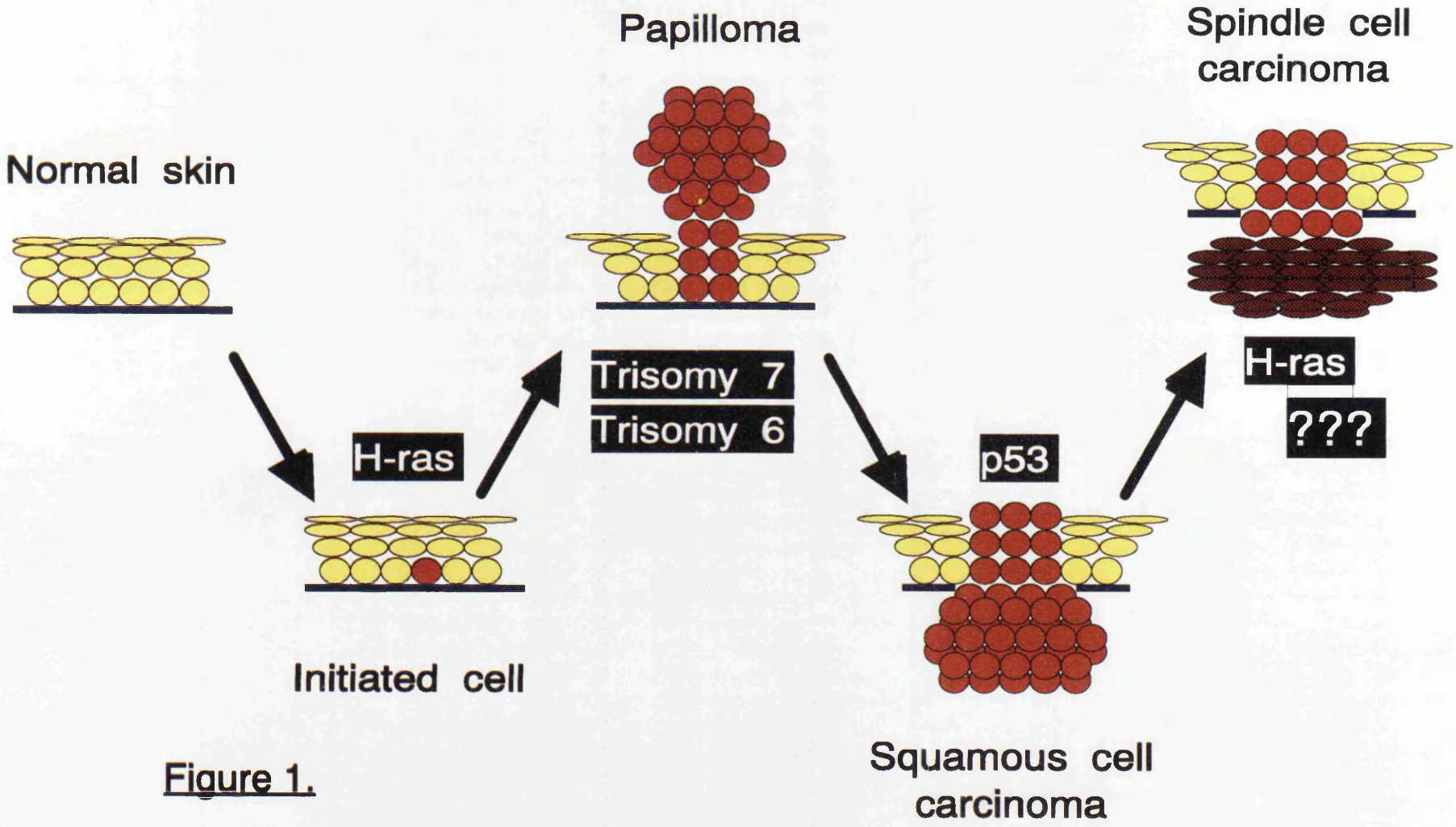


Figure 1.

1.18 Experimental approaches to investigating the role of H-ras in mouse skin tumorigenesis

This thesis outlines two alternative approaches to investigating the function of H-ras in mouse skin carcinogenesis.

The first is an *in vitro* approach: to investigate the effect on cell growth, morphology and tumorigenicity, of altering the gene dosage ratio of mutant:normal H-ras in specific mouse tumour cell lines.

The second approach involves an *in vivo* study of H-ras function. The ultimate aim of this approach is to create a mouse hemizygous for normal ras through the technique of gene targeting. This mouse can then be treated with carcinogens to examine the effect on tumour development of having only one functional copy of the normal H-ras gene.

1.18.1 The *in vitro* approach

To study the function of H-ras in mouse skin tumorigenesis it was decided to experimentally manipulate the levels of normal and mutant ras in specific cell lines. The cell lines that were chosen represent the transition event seen in mouse skin carcinomas, from a well differentiated squamous cell carcinoma to an undifferentiated spindle cell carcinoma.

Single cell clones representing both phenotypes were derived from a carcinoma, MSC11, that had both squamous and spindle components (refer to Results section for more details). Genetic analysis of these cell lines revealed the same H-ras codon 61 mutation and the same p53 mutations independent of cellular morphology. These observations together demonstrated that the spindle carcinoma had developed from the squamous cell carcinoma, whilst indicating that additional genetic alterations were responsible for the transition event. However, the levels of mutant H-ras were substantially different between the squamous and spindle cell lines. There was at least a 5-fold increase of mutant H-ras in the spindle cell line (see the Results section for a more detailed account).

The obvious question that arose from these observations was what was the role for H-ras in this conversion event ? Were alterations in H-ras gene dosage levels alone sufficient for the conversion event, or was another locus involved ? In addition, was the increase in mutant H-ras necessary to overcome a suppressor function of the normal gene, or was it more directly related to increasing the tumorigenicity of the cells ?

It was hoped, that by altering the levels of mutant and normal H-ras in these clonally derived cell lines, we would be able to elucidate a role for H-ras in the distinct transition event, from a well differentiated carcinoma to a undifferentiated more aggressive form.

1.18.2 The *in vivo* approach

The *in vivo* approach to investigating the function of H-ras involves creating a mouse that has only one functional H-ras allele in every cell. This can be acheived through the technique of gene targeting. By utilising the mechanism of homologous recombination in pluripotent embryo-derived stem (ES) cells, it is possible to introduce finite mutations into a specific genomic locus. The mutations most commonly take the form of an inactivating, or "knock-out" mutation, which render the gene of interest functionally obsolete.

ES cells are derived from the inner cell mass of a mouse blastocyst. It is possible to maintain the ES cells in a pluripotent state in culture, either by growing the cells on a feeder layer, or by addition of a growth factor termed LIF (Leukemia Inhibitory Factor) or DIA (Differentiation-Inhibiting Activity)(Smith and Hooper, 1987; Lindsay Williams et al., 1988).

The mutated ES cells can then be reintroduced into a mouse blastocyst, which is then implanted into a pseudo-pregnant mouse. The ES cells colonise the developing embryo resulting in a chimaeric mouse (for a shematic outline of the gene targeting approach see Figure 2).

It is therefore possible, to go from *in vitro* manipulation and mutation of ES cells, to the introduction of the desired mutation into the somatic cells, and in some cases the germline component of a developing mouse (reviewed by Robertson, 1986).

In this way, it has been possible to study the function of many different genes ranging from oncogenes, tumour suppressor genes, as well as genes controlling mouse embryogenesis.

TARGETING APPROACH : OUTLINE

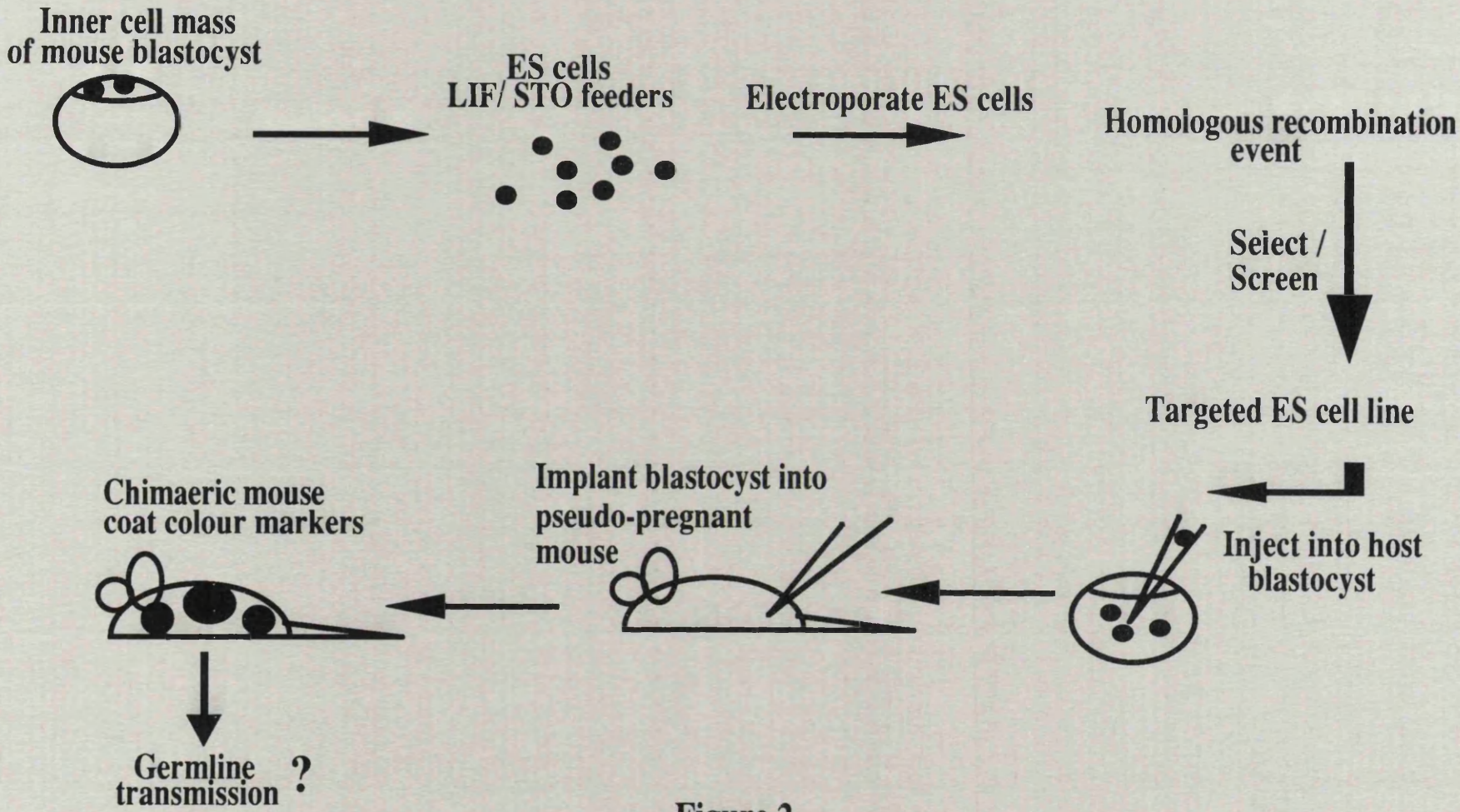


Figure 2.

To study the function of the H-ras gene in mouse-skin tumorigenesis, it was proposed to "knock-out" the H-ras gene, so that it would no longer be functional. Creating a mouse that possessed only one copy or no copies of H-ras should enable a functional analysis of the gene, both in embryogenesis, and in mouse-skin carcinogenesis studies. By treating a hemizygous or homozygous null mouse with chemical carcinogens, it should be possible to determine the role of H-ras in the early stages of tumour progression, in particular the proposed tumour suppressor role assigned to ras. If normal H-ras can suppress one copy of the mutant in the case of an initiated cell, then a hemizygous mouse that is initiated with DMBA would in theory have no functional H-ras to suppress tumour development, and thus should be more susceptible to tumorigenesis.

1.19 Gene targeting by homologous recombination in mouse embryonic stem cells

1.19.1 The mechanism of homologous recombination

Little is understood of the exact mechanism of homologous recombination. It is thought to occur by the Holliday model of recombination (Holliday, 1964). The model outlines a mechanism for reciprocal strand exchange between gapped duplex DNA and homologous linear duplex DNA, provided the linear DNA has an end complementary to the single-stranded DNA in the gap (West et al., 1982). Strand exchange then initiates at the single-stranded region of the gap and proceeds 5' to 3' by a process termed branch migration. Resolution of the recombination structures is thought to occur by specific endonucleolytic cleavage. The RecA protein has been shown to be important in promoting strand exchange in Escherichia coli, while T4 endonuclease VII isolated from the bacteriophage T4 cleaves Holliday junctions *in vitro* (Muller et al., 1990).

Gene targeting was first observed in mammalian fibroblast cells by Lin and colleagues in an artificial locus (Lin et al., 1985), and was subsequently demonstrated to occur at the endogenous β-globin gene by Smithies and colleagues (Smithies et al., 1985).

The homologous recombination events occur fairly rapidly after DNA transfection. In the case of two co-introduced plasmids, recombination was detectable within a 1-hour interval after the introduction of the plasmid DNAs into the cell nucleus (Folger et al., 1985). In the case of homologous recombination between transferred and chromosomal DNA, recombinants were detectable 2 days after transfer (Shulman et al., 1990).

1.19.2 The frequency of gene targeting

In general, the frequencies of gene targeting are relatively low in mammalian cells, in part, due to the competing pathway of random integration.

Random integration of the targeting vector occurs much more readily than the desired homologous recombination event. It is thus necessary to incorporate mechanisms in the vector design to overcome this bias. These will be discussed in more detail in the next section and in the Results section.

Factors that affect the frequency of homologous recombination are many and varied. Early experiments showed that restriction endonuclease cut, linear molecules offered better substrates for homologous recombination (Lin et al., 1984; Wake et al., 1982).

The frequency of recombination has been shown to increase linearly with the length of homology (Thomas and Capecchi, 1987; Shulman et al., 1990), with a two-fold increase in homology enhancing the targeting frequencies 20-fold or 4-fold, respectively.

Transcription has also been included as a factor that may effect the frequency of homologous recombination. Dexamethasone-induced transcription has been shown to stimulate recombination 6-fold over the non-induced levels (Nickoloff and Reynolds, 1990). This data refers to homologous recombination between plasmid vectors introduced into Chinese hamster ovary cells by electroporation. These findings are in direct contrast to the observation that genes such as adipsin and aP2 which are both transcriptionally inactive in ES cells, were none the less readily disrupted by homologous recombination (Johnson et al., 1989). It now appears, that efficient transcription of a gene, is not a prerequisite for it's successful disruption by homologous recombination.

Most recently Berns and colleagues, have reported a 20-fold increase in targeting frequencies at the Rb locus, by utilising isogenic DNA (te Riele et al., 1992). Ensuring that the recipient ES cells are of the same strain as the vector DNA presumably minimises possible mismatches that may occur through strainal divergence.

Factors such as length of homology, length of heterology, the site of linearisation and the source of vector DNA may all play a role in determining the frequency of gene targeting. However, the precise determinants of efficient homologous recombination are still unclear. Careful consideration to the design of the targeting vector is thus critical.

1.19.3 Targeting vectors: design and alternative strategies

A targeting vector is designed to recombine and mutate a specific chromosomal locus. The minimal components of such a vector are homologous sequences and a plasmid backbone. However, since the transfection and targeting frequencies can be quite low, it is desirable to incorporate various selection and enrichment schemes into the design of such a vector.

The use of a positive marker such as the neomycin resistance gene, allows for selection of those cells that receive the plasmid vector during the transfection procedure. The marker gene can also act as a mutagen, for instance, if it is cloned into, or replaces coding exons of the gene of interest.

There are two basic designs of targeting vector, the replacement vector, and the insertion vector. They differ in the site of linearisation and consequently the mechanism of integration.

The replacement vector is linearised in the plasmid backbone, and integrates into the genome by a double cross-over event. The insertion vector, on the other hand, is linearised in the region of homology, and as a result integrates by a single cross-over event (Figure 3). In order to enrich for homologous recombinants, as opposed to random integrants, certain features can be included in the construction of the vector. For instance, removing the promoter or polyadenylation sequences from the vector DNA (Schwartzberg et al., 1990; Charron et al., 1990). In theory, only when properly incorporated into the desired locus, should the positive marker gene be expressed and consequently give a selectable phenotype.

The more commonly employed system to enriching for homologous recombinants is to use a negatively selectable marker. This usually takes the form of the viral Herpes simplex thymidine kinase (HSVtk) gene. Homologous recombination results in loss of the gene from the ends of the vector DNA. However, random integration of the construct, should result in stable incorporation of the HSVtk gene, thus enabling these cells to be killed using the drug Gangcyclovir (Figure 4) (Capecchi, 1989; Thomas et al., 1986; Mansour et al., 1988).

The vectors so far described generally result in the insertion of a positive marker gene into the locus of interest very often as an inactivating mutational event. This results in a disruption (over 1kb in length) of the endogenous locus. Such a rearrangement of the normal locus may complicate the interpretation of any observed phenotypes.

Inorder to create more subtle mutations in the mouse genome, Bradley and co-workers have pioneered a strategy termed the "hit and run" approach (Hasty et al., 1991c). Hit and run vectors are modified insertion vectors that possess a mutation in the homologous sequences. They contain both a positive and a negative selection cassette situated in the plasmid backbone.

Types of targeting vectors

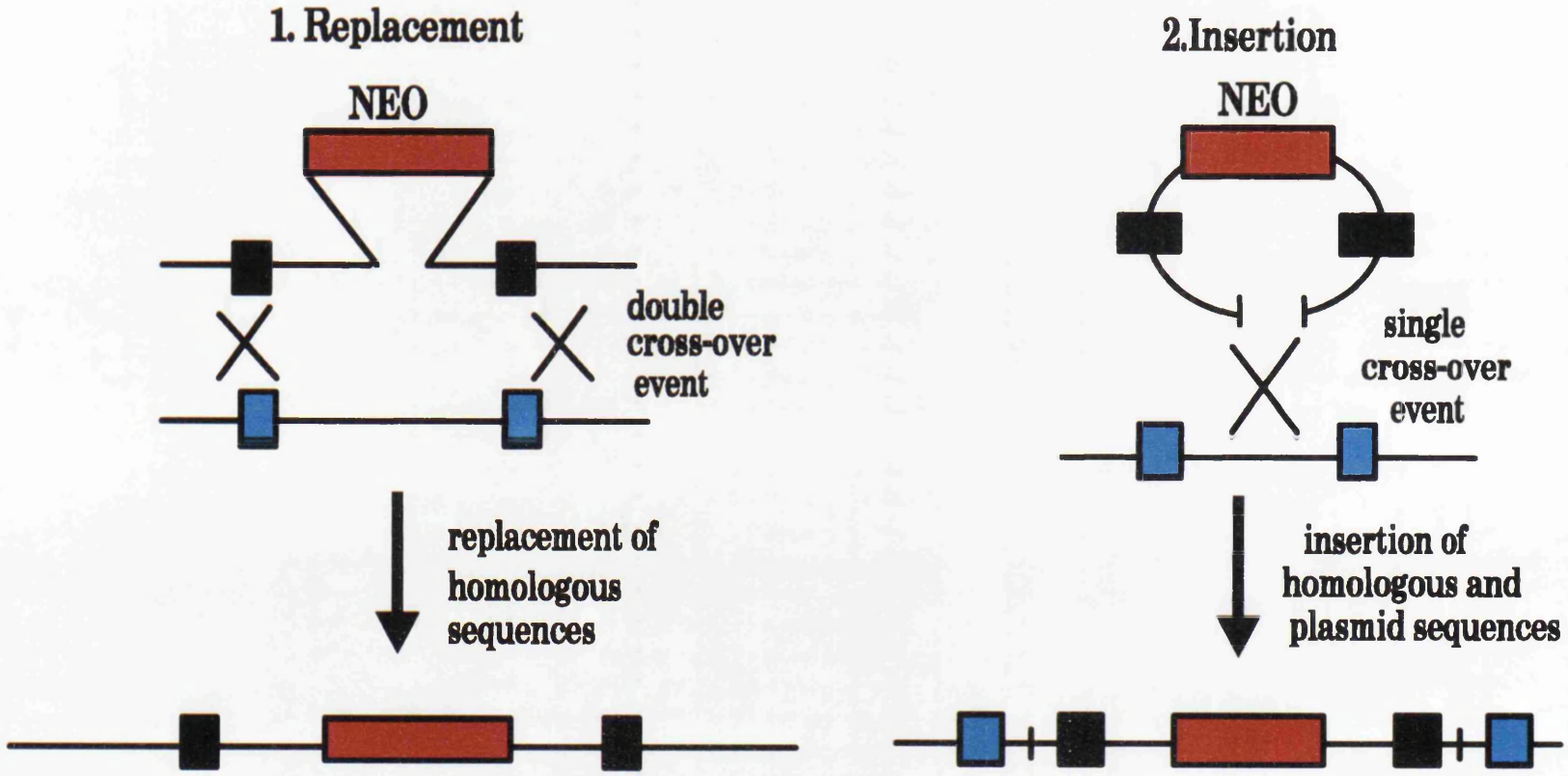


Figure 3.

The positive-negative selection (PNS) strategy

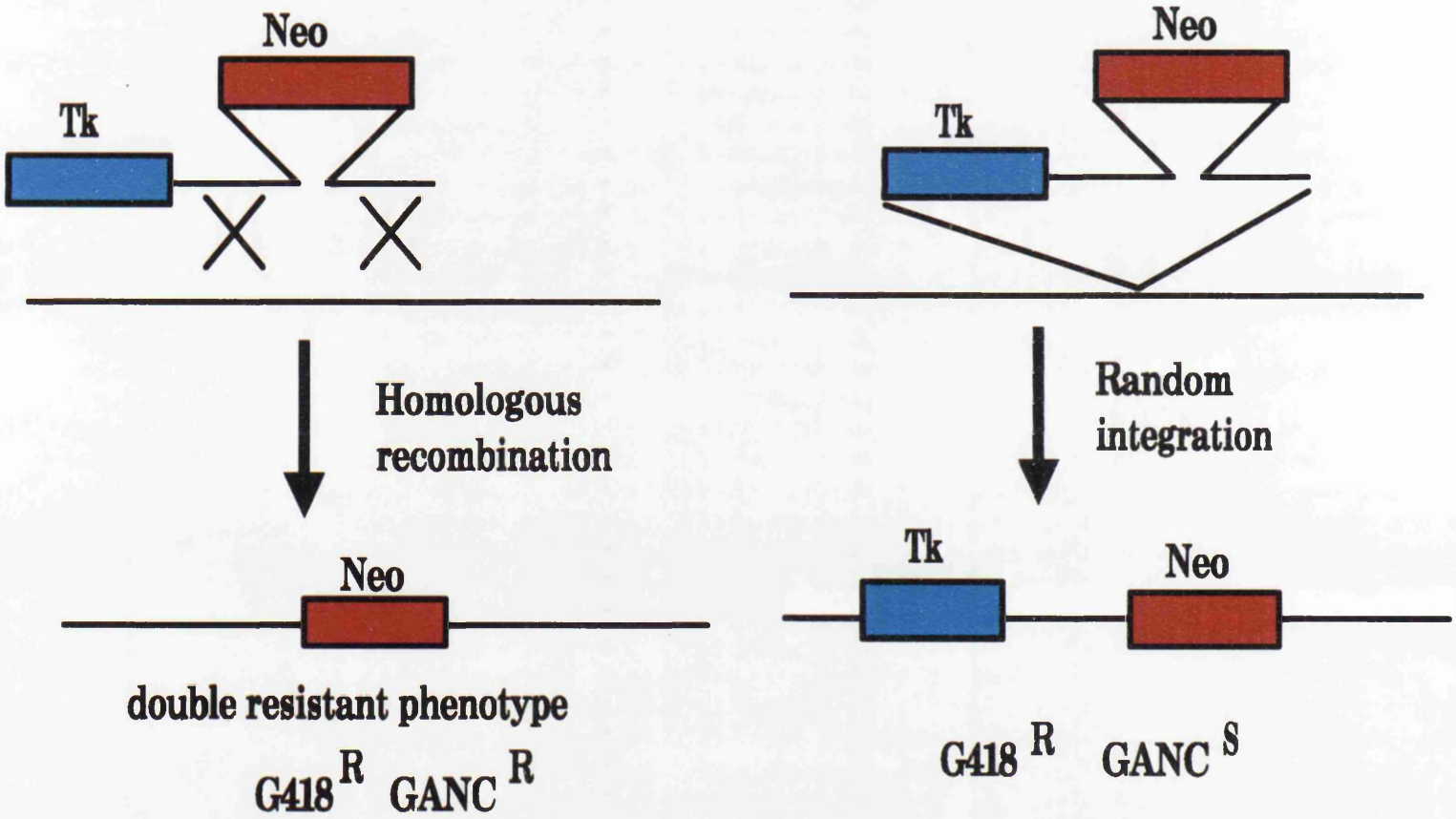


Figure 4.

The vector is first positively selected to encourage an insertion event, and then negatively selected (for example with gancyclovir) for a intrachromosomal "loop-out" event. The end result is reformation of the endogenous locus with the exception of the engineered subtle mutation (Figure 39). This technique has been successfully used to generate a point mutation in the mouse homeobox Hox 2.6 gene (Hasty et al., 1991b).

1.19.4 Phenotypes and the case for redundancy

The various strategies outlined above have been employed to successfully target a variety of genes. In a number of cases the inactivation of a gene has resulted in an anticipated phenotype. This is the case for the c-abl knock-out mice. c-abl shows highest expression in the thymus and spleen and was thought to play a role in lymphopoiesis or haematopoiesis. It was also known that activated c-abl was associated with tumours of the haematopoietic system. Homozygote c-abl$^-$/c-abl$^-$ mice became runted and died 1-2 weeks after birth. Autopsy showed thymic and spleen atrophy and T and B cell lymphopenia (Tybulevicz et al., 1991; Schwartzberg et al., 1991).

Similar expected phenotypes have been reported for the cystic fibrosis gene knock-outs, which mimic several features of the human condition, in particular altered chlorine ion transport (Dorin et al., 1992).

In addition, mice homozygous for the null p53 allele develop normally but are susceptible to the spontaneous development of a variety of neoplasms by 6 months of age (Donehower et al., 1992). This phenotype correlates with the tumour suppressor role allocated to the p53 gene.

However, not all null mice display the predicted phenotypes, and indeed in some cases, there are no observable changes what so ever. This is the case for the HPRT (hypoxanthine-guanine phosphoribosyltransferase) knock-out mouse, which although possesses no functional enzyme, fails to display the characteristic neurological phenotype of the human condition, Lesch-Nyhan deficiency syndrome. The mice are completely viable and die of old age (Hooper et al., 1987)(A.Clarke, personal communication).

A similar story is apparent for the mouse homologue of the *Drosophila engrailed* gene, En-2. Despite being essential for Drosophila embryonic segmentation and development, mice deficient for the En-2 gene showed no obvious defects in embryonic development, but instead show a subtle phenotype in the adult cerebellum. This is explained by the authors as functional redundancy with the related En-1 gene product (Joyner et al., 1991).

It seems therefore, that the predicted phenotype of a null mouse is not always realised. In the case of H-ras it may be speculated that the hemizygous mice would be more susceptible to chemical carcinogenesis. However, in the case of a mouse with no functional H-ras, the phenotype may be homozygous lethal, produce a mouse highly susceptible to tumorigenesis or result in yet another situation of functional redundancy. The actual phenotype of a hemizygous or homozygous H-ras null mouse thus remains to be confirmed.

Chapter 2
Materials and methods

2.1 Materials

Restriction enzymes were obtained from Boehringer Mannheim, Lewes, East Sussex; New England Biolabs (CP Labs, Herts.; Northumbria Biologicals Limited (NBL), Northumberland; or from GibcoBRL, Paisley. Proteinase K, RNase A and Klenow fragment of *Escherichia coli (E.coli)* were obtained from NBL. Polynucleotide kinase was obtained from Pharmacia Ltd., Milton Keynes, Buckinghamshire, and Taq polymerase from Promega, Southampton. The Sequenase^{TM} kit used in double-strand sequencing reactions was from Cambridge Biosciences, Cambridge, and the SP6/T7 transcription kit used in the RNase protection assay was obtained from Boehringer Mannheim.

Deoxynucleotides and mixed hexanuleotides were from Pharmacia. All radioisotopes came from Amersham International P.L.C. , Amersham, Buckinghamshire.

DNA size markers (lambda/HindIII, ΦX174/HaeIII) for gel electrophoresis, and the RNA ladder used in Northern blotting were supplied by Gibco/Bethesda Research Laboratories, Paisley, as were the Ultrapure^{TM} agarose and urea.

Nitrocellulose and nylon membranes were supplied by Sartorius Instruments Ltd., Belmont, Surrey; Gelman Sciences Ltd., Broadmills Northampton and Amersham International.

The Geneclean^{TM} kit for DNA fragment isolation was obtained from Stratech Scientific Ltd., Luton, Beds..

Bacto-tryptone, Bacto-agar and yeast extract were from DIFCO Laboratories, Detroit, Michigan, U.S.A.

Phenol was delivered as a water-saturated liquid from Rathburn Chemicals Ltd., Walkerburn, Peebleshire.

Absolute ethanol was supplied by James Burroughs (F.A.D) Ltd., Witham, Essex.

All other chemicals were obtained from BDH Laboratory Supplies, Poole, or Sigma Chemical Co. Ltd., Poole, Dorset.

Serum, media and supplements for cell culture were obtained from Gibco. The growth factor LIF and the drug Gancyclovir, used in the culturing and selection of embryonic-derived stem cells, were gifts from Martin Hooper, Department of Pathology, Edinburgh.

Plastic-ware for cell culture was supplied by Nunc Intermed, Roskilde, Denmark; Sterlin Ltd., Feltham, Middlesex; or by Falcon^{TM}, Becton Dickinson, Dublin.

Nude mice were from Marlan Olac Ltd., Bichester, Oxon.

2.2 Bacterial transformation

Commercial competent cells, DH5α^{TM}, supplied by GibcoBRL, were used for most purposes, otherwise competent cells were made according to Mandel and Higa,(Mandel and Higa, 1970). All ligations were carried out in Falcon 2063 polypropylene tubes. DNA ligations were diluted 5-fold in 10mM Tris-HCl (pH 7.5) and 1mM Na_2EDTA, and 1-5ul (approximately 10ng) of the dilution was added to 50 or 100ul of thawed competent cells kept on ice. The cells were left on ice for 30 minutes, then heat shocked at 42^{o}C for 45 seconds. The cells were left to recover for 2 minutes on ice, then incubated with 450 or 900ul of L broth (supplemented with 20mM glucose) at 37^{o}C for 1 hour. The tubes were spun at 1K for 5 minutes, the supernatant poured off, and the pellet gently resuspended in residual supernatant. This was spread onto LB plates (7.5g of Bacto-agar in 500ml of L Broth) supplemented with 100ug/ml ampicillin. The plates were incubated overnight at 37^{o}C.

2.3 Preparation of plasmid DNA

2.3.1 Mini-prep method for rapid screening

1-5ml of L broth(with 100ug/ml ampicillin) was inoculated using a loop. The cultures were incubated overnight at 37^{o}C. Cells were spun in 2ml eppendorf tubes. The pellets were resuspended in 100ul of lysis solution (25mM Tris-HCl(pH 8.0); 10mM EDTA; 50mM glucose; 0.5mg/ml lysozyme). 200ul of alkaline/SDS solution (0.2N NaOH, 1% SDS), was added to the tubes and mixed. 150ul of NaOAc (pH4.8) was then added and the tubes vortexed. After incubation on ice for 5 minutes (optional), the tubes were spun, and the supernatant added to a fresh tube containing 400ul of isopropanol. The tubes were spun at 10K for 15 minutes. The pellet was left to air dry and resuspended in 200ul of dH_2O. A further precipitation step with 100% ethanol is optional. RNaseA (100ug/ml f/c) was included at the time of restriction enzyme digestion.

2.3.2 Bulk-prep

A 10ml starter culture was prepared overnight. This was then added to 500ml of L broth containing antibiotic (100mg/ml ampicillin) and incubated for at least 16 hours. The culture was spun in 500ml Sorvall bottles at 5K for 5 minutes at 4^{o}C. The pellet was resuspended in 25mls of ice cold lysis solution (as Section 2.3.1). After 15 minutes on ice 50mls of alkaline /SDS solution (as Section 2.3.1) was added, and the solution mixed vigorously by swirling. Finally 37.5 mls of potassium acetate solution (60mls 5M potassium acetate, 11.5mls glacial acetic acid and 28.5 mls dH_2O) was added and the bottles incubated on ice for approximately 15 minutes. The bottles were spun at 6K at 4^{o}C for 15 minutes. The supernatant was decanted through gauze and isopropanol precipitated with 0.6 volumes of isopropanol. The pellet was resuspended in TE (10mM Tris-HCl; 1mM EDTA pH8.0). Further purification was

carried out using the Cesium chloride-Ethidium bromide gradient method (according to Maniatis (Maniatis et al., 1982), depending on the required quality of the DNA.

2.4 Restriction enzyme digestion of DNA

Plasmid DNA was digested with 5-10 units enzyme/ug DNA for 1-5 hours dependent on suppliers instructions. Genomic DNA was digested under overnight conditions with 10 units/ug DNA. Digestions were analysed for completeness, by gel electrophoresis.

2.5 Agarose gel electrophoresis

Agarose gel electrophoresis of DNA was performed using the appropriate horizontal gel apparatus, with agarose concentrations between 0.7-2.0% w/v, depending on the desired fragment resolution. The agarose was dissolved and cast routinely in TAE buffer (40mM Tris-HCl pH7.8; 20mM Sodium acetate; 1mM EDTA), which was also used as the running buffer. Ethidium bromide (1ug/ml) was either added direct to the molten agarose, or in the case of Southern blotting, the gel was soaked in an ethidium bromide solution, prior to UV illumination and photography.

2.6 Cloning techniques

2.6.1 Purification of DNA fragments from agarose gels

Using UV illumination, the fragment of interest was excised with the minimum amount of agarose. Purification was carried out by two methods depending on the

fragment size. For fragments 500bp-3kb the geneclean^{TM} technique (Stratech Scientific Ltd.) was used efficiently (see manafacturers instructions). For slightly larger DNA fragments 2-10kb spin-X tubes (supplied by Costar) were found to be most effective.

2.6.2 End-fill reactions

The conversion of DNA fragments with protruding 3' and 5' ends, to blunt end species, was carried out according to Maniatis (Maniatis et al., 1982), using the Klenow fragment of *E. coli* DNA polymerase I.

2.6.3 Removal of 5' phosphate groups from DNA

The dephosphorylation of DNA for the purposes of minimising recircularisation of vector DNA was accomplished by treating the restricted DNA with calf intestinal phosphatase (CIP). The protocol for this procedure is outlined in Maniatis (Maniatis et al., 1982).

2.6.4 Ligation of fragments

The ligation of DNA fragments was performed using bacteriophage T4 DNA Ligase. Fragments with sticky complementary ends were ligated overnight at 14^{o}C, whereas, blunt-end ligations were carried out at 4^{o}C for at least 24hrs. The total quantity of DNA per reaction varied, but the molar ratio of insert to vector was always greater than 3:1. The quantitation of vector to insert DNA was accomplished roughly, by gel electrophoresis. The vector DNA was usually phosphatased according to Section 2.6.3, inorder to minimise vector recircularisation. Fresh commercial 5X ligation buffer was routinely used. The volume of the reaction was kept to a minimum, usually 5-10ul.

2.6.5 Ligation of linkers

The linkers supplied in dry form by Promega, either phosporylated or non-phosphorylated, were made up in dH_2O to a concentration of 20pM/ul. The linkers were blunt-end ligated to the linearised DNA (approximately 6ul or 1.2nM linkers to 400ng of a linear fragment, approximately 3kb in size) according to Section 2.6.4.. The excess linkers were removed firstly by ethanol precipitation and then by enzyme digestion. The redesigned fragment was then cloned into the desired restriction site of a phosphatased vector, according to Section 2.6.4.

2.7 Preparation of radioactively labelled probes

Labelled probes from double-stranded DNA were made by the random priming method (Feinberg and Vogelstein, 1984; Feinberg and Vogelstein, 1983). Approximately 50ng of purified insert DNA was boiled for 10 minutes to ensure denaturation, and then labelled in a total volume of 50ul containing 10ul of OLB buffer (see below), 2ul of BSA (10ug/ml stock). Finally, $1.85x10^6$ MBequerel of (α-32P)dCTP and 5 units of Klenow enzyme were added, and the reaction incubated at room temperature for at least 6 hours.

The unincorporated nucleotides were removed by running the probe through a NICK-column^{TM} (Pharmacia), and the specific activity estimated using a scintillation counter.

Oligonucleotide buffer (OLB):

62ul 1M Tris pH 7.4

6ul 1M $MgCl_2$

1ul 2-Mercaptoethanol

2ul 15mM dATP

2ul 15mM dGTP

2ul 15mM dTTP

125ul 2M HEPES pH6.6

50ul 150 OD/ml Calf Thymus Hexanucleotides (Pharmacia)

Stored in aliquots at -20^{o}C.

Probes used in Southern and Northern blot hybridisation:

BS9 probe; 300bp EcoRI insert of BS9 (Ellis et al., 1980).

Neomycin probe; 2kb HindIII insert from pIC20Hneo, derived by cloning the XhoI/SalI neomycin fragment from pMC1POLA (Thomas and Capecchi, 1987) into pIC20H.

pB5 probe; 576bp insert liberated by a BssHII digest of plasmid vector pB5ras, created by PCR amplification of NMRI mouse RNA, generating a cDNA-type probe.

P6KpnI upstream probe; 600bp BamHI/KpnI fragment of original 1.8kb BamHI P6 fragment, created so as to avoid repetitive sequences.

Human H-ras specific probe; 500bp SmaI/HindIII fragment of human ras plasmid pR8.T24 (Bailleul et al., 1988).

2.8 Southern blot transfer of DNA

DNA fragments were separated by agarose gel electrophoresis and transferred to nylon membranes (Hybond-N^{TM} or Biotrace-RP^{TM}) by the method of Rigaud et al, (Rigaud et al., 1987). The gel was soaked in ethidium bromide solution and photographed. It was then soaked in 1.5M NaCl, 0.5M NaOH for 2x 20 minutes, and then in 1M NH_4OAc, 0.02M NaOH for 2 x 30 minutes. The gel was then transferred onto a nylon membrane (Hybond, Amersham, or Biotrace RP), by capillary transfer overnight in NH_4OAc/NaOH transfer solution. Alternatively vacuum transfer of the gel was used according to manafacturers' guidelines.

The membrane was then fixed by baking at 80^{o}C for 2 hours and hybridised as described in Section 2.10.

2.9 Northern blot transfer of RNA

10-20ug of total RNA was freeze dried and resuspended in freshly made sample buffer (50% formamide; 1xMOPS; 16.6% formaldehyde up to 100% with dH_2O). This was incubated at 60^{o}C for 15 minutes, then on ice for 5 minutes. 5ul of "blue juice" (50% glycerol, 10uM Sodium phosphate pH 7.0, and a few bromophenol blue crystals) was added to the samples prior to loading the gel (1% agarose; 1xMOPS; and 18% formaldehyde). The gel was run in 1xMOPS (for 1l of 10xMOPS: 42g MOPS; 6.8g NaOAc; 3.7g EDTA pH 7.0). The RNA ladder was supplied by BRL. The RNA was blotted onto nitrocellulose membrane (Sartorius^{TM}) and hybridised as below, Section 2.10.

2.10 Hybridisation of single copy probes

Filters were prehybridised at 42°C for a minimum of 4 hours in sealed plastic containers with 20-50ml of pre-heated solution {50% formamide; 4xSSPE(20xSSPE is 3.6M NaCl; 200mM NaH_2PO_4 pH 7.4); 0.5%w/v dried milk(marvel); 1% SDS; 10% dextran SO_4; 1mg/ml denatured salmon sperm DNA}. After the alloted prehybridisation time, radio-labelled probe (see section 2.7) was denatured by boiling for 10 minutes and added direct to the pre-hybridisation solution. Hybridisation was normally overnight. Filters were washed on a shaker with 1xSSC/0.1% SDS, then 0.5xSSC/0.1%SDS, at room temperature each wash for 15 minutes. The final wash was with 0.1xSSC/0.1%SDS at 50-65°C for 10-30 minutes with constant monitoring of the background with a geiger counter. The filters were exposed to Kodak X-OMAT AR or X-OMAT S film at -70°C in cassettes containing intensifying screens.

2.11 Western blot transfer of protein

The protein samples were prepared as in Section 2.19. The protein content was estimated using the Bio-rad assay (see manafacturers' guidelines). For the detection of H-ras specific bands the samples were first treated with 50% IgPAS (Protein A sepharose, see below), and then immunoprecipitated with antibody 172 (H-ras specific antibody, Oncogene Science Inc.), as follows: 1mg/ml of protein was made up with lysis buffer (see Section 2.19). 100ul of 50% IgPAS was spun down, and the lysates added directly onto the PAS. These were incubated on a rotator at 4°C for 2 hours. The supernatant was removed and respun to get rid of any residual PAS. To these pre-cleared lysates, a 1:100 dilution of antibody 172 and 1mg/ml f/c BSA were added and incubated at 4°C overnight. 100ul of 50% IgPAS was added over the lysates and incubated for 2 hours at 4°C on a rotator. The tubes were spun and the pellets finally resuspended in a volume of 35ul of western sample buffer { 10% B-mercaptoethanol; 2% (w/v) SDS; 30% (w/v) glycerol; 0.025% bromophenol blue a

few crystals of methyl green in 0.05M Tris-Hcl pH6.8 }. This was boiled for 5 minutes and the supernatant transferred to a fresh tube, which could if necessary be stored at -20^{o}C. The samples were run on a vertical 17.5% polyacrylamide gel (17.5% acrlyamide; 375mM Tris-HCl pH 8.8 ;0.1% SDS; 0.1% Ammonium Persulphate; 1ul/ml TEMED with a 5% polyacrylamide stacking gel) in Tris/Glycine running buffer (for 1l: 3.025g Tris base; 14.4g Glycine; 1g SDS, pH8.5). For size markers, Amersham Rainbow Protein molecular weight markers ranging from MW 2350-46000, were used.

The gel was trimmed to size (using the coloured size markers as a guide) and placed in the electroblotter as folows. Firstly 6 sheets of whatman paper soaked in Anode solution 1 (0.3 Tris/ 20% methanol pH 10.4), then 3 sheets of whatman paper soaked in Anode solution 2 (25mM Tris/ 20% methanol pH 10.4). The nitrocellulose membrane was then placed on top of the sheets of whatman paper followed by the gel itself. Finally 9 sheets of whatman paper soaked in Cathode solution (40mM 6-amino-n-hexonoic acid; 20% methanol pH7.2), were put in place and the electroblotter connected to a 50mA source for 30 minutes. The membrane was incubated with the primary antibody (Pan ras antibody - Oncogene Science Inc.) overnight at room temperature, and then washed in Blotto (5% Marvel in PBS with 0.1% NP40) for 90 minutes. Incubation with the secondary antibody (anti-mouse peroxidase, Amersham International Ltd.) was carried out for 30 minutes at room temperature and was then followed by washing in blotto for 1 hour and TBST (10xTBS for 1l: 12.11g Tris base; 90.0g NaCl pH7.4) for another hour. Luminol solutions (Amersham ECL kit) were mixed in equal proportions and the filter emersed for 1 minute. The filter was then placed in saran wrap and exposed to fast film for 30 seconds to several minutes.

2.12 RNase protection assay

The probe for the RNase protection assay, either mouse H-ras exon 2 or human specific, was generated using the SP6/T7 transcription kit (Boehringer Mannheim) following the manafacturers instructions.

10-20ug of total RNA was dried down in a speedivac, along with $1x10^6$ cpm of labelled probe. This was resuspended in 20ul of 80% FAB (80% formamide; 400mM NaCl; 40mM Pipes pH6.4; 1mM EDTA) and incubated for 16 hours at 50°C. The samples were then placed on ice and 300ul of digestion buffer (10mM Tris pH 7.6; 5mM EDTA; 0.3M NaAc pH 7.0; and 40ug/ml RNaseA, 150U/ml RNaseT1 f/c) was added to each tube. The samples were digested for 1 hour at 30°C. Then 2.5ul of 10mg/ml Proteinase K and 3.2ul of 20% SDS was added to each tube and the mixture incubated at 37°C for 15 minutes. 5ul of yeast tRNA (stock 1mg/ml) carrier was added to each sample. The samples were then phenol/chloroform extracted and precipitated with 100% ethanol, followed by 70% and 100% ethanol washes. The RNA digested samples were freeze dried and resuspended in 5ul of loading buffer (8ml formamide, 1ml 10xTBE, 20ul 0.5M EDTA, 10mg xylenecyanol, 10mg bromophenol blue, and 1ml of dH_2O). The samples may be stored at -70°C, or run on an 8% polyacrylamide gel (8M urea; 1xTBE; and dilution of 40% acrylogel solution, with ammonium persulphate and TEMED added prior to pouring). The samples were heated to 90°C for 5 minutes prior to loading. Gel electrophoresis was at 40-50W for 2 hours. The gel was then dried down under heated vacuum and exposed to film (Kodak X-OMAT AR or S).

2.13 Oligonucleotide synthesis

Oligonucleotides were synthesised on an Applied Biosystema 381A Synthesiser, using the manafacturers protocols and reagents. After deprotection in ammonia at 55^{o}C overnight, the oligonucleotides were precipitated and used without further purification.

2.14 DNA sequencing

Sequencing of double-stranded DNA was carried out using the Sequenase^{TM} T7 DNA polymerase kit and α-35S d-ATP. 10ug of Cesium chloride prepared plamid DNA was digested with the appropriate restriction enzyme, ethanol precipitated and resuspended in 20ul dH2O. 7ul of this was combined with 2ul of the annealing buffer supplied, and 0.5pmol of the designated primer. The protocol and reagents were supplied as for the USB (United States Biochemical) Sequenase kit.

2.15 Amplification by the polymerase chain reaction (PCR)

DNA was amplified according to Saiki and colleagues, (Saiki et al., 1988). A 50ul reaction containing up to 1ug of genomic DNA, or in the case of plasmid DNA, concentrations were in the picomolar range. Commercial 10x buffer supplied by Promega (500mM KCl; 100mM Tris-HCl pH9.0 at 25^{o}C; 15mM $MgCl_2$; 0.1% gelatin w/v; 1% Triton X-100), each amplimer at 7ug/ml and each dNTP at 200uM f/c, were combined in a 0.5ml PCR tube. 2.5 units of thermostable DNA polymerase from *Thermus aquaticus* (Taq polymerase supplied by Promega) was finally added to the mixture. Thermal cycling was controlled by a programmable heating block (Perkin Elmer Cetus, DNA Thermal Cycler 480 or GeneAmp PCR System 9600). Three-step temperature cycling conditions varied, depending on GC content of amplimers, and length of amplification. Optimum amplimers were designed by a computer program (Oligo 4.0-2008 Primer Analysis Software), and made as in Section 2.13. Inorder, to avoid cross contamination of samples, aerosol resistant tips (ART^{TM} supplied by BCL) were employed, and care was taken to ensure that the positive controls were set up last.

Sequence of commonly used amplimers:

Neomycin gene amplimers used in homologous recombination screening:

neo.4: 5'> ctg gat tca tcg act gtg gc <3'

neo262: 5'> gac cgc ttc ctc gtg ctt tac <3'

neo319: 5'> tat cgc ctt ctt gac gag ttc <3'

neo320: 5'> atc gcc ttc ttg acg agt tct <3'

H-ras exon 3 amplimers used in homologous recombination screening:

RC3: 5'> gcg agc agc cag gtc aca c <3'

RC4: 5'> gga tgc cat agc tgc gag caa <3'

RAS365: 5'> ttg gca cat cat ctg aat ctt <3'

RAS366: 5'> att ggc aca tca tct gaa tct <3'

RAS372: 5'> agc acc att ggc aca tca tct <3'

RAS373: 5'> cag cac cat tgg cac atc atc <3'

Microsatellite primer pairs 21 and 28 as used in microsatellite analysis of cell line DNA were gifts from J. Todd and described as in original paper N.A.R. 18:4123-4130 (1990), with original names Igh (T161):pp21 and Il-1b (T49):pp28.

2.16 General culture of mammalian cell lines

2.16.1 Medium and supplements

Cell lines were grown routinely in Special Liquid Medium (GibcoBRL) supplemented with 10% foetal bovine serum (FBS), 4mM glutamine, and 5ug/ml penicillin. Cells were grown in 25, 75 or 175cm^3 flasks or 9cm dishes gased with 5% CO^2, and placed in 5% CO_2 ; 37^oC incubators. Cells were washed with phosphate buffered saline or PBS (0.14M NaCl; 27mM KCl; 10mM Na_2HPO_4; and 15mM K_2HPO_4). Trypsin for cell disagregation was used at 0.025% w/v in PE (phospate buffered saline; EDTA pH 7.8). Trypsin was inactivated with 10-fold volume of serum containing medium. Cell numbers were estimated using a Coulter counter.

Rat1 cells, used as a positive control cell line in the soft agar assays, were grown in Dulbeco modified Eagles medium (DMEM), for recipe see Section 2.21.

2.16.2 Freezing and storage of cell lines

Cells for freezing were grown to near confluence, typsinised and spun at 1K for 5 minutes. The cells were resuspended in equal proportions of growth medium and freezing medium (25% FCS; 20% DMSO; 55% growth medium). Cells were stored in 1-2ml nunc vials in liquid nitrogen.

2.17 Transfection of eukaryotic cells

2.17.1 Calcium phosphate technique

5ug of supercoiled plasmid DNA was mixed with 35ug of carrier DNA (normally Salmon Sperm DNA) in a solution of TE (1.0mM Tris pH8.0; 0.1mM EDTA pH8.0) to a final volume of 500ul. To this was added 400ul of TE(as above) and 100ul of 2.5M $CaCl_2$ (refractive index 1.401). This mixture was added slowly to 1ml of 2X HBS:(280mM NaCl; 50mM HEPES; 1.5mM $Na_2HPO_4.2H_2O$:pH 7.12-Critical) and left for 30 minutes at room temperature to form a precipitate. This was then added to 4ml of medium, mixed gently and placed over a sub-confluent culture of cells. After 24 hours the transfection medium was replaced with fresh growth medium and incubated at 37^{o}C for a further 1-2 days. The cells were then split 1:10 and selection applied as appropriate.

2.17.2 Lipofection technique

The technique of cationic liposome mediated transfection as described by Felgner and Holm (Felgner et al., 1987)(GibcoBRL Focus (11)2, 1989) was used to transfect a number of mouse epithelial cell lines. The protocol was as for the DOTAP^{TM} reagent supplied by Boehringer Mannheim.

2.18 Preparation of DNA and RNA from cultured cell lines

Genomic DNA was prepared by treating adherent cultured cells grown in 75 or 175cm^3 flasks with 2 or 5mls of lysis buffer (50mM Tris pH8.0; 50mM EDTA; 100mM NaCl; 5mM dithiothreitol; 1% SDS and 0.5mM spermidine) respectively. The cell lysate was scraped from the flask using a sterile scraper, and placed in a 15ml Falcon tube, to which 200ul (10mg/ml solution) of proteinase K was added. The tubes were incubated at 37^{o}C in a rotator, overnight. The contents were then phenol/chloroform extracted, and subsequently isopropanol precipitated. The gloppy pellet was spooled and washed in 70% then 100% ethanol, then allowed to air dry. The genomic DNA was finally resuspended in 200-400ul of TE (10mM Tris pH7.4; and 1mM EDTA).

RNA was prepared using the RNAzol^{TM} protocol (Biogenesis Ltd.). To 10^6 adherent cells, 2ml of RNAzol was added directly to the flask, and the lysate scraped off using a sterile scraper. This was placed in a 15ml Sorvall tube and one tenth volume of chloroform added. After shaking, the tube was spun at about 5K at 4^{o}C for 15 minutes. The colourless upper phase was transferred to a fresh tube, and the RNA precipitated using an equal volume of isopropanol. The RNA was then pelleted and washed with 75% ethanol and allowed to air dry. The pellet was then resuspended in Diethylpyrocarbonate (DEPC) treated water. Care was taken to ensure that all solutions used were RNase free. RNA should be stored at -20^{o}C.

2.19 Preparation of protein from cultured cell lines

A 9cm dish of subconfluent cells was washed three times with ice cold PBS. 1ml of lysis buffer (1% Triton X100; 0.5% Deoxycholate.Na; 0.1% SDS; 100mM NaCl; 50mM Tris-HCl pH7.4; 5mM MgCl2; and 3mM PMSF added just before use) was added direct to the cells and left for 15 minutes on ice. The lysate was scraped off the

dish and transferred into an eppendorf tube. After a 15 minute spin at $4^{o}C$ the supernatant was transferred to a fresh tube and stored at $-20^{o}C$. Preparation of protein samples for Western analysis is described in Section 2.11.

2.20 Virus preparation and cell infection

Virus producer cell lines were grown until 80% confluent. The medium was replaced with the minimum volume of growth medium to ensure coverage. This was left for 24 hours removed and filtered through a series of syringe filters 1.2 -0.2 microns. 24 hours prior to harvesting the viral supernatant, $5x10^{5}$ cells (cells for infection) were seeded per $25cm^{3}$ flask. To each flask was added a mixture of 1ml of viral supernatant, 210ul of polybrene (0.8mg/ml), made up to 7ml with growth medium. After 48 hours the medium was changed with normal growth medium and 2 days later the cells were split into 9cm dishes and selection with geneticin commenced if appropriate.

To estimate the titre of a virus, the NRK focus forming assay was used. Early passage NRK cells were seeded into 24 well plates, at 10^{3} cells per well. The next day the medium is replaced by limit medium (5% FCS; 1% DMSO; 6ug/ml polybrene in 1x DMEM). Then the following day virus dilutions (5^{0}-5^{-8}) of the filtered virus stock were added direct to the wells (33ul per well). After 4 days the plates were refed with limit medium minus polybrene. After another 5 days the foci were counted and the titre of the virus (in focus forming units per ml, or ffu/ml) calculated.

2.21 Growth in soft agar

Soft agar assays were used as an indicator of anchorage independent growth. 40ml of 2.5% Difco bacto-agar in a 200ml Duran bottle was melted in a microwave and cooled to 44°C. 40ml of 2xDulbeccos Modified Eagles medium or 2xDMEM (for 100mls :20mls 10xDMEM; 2ml 200mM glutamine; 2ml 100mM pyruvate; 10ml 7.5% NaHCO3; 4ml 1M HEPES made up with dH2O plus antibiotics) and 20ml newborn calf serum was added to the cooled agar, and the volume made up to 200ml with 100ml 1XDMEM. The resulting 0.5% agar solution was maintained at 44°C for pouring (about 5ml per 60mm plate). Base plates were allowed to set at room temperature or 4°C. 10^5 cells were resuspended in 0.5ml of growth medium and mixed in a plastic universal with 1ml of cooled (37°C) base agar. A base plate was carefully overlayed with this soft agar/cell mix and allowed to set at room temperature. Plates were set up in triplicate and examined after 2 weeks incubation at 37°C.

2.22 Culture of primary tumour explants

Tumour samples for explants were cut up into fine pieces and incubated in Special Liquid Medium supplemented with 20% v/v foetal bovine serum (FBS); 4mM glutamine; 5ug/ml penicillin; 100ug/ml streptomycin in 5% CO_2 at 37°C. The concentration of FBS was reduced to 10% as soon as the tumour cell line was established.

2.23 Specialised culturing of embryonic stem (ES) cells

2.23.1 Electroporation of ES cells

A 175cm^3 flask of confluent ES cells were trypsinised and the trypsin inactivated with serum containing medium. The cells were first washed with Phosphate Buffered Saline (PBS), and then resuspended in about 0.4ml of PBS. In an eppendorf tube 75-150ug of linearised plasmid DNA was resuspended in 0.4ml of PBS and mixed with the cell suspension. The contents were transferred to a sterile Bio-Rad electroporation cuvette. The cuvette was placed in the Bio-Rad Gene Pulser^{TM}, ensuring that the electrodes were connected. The capacitance was set at 3uF (not on extender), whilst the voltage was set at 0.8kV. Both pulse buttons were held in until the buzzer sounded acknowledging that the pulse has been delivered. The cuvette was removed and the contents added to 50-100ml of prewarmed growth medium and plated into 9cm dishes. Selection was begun 24-48 hours after electroporation.

2.23.2 Culturing and Selection of ES Cells

The ES cells were grown in Glasgows Modification of Eagles medium, supplemented with non-essential amino acids and 0.1mM β-mercaptoethanol. In addition, the growth factor LIF was added to a final concentration of 10^3 units/ml (stored as a 10^5/ml stock in medium at -20^{o}C). Selection was carried out using geneticin at a final concentration of 200ug/ml and Gancyclovir (or Cymevene, Syntex Corp.) at a final concentration of 2uM (stored as a 10,000-fold stock at 4^{0}C).

2.23.3 Picking ES cell clones

After about 2 weeks in selection, the clones that developed were picked and expanded. This was accomplished using a drawn-out pasteur pipette connected to a

length of tubing with a mouthpiece and an in-line-filter. With the aid of a stereo-microscope the colony was scraped and sucked up into the pipette, and transferred to a drop of trypsin on a 9cm plastic plate. Care was taken to minimise the amount of medium sucked up as this inactivates the trypsin. The plate was covered and incubated for approximately 10 minutes. The dispersed colony was then transferred using the drawn out pasteur into a 24 well plate and grown up as desired.

2.23.4 Freezing ES cells

Freezers were made from half a confluent $25cm^3$ flask. The cells were spun down and resuspended in freezing medium(8mls growth medium; 1ml FCS; 1ml DMSO), and frozen in 1ml nunc cryotubes. Storage was in liquid nitrogen.

2.23.5 Preparation of DNA from ES cells

DNA was made from half a confluent $25cm^3$ flask using the rapid lysis method described in Section 2.18. The remaining half of the flask was frozen down (as Section 2.23.4) and stored at -70^oC.

2.24 Assay for tumorigenicity

Cell lines were tested for their degree of tumorigenicity by sub-cutaneous injection of nude mice. Cells were trypsinised, counted and resuspended in SLM at a concentration $1x10^7$ cells/ml. 100ul volume was injected per site, two sites per mouse. The mice were checked regularly for tumour development.

2.25 Tumour histology

Tumours were fixed in 5% formalin before embedding in paraffin sectioning, and staining in hematoxylin and eosin.

Results

Introduction: Tumour progression is associated with an increase in the H-ras mutant:normal gene ratio

The mouse skin carcinogenesis model has been used extensively to study the biological and biochemical features of tumour initiation and progression.

Skin tumours induced by the standard DMBA/TPA protocol display an A:T -> T:A transversion at codon 61 of the H-ras gene. This mutation introduces a novel XbaI restriction site, generating a tumour-specific RFLP.

Previous studies have suggested an important role for the oncogenic mutation of H-ras, and duplication of the chromosome carrying the mutation, in early stages of mouse skin tumorigenesis. Such alterations are thought in some way, to provide a selective environment to the initiated cell, permitting clonal expansion to produce a benign papilloma.

Genetic alterations at the H-ras locus have been shown not only to be associated with early events in tumorigenesis. RFLP studies of carcinomas generated by the DMBA/TPA protocol show a strong correlation between the increase in mutant:normal gene ratio and the generation of the anaplastic phenotype. However, loss of heterozygosity studies were unable to conclude whether the main determinant of these consistent chromosomal changes was H-ras itself, or a linked tumour suppressor gene. More recent studies, using *spretus/musculus* hybrid mice have shown that it is always the parental chromosome carrying the H-ras point mutation that is duplicated (C.J. Kemp, personal communication). Although these observations suggest a causative role for H-ras, it is still impossible to rule out the involvement of another linked gene. To try to dissect a function for H-ras in tumour progression, we have chosen two alternative approaches.

The first, termed the *in vitro* approach concentrates on the rather distinct transition event between the well-differentiated squamous carcinoma, and the much more undifferentiated, refractile spindle cell tumour.

Previous PCR studies using paraffin sections of carcinomas of defined histological types suggested a role for H-ras in this transition (Buchmann et al., 1991). In four cases, where both squamous and spindle cell components could be identfied within the same tumour, the spindle component had a much higher mutant:normal gene ratio. In addition cell lines representing both the squamous and spindle phenotypes, have been isolated from a single carcinoma, MSC11. These clonally derived cell lines have offered a unique *in vitro* system for analysing the role of H-ras in the later stages of tumour progression.

The alternative approach, which we have termed the *in vivo* approach, uses the novel technique of gene targeting. This enables one to study the function of genes in mammalian development, or in our case, the consequence of loss-of-function mutations in the H-ras gene for tumour development. The results from these alternative approaches to examining the role of H-ras in tumorigenesis are split into the following chapters 3 and 4 of this thesis.

Results
Chapter 3

3.1 The role of Harvey-ras in the squamous to spindle conversion

3.1.1 The squamous to spindle conversion is a discrete step in tumour progression

The analysis of the role of H-ras in tumour progression and loss of differentiation, has been facilitated by the isolation of cell lines representative of specific stages of tumour development.

One such cell line MSC11 was explanted from a carcinoma generated by treating a *Mus spretus/ Mus musculus* hybrid mouse with multiple treatments of DMBA (see Figure 5). It was noticed from histological analysis, that two distinct cellular morphologies existed within the same tumour. Three single cell clones, representing both morphologies were derived from the parental MSC11 cell line. B9 denotes the squamous cell component, while A5 and D3 represent the spindle cell phenotype (see Figure 6).

Southern blotting analysis of the three cell lines, utilising the XbaI polymorphism, indicated that all three clones had the same codon 61 H-ras mutation in the *Mus musculus* allele. In addition, sequence analysis of the conserved regions of the p53 locus showed that the cell lines of both morphologies, had the same p53 point mutations, in the *musculus* allele at codon 241 (Asn->Ile) and in the *spretus* allele at codons 236 (Cys->Phe), and 246 (silent change) (Burns et al., 1991). These data together, provided clear proof that the spindle cell phenotype arose from the squamous component of the same tumour. It also indicated, that genetic alterations of the p53 locus occur prior to, and are not responsible for, the squamous to spindle transition. Thus alterations of another locus or loci must be involved.

Isolation of squamous and spindle cell lines
from mouse-skin carcinoma MSC11

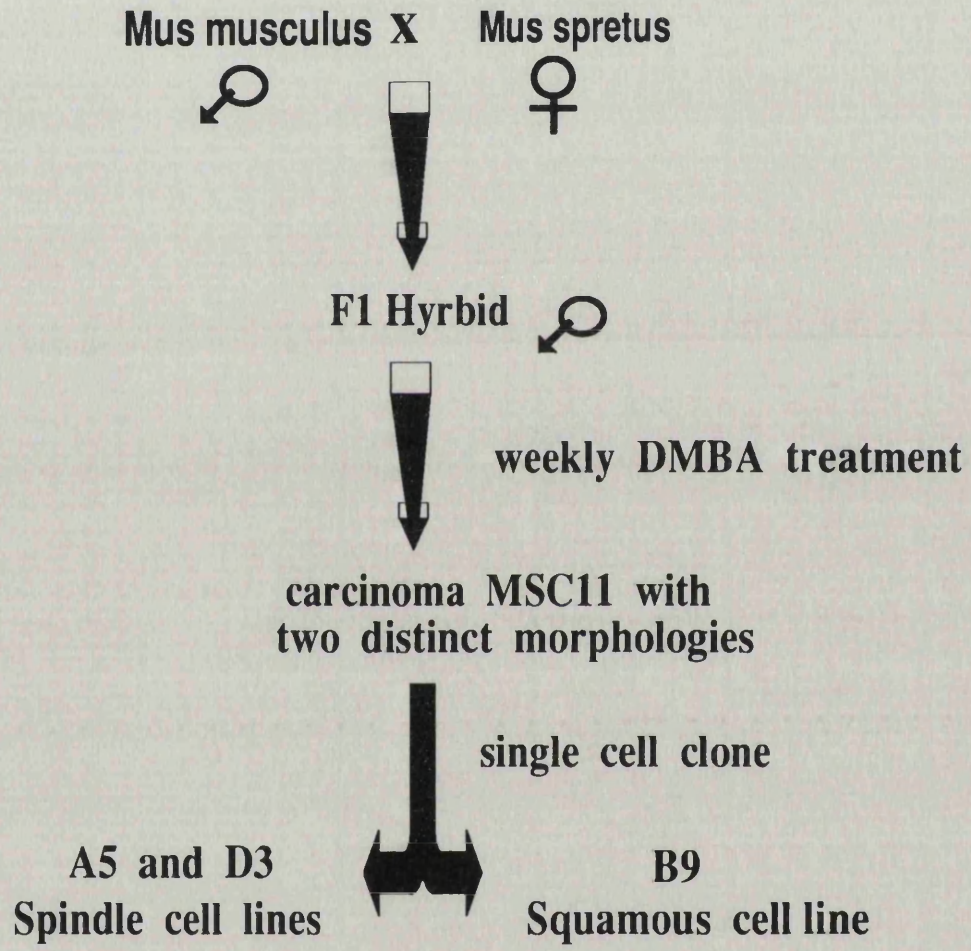


Figure 5.

Figure 6.

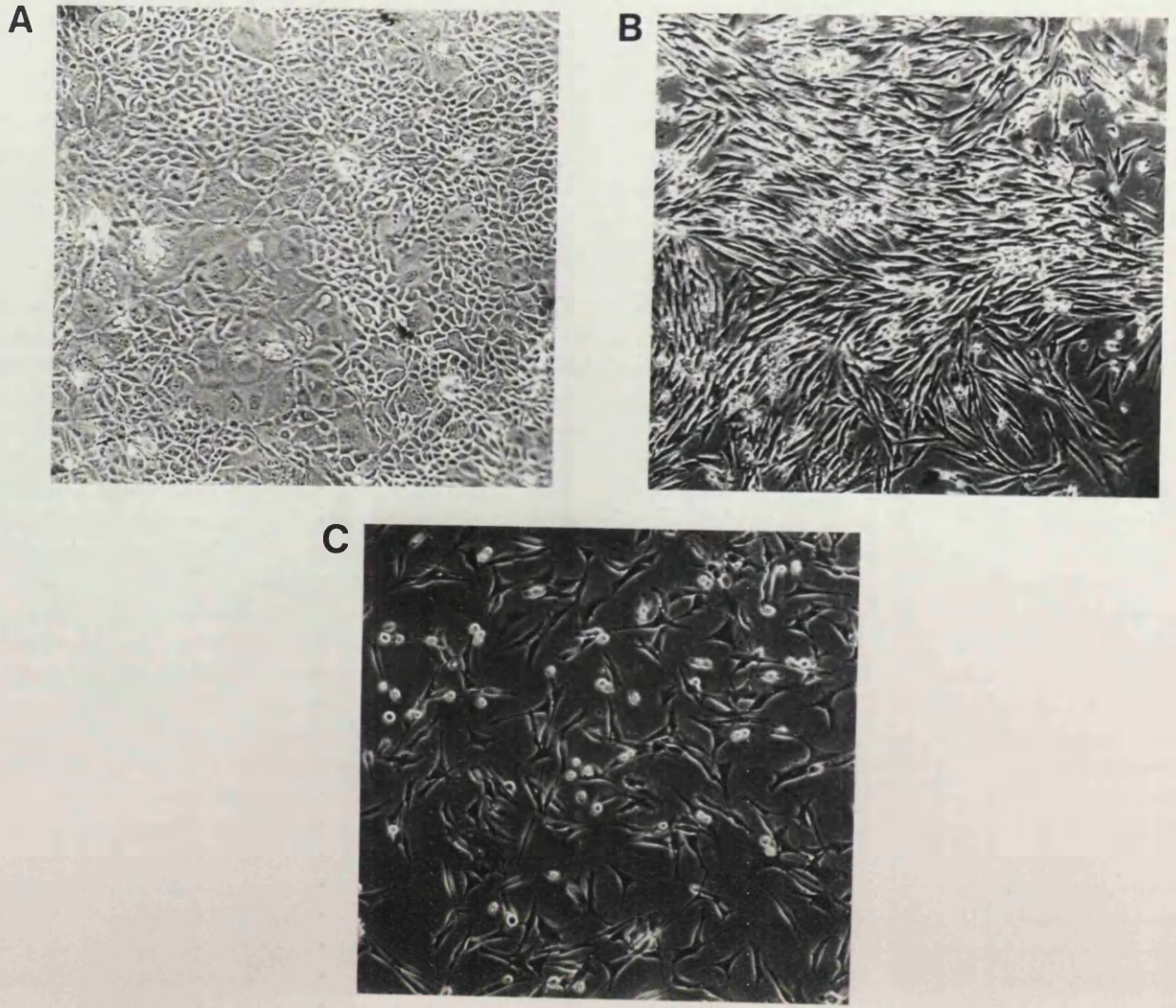
Morphology of MSC11 cell clones.

(A) MSC11 single cell clone B9

(B) MSC11 single cell clone A5

(C) MSC11 single cell clone D3

(25 x objective)


A
B
C

3.1.2 The spindle cell lines are more tumorigenic and histologically distinct compared to their squamous counterparts

Just as with PDV and PDVC57 the ratio of mutant:normal H-ras, directly correlates with the tumorigenicity of the cell lines. B9 is much less tumorigenic than both A5 and D3. A mixing experiment was carried out using 10^4 B9 cells along with varying numbers of A5 cells. As few as 10 A5 cells along with 10^4 B9 cells, when injected subcutaneously into a nude mouse, had a discernible effect on the latency of tumour development. After a month, only one out of the four B9 injected mice showed a small flat nodule at the site of injection, whereas the B9 cells combined with the equivalent of only about 10 A5 cells showed 5-7mm nodules in 2/4 mice and smaller nodules in the remaining two mice.

Subcutaneous injection of nude mice with 10^6 B9 cells resulted in 1cm tumours, after a latency period of about 6 weeks. On the other hand, A5 and D3 cell lines produced similar sized tumours in only 3 weeks. Histological analysis of tumour sections, revealed characteristically well differentiated tumours in the case of the B9 squamous cell line, but very disorganised, aggressive tumours in the case of the spindle cell lines A5 and D3 (Figure 7).

There was a slight difference in tumorigenicity between A5 and D3, with A5 having a marginally shorter latency period. This difference was observed over several nude mouse injection experiments.

3.1.3 The squamous to spindle conversion is concomitant with an increase in the copy number and level of expression of mutant H-ras

Examination of an XbaI Southern blot of the three clones showed a clear increase in copy number of the mutant H-ras gene between B9 and A5/D3 (Figure 8A). The normal *musculus* H-ras gene resides on a 12kb XbaI fragment. However, due to the XbaI polymorphism at codon 61 of H-ras, XbaI restricts the mutant *musculus* allele

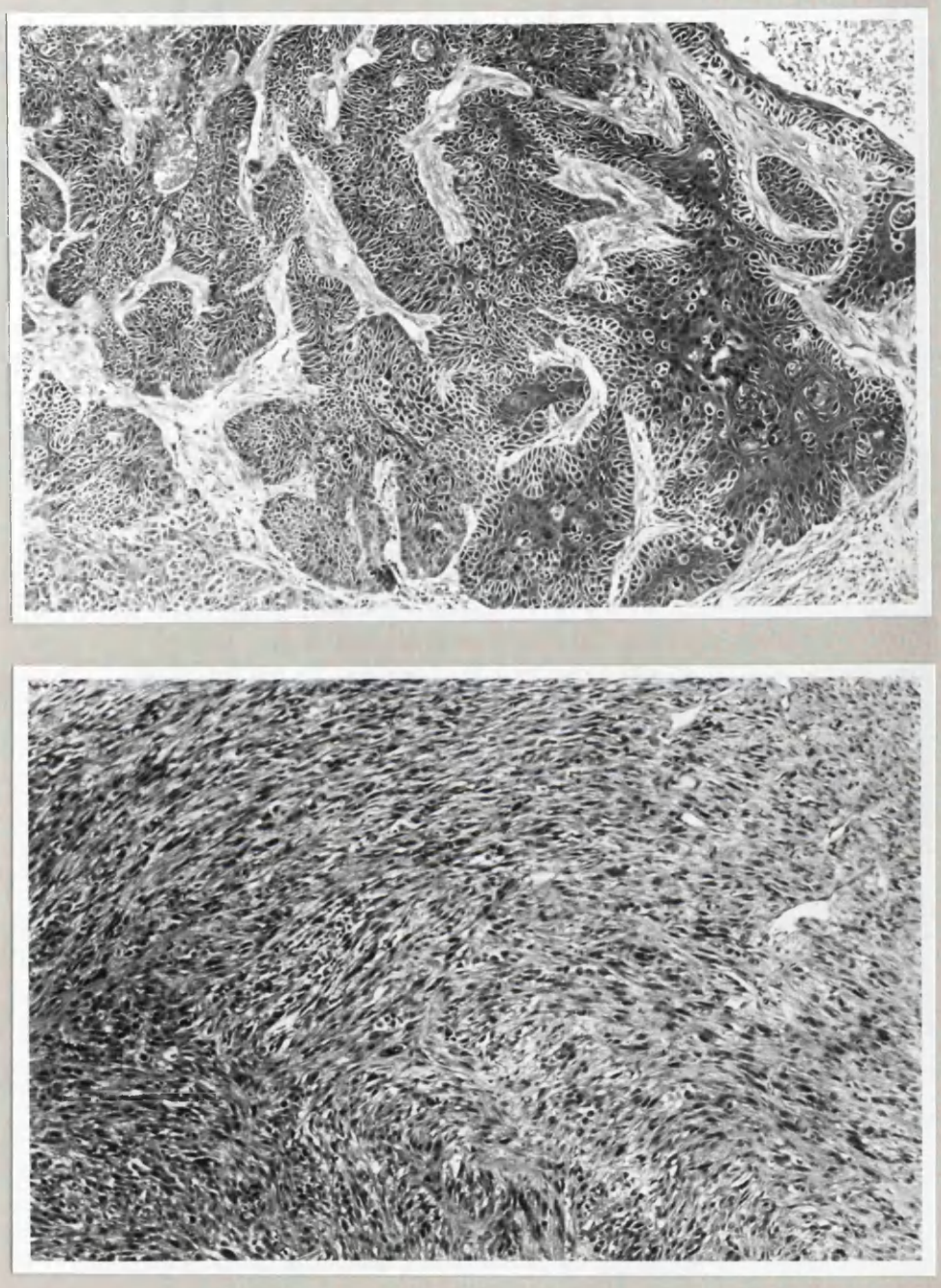


Figure 7.

Histology of MSC11 cell clones injected sub-cutaneously into nude mice.

(a) Tumour histology resulting from injection of MSC11 B9 cells

(b) Tumour histology resulting from injection of MSC11 A5 cells

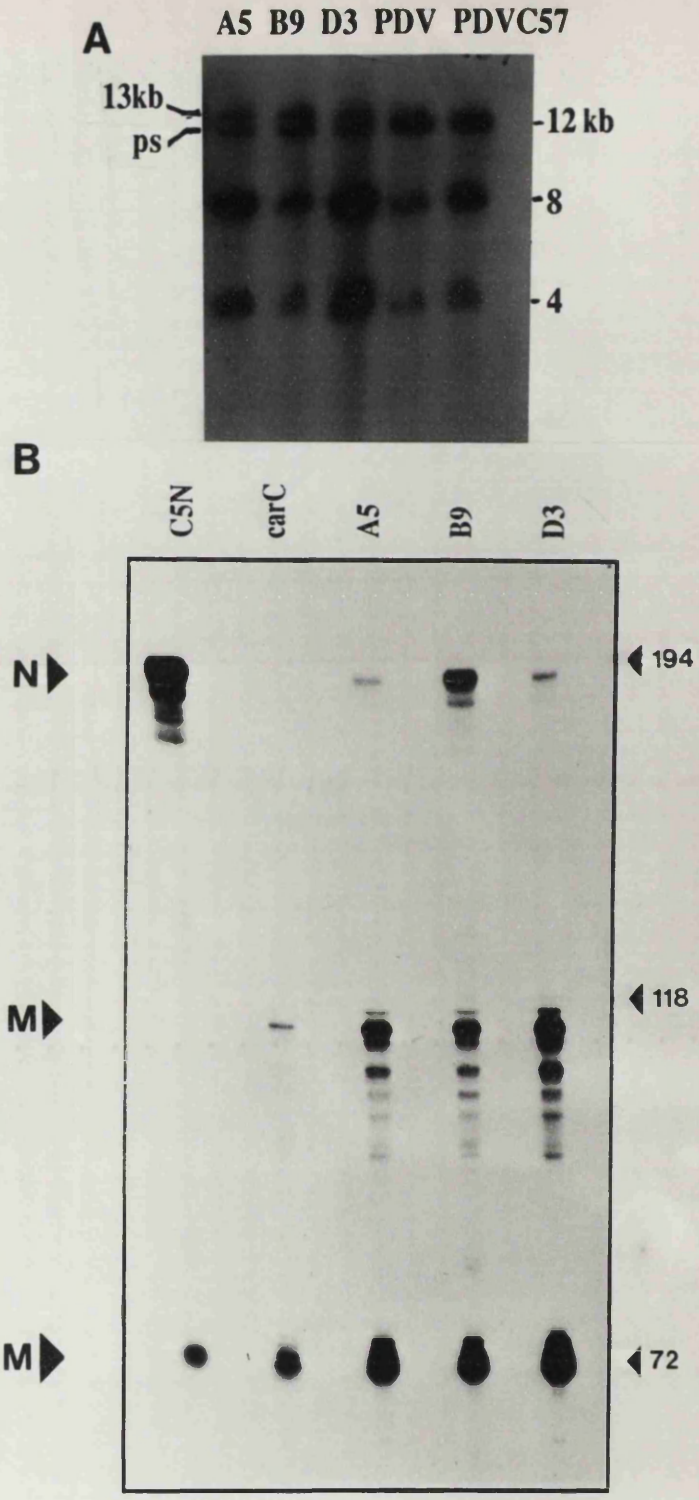
Magnification x100, (Photo courtesy of P.A. Burns).

Figure 8.

Relative copy number and expression of H-ras mutant and normal genes in MSC11 B9, A5 and D3 cell lines.

(A) XbaI Southern blot of cell lines, 10ug genomic DNA.
PDV and PDVC57 cell lines included as controls with relative mutant:normal H-ras ratios of 1:2 and 2:1 respectively.
Size markers as shown, (ps) spretus H-ras pseudogene
Refer to figure 9. for schematic details.

(B) RNase protection analysis of MSC11 cell clones.
20ug of total RNA from each cell line was hybridised to mouse H-ras exon 2 specific probe. C5N and carC represent control cell lines which express only normal or mutant H-ras transcripts, respectively.
(N) position of H-ras normal transcript
(M) positions of mutant specific protected fragments
size markers in nucleotides.
(Data courtesy of S.Haddow)


A
A5 B9 D3 PDV PDVC57
13kb
ps
12 kb
8
4
B
C5N
carC
A5
B9
D3
N
M
M
194
118
72

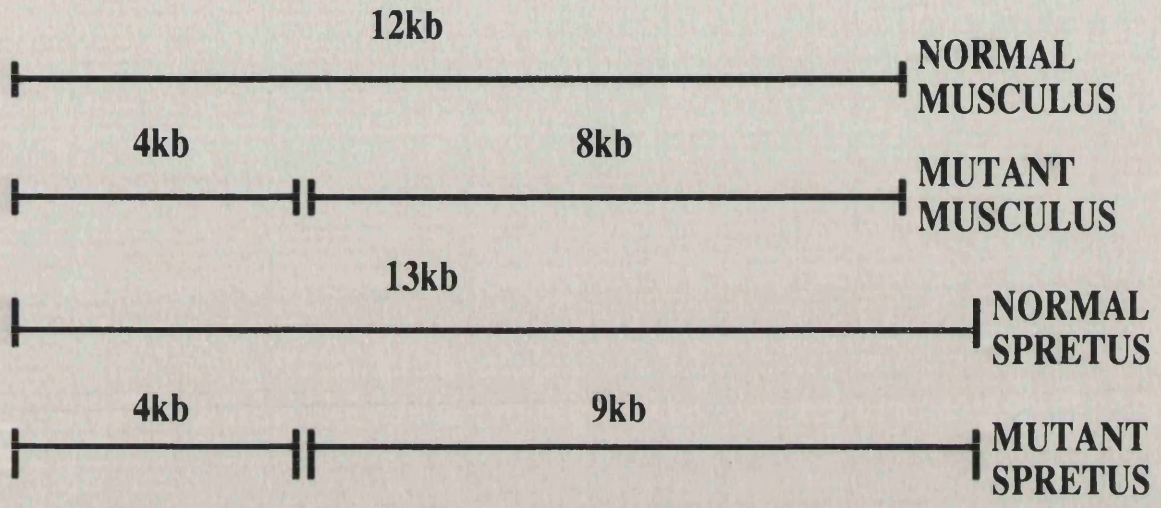
into a 4 and a 8kb fragment. However, the XbaI fragment representing the spretus normal allele is 13kb in size. XbaI digestion of the spretus DNA carrying the codon 61 mutation results in a 4kb and a 9kb fragment (Figure 9). The XbaI Southern blot in Figure 8A, shows the cell lines B9, A5 and D3 as well as PDV and PDVC57 included as controls. The top band of the B9/A5/D3 lanes represents the 13kb normal spretus allele and the band just below, the spretus H-ras pseudogene. The mutant bands are 8kb and 4kb in size indicating that these cell lines have required a codon 61 mutation in the musculus allele. Comparison of copy number can be made with the cell lines PDV and PDVC57, which have mutant:normal H-ras gene ratios of 1:2 and 2:1, respectively (Quintanilla et al., 1991). These particular cell lines were derived from a *musculus* background, and therefore display the normal 12kb and mutant 8kb and 4kb bands. It can be estimated that the ratio of mutant:normal in B9 is about 2:1 similar to PDVC57. However, the ratio is much higher in the case of A5 and D3, in the range of 5:1 to 10:1. Such a dramatic increase in copy number cannot be readily explained by imbalance of chromosome 7 in favour of the mutant. It seems more likely the result of a local genetic amplification event. Data to support this notion, has come from PCR microsatellite studies (C.J.Kemp, personal communication). The ratio of microsatellite markers flanking the H-ras locus on chromosome 7 correlated with the expected trisomic status.

Interestingly, independent evidence for a possible amplicon on mouse chromosome 7, that may include H-ras, has come from studies of human breast and squamous cell cancers which show amplification of the PRAD1 oncogene (Lammie et al., 1991). PRAD1 maps to human 11q13 and to the syntenic region in mouse 7 close to the H-ras locus (Rosenberg et al., 1991).

However, preliminary data (S.Haddow, unpublished) has shown no differences in PRAD1 copy number or expression levels between B9 and A5/D3.

This increase in mutant copy number correlates with the expression data of the mutant gene as determined by the RNase protection assay (Figure 8B). 10ug of total RNA from the cell lines B9, and A5/D3 were treated as in Section 2.12 of the Materials and

XBA I POLYMORPHISM IN MUSCULUS / SPRETUS HYBRIDS



XBAI SOUTHERN BLOT REPRESENTATION

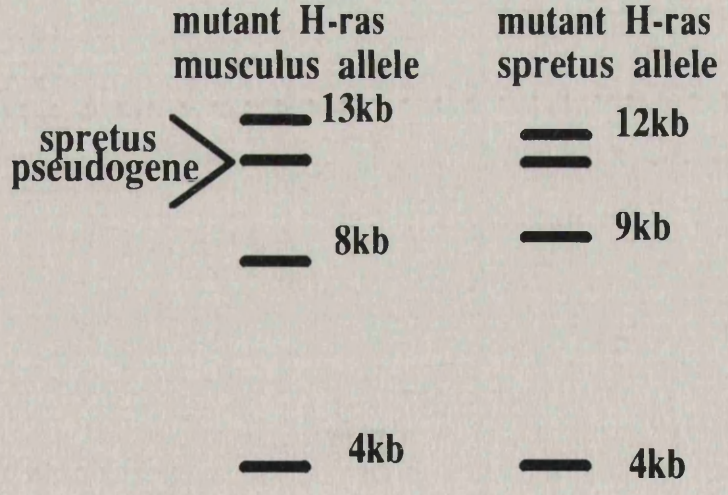


Figure 9.

Methods. The cell lines C5N and carC were included as controls. C5N expresses only normal H-ras, whereas carC only expresses mutant. The protected fragments which hybridise to the mouse H-ras exon 2 probe, represent the normal transcript (N), and the mutant (M), respectively. From Figure 8B the most striking difference between B9 and A5/D3 appears to be a decrease in the expression level of the normal gene. This is dicussed in the following Section 3.1.4. In addition, there is an increase in the amount of mutant transcript, concurrent with the squamous to spindle conversion. However, the increase represented by this particular RNase protection assay, is not as dramatic as the Southern blot suggests. It is however difficult to quantify the exact ratios, without incorporating loading controls. Examination of the protein levels of the normal and mutant p21 species in the squamous and spindle cell lines, by Western blot analysis, correlates in general with the RNase protection data, with an increase in the mutant $p21^{leu61}$ species (Figure 10), and a decrease in the normal p21 protein. However, the increase in the amount of mutant protein present in the spindle cell lines appears to correlate with the Southern blot data, rather than the more modest increase suggested by the RNase protection results. However, once again, the lack of a measure for internal loading, implies that care must be taken in interpreting the precise ratios of normal to mutant protein levels in the cell lines analysed.

3.1.4 The squamous to spindle conversion correlates with an apparent decrease in level of expression of normal H-ras

There is substantial data to support the correlation between tumour progression and the increase in the copy number and level of expression of mutant H-ras.

However, there is also evidence to suggest that the normal allele is lost or down-regulated in many carcinomas. In the PCR studies of paraffin sections of carcinomas, the squamous to spindle transition correlated not only with an increase in mutant but also a decrease in the amount of normal H-ras alleles present (Buchmann et al., 1991).

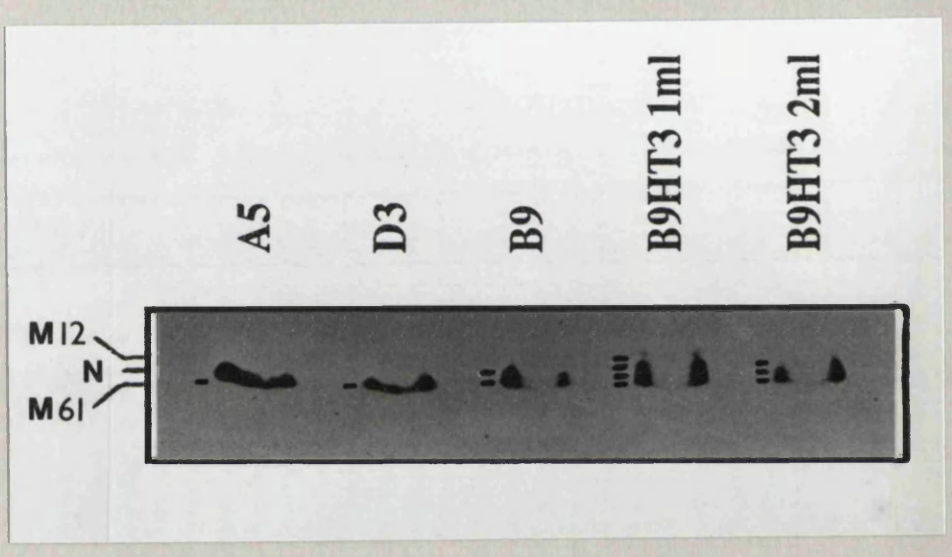


Figure 10.

Western blot analysis of MSC11 cell clones and B9 cells infected with HaMSV.

Separation of H-ras p21 species, highlighted by dashes, as follows:
(M12); Mutant H-ras Arginine substitution at codon 12 of HaMSV
(N); normal p21 H-ras species
(M61);Mutant H-ras Leucine substitution at codon 61.
Additional top band in B9HT3 1ml represents phosphorylated mutant Arg12

In the transition from B9 to A5/D3 there appears to be a reduction in the level of expression of the normal gene as determined by the RNase protection data (Figure 8B). This reduction in the level of the normal transcripts appears quite dramatic, whereas the copy number of the normal gene does not appear to change from B9 to A5/D3. This suggests transcriptional down-regulation of the normal H-ras gene rather than complete gene loss. Western blot analysis correlates with the RNase protection data and shows the apparent loss of the band representing the normal p21 species (Figure 10).

3.1.5 Conclusions

In summary, the data indicate that the transition from a well differentiated squamous carcinoma to an undifferentiated spindle cell carcinoma represents a discrete step in mouse skin tumorigenesis. Analysis of the clonally derived cell lines B9 and A5/D3, representing the squamous and spindle phenotypes respectively, excludes a role for p53 as a critical factor in the transition event. Instead, the conversion of squamous to spindle cells correlates with a dramatic increase in the mutant H-ras gene copy number and corresponding increase in the levels of mutant transcript and protein. The increase in levels of mutant copy number (5-10 fold) are best explained by genetic amplification of a region on chromosome 7 including the H-ras locus.

In addition, there appears to be a reduction in expression of the normal H-ras gene and levels of protein.

On the basis of these results, the spindle cell phenotype may arise by alterations in the mutant:normal H-ras gene dosage ratio. Inorder to investigate this theory further, it was proposed to experimentally alter the H-ras gene dosage ratios in squamous and spindle cell lines.

Perhaps, by increasing the level of mutant H-ras in a squamous cell line it would be possible to convert it to a spindle cell phenotype. The converse may also be feasible. By increasing the levels of normal H-ras in a spindle cell line it may be possible to

induce a reversal of the spindle phenotype to a more differentiated squamous form. Both of these experimental approaches are outlined in the following sections of this chapter.

3.2 Experimentally increasing mutant H-ras in squamous cell lines

The squamous carcinoma cell line B9 was transfected using the lipofection protocol (see Section 2.17.2 of Materials and Methods), with the plasmid construct pHO6T1 (Spandidos and Wilkie, 1984). This contains a 6.6kb BamHI fragment of the human T24 H-ras oncogene (carrying the codon 12 mutation) is under the transcriptional control of its own promoter sequences. The plasmid also contains a SV40 enhancer region, and a neomycin resistance gene under the control of the Moloney murine sarcoma virus long terminal repeat (MoLTR).

3.2.1 Mutant H-ras appears cytotoxic to the cells

Stable transfection of this construct appeared cytotoxic to the cells. Even when the transfection efficiency was high (as established using a neomycin resistance plasmid, Homer6 (Spandidos and Wilkie, 1984), very few mutant transfected colonies survived selection. This correlates with other transfection experiments carried out in our group, using mutant H-ras constructs (K.Brown and W.Lambie, unpublished data). Two clones of B9 transfected cells (pHO6T1 II and III) were ring-cloned and grown-up as cell lines, along with control clones, transfected with the neomycin vector (Homer 6) alone.

In addition, to try to avoid "stressing" the cells, by G418 selection using an enhancer based plasmid construct, a high titre non-selectable Harvey murine sarcoma virus (HaMSV), was used to infect a pool of B9 cells. The producer cell line HT3 was

generated by infecting HaMSV along with a helper virus into NIH3T3 cells. Supernatant from the producer cell line HT3 was collected and used directly to infect B9 cells using the polybrene method outlined in Section 2.20 of the Materials and Methods. Two separate pools of infected cells were isolated, B9HT3 1ml and B9HT3 2ml.

Figure 10 shows Western blot data on the two independent pools of infected cells B9HT3 1ml and B9HT3 2ml. The codon 12 mutant form of p21, representing the introduced HaMSV species, can be seen as a specific band in particular in the pool B9HT3 1ml. The separation of the different proteins is perhaps not ideal. In addition, the pattern of dots rather than homogeneous bands appears to be a problem with this particular Western blot protocol. The lines to the left of each lane help to define the position of the bands. However, it can still be seen that B9HT3 1ml shows the highest level of expression with an additional fourth band representing the phosphorylated species of v-ras p21 (Figure 10). B9HT3 2ml does appear to express the virus specific codon 12 species but at a lower level than B9HT3 1ml.

3.2.2 The morphology and growth characteristics of the transfectants and infectants remain characteristically squamous in vitro

The morphology of both transfected cell lines, pHO6T1 II and III remained characteristically squamous in appearance (Figure 11). However, they formed less regular "cobblestone" colonies than did the B9 Homer6 control transfectants. These slight irregularities in colony distribution were also seen in the virally infected clones (data not shown). It was also apparent that many of the cells formed what appeared to be vacuoles in their cytoplasm. This can be seen in figure 11 and an extreme situation in figure 12. These intracellular vesicles were more noticeable in subconfluent rather than confluent cultures. The significance of these observations will be discussed at a later date.

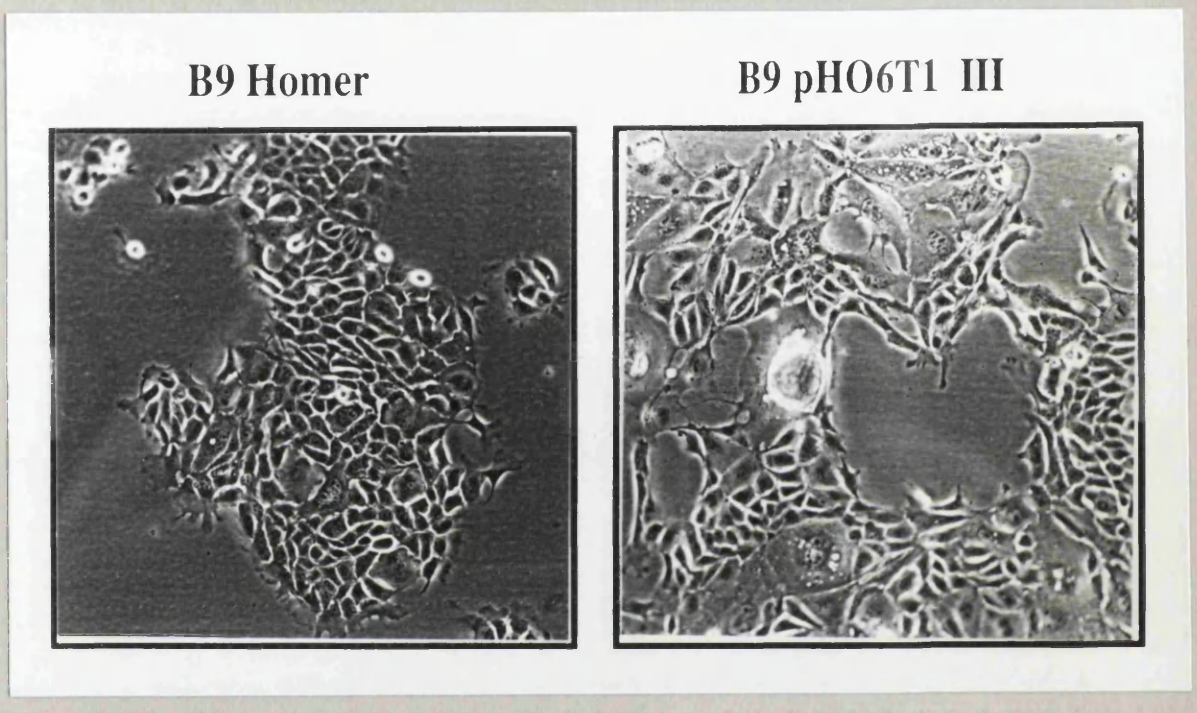


Figure 11.

The morphology of B9 cells overexpressing mutant H-ras remains characteristically squamous

Figure shows morphology of control B9 cells transfected the neomycin vector Homer6 and experimental clone transfected with human codon 12 mutant H-ras plasmid pHO6T1.

(25 x objective)

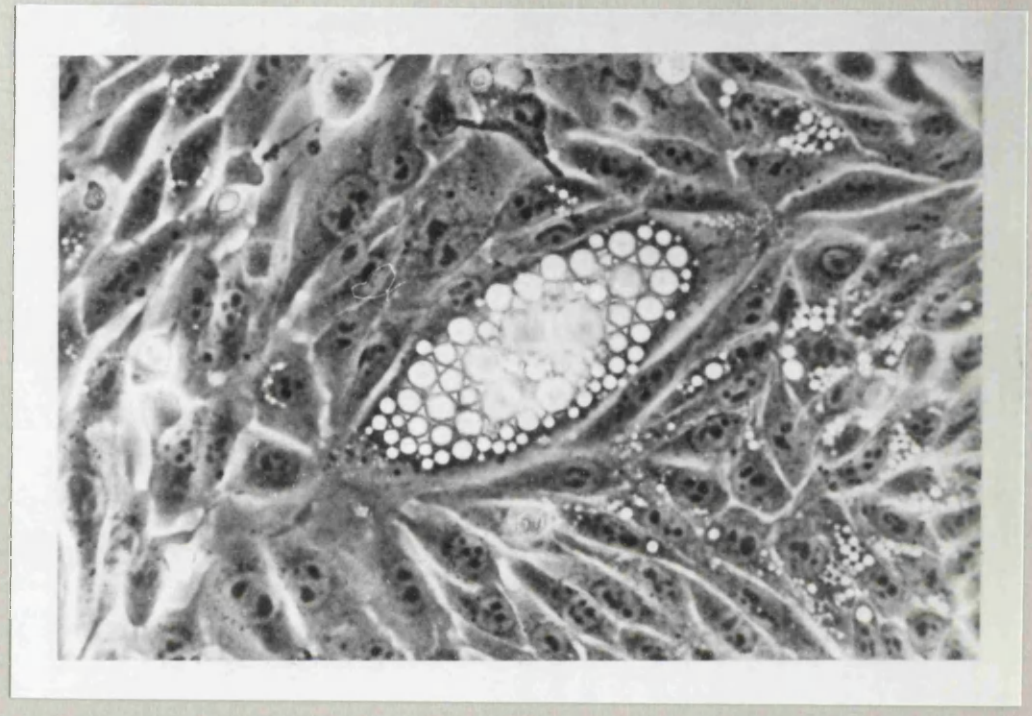


Figure 12.

Vacuolation of B9 cells overexpressing mutant H-ras

Cells as shown, B9HT3 1ml.
(25x objective).

To examine the growth characteristics of B9 cells overexpressing mutant H-ras, the highest expressor clone, B9HT3 1ml, was plated under anchorage-independent conditions. Parental B9 and A5 cells along with src transformed rat fibroblasts were also seeded in soft agar, according to Section 2.21 of the Materials and Methods.

Both parental B9 and B9HT3 1ml were unable to form colonies under anchorage independent conditions. However the spindle cell line, A5, formed colonies in the soft agar but to a lesser degree than the positive control Rat-1 src-transformed fibroblasts. A comparative field of view of both the Rat-1 and A5 seeded soft agar plates can be seen in figure 13.

The cell lines overexpressing mutant H-ras did not appear to grow at appreciably different rates compared to the parental B9 cells, although no detailed kinetic growth studies have been carried out.

3.2.3 Increasing mutant ras correlates with increased tumorigenicity

Tumorigenicity was assayed by subcutaneous injection of 10^6 cells into nude mice (as in Materials and Methods).

The transfectant clones, B9pHO6T1 II and B9pHO6T1 III, and the infected pool B9HT3 1ml were all determined to be more tumorigenic than parental B9 cells. After 4 weeks post-injection, parental B9 cells produced only one large nodule on one mouse, and four small nodules on two mice with one mouse showing apparently nothing. Whereas, injection of B9HT3 1ml resulted in large tumours mostly between 8-10mm in size. Similar sized tumours were seen in the B9 pHO6T1 transfectants whereas B9HT3 2ml showed only one large tumour between four mice. In general therefore, the degree of tumorigenicity of the clones correlated with the expression level of mutant H-ras.

Figure 13.

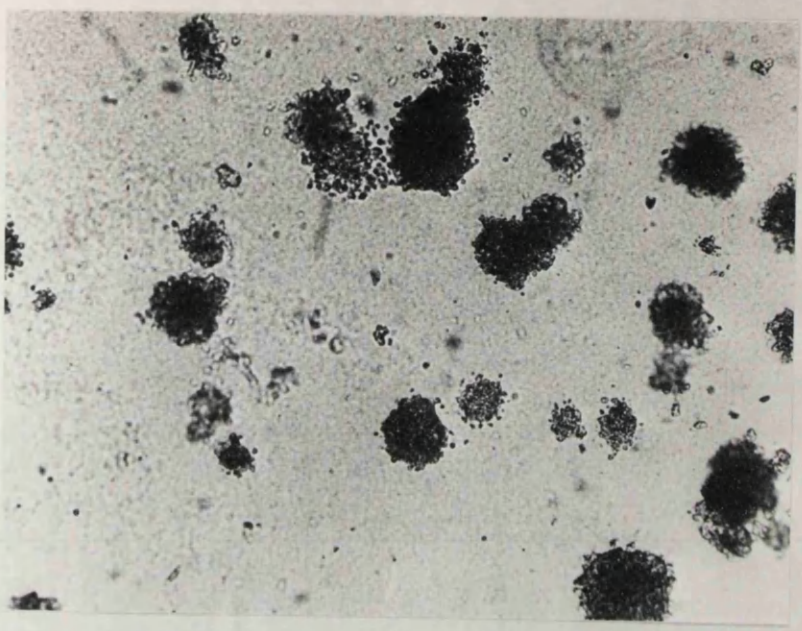
Growth of spindle A5 cells in soft agar.

Comparison with Rat-1 src transformed cells

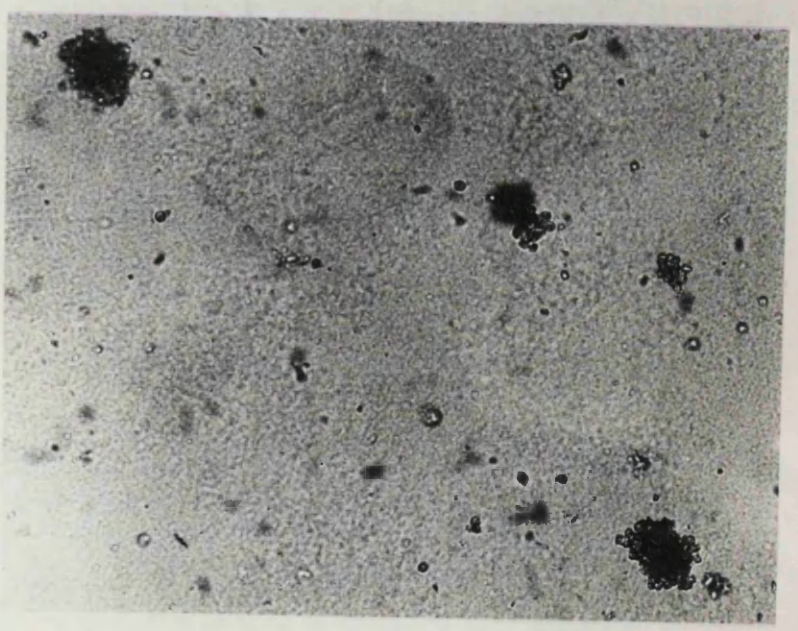
seeded at same cell density, 10^5 cells per 60mm plate

(10 x objective)

SOFT AGAR ASSAY



RAT 1
Src



A5

3.2.4 The histology of tumours induced by B9 virus infected cells shows two distinct components

The tumour histology of the B9pHO6T1 nude mouse tumours showed a differentiated organisation characteristic of the parental B9 squamous cell carcinomas. Thus although the tumorigenicity, as measured by the latency period for a specfic sized tumour to develop, had increased, the resultant tumours were still squamous in phenotype.

However, the data on the histology of the B9HT3 nude mouse tumours appeared intriguing. There was evidence of dimorphism, with what appeared to be spindle and squamous components within an individual tumour. On the other hand, tumours that resulted from subcutaneous injection of 10^6 B9 or B9 Homer6 cells have repeatedly shown only a squamous well-differentiated structure. It appeared therefore, that injection of the virus infected mutant overexpressor clones B9HT3 1ml and B9HT3 2ml, were able to convert to a spindle morphology when presented with an *in vivo* environment. Several tumours were taken and explanted in culture as described in Section 2.22 of the Materials and Methods.

Explants from the B9HT3 tumours, reflected two very different morphologies, one squamous and the other characteristically spindle in phenotype. Some of the explanted colonies showed both morphologies emanating from the same tumour section, whereas others showed only squamous or spindle (Figure 14). The initial 20% v/v foetal bovine serum concentration was reduced to 10% as soon as the explants had taken.

Ring-clones representing both phenotypes were isolated and grown up as cell lines.

Figure 14.

Morphology of nude mouse tumour explants generated by sub-cutaneous injection of B9 cells infected with HaMSV.

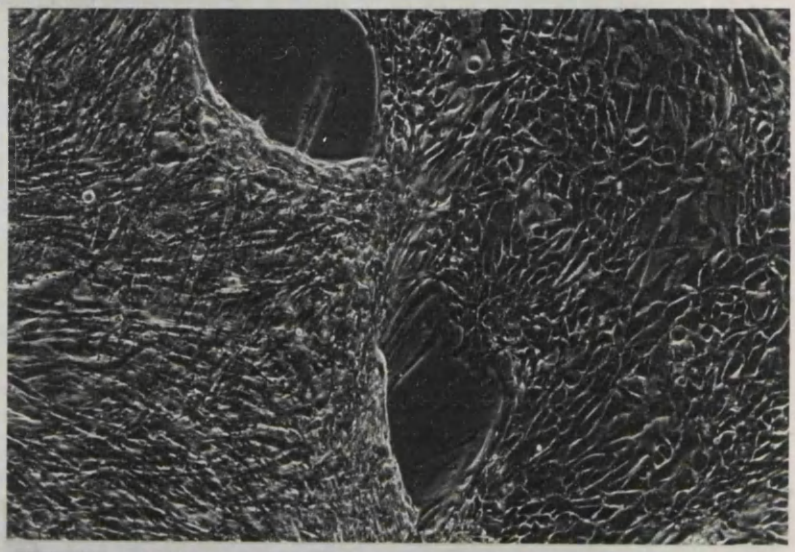
(25 x objective)

(a); squamous /spindle explant

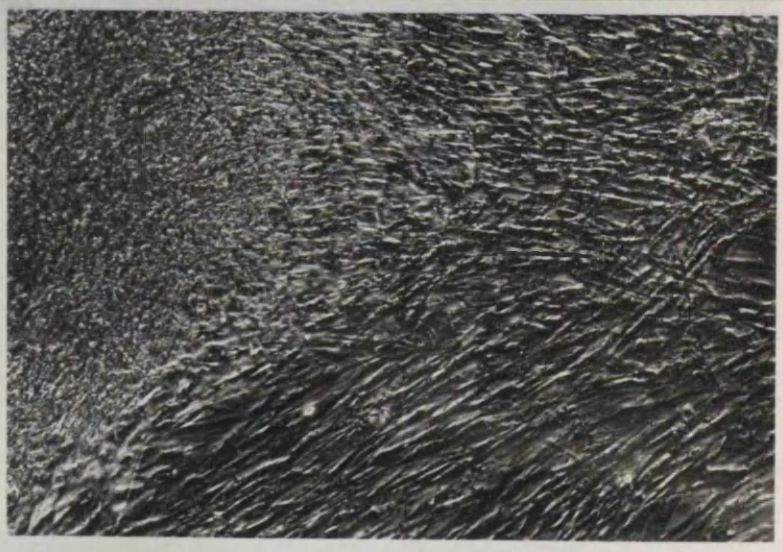
(b); spindle explant

B9HT3 TUMOUR EXPLANTS

a.



b.



3.2.5 The"spindle" cell lines derived from the tumour explants are in fact host fibroblasts

After about a week in culture the "spindle" looking cell lines began to take on a much flatter appearance.

3/7 of the "spindle" cell lines continued to grow with a flat fibroblast appearance, the others began to senesce displaying characteristic features such as stress fibres and very flat "kite" shaped cells.

With the possibility that these spindle-shaped cells were in fact of host origin, i.e. nude mouse fibroblasts, it was considered important to examine the origin of these cells. DNA was made from both squamous and "spindle" cell lines derived from the same tumour. These samples were analysed by the PCR microsatellite assay. Owing to the fact that B9 was derived from a *Mus spretus / Mus musculus* hybrid mouse, the microsatellite approach was able to distinguish between the alleles, displaying two bands on an agarose gel. Figure 15 shows an agarose gel with products of the PCR amplification reactions clearly resolved. Panel A and B represent the same DNA samples but with different polymorphic microsatellite primer pairs, pp21 and pp28, located on mouse chromosome 12 and 2 respectively. The figure shows four DNA samples from cell lines from squamous tumour explants (sq), and four DNA samples from the so-called spindle cell lines (sp?). Control samples include pure *musculus* DNA (m), *spretus* DNA (s), and DNA from the spleen of a *musculus/spretus* hybrid mouse (h).

Analysis of the squamous tumour DNA samples shows the expected polymorphism and appearance of two distinct bands, characteristic of the *musculus* and *spretus* alleles respectively. Analysis of the so-called "spindle" DNA samples shows a clear absence of the spretus alleles (Figure 15). This is evident in PCR reactions with both primer pairs. Therefore, the loss of the spretus allele from the "spindle" cell lines cannot be readily explained as loss of a spretus chromosome, since the primer pairs employed reside on different mouse chromosomes. It seems therefore, that

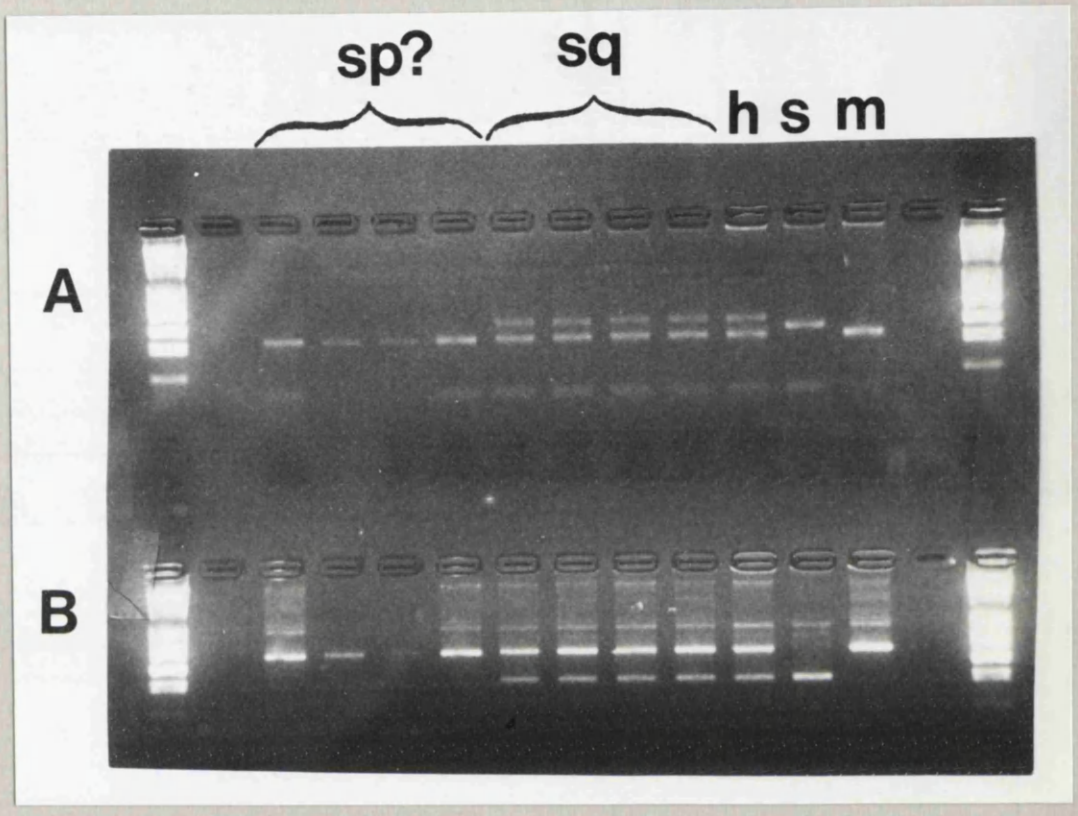


Figure 15.

Host fibroblast origin of spindle-like cell lines from tumour explants (as shown in figure 14.). Nude mouse tumours generated from HaMSV infected B9 cells.

PCR microsatellite analysis of both squamous (sq),and spindle (sp?) cell lines. PCR products electrophoresed on 1.7% agarose gel.

(h); musculus/spretus hybrid control DNA

(s); spretus control DNA

(m); musculus control DNA

A and B represent PCR reactions with primer pairs, pp21 and pp28 respectively.

these spindle-shaped cells, infiltrating the squamous tumours induced by sub-cutaneous injection of B9HT3 1ml and 2ml, are in fact host derived fibroblasts. This was intriguing since only the virus infected clones had shown this effect. One possible explanation for host fibroblast infiltration, was the infection of surrounding host fibroblasts by HaMSV produced by the injected cells.

3.2.6 The B9HT3 cells produce infective virus

For this reason the cell line B9HT3 1ml was analysed for the production of infectious virus, using the NRK assay.

Conditioned medium from the B9HT3 1ml cells was used to transform early passage NRK cells. Focus formation was measured against serial dilutions of the conditioned medium as described in Section 2.20 of the Materials and Methods. The titre of the producer line HT3 was calculated as $1.2x10^5$ focus forming units/ml whereas B9HT3 1ml showed a five-fold lower titre of $2.4x10^4$ ffu/ml. Nevertheless, B9HT3 1ml was capable of virus production, which was not surprising due to the fact that the producer cell line possessed a helper virus which enables packaging of the virus particles.

3.2.7 The apparent absence of integrated virus in host fibroblasts

The possibility that integration of the HaMSV was responsible in some way for recruitment of the host fibroblasts was examined. Two Southern blots of several squamous and three immortal fibroblast cell line DNA samples, hybridised with the H-ras probe (BS9), can be seen in figure 16 . Figure 16A shows 16 squamous DNA samples restricted with PstI (top panel) and BglII (bottom panel). The same quantity of DNA of each sample was used in both enzyme digests, with the lanes lined up according to the DNA sample. PstI cuts once in the HaMSV and consequently shows the pattern of integration sites. BglII cuts internally to the HaMSV, and thus gives an indication of the copy number of the virus. The 2kb PstI band represents the endogenous H-ras PstI fragment, and therefore provides a rough means of controlling for loading between samples. However, it was apparent from examination of the agarose stained gel, that sample 23 had very different amounts of DNA in the PstI and the BglII lanes. This was probably due to a loading problem or a leaking well in the agarose gel. This explains the stark contrast between the amount of DNA indicated by the 2kb endogenous PstI band, and the copy number of the virus

Figure 16.

Stable integration of HaMSV in the squamous tumour explant cell lines, but not in the fibroblast cell lines

Southern blot analysis, (A): top panel; PstI digest, bottom panel; BglII digest. 10 ug of genomic DNA of each of cell lines 1-23: squamous tumour explant cell lines, HT3(virus producer cell line), and B9HT3 1ml (B9 infected with HaMSV).

(B): PstI digest of three fibroblast cell lines 10/13/22, derived from virus tumour explants and squamous explant cell lines 6-9

BS9 insert used as probe.

A
HT3
B9HT3 1ml
23
21
20
19
18
17
16
15
14
9
7
5
4
3
2
1
2kb
1·8kb
B
9
8
7
6
22
13
10
2kb

as indicated by the 1.8kb BglII band. The producer cell line HT3 shows multiple integrations of the virus as one may expect. B9HT3 1ml represents a pool of virus infected B9 cells and consequently the individual integration patterns are too faint to be seen in the Southern blot. The squamous tumour explant cell lines show on average 1-3 different virus integrations. The bottom panel of figure 16A showing the BglII digest of the same DNA samples indicates that the HT3 producer cell line as expected from the pattern of integrations has a high copy number of integrated virus. The B9HT3 1ml on the other hand shows a very faint 1.8kb band. Because the endogenous 2kb PstI band provides a rough internal loading control, comparison of tumour cell lines 15-18 which have about the same amount of DNA as B9HT3 1ml show a much higher copy number of the virus. It seems therefore, that there may be a selection for the cells possessing the virus *in vivo*. Figure 16B shows a similar PstI Southern blot with three host derived fibroblast explant DNA samples and four squamous explant DNA samples. It can be seen that while there are viral integrations in the DNA from squamous explants there appears to be a complete absence of HaMSV in the host recruited fibroblasts.

Further experiments have been carried out, using an alternative virus producer cell line, H9. This producer cell line does not possess a helper virus and should therefore be non-replicative. This should therefore further clarify the question of virus infection of host fibroblasts. In addition, an alternative virus construct RPDpsi6 (gift from D. Wynford-Thomas) which possesses the codon 61 mutation and the neomycin resistance gene, was used to infect both B9 cells and another squamous cell line SN161. It will be interesting to compare the characteristics of squamous cells overexpressing mutant codon 12 H-ras with those overexpressing the codon 61 mutation. These cell lines have been selected, ring-cloned and frozen-down. Data including expression analysis of these clones should provide a clearer picture of the role of overexpressing mutant H-ras in squamous cell carcinomas.

3.2.8 Summary

These data indicate that increasing the levels of mutant H-ras in the squamous cell line B9 is unable to convert the cells to a spindle phenotype. Overexpression of the mutant H-ras gene appears detrimental to the cells causing cell death in the case of G418 selected transfectants. In addition, there appear to be some minor morphological changes in the transfectant and infectant clones, with overexpression of mutant H-ras inducing a more irregular cellular distribution. The appearance of vacuoles in the cytoplasm was seen both in the plasmid transfected colonies and the virally infected pools. It should be pointed out that the same vacuolation was seen in parental B9 cells and B9 control Homer6 clones but at a vastly reduced frequency.

The cells overexpressing the codon 12 mutant HaMSV were unable to grow in soft agar as were the parental B9 cells. However, the spindle cell line A5 grew in anchorage independent conditions and may, in the future, provide a useful means for selecting for the isolation of novel spindle cell lines.

As expected, increasing mutant H-ras in the squamous cell line correlated with a slight increase in tumorigenicity as assayed by nude mouse injections. However, on the whole the histology of the resultant tumours remained characteristically squamous. The exception occured when the virus infected B9 cells were injected into nude mice. The result, was a tumour which possessed two distinct histological components. The "spindle" looking cells in the tumours showed a distinct morphology when initially explanted but soon began to senesce when the serum (FBS) concentration was reduced from 20% to 10% v/v. These "spindle" looking cells were identified as being host fibroblasts. The mechanism of host fibroblast recruitment remains uncertain. However, dimorphism within B9 induced nude mouse tumours has not been identified before, both with parental B9 cells and with the B9 mutant plasmid transfectants. A role for the HaMSV in infection and infiltration of the host fibroblasts into the tumour mass was examined, but appeared not to be involved. These observations will be discussed in more detail in Chapter 5, Section 5.4.

3.3 Overexpressing normal H-ras in spindle cell lines

The converse of the previous experiments would be to increase the level of normal H-ras p21 in a spindle cell, with the aim of reverting its morphology to that of a more differentiated squamous phenotype.

A5 and D3 were transfected with the constructs pHO6N1 and pAGN1 (Spandidos and Wilkie, 1984).

pHO6N1 contains a 6.4kb BamH1 fragment of the normal human H-ras gene from fetal liver. This was cloned into the BamH1 site of Homer6 which contains the bacterial aminoglycoside phosphotransferase (aph) gene under the control of the MOLTR sequences. The H-ras gene is under transcriptional control from its own promoter sequences. The pHO6N1 construct in addition possesses a SV40 enhancer region.

The pAGN1 vector contains the 6.4kb fragment cloned into the BamH1 site of pAG60. This has the aph gene under conrtrol of the HSV TK promoter. SV40 enhancer sequences are absent from this construct.

These constructs were lipofected (according to Section 2.17.2 of Materials and Methods) into A5 and D3 cells and selected at 400ug/ml of G418 for 2 weeks. Resistant colonies were ring-cloned and grown up as individual cell lines. A5 and D3 cells transfected with the neomycin resistance plasmid, Homer6, were selected and ring-cloned as controls.

3.3.1 The presence and expression of normal ras

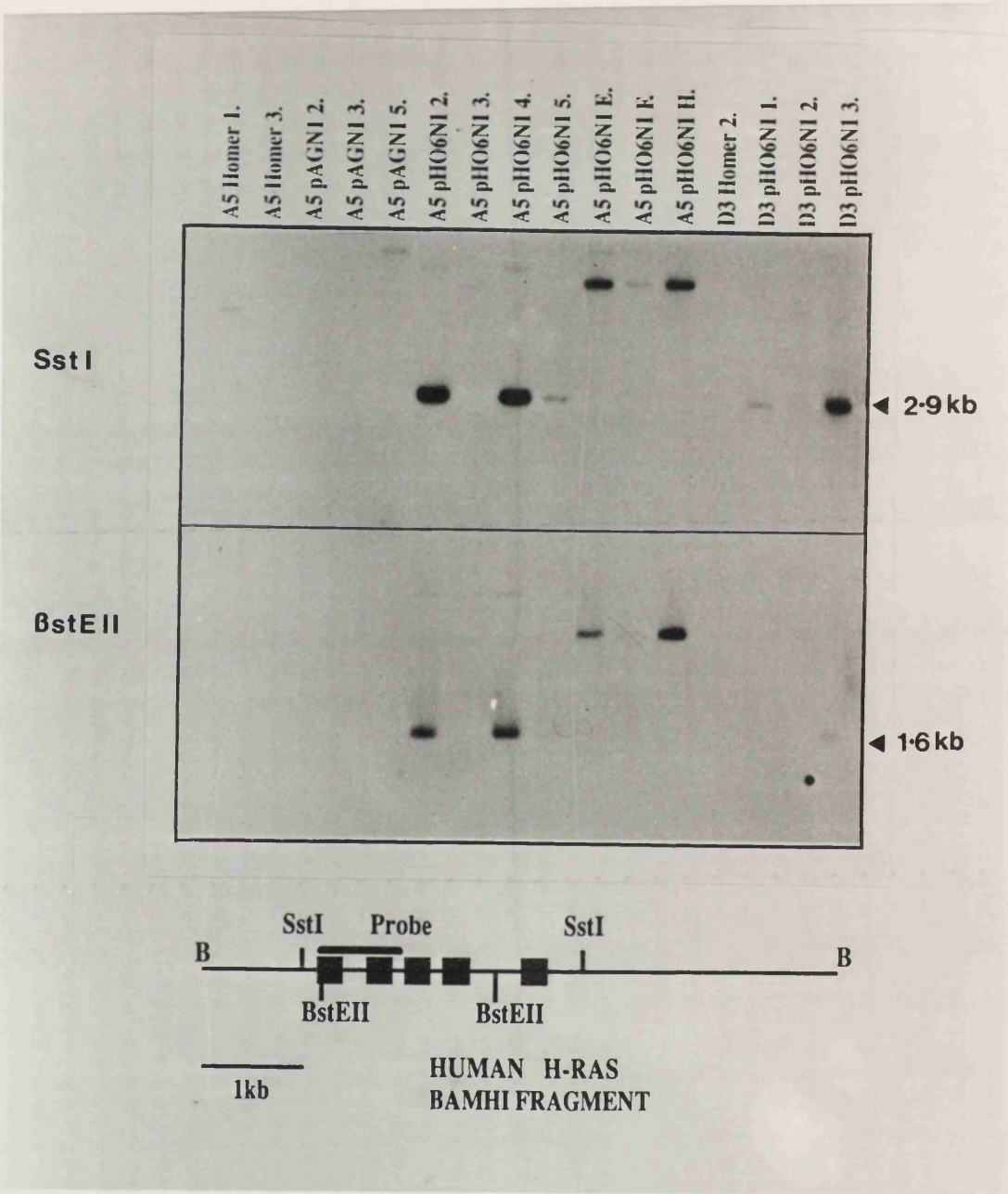
The Southern blot of the transfectants shows the stable integration of the human normal H-ras plasmid in several of the clones (Figure 17).

The SstI digest should cut out an internal fragment of 2.9kb whereas the BstEII cuts out a smaller 1.6kb segment of the gene. As can be quite clearly seen, A5 pHO6N1 2 and A5 pHO6N1 4 showed the highest copy number of integrated plasmid.

Figure 17.

Copy number of normal H-ras expressing plasmids, in A5 and D3 transfectants

10ug of each transfectant digested with SstI and BstEII as indicated.
Restriction map of the human H-ras BamHI fragment incorporated into plasmids pHO6N1 and pAGN1 shown beneath. Expected sizes are 2.9kb and 1.6kb for SstI and BstEII digestion respectively
Human T24 specific probe used in the hybridisation of the blot.


A5 Homer 1.
A5 Homer 3.
A5 pAGN1 2.
A5 pAGN1 3.
A5 pAGN1 5.
A5 pHO6N1 2.
A5 pHO6N1 3.
A5 pHO6N1 4.
A5 pHO6N1 5.
A5 pHO6N1 E.
A5 pHO6N1 F.
A5 pHO6N1 H.
D3 Homer 2.
D3 pHO6N1 1.
D3 pHO6N1 2.
D3 pHO6N1 3.
Sst I
BstE II
2·9 kb
1·6 kb
SstI
Probe
SstI
B
B
BstEII
BstEII
1kb
HUMAN H-RAS
BAMHI FRAGMENT

pHO6N1 E /F and H all showed altered fragment sizes. The BstEII fragment was calculated to be about 3.5kb and a BamHI Southern gave a fragment of approximately 12kb (data not shown). This suggests a rearrangement may have involved a head to head repeat with loss of the intervening BamHI, SstI and BstEII sites.

The D3 clones pHO6N1 1-3 all showed the correct sized restriction fragments with varying copy numbers.

Expression of the exogenous as well as the endogenous H-ras genes was examined by RNase protection analysis. The RNase protection assay was carried out using two probes, one specific for the mouse transcript and the other for the human (for details refer to Materials and Methods, Section 2.12). An estimated 10ug of total RNA was used in the assay. Figure 18 shows the protected fragments which hybridise to both the human-specific (H), and the mouse-specific probes, representing both the endogenous normal (N) and mutant (M) transcripts.

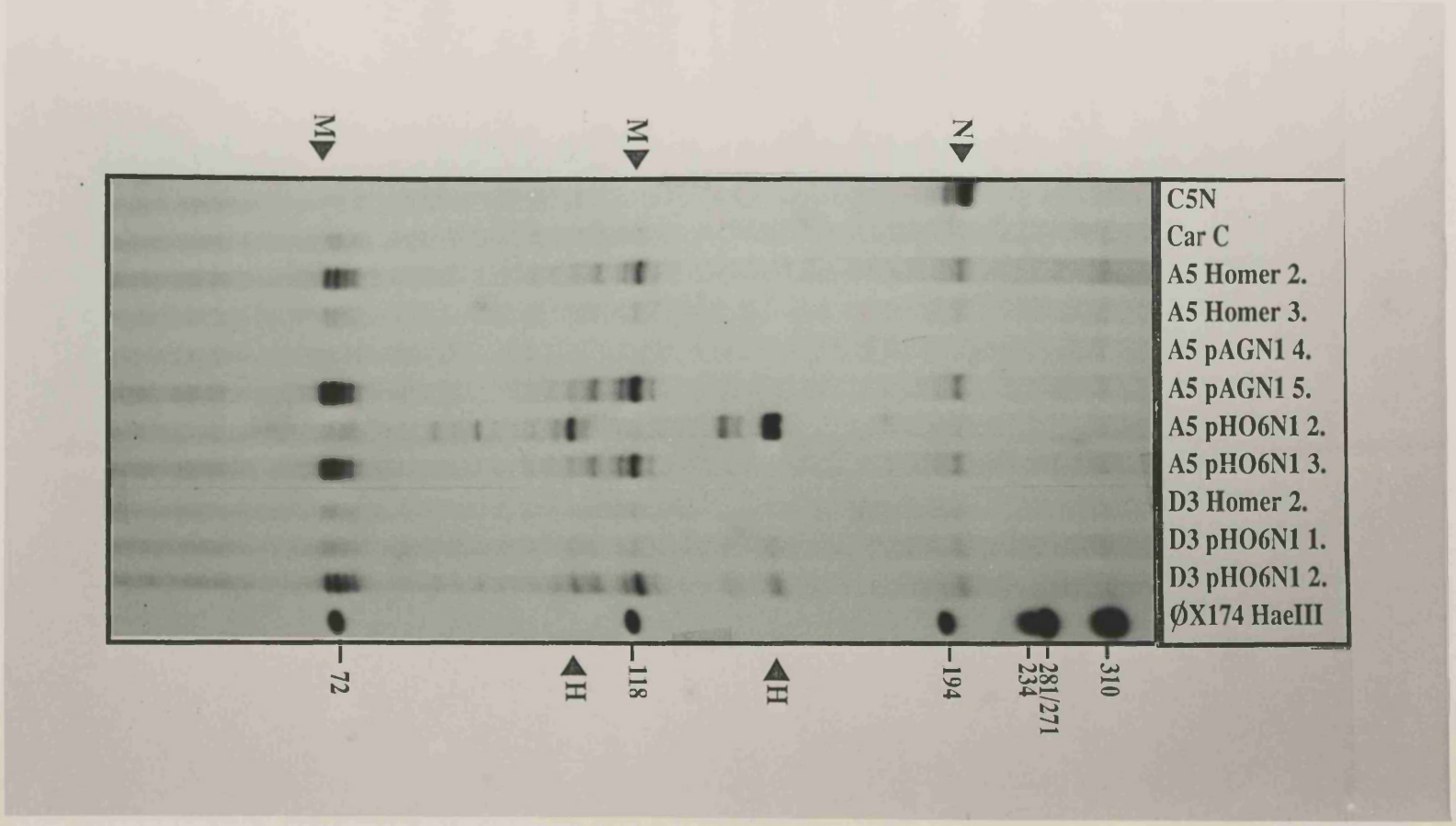
The expression data correlates with the Southern blot analysis, with clone pHO6N1 2 showing the highest level of expression of the human specific H-ras transcript.

An interesting observation that came from the RNase protection data, was the apparent down regulation of the mutant gene in several of the clones. This appeared most evident in the clones which over-expressed the exogenous normal H-ras. However, the same apparent down-regulation was also seen in the control A5 Homer 3, and D3 Homer 2 clones.

Figure 18.

Expression levels of the exogenous and endogenous H-ras genes, in A5 and D3 transfectants

RNase protection analysis of cell line RNA, 10ug in each lane
Details as for figure 8, with the addition of a human specific probe (H); human specific fragments.


M
M
N
C5N
Car C
A5 Homer 2.
A5 Homer 3.
A5 pAGN1 4.
A5 pAGN1 5.
A5 pHO6N1 2.
A5 pHO6N1 3.
D3 Homer 2.
D3 pHO6N1 1.
D3 pHO6N1 2.
ØX174 HaeIII
72
H
118
H
194
234
281/271
310

Another RNase protection assay was carried out on additional clones, with some overlap with the previous samples (Figure 19). Although digestion of the RNA is not complete in this particular experiment (as apparent from the ratio of the endogenous normal to mutant bands), overall the same conclusions can be made from examination of the data. In addition, inclusion of a GAPDH Northern blot of the same samples, provided a means of controling for the loading between lanes. If one concentrates on the lanes A5 pHO6N1 2, 4 and 5, once again the levels of mutant are reduced in A5 pHO6N1 2 and 4 compared to A5 pHO6N1 5, despite there being overall less RNA in the A5 pHO6N1 5 lane. In addition, there appears to be differences in the levels of endogenous normal transcripts between certain clones. For instance clones A5 pHO6N1 2 and 4, have distinctly less endogenous normal transcripts than A5 pHO6N1 5.

The precise mechanism for the reduction in the level of expression of the endogenous mutant H-ras gene was unknown. The fact that it occurred even in clones not overexpressing the exogenous normal gene, suggested that it was not due to some form of transcriptional competition between the introduced and mutant genes.

3.3.2 Spindle cells overexpressing normal H-ras remain characteristically spindle in nature

Figure 20 shows a representative view of two different transfectant clones. A5 pHO6N1 5 shows very little expression of the exogenous normal H-ras gene (Figure 19), whilst A5 pHO6N1 2 was one of the highest normal expressor clones. It can be clearly seen, that both cell lines are characteristically spindle in morphology. This was the same for all the transfected clones independent of copy number and levels of expression of the normal H-ras gene.

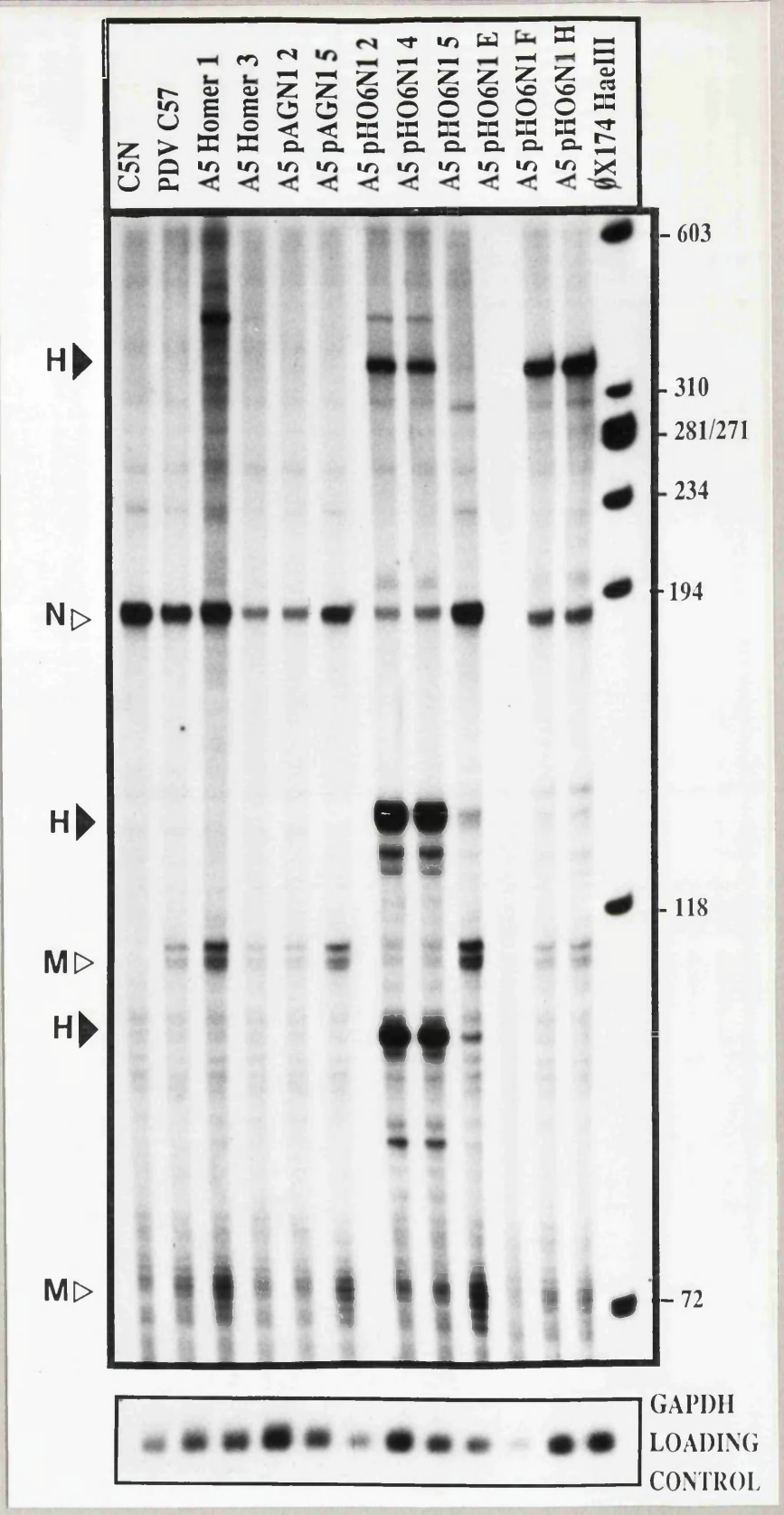


Figure 19.

Expression levels of the endogenous and exogenous H-ras genes in additional A5 transfectants

As for figure 18. Lower panel shows Northern blot of 5ug from same cell lines hybridised with GAPDH probe.

Figure 20.

The morphology of the transfectants overexpressing normal H-ras remain characteristically spindle

Transfectant A5pHO6N1 2. shows a high copy number (figure 17), and level of expression (figure 18) of the normal human H-ras gene, whereas A5pHO6N1 5 does not.

(25 x objective)

3.3.3 Reduction in tumorigenicity correlates with loss of endogenous mutant

In order to determine the tumorigenicity of the transfectants, a number of the clones were injected subcutaneously into nude mice (see Section 2.24 Materials and Methods). It became apparent at a very early stage (within 7 days post-injection), that there were marked differences in tumour development between the clones.

At first sight it seemed that the clones that overexpressed normal H-ras were those that showed a vast reduction in tumorigenicity. However, one of the control Homer transfectants, A5 Homer 3, and two cell lines that were transfected with the plasmid, pAGN1, but by Southern analysis were shown not to contain any human H-ras sequences, also showed suppression.

The nude mouse injections were repeated with the addition of several other clones. The results were conclusive. The same cell lines displayed a reproducible degree of suppression.

The data was intriguing, in particular because a similar experiment with overexpression of normal H-ras in another spindle cell line, carB, was unable to induce any differences in terms of tumorigenicity (S.Haddow and A.Balmain, data not shown).

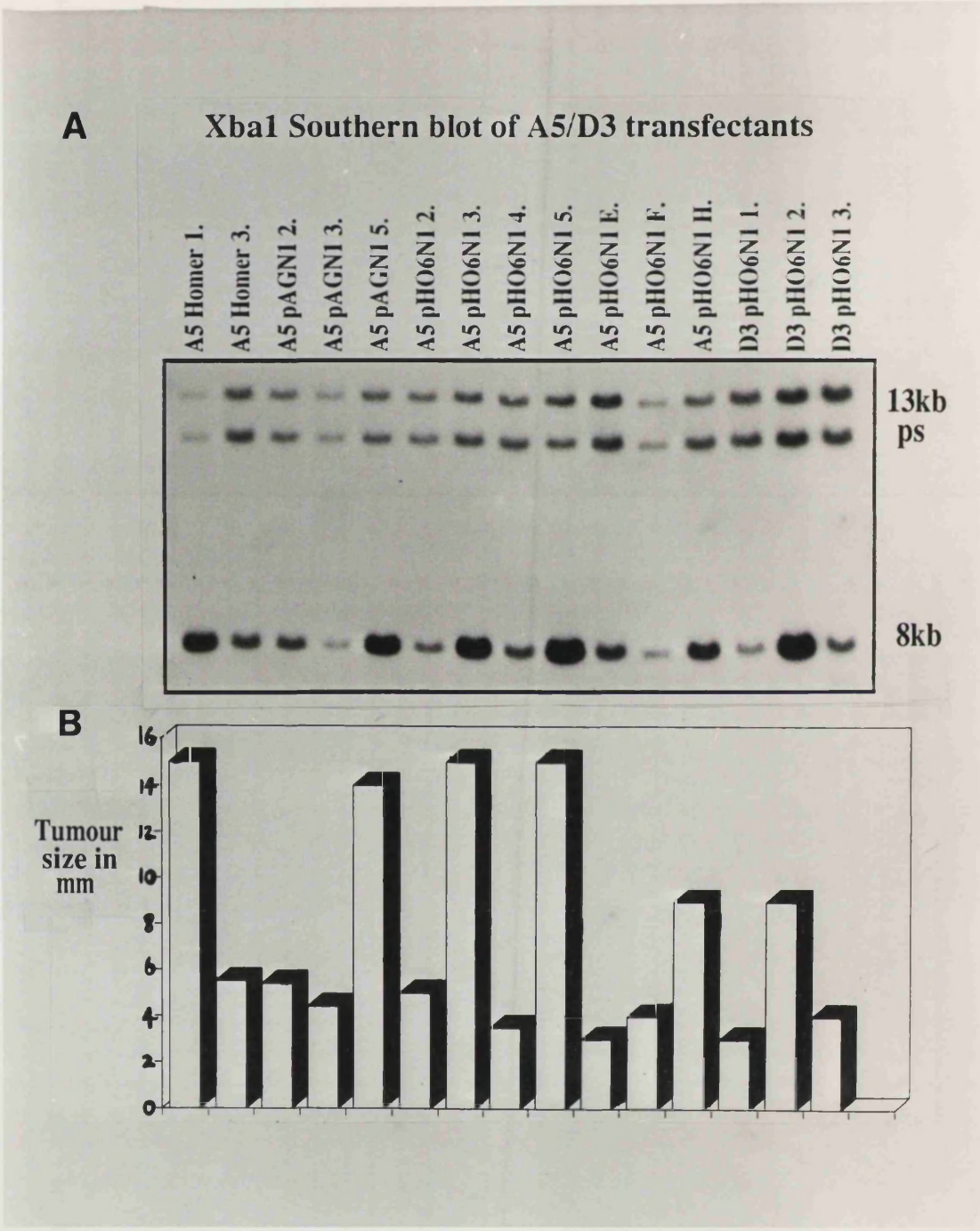
It was realised, that because of the lack of correlation of tumorigenicity with the overexpression of the exogenous normal H-ras, and the apparent down-regulation of the endogenous mutant gene, that it was important to examine the genetic content of the endogenous locus. An XbaI Southern blot of the transfected clones is shown in Figure 21A. From top to bottom, the figure shows the 13kb normal *spretus* H-ras containing fragment, the *spretus* pseudogene and the 8kb fragment representing one of the mutant *musculus* allele fragments. The samples could be roughly calibrated for loading using the intensity of the H-ras pseudogene band. The variation in the levels of the mutant H-ras alleles was striking. For example if one concentrates on the lanes marked A5 pHO6N1 3, 4, and 5, although they have similar quantities of DNA, as

Figure 21.

Reduction in tumorigenicity of some transfectant clones correlates with loss of the endogenous mutant H-ras gene.

(A) XbaI Southern blot of clones transfected with plasmids pAGN1, pHO6N1, and control neomycin plasmid Homer.
Details as for figures 8A and 9.

(B) Graphic illustration of the tumorigenicity of the clones, as measured by sub-cutaneous injection of nude mice. Each bar represents the average tumour size in mm of 10 separate injection points, three weeks post-injection.


A
Xba1 Southern blot of A5/D3 transfectants
A5 Homer 1.
A5 Homer 3.
A5 pAGN1 2.
A5 pAGN1 3.
A5 pAGN1 5.
A5 pHO6N1 2.
A5 pHO6N1 3.
A5 pHO6N1 4.
A5 pHO6N1 5.
A5 pHO6N1 E.
A5 pHO6N1 F.
A5 pHO6N1 H.
D3 pHO6N1 1.
D3 pHO6N1 2.
D3 pHO6N1 3.
13kb
ps
8kb
B
Tumour size in mm
16
14
12
10
8
6
4
2
0

indicated by the intensities of the spretus pseudogene band, the intensities of the mutant specific band are obviously very different. Clone A5 pHO6N1 4 shows a vast reduction in copy number of the mutant gene, compared to A5 pHO6N1 3 and 5, which are more akin to the A5 parental pattern shown in figure 8A.

Figure 21B provides a graphic illustration of the average size of the nude mouse tumours in millimetres, 3 weeks post-injection. These measurements are an average of 10 injection points, two injection points per mouse. The clones represented on the Southern blot are lined up with their corresponding tumorigenicity measurements.

It was immediately obvious that the cell lines which were less tumorigenic displayed a dramatic loss in copy number of the mutant H-ras gene. The correlation was strong, even to the extent that clone A5pHO6N1 H which showed an intermediate reduction in the number of copies of mutant H-ras, showed a corresponding intermediate level of tumour suppression.

A5 Homer 3 and clones A5 pAGN1 2 and 3 which had a reduction in the level of tumorigenicity, but the conspicuous absence of any exogenous normal ras, showed a decrease in the amount of mutant H-ras present.

The relative levels of normal:mutant p21 in the clones were analysed by the Western blotting technique (Figure 22). Although the separation between normal and mutant p21 was not ideal, and the loading difficult to quantify, the results were interesting.

In clone A5pHO6N1 4 which showed the highest level of expression of the exogenous normal construct, there was a correspondingly high level of normal p21H-ras. In addition, there appeared to be a reduction or loss in the mutant p21H-ras species, which correlates with the Southern blot and RNase protection data on loss and corresponding reduction in expression levels of the endogenous mutant alleles.

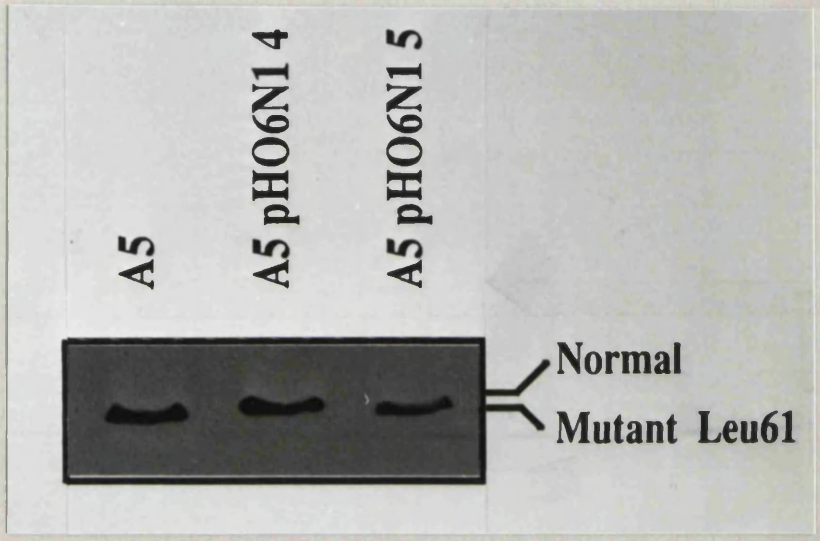


Figure 22.

Western blot analysis of A5 transfectants

Position of normal p21 H-ras and mutant codon 61 Leucine species are indicated.

3.3.4 The histology of the "suppressed" tumours remained spindle

Histology sections from the "suppressed" tumours displayed an overall spindle phenotype. Figure 23 shows two tumour sections taken from tumours about 4mm in size, 3 weeks post-injection of clones A5 pHO6N1 4 (a. and b.) and A5 pHO6N1 E (c. and d.). Areas of more differentiated cells are visible amongst the typically aggressive swirlling pattern of the spindle tumour cells (Figure 23 a and b)(Compare to histology of parental A5 cells, Figure 7). Whereas injection of clone A5 pHO6N1 E generated a tumour which shows a large degree of cell death, perhaps suggestive of a more differentiated phenotype.

However, if the "suppressed" tumours were left to grow for an additional 2 weeks, they increased in size, in some cases to form quite large tumours (about 1cm). Tumour suppression was therefore not complete, but rather resulted in an increase in the latency period.

3.3.5 Summary of data

Overexpression of normal H-ras in the spindle cell lines A5 and D3 is unable to alter the morphology of the cells. Even the clones which displayed high integrated plasmid copy number, high exogenous transcript levels, and elevated amounts of normal p21H-ras remained characteristically spindle in morphology.

When the cell lines were injected into nude mice, dramatic differences were seen in tumour latency between some of the clones. However, variation in tumour size within a group of mice injected with the same clone of cells was minimal. Initially, it was thought that the reduction in tumorgenicity of individual clones, was due to overexpression of the normal gene. However, because of the number of clones included in the experiments, and the addition of several control cell lines, it became clear that something other than the level of expression of the exogenous normal H-ras gene was responsible for the reduction in tumorigenicity.

Figure 23.

The histology of the "suppressed" tumours remains characteristically spindle

a. and b. Histology section from 3-5mm nodules 4 weeks post-injection of A5 pHO6N1 4.

a. 10x objective

b. 40x objective

c. and d. Histology section from 3-5mm nodules 4 weeks post-injection of A5 pHO6N1 E.

c. 10x objective

d. 40x objective

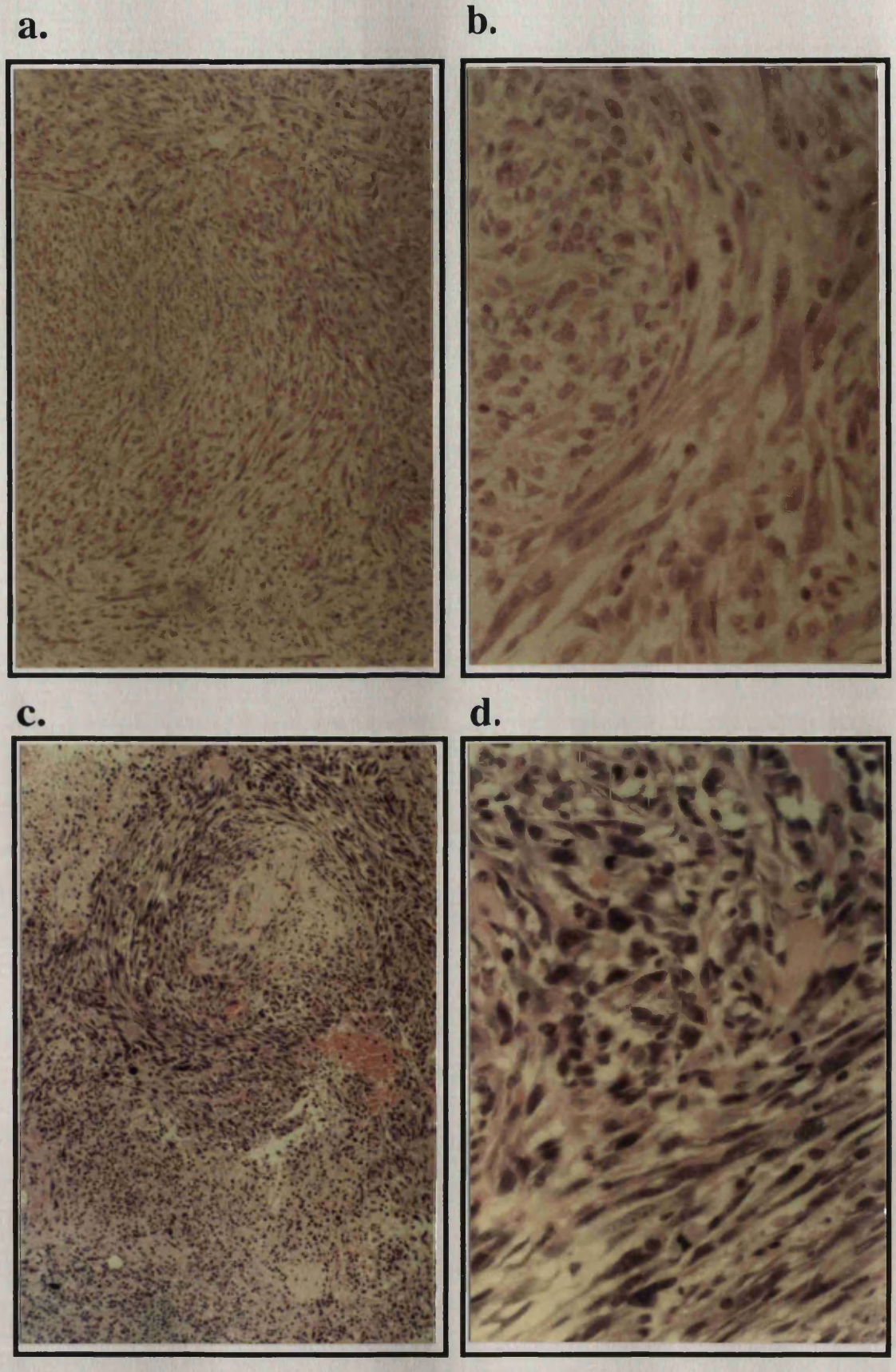


Figure 23.

Examination of the ratio of the H-ras alleles, by an XbaI Southern blot, showed there were marked differences between clones in the quantity of mutant but not normal alleles. This variation was due to the loss of mutant genes in many of the clones, and appeared independent of the copy number and levels of expression of the transfected normal H-ras gene. The clones which displayed loss of mutant H-ras correlated with those which showed a reduction in tumorigenicity as measured by subcutaneous injection of 10^6 cells into nude mice.

These observations explain the RNase protection data, where a reduction in the level of endogenous mutant transcripts seen in several clones may be as a direct result of loss of the mutant alleles. However, an explanation for the apparent reduction in expression of the endogenous normal H-ras genes, observed in figures 18 and 19, was more difficult to account for since there appeared to be no loss of normal H-ras alleles in the corresponding clones, as determined from the XbaI Southern blot (Figure 21). This anomaly may be explained if one considers that incomplete digestion of the mutant alleles would contribute to the intensity of the endogenous normal band. Loss of mutant H-ras in several clones would therefore result in a reduced intensity of the endogenous normal band.

The histology of the repressed tumours remained spindle, even when removed and examined at an early stage. Thus suppression of tumorigenicity correlates with an increase in the latency period of tumour development, but not with the overall gross morphology of the tumours.

Further experiments with increasing the levels of normal H-ras in the spindle cell lines A5 and D3, were carried out using a selectable virus, psi2wt. Conclusive expression data on these clones is not yet available. However, injection of these clones showed similar variability in tumour latency, with 3 out of 5 clones displaying some reduction in tumorigenicity..

It therefore seems clear that overexpression of the normal gene is unable to convert the spindle cell to a more differentiated phenotype. In addition, the spontaneous loss of mutant H-ras from the spindle cells does not alter the cellular morphology

Thus the critical factor in determining the tumorigenicity of the individual clones, was the level of the endogenous mutant H-ras gene. The morphology of the cells is clearly an independent factor and has at least in this particular case little bearing on tumorigenicity as measured by the nude mouse assay.

Spontaneous loss of the mutant alleles occurs quite frequently, in about 70% of the ring-cloned A5 and D3 cell lines.

The intrinsic instability of these spindle cell lines, may offer a clue as to the mechanism for increasing mutant H-ras in the conversion from a squamous to a spindle carcinoma. If the increase in mutant H-ras occurs by a genetic amplification event, as has been previously suggested, this may provide an explanation for the degree of genetic instability encountered, particularly if such an amplicon resided on an extra-chromosomal double minute. The fact that one cell line, A5pHO6N1 H, showed an intermediate reduction in the level of mutant H-ras and a corresponding intermediate level of tumorigenicity, may indicate the presence of several such double minutes.

Indeed, preliminary data with a chromosome fluorescence technique has identified several such extra-chromosomal bodies, most likely double minutes, in the spindle cell line D3 (N.Keith; personal communication).

This data together provides the first indication that H-ras resides on an amplicon which may be in the form of double minutes. In addition, genetic amplification of mutant H-ras has been shown to be a critical event in the squamous to spindle transition. However, this does not appear to be the only event that is necessary to create the overall spindle phenotype, since complete or partial loss of these amplicons does not alter the morphology of the cells.

These observations will be discussed in more detail in the following discussion chapter.

3.4 Increasing normal H-ras in squamous cell lines

Although, overexpression of normal H-ras was unable to alter the phenotype of the spindle cells, it still does not rule out a suppressive role for normal H-ras at an earlier stage of tumorigenesis. It was decided to include an additional set of experiments. To overexpress the normal H-ras gene in the squamous cell lines B9 and SN161. SN161 was derived from a DMBA/TPA lymph node metastasis, squamous in morphology. Analysis of chromosome 7 RFLPs showed that this cell line had completely lost the normal H-ras and Int-2 alleles, indicative of a mitotic recombination event (Bremner and Balmain, 1990).

The same pHO6N1 normal H-ras plasmid construct as before, was used to transfect the squamous cell lines. In addition, a virus, psi2wt, expressing the normal gene (gift from D. Wynford-Thomas), was used to virally infect the squamous B9 and SN161 cell lines. The virus contained a neomycin resistance gene, allowing selection for the cells infected with the virus.

Preliminary tumorigenicity data, indicates that some clones, both transfectants and viral infectants, showed a reproducible degree of suppression. Further data including expression analysis of these clones is necessary before any conclusions can be made concerning the suppressive effect of normal H-ras on the squamous phenotype.

Chapter 4
Results

Altering ras gene dosage levels by homologous recombination in ES cells

4.1. Introduction

Homologous recombination enables an exogenous gene construct, introduced into a cell most commonly by means of electroporation, to pair with an endogenous homologue in the cellular genome and to undergo a reciprocal exchange event.

With the use of pluripotent embryo-derived stem (ES) cells it is possible to introduce a genetic mutation by the mechanism of homologous recombination into a specific genomic locus. The mutated ES cells can then be returned to a host blastocyst where they are able to colonise the developing embryo, in some cases contributing to the germline of the resultant mouse.

In this way the ES cells can be modified by homologous recombination *in vitro*, and the mutational event introduced into the mouse germline *in vivo*.

Through the use of this technique it was hoped to be able to generate a null allele of H-ras by the disruption of the coding region of the gene, most commonly by the introduction of a neomycin resistance gene.

Creating an ES cell line that possesses only one functional H-ras gene, and the ultimate goal a transgenic mouse hemizygous for H-ras should provide the clearest means to study the effect of the normal allele in early tumour development.

If the normal allele functions as a suppressor in mouse skin tumorigenesis, then carcinogenesis studies on the transgenic mice carrying only one functional allele of H-ras should theoretically result in a more susceptible phenotype, producing an initiated cell with no normal H-ras alleles.

The H-ras "knock-out" mouse if viable, can be bred to homozygosity enabling an examination of the effect on mouse development and tumorigenesis of having no functional H-ras.

Altough the targeted ES cells are undoubtedly the most useful, it was hoped that if successful, the same targeting constructs would be used to target the H-ras alleles of other cell lines. Cell lines such as PDV and PDVC57 which possess H-ras gene dosage ratios of 2:1 and 1:2, normal:mutant respectively, were considered particularly relevant. Although both are characteristically epithelial in morphology, PDVC57 is much more tumorigenic than PDV. Depending on the particular allele inactivated, it was hoped that changes in tumorigenicity or perhaps cellular morphology may occur.

4.2 Alternative strategies for gene targeting

Several techniques for gene targeting utilising homologous recombination have been detailed. The main differences between the methods involves the design of the targeting vector, and the system of delivery of the construct into the cell.

Alternative strategies were employed to attempt to target the H-ras gene.

Direct nuclear micro-injection of a targeting construct was reputed to dramatically increase the overall gene-targeting frequency, presumably by delivering the vector by a more direct route. The H-ras targeting vector constructed for microinjection was based on the vector design used by Gruss and colleagues (Zimmer and Gruss, 1989). The vector contained no selectable marker but an oligonucleotide insert which introduced several in frame translation stop codons into the coding sequences of H-ras exon 1 as shown in figure 24. The region of the vector including the engineered oligo stretch was sequenced using the Sequenase kit as Section 2.14 of Material and Methods. It was intended, that screening for correct homologous recombinants would be by PCR amplification. Nuclear micrinjection of cells, was accomplished using an Eppendorf pneumatic injector. The system was semi-automated, with the movement of the needle controlled by a micro-processor. However, despite great efforts to obtain viable clones after micro-injection of the cells, the approach was eventually abandoned.

Figure 24.
Abbreviations for restriction endonuclease sites:
P - PstI
H - HindIII
B - BamHI

MICROINJECTION CONSTRUCT

complimentary oligonucleotides creating NotI, XbaI restriction sites and three in-frame stop codons cloned into HindIII site in exon 1 of NPR plasmid

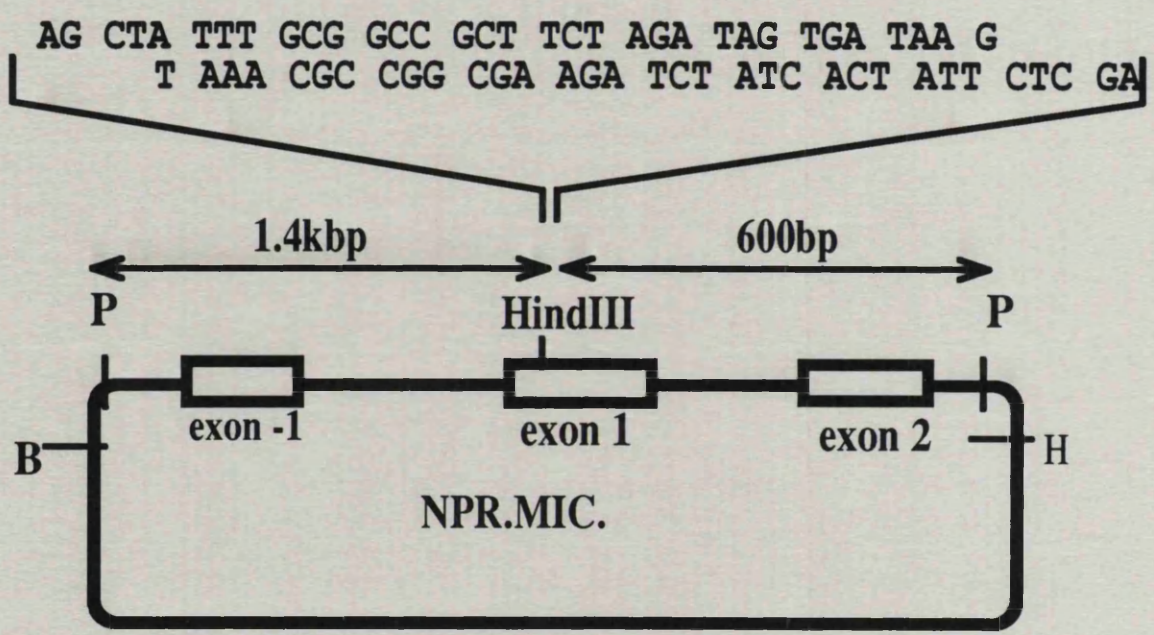


Figure 24.

An alternative technique to increase the gene targeting frequency was to use a promoterless neomycin resistance gene construct. Cloning of such a construct was carried out as outlined in figure 25. In theory, only when the construct was correctly integrated at the homologous site, or when the construct was randomly integrated adjacent to an active cellular promoter, would the clone survive selection.

Despite several electroporations and selection regimes, it was never possible to obtain viable ES cell colonies after G418 selection. However, the same construct electroporated into PDV epithelial cells provided a selectable phenotype.

A Northern blot of total ES cell RNA compared to PDV RNA showed a much lower level of H-ras expression (Figure 26). This pattern was reproduced in a subsequent Northern blot of total RNA from ES, PDV and C5N cells (data not shown). This may in part explain the inability to derive resistant clones, because the endogenous level of H-ras in embryonic stem cells was insufficient to drive a selectable phenotype. However, this cannot account for the total lack of resistant clones, since random integrants should be independent of the endogenous H-ras transcriptional activity. It was decided to however, abandon this approach in favour of a strategy independent of endogenous expression levels.

4.3 The positive-negative selection strategy

The positive-negative selection (PNS) protocol has been widely used to target many different genes (for review Capecchi, 1989). It has the advantage of allowing genes which are expressed at moderate levels, or not expressed at all in ES cells, to be successfully targeted.

Figure 25.
Abbreviations for restriction endonuclease sites:
S - SmaI
H - HindIII

Promoterless H-ras targeting construct : Design and cloning strategy

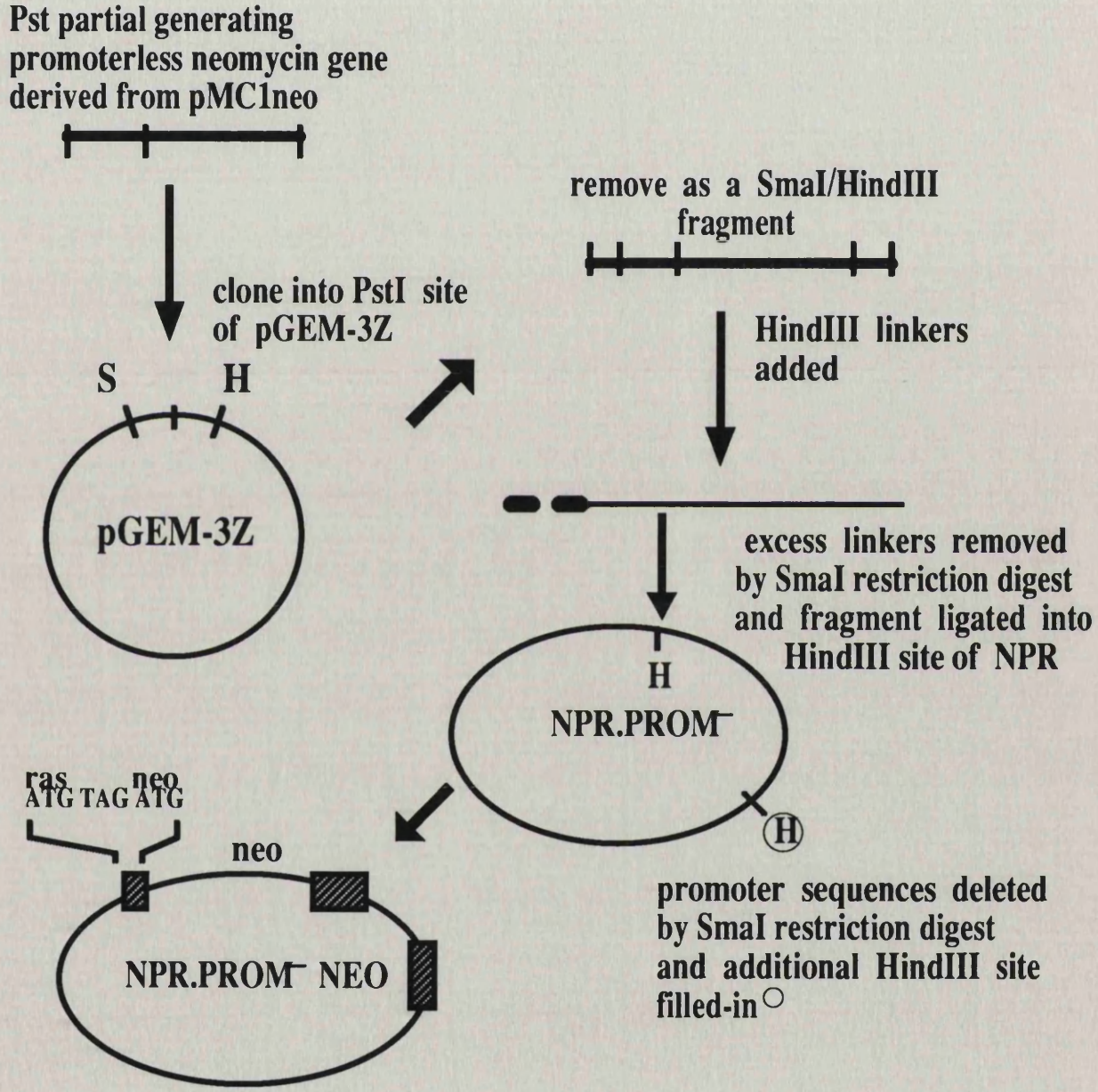


Figure 25.

Figure 26.

Low level expression of H-ras in ES cells

10ug of total RNA from PDV and ES cells

Hybridisation was carried out with H-ras probe BS9.

A functional HSV-tk gene is positioned at the end of the linearised targeting vector. Random integration of the vector results in incorporation of the HSV-tk gene (Figure 4). Homologous recombination of the construct, on the other hand, results in the loss of the HSV-tk sequences. Cells that contain the tk gene can then be selected against, using the anti-viral agent, gancyclovir (GANC). ES cells containing targeted disruptions can be enriched several fold, presenting a double resistant $G418^R/GANC^R$ phenotype (Figure 4).

4.3.1 Cloning of a positive-negative selection (PNS) vector

The cloning strategy for a positive-negative selection construct is outlined in figure 27. The neomycin resistance gene under the transcriptional control of the HSV tk promoter, was isolated as an XhoI/SalI fragment from the plasmid Pmc1PolA (Thomas and Capecchi, 1987). The ends were converted to HindIII ends by subcloning into the polylinker sequences of pIC20H. This neomycin HindIII fragment was then cloned into the HindIII site in exon 1 of H-ras. The H-ras clone pB4 was used as the basic backbone of the vector (see figure 28). This provides about 3kb of H-ras homology. The HSV tk gene under the control of the phosphogluco-kinase (pGK) promoter was blunt-end ligated into the unique HpaI site in the extraneous lambda charon sequences of pB4. The vector termed the PNS construct was linearised prior to electroporation with the restriction enzyme NotI.

The ES cells were electroporated as outlined in the Materials and Methods, Section 2.23.1. Selection was at 200ug/ml of G418 initially for 4-7 days with the subsequent addition of gancyclovir at a final concentration of 2uM. Colonies were picked at day 12-14 of selection using drawn out glass pasteur pipettes and seeded into 24 well plates. The cell lines were further expanded into $25cm^3$ flasks from which DNA and frozen aliquots of cells were prepared.

Figure 27.

Abbreviations for restriction endonuclease sites:

X - XhoI

S - SalI

H - HindIII

E - EcoRI

N - NotI

PNS strategy construct

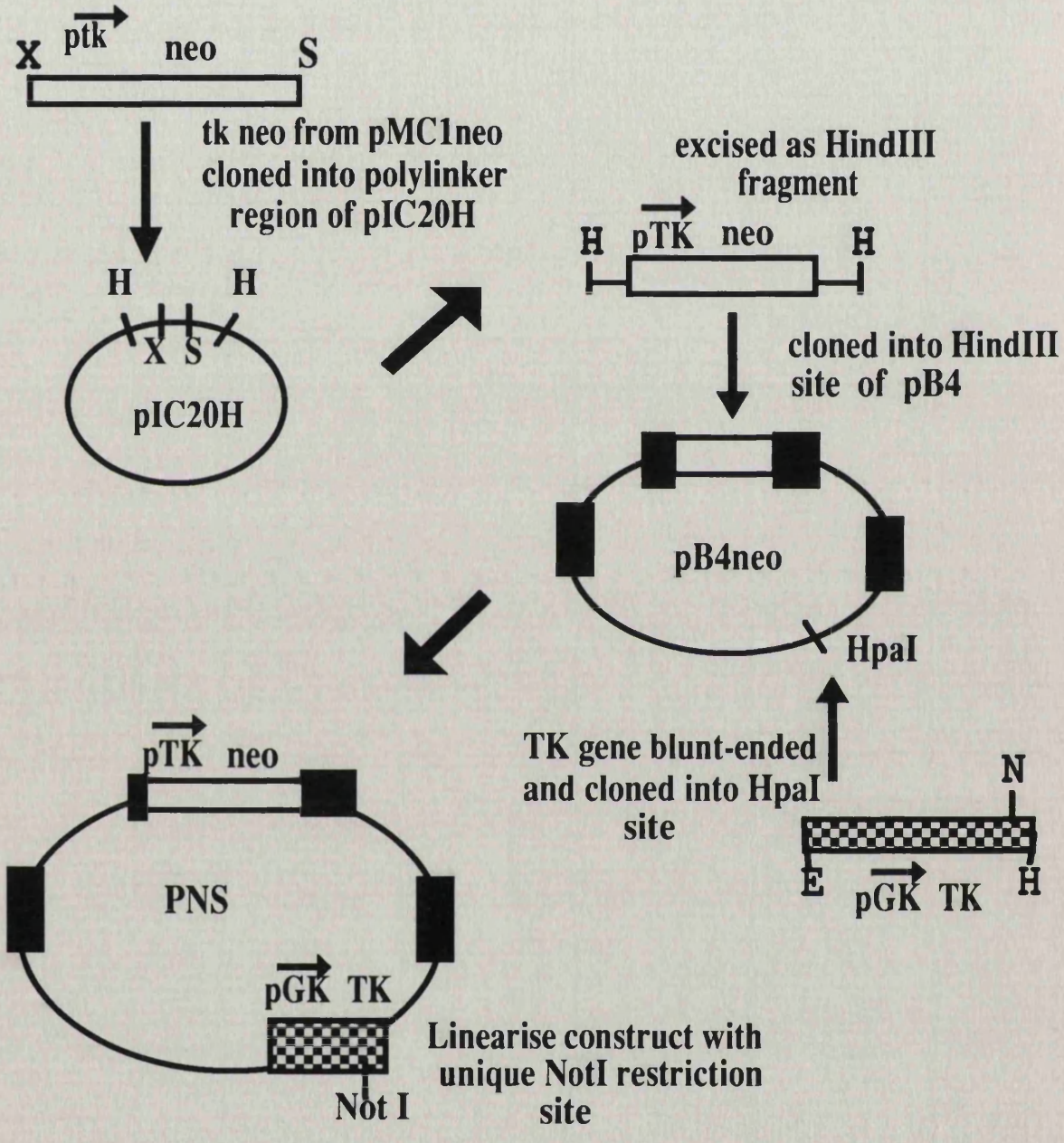


Figure 27.

Figure 28.

Abbreviations for restriction endonuclease sites:

B - BamHI	**Hp - HpaI**
Bg - BglII	**P - PstI**
E - EcoRI	**S - SmaI**
H - HindIII	**Sa - SalI**

H-RAS SUBCLONES USED IN VECTOR CONSTRUCTION

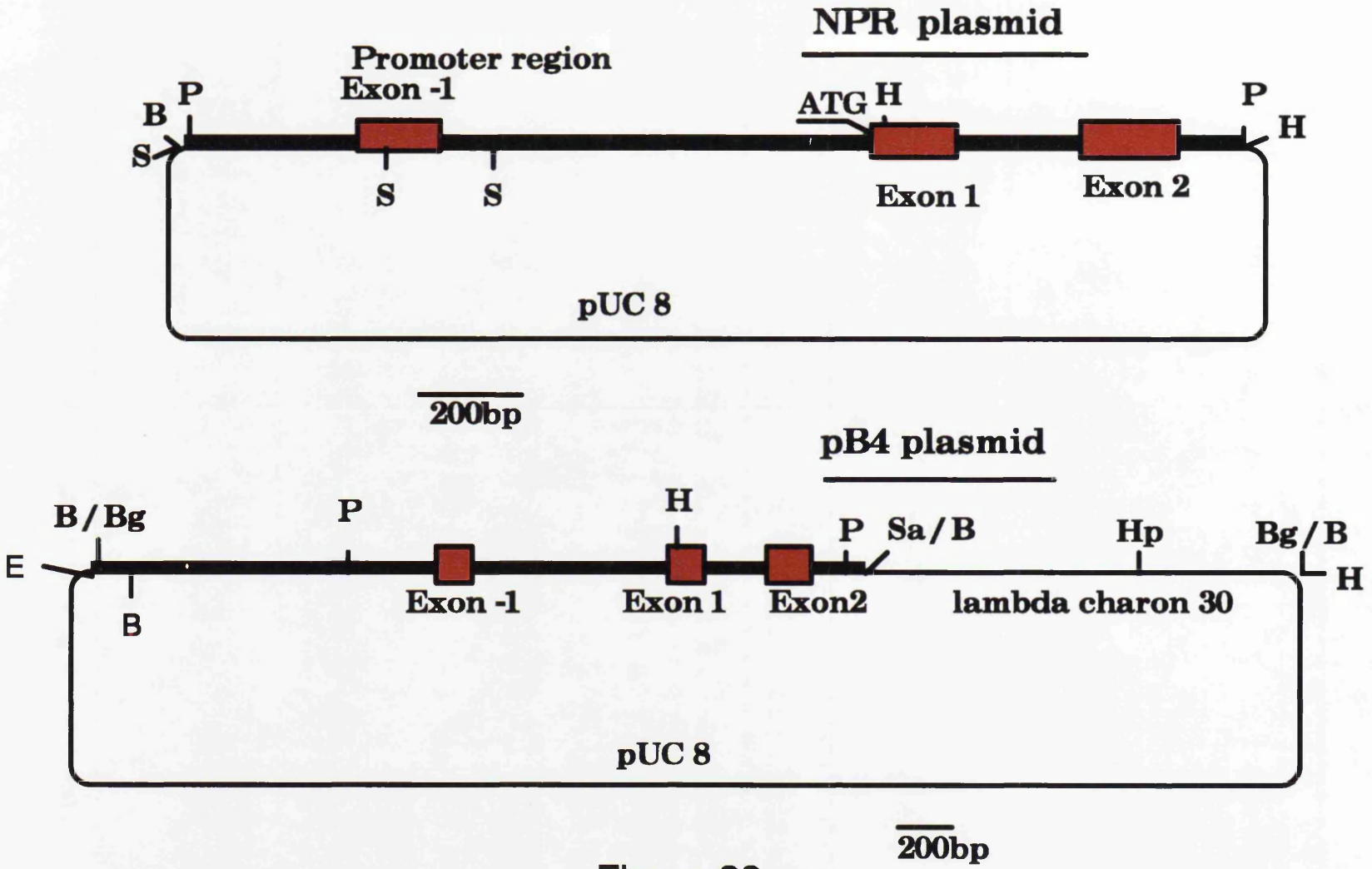


Figure 28.

4.3.2 Screening the ES cell clones by Southern blot hybidisation

The general strategy for screening the ES cell clones for a homologous recombinant, by Southern blot hybridisation, is outlined in figure 29.

In the first transfection experiment a total of 180 double selected ($G418^R/GANC^R$) colonies were picked and analysed by Southern blot hybidisation.

The DNA was made by the rapid lysis method outlined in the Materials and Methods, Section 2.18.

Restriction digests were performed overnight using enzymes considered to be informative. These included BamHI, EcoRI, KpnI, and XbaI.

Figure 30 shows a BamHI Southern blot of 33 samples with a restriction map of the endogenous and expected targeted loci. The upper panel shows the blot hybridised to the H-ras probe, pB5. The pB5 insert, was made by PCR amplification of the H-ras cDNA from mouse RNA (W. Lambie). The cDNA type probe covered exons 1-4 of the H-ras gene. The lower panel represents the same blot rehybridised with an insert from the neomycin resistance gene.

A correctly targeted clone should display an additional 1kb BamHI fragment that should hybridise to the H-ras probe. Although the clone indicated by an arrow shows a faint band of the right size, this was discounted as being a targeted event by additional Southern blot and PCR analysis.

Rehybridistion of the blot with the neomycin probe shows a 3.6kb band indicative of random integration of the construct. However, reprobing the BamHI blot with neomycin is not strictly informative, since a targeted event would produce a similar sized band on the Southern. In addition, it appeared that not all of the clones possessed the marker gene, despite almost 2 weeks under selection. One possible explanation for this observation, may be that the randomly integrated constructs, including the neomycin resistance gene, resided on large genomic fragments that were not properly transferred onto the membrane. Another possibility was that the

SOUTHERN BLOT SCREENING FOR
HOMOLOGOUS RECOMBINANTS

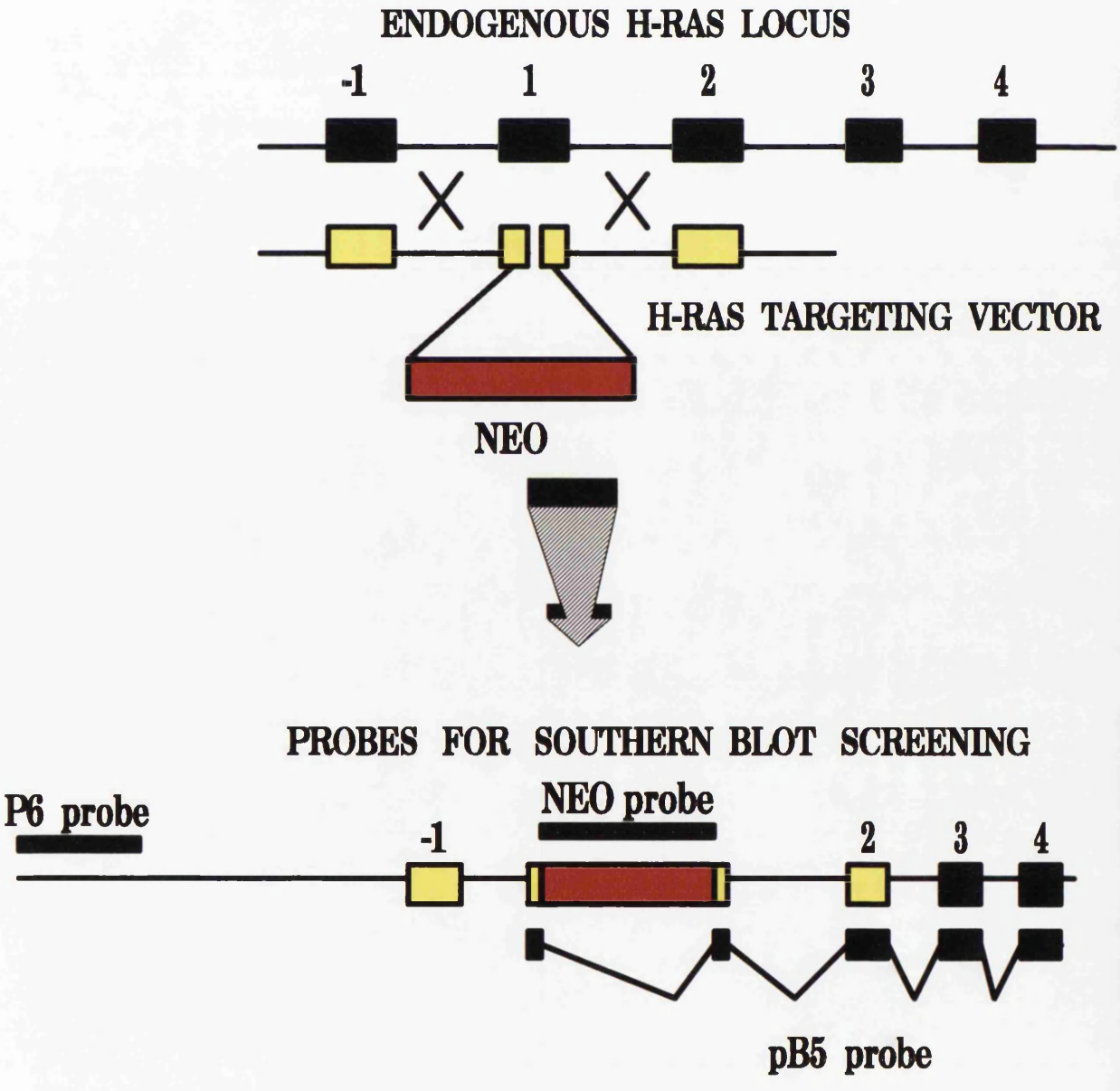
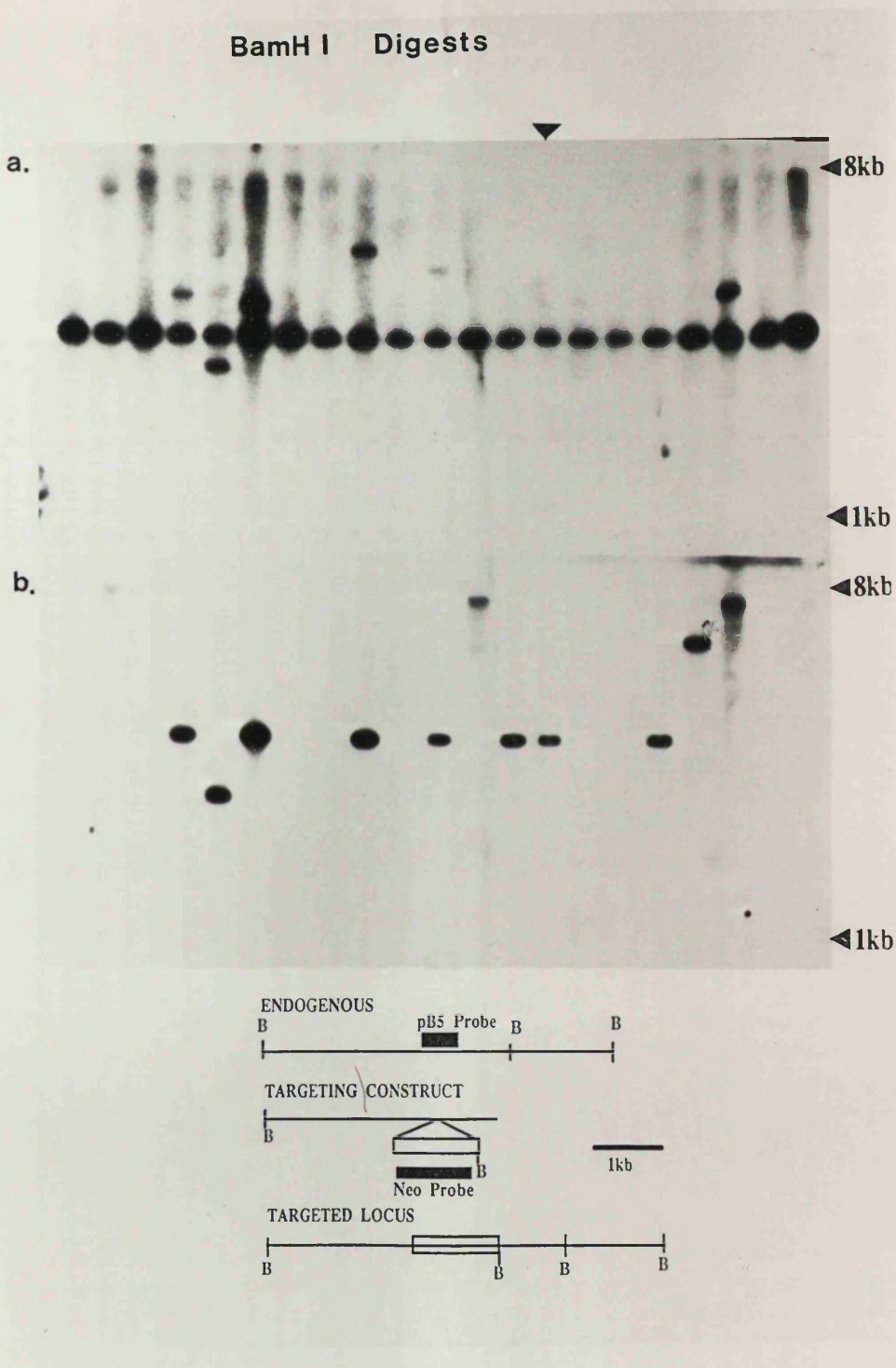


Figure 29.

Figure 30.
BamHI Southern blot screening of ES cell clones electroporated with the PNS targeting vector.

(a); Filter hybridised with H-ras pB5 probe
(b); Filter reprobed with neomycin insert
Size markers shown in kb with BamHI restriction map of the endogenous locus, targeting vector and expected structure of the region if properly targeted


BamH I Digests
a.
8kb
1kb
b.
8kb
1kb
ENDOGENOUS
B
pB5 Probe
B
B
TARGETING CONSTRUCT
B
B
Neo Probe
1kb
TARGETED LOCUS
B
B
B
B

geneticin (G418) selection was not optimal. Fresh geneticin stocks were prepared for future experiments.

A further electroporation and selection was carried out with the same PNS construct, and an additional 154 double resistant, $G418^R/GANC^R$, clones were isolated.

These were screened in the same way as before. Figure 31 shows an XbaI Southern blot of 38 clones which were hybridised to the H-ras probe, pB5, (panel a and c), and the same membranes stripped and reprobed with an insert from the neomycin gene (panel b and d).

In the case of a targeted event, the endogenous 12kb XbaI fragment should be increased in size by about 1kb, with the insertion of the neomycin gene. On a Southern therefore, the same 13kb fragment should hybridise with both the H-ras and neomycin probes. Two clones (indicated by arrows) showed bands of about the right size i.e. 13kb. However, when further analysed by PCR and by Southern blotting using alternative restriction enzyme digests such as EcoRI, they proved negative. The neomycin reprobed filters, Figure 31 panels b. and d. show that almost all of the clones have integrated neomycin genes (in contrast to the previous experiment), which on the whole correspond with the same fragments that hybridise to the H-ras probe, as one would expect for randomly integrated copies of the vector.

Figure 32a shows an EcoRI Southern blot of 17 different clones, hybridised to the H-ras, pB5 probe. A double cross-over event should disrupt the endogenous EcoRI 23kb H-ras fragment into two smaller fragments, 9 and 15kb in size (since the neomycin gene contains an EcoRI site). As can be seen from the Southern there were no smaller bands of the right size only some larger bands of approximately 25kb in size. When the blot was rehybridised with the H-ras probe, P6 KpnI (KpnI sub-fragment of the BamHI P6 probe), derived from sequences upstream of the targeting homology, (termed an external probe), these same bands did not rehybridise. This indicates that these larger 25kb fragments represented randomly integrated constructs and not some homologously recombined variant.

Figure 31.

XbaI Southern blot screening of ES cell clones electroporated with the PNS targeting vector

(a); Filter of 20 ES cell clones hybridised with pB5 H-ras probe. (b) same filter reprobed with an insert from the neomycin resistance gene. (c); Filter of 18 ES cell clones hybridised with pB5 H-ras probe. (d) same filter reprobed with neomycin probe. Position of endogenous H-ras 12kb XbaI band is marked as well as some bands of about the expected size (13kb) for targeted locus.

Xba I Digests

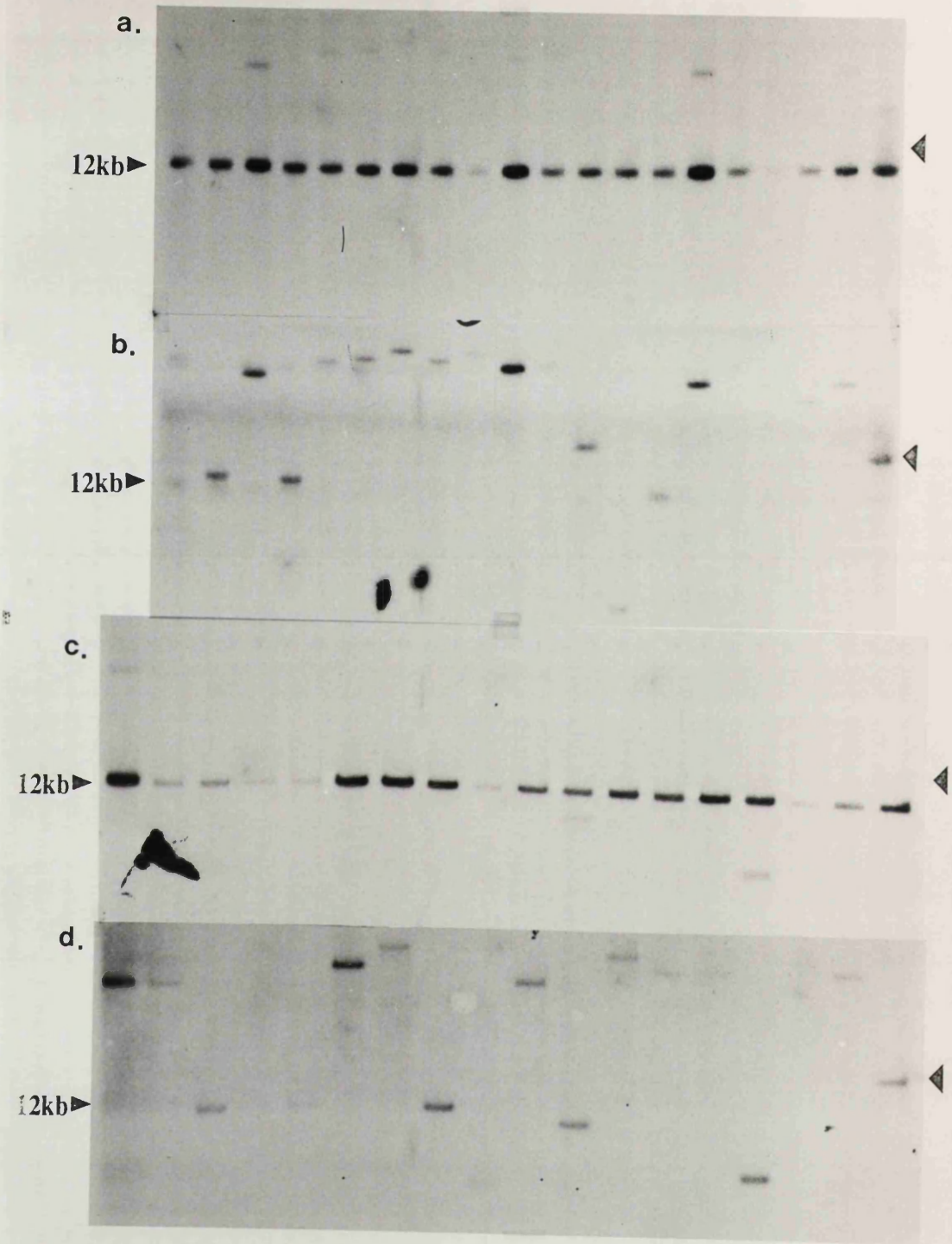


Figure 32.

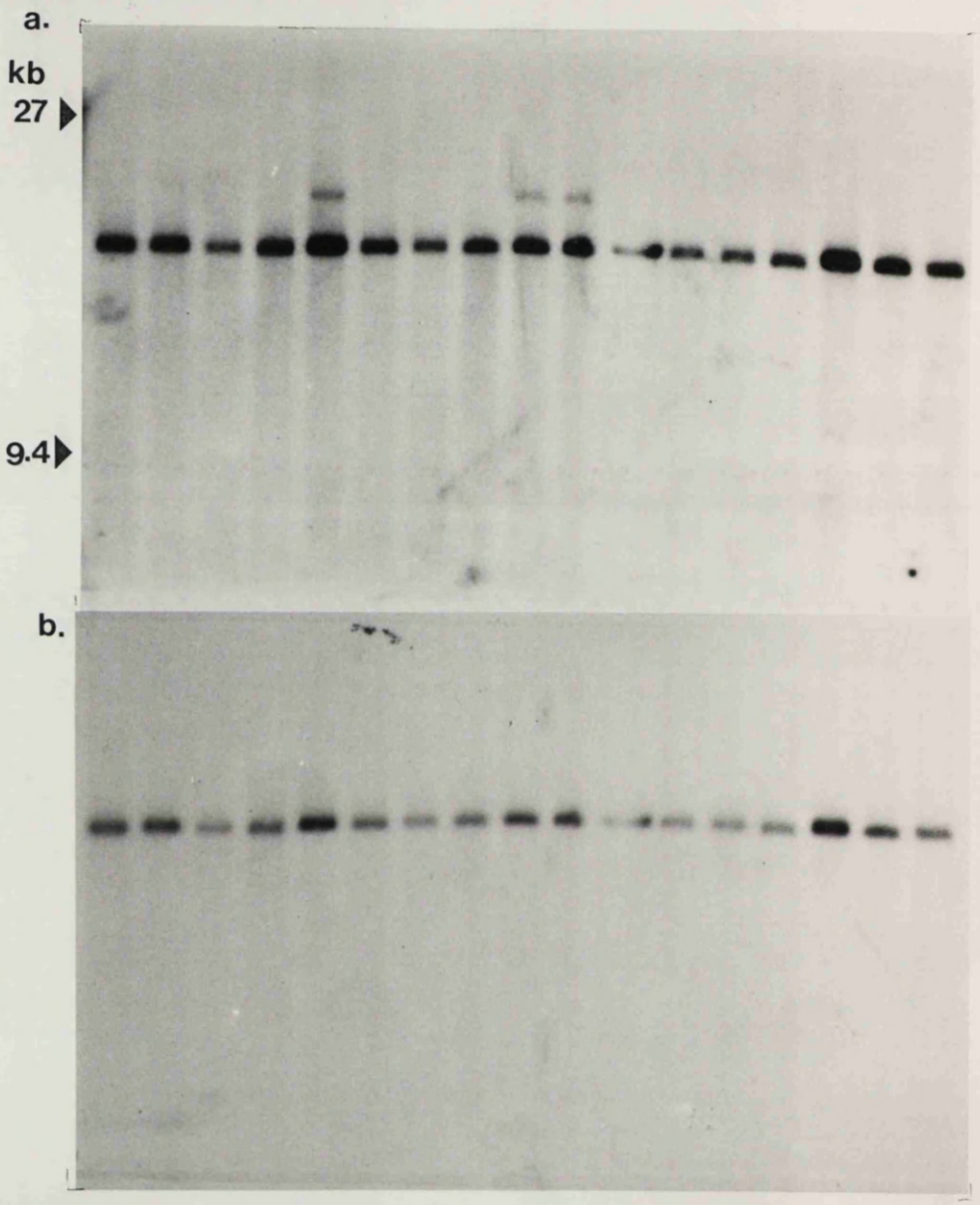
EcoRI screening of ES cell clones electroporated with PNS vector.

(a) Filter hybridised with pB5 H-ras probe

(b) Filter hybridised with upstream P6 H-ras probe

Size markers lambda/HindIII as indicated

EcoRI Digests



Although the Southern blotting protocol is the most definitive method for confirming that a targeted event has taken place, it was in practice tedious and time consuming. An alternative to the Southern screening protocol, was to use the PCR technique, which offers a more rapid approach to the initial screening of large numbers of cell lines (Joyner et al., 1991).

4.3.3 Screening by the PCR technique

The general strategy for screening the ES cell clones for a homologous recombinant using the polymerase chain reaction (PCR) assay is outlined in figure 33.

PCR amplification was carried out according to the conditions outlined in the Materials and Methods, Section 2.15. Several amplimers were made from sequences in the neomycin gene, and from sequences in H-ras exon 3 external to those contained in the targeting construct. In theory, only in the case of a legitimate recombination event should the neomycin and exon 3 sequences be contiguous. This should enable PCR amplification, across the 3' recombination boundary, of a specific sized fragment.

It was realised that it was essential to provide a positive control for the PCR screening of ES cell clones. It was necessary therefore, to artificially create a H-ras homologous recombination event. The positive control construct was engineered as outlined in figure 34. Using amplimers complementary to the neomycin gene, to exon 2 and to exon 3, the construct was amplified in two halves. The 5' half was amplified from the targeting plasmid and the 3' half using genomic DNA as the template. The exon 2 amplimers were specially designed with 20 bp overlapping tails. These tails were complementary to each other, and when properly annealed were designed to form a unique EcoRI restriction site. This was included in the design, so that in the likelihood of plasmid contamination of ES cell clones with the positive control plasmid, it would be possible to distinguish plasmid contamination from legitimate recombination. The positive construct was generated as figure 34

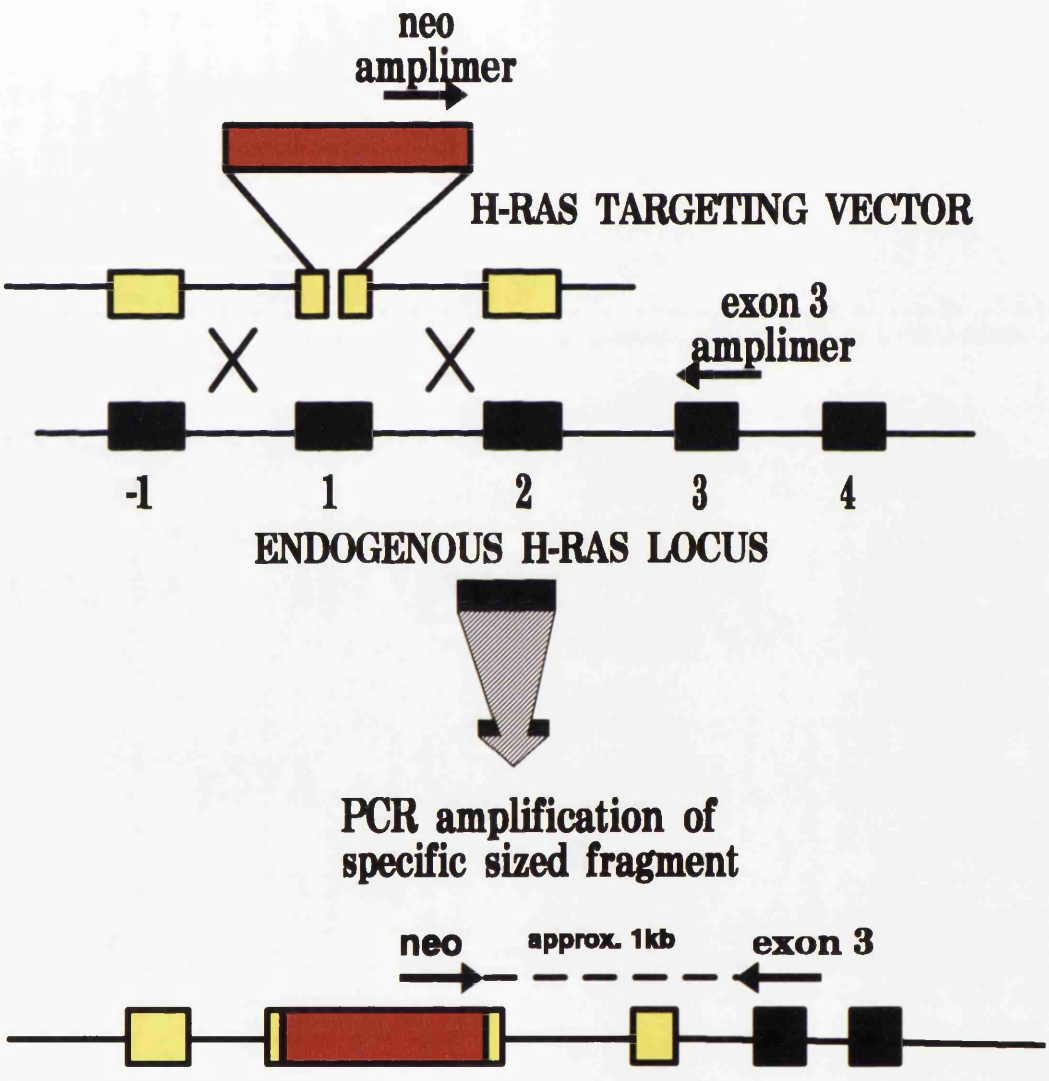

PCR SCREENING FOR
HOMOLOGOUS RECOMBINANTS
neo
amplimer
H-RAS TARGETING VECTOR
exon 3
amplimer
-1
1
2
3
4
ENDOGENOUS H-RAS LOCUS
PCR amplification of
specific sized fragment
neo
approx. 1kb
exon 3

Figure 33.

GENERATING A PCR POSITIVE CONSTRUCT
FOR HOMOLOGOUS RECOMBINATION.

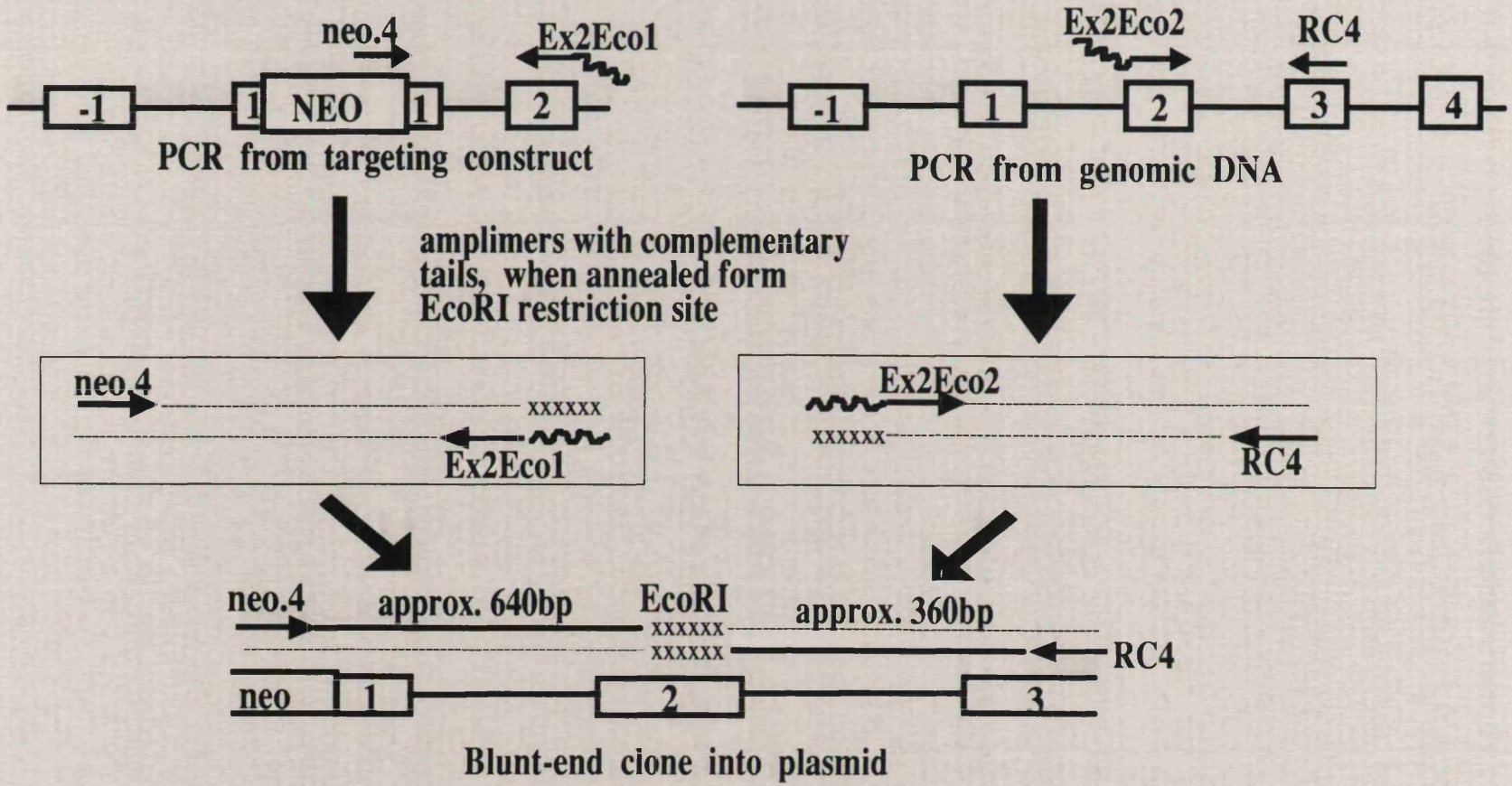


Figure 34.

outlines. In the first set of PCR reactions, amplimers NEO.4 and ExEco1 were used along with the targeting construct as template DNA, to amplify a fragment from the 3' end of the neomycin gene to the middle of exon 2. The amplimers ExEco2 and RC4 were used in a separate reaction, with mouse genomic DNA as the template, to amplify a fragment from H-ras exon 2 to exon 3. In the final PCR reaction the PCR products from the previous reactions were mixed in equimolar amounts and amplimers NEO.4 and RC4 added to the PCR reaction mix. Annealing of the complementary tails and initial extension from the 3' ends of the two template fragments resulted in a full length template. Subsequent rounds of amplification occurred across the bridged fragments from NEO.4 to RC4. The final product was identical to the expected sequences produced by a legitimate homologous recombination event with the exception of the 20bp introduced EcoRI linker sequences. This fragment was then blunt-end cloned into the SmaI site of pUC19.

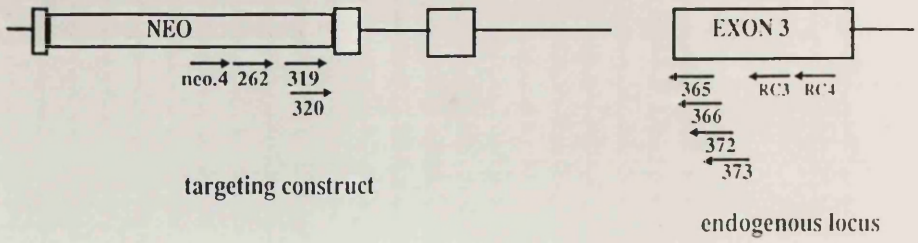
PCR reactions were carried out with this positive control plasmid. Reactions were set up with the same amplimers and amounts of ES cell genomic DNA with the addition of serial dilutions of the control plasmid DNA to the reaction mix. Figure 35 shows a typical PCR screen of 25 ES cell clones. Included was a reaction with the same quantity of ES genomic DNA (typically 0.5-1ug) but spiked with 1ng of plasmid DNA. 20ul of each 50ul reaction was run on a 1.7-2.0% agarose gel. The gel was blotted and the membrane hybridised with a H-ras probe. The result can be seen in figure 35, with the positive control samples producing a very intense signal. Nothing could be visualised in the other lanes with the exception of the lane marked with an arrow. A very faint band of approximately the right size could be seen on the original autorad. However, further PCR reactions and Southern blot hybridisation proved negative. Figure 35, panel c, shows a similar screen of 8 ES cell clones. The three lanes with strong signals indicate PCR amplification with 0.1ng of positive control plasmid DNA. The middle of these lanes shows the same positive control except that before being run on the gel it was restricted with EcoRI. This shows that the original fragment was digested into two smaller fragments with the restiction enzyme EcoRI,

Figure 35.

PCR screening of ES cell clones using amplimers complimentary to neomycin and H-ras exon 3 sequences.

(a); Position of amplimers used in screening
(b); Southern blot of PCR products run on 1.7% agarose gel and hybridised with pB5 H-ras probe. Intense signal from positive control plasmid (1ng)
(c); as for b. except positve control product was also restricted with EcoRI. (middle lane of signals)

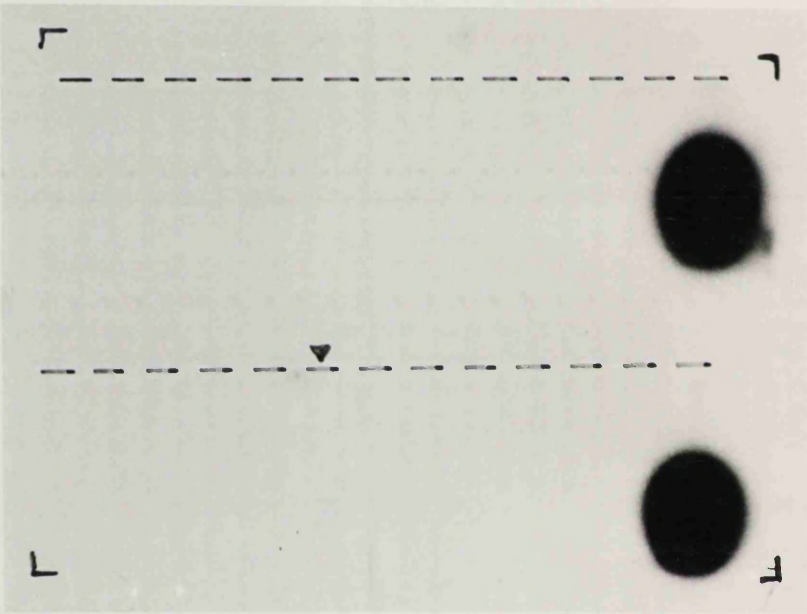
a.



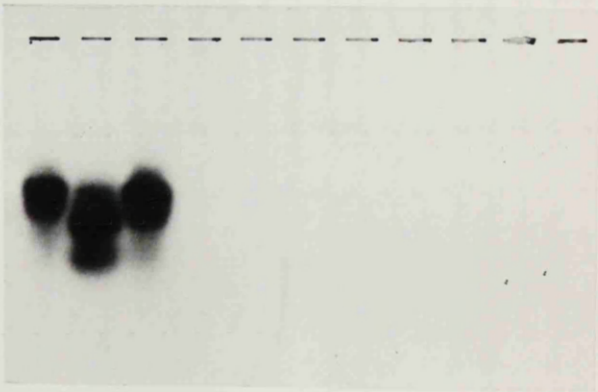
targeting construct

endogenous locus

b.



c.



and thus verifies the method for distinguishing the positive control fragment from a homologous recombinant.

4.3.4 PCR reconstruction experiment

Inorder to confirm the sensitivity of the PCR approach to the screening of ES cell clones, a reconstruction experiment was carried out using the positive control plasmid. Serial dilutions of positive plasmid DNA (from 500pg to the equivalent of 5pg) were mixed with a fixed quantity of genomic DNA (about 1ug). Figure 36 shows the results of PCR
amplification, gel electrophoresis and blotting of the PCR products. The membrane was hybridised with the H-ras pB5 probe. Although this experiment demonstrates that efficient hybridisation of a signal can be visualised from a very small quantity of control plasmid DNA it is perhaps not analogous to the PCR amplification of genomic DNA. It is thus intended to transfect the positive plasmid into ES cells, and use the resultant pool of cells as a more realistic model for the PCR screening approach.

4.3.5 Summary of the data using the Positive-Negative Selection (PNS) vector

A total of about 350 double resistant ($G418^R/GANC^R$) ES cell clones, electroporated with the Positive-Negative Selection (PNS) vector, have been screened by the Southern and PCR technique with an additional 70 by PCR alone. So far, all the clones which have been screened by both the Southern protocol and the PCR method have proved negative.
The reported targeting frequencies vary considerably between different genes. However, targeting frequencies such as 5% of the total double resistant clones using the positive-negative approach, have been reported for both int-2 (S.L. Mansour et al, 1988) and Rb genes (E.Y.-H.P. Lee et al, 1992), and 2% in the case of the p53 locus

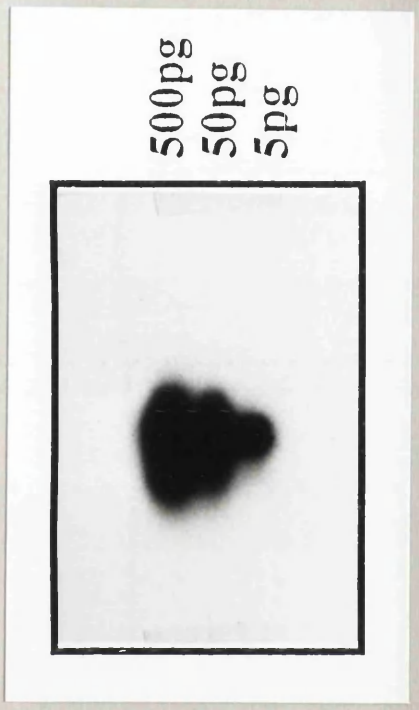


Figure 36.
Serial dilution of the positive control plasmid used in PCR screening for an homologous recombinant
10-fold serial dilutions of plasmid (as shown), mixed with approximately 1ug ES genomic DNA. Blot hybridised with pB5 H-ras probe.

(L.A. Donehower et al, 1992). According to these frequencies one should expect to identify in the region of 8-20 positive targeted events, from the number of clones that have been screened to date. However, the targeting frequencies of different genes cannot be easily compared. It appears evident from the literature, that certain genes are intrinsically more difficult to "knock-out" than others. One of the factors considered important in determining the frequency of homologous recombination with a specific vector, is the total length of homology the vector possesses (Thomas and Capecchi, 1987; Hasty et al., 1991c). The PNS construct has 3kb of total homology to the H-ras locus, which is considered to be close to the minimum end of the scale. For this reason it was decided to increase the size of H-ras homology with the hope of increasing the overall gene targeting frequency.

4.4 Modification of the PNS construct by increasing the length of homology to the H-ras genomic locus

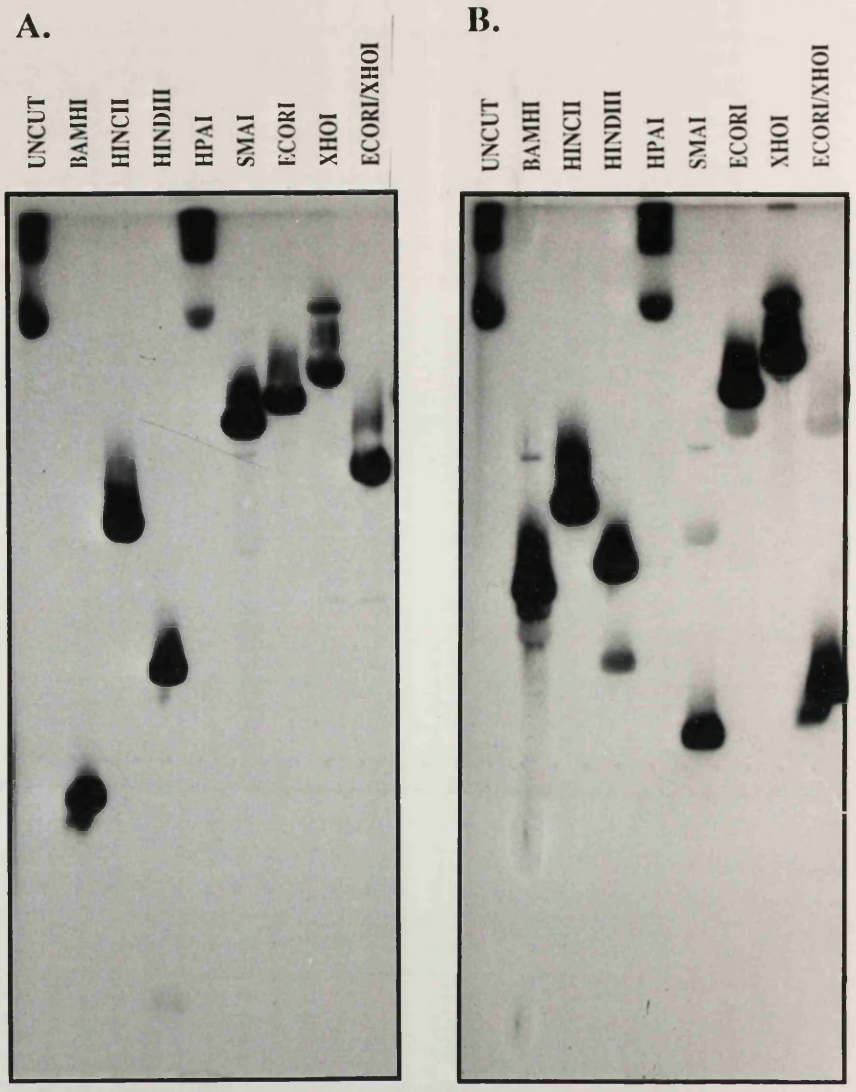
4.4.1 Subcloning upstream H-ras sequences

In order to increase the length of H-ras homology in the targeting vectors, it was necessary to subclone sequences 5' to the H-ras gene. It has been impossible to isolate clones that span the 3' region of the gene despite a great deal of effort. However, a very large segment of 5' homology (about 21kb), including the H-ras exons -1, 1 and 2 has been cloned into lambda charon 30, termed the λN1 clone.
λN1 was restricted with HpaI and a large fragment approximately 15kb in size was subcloned into the HincII site of the cloning vector pGEM -3Z (Promega). This clone, pGEMHpa was digested with a variety of restriction enzymes inorder to determine a rough restriction map of the region. Figure 37 shows a Southern blot of several digests of pGEMHpa. Panel a. represents the membrane hybridised to the

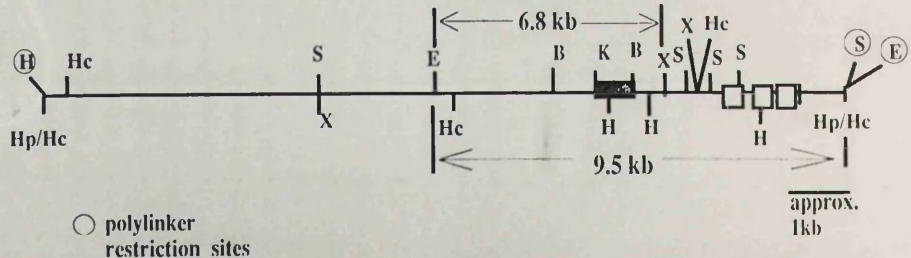
Figure 37.

Restriction mapping of a large upstream H-ras fragment isolated from the lambda N1 clone.

Southern blot of plasmid digests of pGEM.HPA subclone (A);Filter probed with P6 probe (KpnI/BamHI insert) represented as solid box in restriction map.
(B); Filter reprobed with pB5 H-ras probe (represents coding exons of H-ras).
Restriction map of pGEM.HPA subclone with fragment sizes in kb as measured from the blots



Restriction map of HpaI fragment cloned into pGEM vector



○ polylinker restriction sites

pGEM.HPA RESTRICTION FRAGMENT SIZES (IN KB):

	PROBED WITH pB5	PROBED WITH P6
EcoRI/XhoI	2.7	6.8
XhoI	12.5	12.0
EcoRI	9.5	9.5
SmaI	2.2	8.6
HindIII	4.3/2.9	2.9/0.6
HincII	5.5	5.2
BamHI	3.9	1.7

upstream H-ras probe, P6KpnI (600bp KpnI/BamHI fragment of P6). Panel b. represents the same filter reprobed with the H-ras probe, pB5.

A 9.5kb EcoRI fragment and a 6.8kb EcoRI/XhoI fragment were subcloned into pUC19 and pIC20H respectively, to produce H-ras subclones, pUC9.5Eco and pIC6.8E/X. These were further analysed using a variety of restriction enzymes.

4.4.2 Increasing the length of homology of the PNS vector

The length of homology of the original PNS construct was extended as outlined in figure 38. A 3.9kb EcoRI/BamHI fragment was isolated from a double digest of pUC9.5Eco. The PNS vector was digested with EcoRI/BamHI and the purified 3.9kb fragment was ligated to the linearised vector. Finally, the neomycin resistance gene and exon -1 and exon 1 sequences were replaced as a BamHI fragment. This 3.9kb extension of the original PNS construct termed PNS.EXT, possesses approximately 7kb of total H-ras homology. This vector has been electroporated into ES cells and double resistant colonies grown up and screened. To date 73 $G418^R/GANC^R$, double resistant colonies, transfected with the PNS.EXT construct, have been screened. As yet no positive clones have been isolated using this targeting vector. Experiments are continuing in Edinburgh in collaboration with M. Hooper and A. Clarke, Department of Pathology.

4.5 The "hit and run" approach to gene targeting

An alternative to the replacement-type targeting vector represented by the PNS and PNS.EXT constructs, is the insertion-type vector. This type of vector is linearised in the region of homology, and inserts itself into the genomic locus by a single cross-over event (Figure 3). Through the use of such a vector Allan Bradley and colleagues have devised a unique strategy (termed the "hit and run" strategy), for

Extending the length of homology of the PNS vector

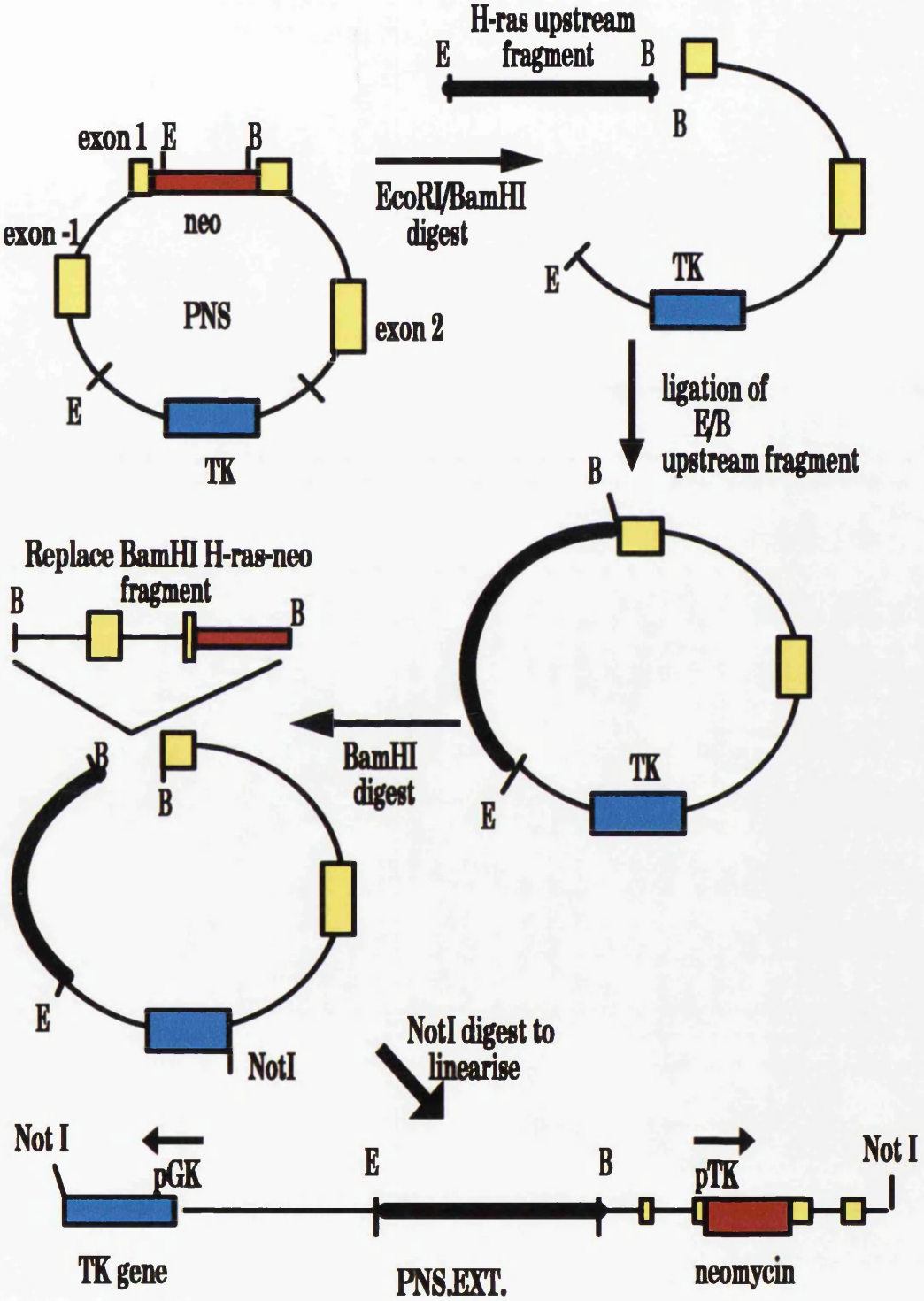


Figure 38.

introducing subtle mutations into a specific genomic locus (Hasty et al., 1991a). The so-called "hit" event describes the insertion of the vector sequences, selected for by the introduction of the neomycin gene. The "run" event is selected using the negative selectable TK gene (Figure 39). Loss of the TK gene, neomycin gene, and plasmid sequences occurs by a looping-out and intrachromosomal recombination event. The end-point is restoration of the genomic locus with the only difference being the introduction of the specific mutation. The insertion-type vector has been reported to target up to nine-fold more frequently than a replacement vector with the same length of homologous sequence (Hasty et al., 1991c). By generating a mutation that introduces in frame stop codons into the coding sequences of a gene, they have successfully inactivated the hprt and hox 2.6 genes in mouse ES cells (Hasty et al., 1991b).

It was hoped, that by creating a mutation that alters the open reading frame of the H-ras gene, and causes premature translational termination, a null allele of H-ras would be achieved. Figure 39 depicts the H-ras "hit and run" vector that was constructed. The mutation was made by filling-in the HindIII site of exon 1 and religation of the blunt-ends. This creates a translational frameshift, in theory resulting in premature translation at nearby termination codons. The basic vector was constructed from the 9.5kb EcoRI fragment subcloned into pUC19, or pUC9.5Eco. The HindIII fill-in mutation was recovered as a short NsiI/BbrPI fragment from another H-ras clone (NPR with the exon 1 HindIII site end-filled, see figure 28 for a map of NPR) . The normal NsiI/BbrPI fragment was excised and the mutant fragment inserted. Finally the 9.5kb EcoRI fragment carrying the mutation was cloned into the EcoRI site of the plasmid PNT (gift from A. Clarke). This plasmid carries a TK gene and a neomycin resistance gene both driven by pGK promoters. The fidelity of the HindIII fill-in was verified by sequence analysis, using the H-ras primer 12A and the method outlined in Section 2.14. Experiments with this H-ras "hit and run" vector are currently in progress in Edinburgh.

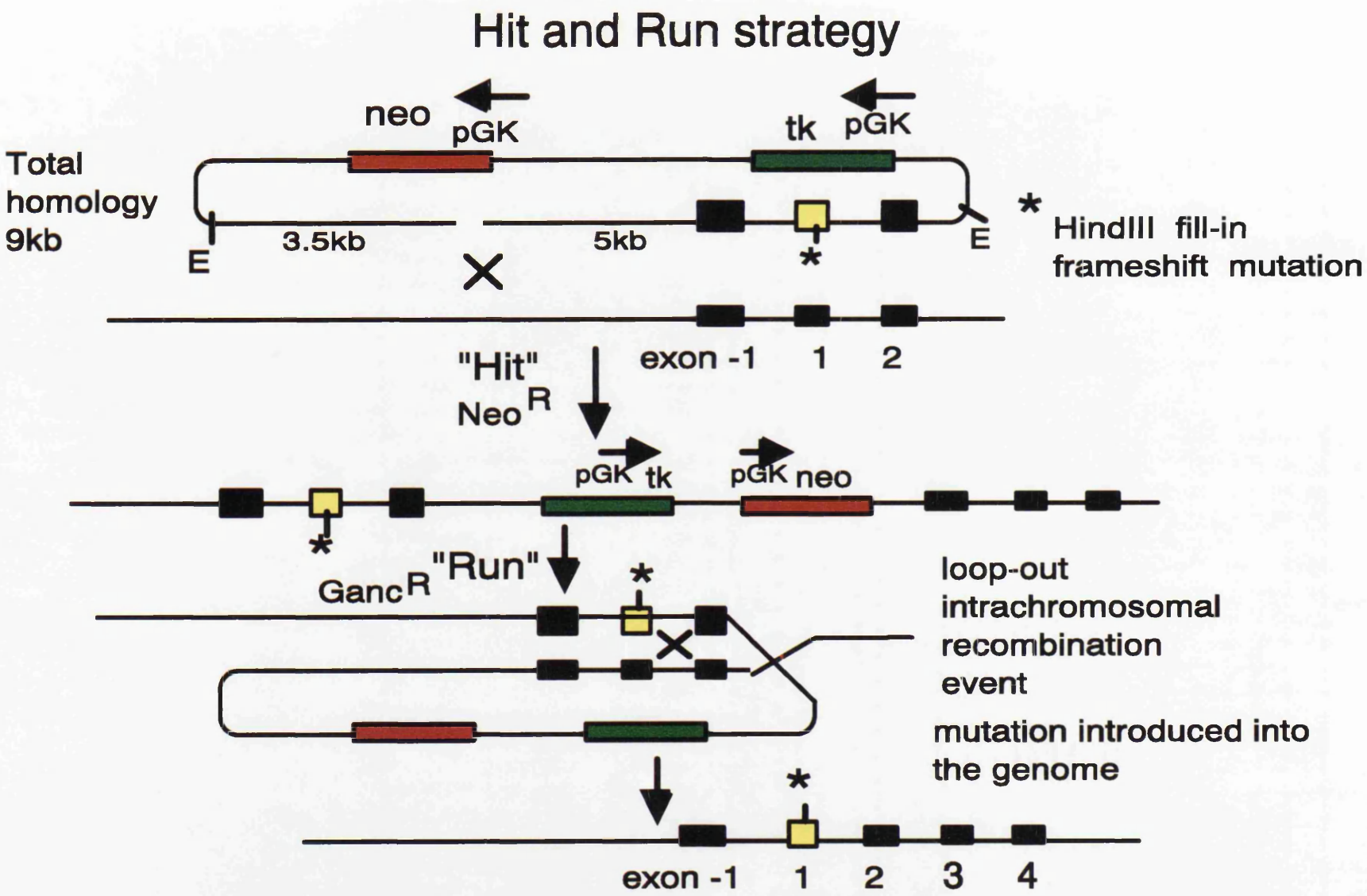

Hit and Run strategy
neo
pGK
tk
pGK
Total
homology
9kb
E
3.5kb
5kb
E
*
HindIII fill-in
frameshift mutation
"Hit"
Neo R
exon -1
1
2
pGK tk
pGK neo
Ganc R
"Run"
loop-out
intrachromosomal
recombination
event
mutation introduced into
the genome
exon -1
1
2
3
4

Figure 39.

4.6 Summary of the gene targeting data

As yet no targeted allele of H-ras has been isolated in ES cells. Initial experiments with direct nuclear microinjection of a specially designed construct were unsuccessful due to technical difficulties. Attempts to electroporate and select ES cells with a promoterless neomycin construct also failed, most probably due to the low endogenous level of expression of the H-ras gene in ES cells.

The majority of the electroporation and selection experiments were carried out with the positive-negative selection (PNS) vector. This vector has 3kb of H-ras homologous sequences, a positive selectable neomycin resistance gene driven from a TK promoter, and a negative selectable TK (thymidine kinase) gene driven from a pGK (phosphogluco kinase) promoter. This construct has been electroporated into ES cells on a number of occasions and selected with both geneticin (G418), and the drug gancyclovir (GANC). Over 400 double resistant colonies have been grown up and analysed by Southern blot hybridisation and PCR analysis. As yet, no positive clone has been identified. In an attempt to increase the targeting frequency, the length of homology of the PNS vector, was increased from 3kb to 7kb. The length of homology has been reported to increase linearly with the targeting frequency of a particular gene (Thomas and Capecchi, 1987; Hasty et al., 1991c). This extended PNS construct, PNS.EXT, has been electroporated into ES cells but as yet only 73 double resistant colonies have been screened, all so far negative.

In addition to the replacement strategy of gene targeting, an insertion vector, with 9kb of H-ras homology has been designed and cloned. The insertion-type vector has been reported to increase the targeting frequency up to nine-fold compared to a replacement vector with the same homologous sequences. Bradley and colleagues have utilised a modified version of the insertion vector in their "hit and run" strategy. It is hoped that with the use of the H-ras "hit and run" vector a subtle mutation can be introduced into the H-ras locus that will result in premature termination of translation. The predicted product should be a short 34 amino acid peptide with only the first 5 amino acids the same as H-ras. This should therefore create a null-allele of H-ras. This vector is currently being electroporated into ES cells in Edinburgh.

Preliminary experiments in cells other than ES cells, such as PDV, have been carried out (data not shown), with as yet no positive targeted clones. Most promising perhaps are experiments with the mouse cell line, PECA (gift from J.Schweizer, Heidelberg). This cell line is derived from the mouse strain (NMRI), the same as that from which our H-ras genomic clone was obtained. Berns and colleagues have reported that the frequency of gene targeting increases dramatically when isogenic DNA is used in the construction of the targeting vector (te Riele et al., 1992). This may imply that with this particular cell line, the frequency of gene targeting will be greatly enhanced. However, preliminary transfections using both electroporation and lipofection protocols have proved unsuccessful. Further refinements to the transfection protocol for these particular cells is apparently necessary.

Through the use of the new, "improved" vectors, it is hoped that the H-ras gene can be inactivated by a legitimate homologous recombination event in ES cells, enabling the creation of a transgenic mouse, hemizygous or nullizygous for H-ras. These mice should provide the clearest answer to the role of normal H-ras both in mouse development and the early stages of tumorigenesis.

Chapter 5
Discussion

5.1 Introduction

The results section of this thesis has been split into two chapters, outlining the alternative approaches to the study of the H-ras proto-oncogene in mouse skin carcinogenesis. The *in vitro* approach has taken advantage of a number of unique carcinoma cell lines isolated in our group, which have provided an interesting insight into the final stages of tumour progression. In addition, we have embarked on the novel technique of gene targeting which at the time this thesis was begun, was still in its infancy. Much has since been learnt about the parameters affecting the frequency of homologous recombination in ES cells and the intricacies in the design of the targeting vector. In the following sections of this thesis, it is my intention to summarize and speculate upon both the successes and failures of these alternative approaches.

5.2 The significance of the spindle cell phenotype

Through analysis of the mouse-skin model system, we have been able to define discrete stages in the multi-step process of tumorigenesis. In addition, we have been able to determine the genetic alterations associated with primary lesions and show that these correlate with those found in mouse tumour cell lines of the same stage. It has therefore been possible, to identify candidate genes responsible for specific stages of tumour progression *in vivo*, manipulate these genes *in vitro*, and ultimately return them to an *in vivo* environment by injecting the cells into nude mice.

Perhaps the most useful of the cell lines isolated to date, are those that represent the squamous to spindle transition event described in previous sections of this thesis. Derived from the same mouse skin carcinoma, MSC11, the cell lines B9, A5 and D3 were isolated by a process of single cell cloning. B9, the squamous counterpart, showed a characteristically well-differentiated histology and was unable to grow in soft agar (Figure 7). However, the spindle cell lines A5 and D3 produced undifferentiated, aggressive tumours in nude mice, and in addition, grew in anchorage independent conditions (Figures 7 and 13). There were marked differences in tumour latency between the squamous and spindle cell lines. Sub-cutaneous injection of 10^6 B9 cells produced 1cm tumours after about 6 weeks, whereas the spindle A5 and D3 cell lines produced similar sized tumours after only 2-3 weeks. A discrete conversion event had occurred *in vivo*, which resulted in the spindle cell variants.

This transition from an epithelial to spindle derivative is not unique to the mouse-skin system. As discussed in Section 1.17 of the introduction to this thesis, spindle cells have been characterised as components of a number of different human tumours (Matsui and Kitagawa, 1991; Kaufman et al., 1983; Steeper et al., 1983).

Indeed changes in the state of differentiation and morphology of a cell are known to play important roles not only in carcinogenesis but embryogenesis as well.

The first tissues to emerge in the early vertebrate embryo are epithelial in nature. The mesenchymal cells are all derived from the embryonic epithelia. The epithelial cells exhibit an assymmetry that is referred to as apical-basal polarity. It now seems evident that this polarity can be modulated by a variety of signals including growth factors (Boyer et al., 1989a), tumour promoting phorbol esters (Ojakian, 1981), or oncoproteins such as v-src (Warren et al., 1988), and ras (Schoenenberger et al., 1991). These changes in polarity result in a more motile, fibroblastic-type cell, and correlate with changes in the cytoskeletal organisation with the mesenchymal cells no longer producing cytokeratin filaments but instead vimentin intermediate filaments.

In addition, the cell-cell adhesion of the epithelial cells, mediated by specific cell adhesion molecules is decreased in the transition. These observations have led to the concept of an epithelial-mesenchymal transition, defined as a reversible process (Boyer et al., 1989b).

This plasticity in cell type, is clearly important in terms of the study of cancer. It offers an explanation for tumour cell heterogeneity, whereby a differentiated carcinoma cell can convert to a less differentiated spindle form within an isolated tumour mass, as seen in many human tumours and our example in the mouse carcinoma, MSC11. It is also thought to play a role in tumour metastasis, whereby a motile tumour cell can escape the confines of the primary tumour. A molecular understanding of the changes associated with this transition to a dedifferentiated state is therefore clearly important to our understanding of cancer biology as a whole. *In vitro* models now exist which reflect the intrinsic motility of a tumour cell. Thiery and colleagues have shown that the bladder carcinoma cell line, NBT-II, when grown in the presence of the serum substitute, Ultroser-G, displays a conversion from an epithelial cell type to a motile, spindle-like cell (Boyer et al., 1989a). This transition correlates with redistribution of desmosomal plaque protein, cytokeratin and actin filament systems, as well as *de novo* expression of vimentin (characteristic of a mesenchymal cell). Furthermore this transition is reversible with re-expression of the differentiated phenotype upon removal of the inducing factors (Boyer et al., 1989b).

The presence or absence of the calcium-dependent cell-cell adhesion molecule, E-cadherin (or uvomorulin), has been shown by a number of investigators to tightly correlate with this conversion process (Frixen et al., 1991; Vleminckx et al., 1991; Navarro et al., 1991). Canine epithelial (MDCK) cells transformed with Harvey and Moloney sarcoma virus acquired fibroblastic, invasive properties and were consequently shown to have lost E-cadherin. Similar phenotypes were produced by the addition of antibodies against E-cadherin. This down-regulation of E-cadherin was shown to be reversible, in that an epithelial MDCK-ras clone which stained positive for E-cadherin when injected into nude mice produced tumours with a heterogeneous E-cadherin profile. Prolonged culture of these cells from the primary tumour eventually resulted in reversion to a E-cadherin positive phenotype (Mareel et al., 1991). It seems that host factors present in the *in vivo* environment were capable of down-regulating E-cadherin. Indeed analysis of tumour sections from squamous cell carcinomas (SCCs) of the head and neck indicated a similar correlation between the state of differentiation and the expression of E-cadherin whereby the most poorly differentiated SCCs were E-cadherin negative (Schipper et al., 1991).

Other extracellular matrix components have also been shown to influence the epithelial morphology of tumour cells. Reexpression of the cell surface proteoglycan syndecan in spindle cells (created by steroid treatment of mouse mammary epithelial cells), has been shown to restore the epithelial morpholgy of the cells (Leppa et al., 1992).

In addition, the importance of c-Fos in the modulation of the epithelial phenotype has been reported in a recent paper (Reichmann et al., 1992). Prolonged activation of c-Fos using an inducible estrogen receptor fusion construct in mammary epithelial cells led to an invasive spindle phenotype. Loss of epithelial characteristics such as disruption of tight junctions and disruption of E-cadherin distribution were noticeable as well as *de novo* expression of vimentin. This may represent the mechanism by which v-src and ras exert their effect on cell polarity, since it has been shown that these oncogenes induce c-Fos expression.

Thus in summary, although the exact mechanisms controlling the epithelial to mesenchymal transitions remain unclear, the squamous to spindle transition represented by B9 and A5/D3 is clearly not an isolated example, but rather typifies a more common state of dedifferentiation seen in both embryogenesis and carcinogenesis.

5.3 The squamous to spindle transition correlates with an increase in mutant to normal H-ras gene dosage ratio

Molecular analysis of the squamous to spindle cell lines isolated from MSC11 have led to a number of interesting observations. A role for p53 in the squamous to spindle carcinoma transition event was discounted, since all three clones already possessed the same p53 mutations (Burns et al., 1991). The most obvious difference between the squamous and spindle cell lines, as outlined in the results section of this thesis, was the level of mutant H-ras (Figure 8). The copy number of mutant H-ras increased 5-10 fold in the conversion to a spindle cell carcinoma. This increase in copy number of the oncogenic species was best explained by a local genetic amplification event. Data to support this has come from PCR microsatellite studies with markers flanking H-ras indicating a trisomy of chromosome 7, as expected (C.J.Kemp, unpublished results). Expression data in the form of RNase protection and Western blot analysis correlated with this increase in mutant H-ras (Figures 8B and 10).

However, in addition to the increase in mutant p21 H-ras, there was a reduction in the level of expression of the normal gene. This appeared to occur by a mechanism involving transcriptional down-regulation rather than loss of genetic material as determined by the RNase protection data, and Southern blot analysis (Figure 8 A and B). Examination of the ratios of the p21 species on a Western blot agreed with the RNase protection data. Namely that concurrent with the transition to a spindle cell phenotype, was an increase in the mutant leu61 p21 species, and an apparent decrease in the normal p21 H-ras species (Figure 10). The mechanism for this down-regulation of the normal gene remains unclear, but may involve some form of competition possibly for a limited supply of transcription factors. In this way a 5-10-fold genetic amplification of the mutant H-ras gene, may result in down-regulation of the normal gene by competing out any available transcription factors.

Regardless of the exact mechanism involved, the overall result is an increase in the mutant to normal gene dosage ratio concurrent with the squamous to spindle conversion.

The obvious question that arises is whether alteration of the mutant:normal gene dosage ratio is sufficient to drive the transition event ?

5.4 Overexpression of mutant H-ras in the squamous B9 cell line is unable to convert it to a spindle phenotype, but induces distinct changes in cell morphology

Despite overexpression of mutant H-ras both in the form of viral and plasmid constructs, the B9 cell line remained squamous in character (Figure 11). There was an increase in tumorigenicity of the overexpressors when injected sub-cutaneously into nude mice, however the tumours remained well differentiated, characteristic of the squamous parental cell line B9. In addition, the overexpressor cell line B9HT3 1ml, when plated under anchorage independent conditions failed to produce colonies.

It seemed therefore, that overexpression of mutant H-ras in a squamous environment was insufficient to alter the overall phenotype.

However, overexpression of mutant H-ras in the B9 squamous cell line appeared to be cytotoxic to the cells. This was particularly noticeable in the plasmid transfectants, perhaps because of the forced selection for presence of the plasmid. Despite efficient transfection frequencies with neomycin plasmid vectors, very few mutant H-ras transfectant colonies survived selection. Those two clones that did survive selection, showed relatively low levels of mutant H-ras expression as compared to the non-selected HaMSV infected pool of B9 cells, B9HT3 1ml. Therefore despite an overall higher level of mutant H-ras, the non-selected pool of infected B9 cells, B9HT3 1ml, showed less cell death. This perhaps may be because of the presence of uninfected cells which somehow minimise the cytotoxic effect of mutant H-ras.

Growth arrest induced by mutant H-ras has been documented in the past. Hirakawa and Ruley described the temperature-dependent transformation of established rat embryo fibroblasts (REF52), with a temperature-sensitive SV40 large T antigen, in collaboration with the T24 H-ras oncogene (Hirakawa and Ruley, 1988). Two thirds of the clones became growth arrested in G2, or late S phase, when shifted to a nonpermissive temperature for T antigen stability. Thus, expression of the large T antigen allowed the cells to tolerate higher levels of mutant H-ras.

Although the resultant clones overexpressing mutant H-ras were still characteristically squamous in appearance, there were however consistent differences in the morphology of the cells. Small cytoplasmic vacuoles could be seen only very occasionally in the parental B9 cells. In the overexpressor clones they were noticeably more numerous, occupying the entire cytoplasm of some cells.

A paper by Russo and colleagues describes the transfection of an immortalised breast epithelial cell line, MCF-10A, with the same plasmid constructs used in the present studies, pHO6T1, and pHO6N1 (Russo et al., 1991). Transfection of the normal construct produced discrete changes in cell shape, and cytoplasmic appearance. These changes were made more prominent by transfection with the mutated H-ras oncogene. The features described include the lengthening and thickening of cell surface microvilli, formation of blebs, and the emission of filopodial projections. Cytoplasmic changes included the formation of intracellular lumina, an increase in the number of lysosomes, mitochondria and glycogen content. These observations together indicate a more metabolically active cell (Russo et al., 1991).

Similar alterations in cell structure have been documented by Bar-Sagi and colleagues. Microinjection of human H-ras oncogenic protein into quiescent rat embryo fibroblasts stimulated membrane ruffling and fluid-phase pinocytosis as early as 30 minutes post-injection (Bar-Sagi and Feramisco, 1986). These effects persisted for more than 15 hours after injection. Microinjection of the proto-oncogenic p21 species produced similar phenotypes but with a more transient 3 hour lifespan. In addition, it has been shown that certain mitogens, hormones and immunomediators such as, EGF (Haigler et al., 1979), PDGF (Davies and Ross, 1978), NGF (Connolly et al., 1979), and insulin (Goshima et al., 1984), produce a similar series of membrane responses in certain target cells. However, these effects are more rapid, occurring within minutes of exposure, and last for only 1-2 hours, maximum. The fact that both oncogenic and proto-oncogenic p21ras were able to induce similar changes in cell structure, suggests they share common cellular target(s), except that the oncogenic species produces a more constitutive phenotype.

In addition to the mammalian proto-oncogenes, other members of the ras superfamily namely the rho and rab genes have been shown to influence both cytoskeletal organisation and vesicular transport, respectively (for reviews see Hall, 1992; and Balch, 1990).

Microinjection of the activated form of rhoA, Val14rhoA, induces rapid morphological changes when injected into Swiss 3T3 cells (Paterson et al., 1990). These include stress fiber and focal adhesion plaque formation (Ridley and Hall, 1992a). Growth factors can induce rho-dependent responses, most likely by directly increasing the level of GTP-bound rho in the cell, either by inhibiting rhoGAP or by stimulating a rho GDP-exchange factor (for more details see Section 1.9.4 of the Introduction to this thesis). However, these cellular alterations are distinct from those induced by another rho-family member, Rac1. Introduction of activated rac1 (Val12rac1) into fibroblasts creates ruffling of the plasma membrane 5-15 minutes post injection (Ridley et al., 1992). Within 6 hours of microinjection of the protein, intracellular vesicles appear. The vesicles gradually accumulate until the entire cytoplasm is full and the cell simply rounds up. Microinjection of confluent fibroblasts on the other hand rarely induces similar vesicle formation. These observations are particularly interesting in terms of this thesis, since the rac1 double-mutant, Val12 Asn17, has been shown to inhibit the qualitatively similar changes induced by oncogenic Val12H-ras. This suggests that rac may act downstream of H-ras. However, prior blockage of ras action by injection with antibody Y13-259, or the Asn17 H-ras dominant inhibitory mutant (Feig and Cooper, 1988a), did not interfere with PDGF, EGF or Val14rhoA induced stress fiber or membrane ruffling (Ridley et al., 1992).

A model to explain these results has been offered by Hall and colleagues (Figure 40). H-ras and PDGF appear to act upstream of rac, to induce actin polymerisation and membrane ruffling. Rho on the other hand, can be stimulated by rac1, or independently of rac, by bombesin and LPA, leading to the formation of focal adhesions and stress fibers. This suggests that there are parallel but interconnecting pathways involving H-ras and ras-related small GTP-binding proteins, that influence the organisation of the actin cytoskeleton.

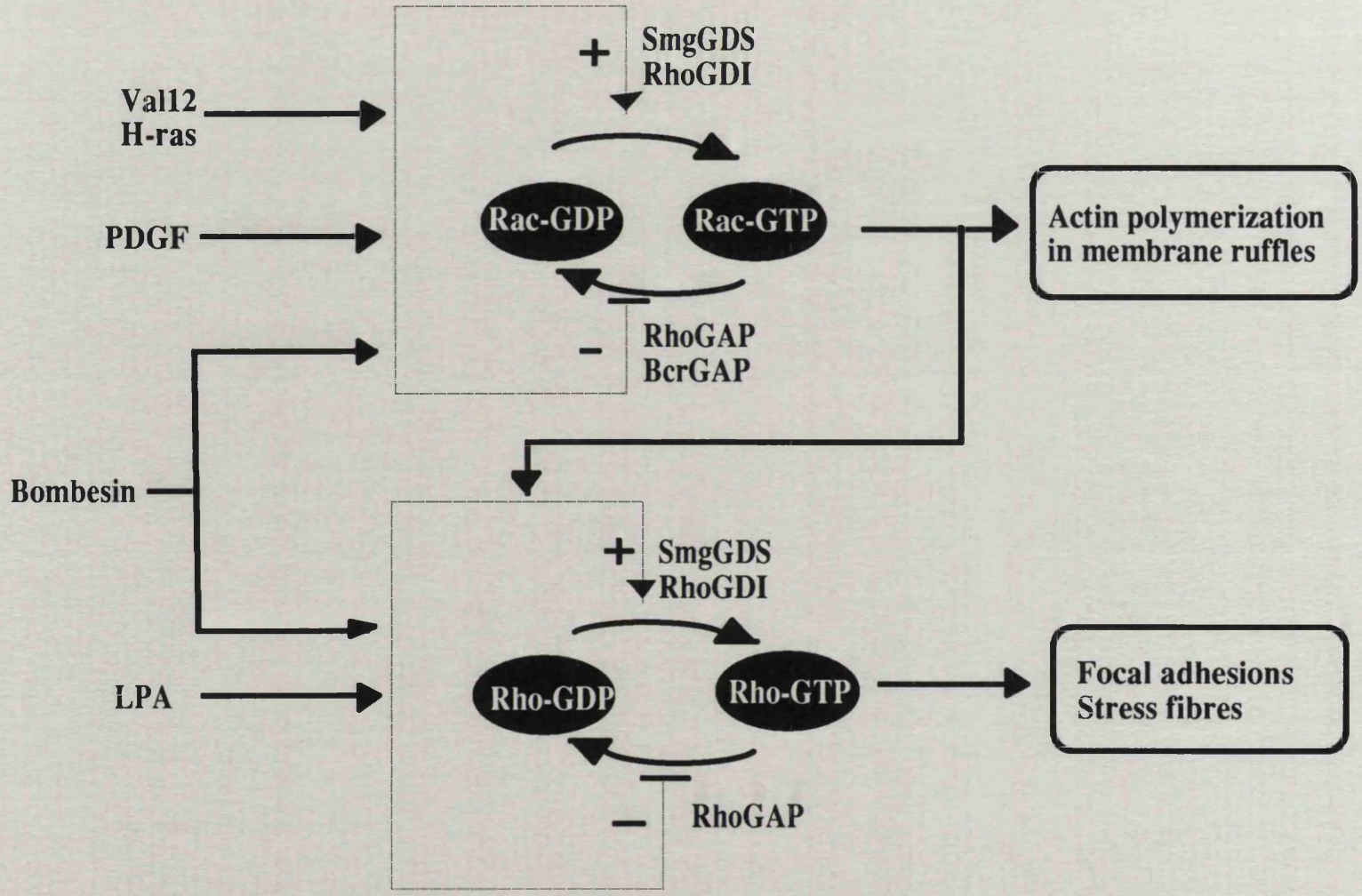


Figure 40. (From Ridley and Hall, Cell 70:401, 1992)

The significance of this data to the present studies emanates from the observation that introduction of mutant H-ras into the squamous carcinoma cell line B9, produces similar alterations in cell morphology compared to Val12rac1 injected subconfluent fibroblasts (Ridley et al., 1992). It was noticed that vesicles accumulated in the cytoplasm of the B9 transfectant and infectant clones. In extreme cases, the entire cytoplasm was filled, and the cells simply detached from the dish. It was also recognised that the number of vesicles were far more numerous in subconfluent cell cultures than in confluent cultures. It is interesting to speculate that the phenotype seen in two cell lines of very different origins, results from a similar disruption of the ras-rac pathway. Examination of the cells by high power microscopy may reveal distinct alterations in the plasma membrane characteristic of fibroblast membrane ruffling induced by Val12H-ras (Bar-Sagi and Feramisco, 1986) and Val12rac1, and the filopodial projections seen in the MCF-10A epithelial cell line transfected with oncogenic H-ras (Russo et al., 1991).

In addition further investigations may reveal that the phenotype induced in the B9 cells by overexpression of mutant H-ras can be blocked by co-transfection with the Asn17rac1 inhibitory mutant, thus verifying a link between the rac and ras pathways in our epithelial cells.

5.5 Infection of the squamous cell line B9 with HaMSV results in a cell line capable of *in vivo* host fibroblast recruitment

Despite the inability to alter the morphology and *in vitro* growth characteristics of the squamous B9 cell line, sub-cutaneous injection of the two clones, B9HT3 1ml and B9HT3 2ml, infected with the HaMSV construct, resulted in tumours displaying two distinct components. Explants of these tumours produced cell lines with squamous and spindle-like morphologies (Figure 14). However, after 1-2 weeks in culture, and a reduction in serum concentration, the "spindle" cell lines began to take on a much

flatter appearance, characteristic of senescing fibroblasts. The origin of these cells was examined using the PCR microsatellite approach. Since the parental B9 cells were originally from a hybrid *musculus/spretus* mouse, the two alleles could be distinguished using polymorphic microsatellite primers in PCR amplification reactions. PCR amplification followed by gel electrophoresis resolved two bands representing both the *musculus* and *spretus* alleles from the squamous tumour cell lines (Figure 15). However, the spindle-like tumour cell lines showed only one band representing the *musculus* allele. This suggests that these cells were indeed of host fibroblast origin. From an NRK assay it was shown that the B9 infected cells were capable of producing infective virus particles. It was therefore important to examine the involvement of virus infection of the surrounding fibroblasts. A Southern blot of squamous and fibroblast cell lines from the tumour explants was probed with a H-ras specific probe. No copies of the HaMSV could be seen in the fibroblast cell lines (Figure 18). It seems therefore, that virus integration is not responsible for recruitment of the nude mouse fibroblasts into the tumour mass. Despite several injection experiments of parental B9 cells, and plasmid transfected B9 pHO6T1 II and III clones, similar dimorphism has not been encountered. Thus this fibroblast recruitment appears unique to the virus infected clones. One possible explanation for this phenomenon, is that the cells overexpressing the viral p21 are secreting a factor which recruits the surrounding host fibroblasts into the tumour mass. Several factors that enhance the plasticity and hence the motility of cells have been identified. These include the autocrine motility factor (AMF) (Liotta et al., 1986), produced by human metastatic melanoma and ras-transformed NIH-3T3 cells, and the migration-stimulating factor (MSF), an autocrine agent that is produced by normal fetal and adult malignant fibroblasts (Grey et al., 1989). The paracrine factor, scatter factor (SF), was originally identified as a non-dialyzable, heat-labile, trypsin-sensitive agent that promotes the dispersal of MDCK epithelial cells by disruption of the junctions between the epithelial cells (Stoker and Perryman, 1985). Growth factors such as IGF-II, EGF, aFGF and TGF-α have also been implicated in the stimulation of cell

motility and invasiveness (for a review Jouanneau et al., 1991). In the present case, TGF-α is perhaps the most likely candidate factor, since it has been shown that viral transformation of various cell lines induces the expression of TGF-α (DeLarco and Todaro, 1978). In addition, transfection of immortalised rat fibroblasts with a human TGF-α cDNA induces fibroblast transformation, creating clones that form tumours in nude mice at a much higher frequency than untransfected controls (Rosenthal et al., 1986). Further experiments are clearly necessary to establish the mechanism of fibroblast recruitment.

5.6 Overexpression of normal H-ras in spindle cells is unable to alter the spindle phenotype

Transfection of two spindle cell lines A5 and D3 with normal H-ras plasmid constructs resulted in transfectant clones some of which overexpressed large quantities of normal p21 H-ras (Figure 18). The morphology of these clones remained spindle in appearance (Figure 20), and the histology of the tumours induced when the cells were injected sub-cutaneously into nude mice was undifferentiated, characteristic of the parental spindle cell lines (Figure 23). These results correlate with additional findings from our group and others, where overexpression of normal H-ras in the spindle carcinoma cell line, carB, was unable to induce a morphological reversion despite displaying high levels of normal p21 H-ras (S.Haddow and A.Balmain, unpublished results). Similarly, data from Paterson and co-workers has shown that overexpression of normal N-ras in HT1080 cells (which carry an activated N-ras allele), was also unable to cause a phenotypic reversion, as judged by cellular morphology and growth in soft agar (Paterson et al., 1987).

5.7 Spontaneous loss of mutant leads to a dramatic reduction in tumorigenicity of the spindle A5 and D3 cells

Expression data on a number of A5 and D3 clones transfected with normal H-ras, showed varying levels of expression of the exogenous H-ras gene as well as of the endogenous mutant gene. Initially, it was considered that overexpression of the exogenous normal H-ras gene resulted in down-regulation of the endogenous mutant, perhaps by some form of competition mechanism. However, clones which appeared to have no exogenous H-ras sequences and control clones with only a neomycin resistance gene (Homer 6), showed similar decreases in the levels of endogenous mutant H-ras. Immediate differences in tumour latency were recognised when the various transfectant clones were injected into nude mice. The latency period directly correlated, not with the level of exogenous normal H-ras in the cells, but with the level of endogenous mutant (Figure 21). The spindle cell lines appeared to have spontaneously lost copies of the mutant H-ras gene, in the process of selection and isolation as individual cell lines. Indeed, this instability was seen in the majority of clones, approximately 70%, and has since been noticed by others working with the same cell lines. These observations confirm the initial belief that a local genetic amplification event best explains the increase in ratio of the mutant alleles in the squamous to spindle transition. Indeed, recent data with a chromosome fluorescence technique, has identified the presence of numerous extra-chromosomal bodies in the spindle cell line, D3 (N.Keith and A.Balmain, unpublished results). Although not yet confirmed, this may suggest that H-ras resides on these double minute-like structures, providing an explanation to the frequency of loss of mutant H-ras in these particular cell lines.

Thus the spontaneous loss of mutant alleles from many of the A5 and D3 transfectants, provides an explanation for the apparent down-regulation of the endogenous mutant gene in the corresponding clones, as indicated by the RNase protection data (Figures 18 and 19).

Despite the reduction in levels of mutant and overexpression of normal H-ras in certain clones, such as A5 pHO6N1 2 and 4, the cells remained spindle in morphology (Figure 20). In addition, the histology of the "suppresssed" tumours remained characteristically spindle in nature (Figure 23). It thus appears, that alterations of the mutant:normal gene dosage ratio are not sufficient to revert the overall phenotype of the spindle cell carcinoma. In agreement with these results, are cell fusion studies from our group, which indicate that the spindle cell phenotype is recessive (A.Stoler and A.Balmain, in press). Fusion experiments between the spindle and squamous cell lines showed that the hybrids took on the phenotype of the squamous parent, as determined by cell morphology, and expression of keratins and E-cadherin. These data suggest that the spindle phenotype involves functional loss of a gene(s), which controls the epithelial differentiation program.

The present studies imply that the tumorigenic potential of the spindle cell carcinoma is independent of cellular morphology, since even the histology of the "suppressed" nude mouse tumours remained spindle in appearance. The critical factor that appears to control the tumorigenic potential of these cells therefore, is the copy number and expression levels of mutant H-ras.

The correlation between increasing levels of oncogenic ras and the induction of neoplastic transformation *in vitro*, and the enhancement of tumorigenicity *in vivo*, has been reported by a number of investigators (Colletta et al., 1983; Yoakum et al., 1985; Fetherston et al., 1989; Aguilar-Cordova et al., 1991; Compere et al., 1989; Paterson et al., 1987). Infection of mouse epidermal keratinocyte cell lines with BALB or Kirsten murine sarcoma viruses resulted in the rapid acquisition of EGF-independent growth and loss of sensitivity to calcium-induced terminal differentiation (Weissman and Aaronson, 1983), whereas the introduction of the EJ ras oncogene into chemically induced benign mouse skin papilloma cell lines resulted in transfectants that form rapidly growing, anaplastic carcinomas in nude mice (Harper et al., 1986).

Similar results have been seen in immortalised human cell lines. Retroviral infection and plasmid transfection of an SV40 immortalised bronchial epithelial cell line, with H-ras and K-ras vectors, produced clones that displayed an increase in ploidy (Amstad et al., 1988), were highly tumorigenic when injected into nude mice, and were insensitive to the growth inhibitory effects of TGF-β (Reddel et al., 1988). Similarly cervical squamous cell lines immortalised by HPV-16 or 18 were also made tumorigenic by the introduction of activated H-ras (DiPaolo et al., 1989).

Experiments by Paterson and colleagues support these findings. Isolation of flat revertants of the HT1080 fibrosarcoma cell line, showed a reduction in the level of tumorigenicity which corresponded with a loss or under-representation of endogenous mutant N-ras. Retransformants derived from nude mouse tumours that developed after a long latency period, were shown to have regained their transformed phenotype and had correspondingly increased levels of mutant N-ras. Furthermore, transfection of the revertants clones with mutant N-ras or the EJ oncogene, was shown to retransform the cells and regain the tumorigenic potential of the parental HT1080 cells (Paterson et al., 1987).

Thus from these experiments and the work of others, it appears that the tumorigenic potential and growth factor independence of the cells, directly correlate with the expression levels of oncogenic ras. However, this correlation may not be as steadfast as suggested. Boukamp and colleagues transfected a spontaneously immortalised human keratinocyte cell line with the EJ oncogene (Boukamp et al., 1990). The transfectants were categorised into separate classes according to their *in vivo* tumorigenicity values. Some clones formed slowly growing papillomatous-type benign tumours whereas others formed highly differentiated locally invasive squamous cell carcinomas. However, on the basis of Western blot analysis of the transfectants, some of the so-called malignant clones showed lower levels of H-ras p21-val12 than one of the benign transfectants.

A similar lack of correlation has been seen by our group with two cell lines, HT1 derived by transfection of the immortalised keratinocyte cell line MCA-3D, and a NIH3T3 transformant, 1d1A. Both cell lines displayed similar levels of mutant p21 ras but whereas the NIH3T3 transformants produced tumours within 1 week of injection, HT1 produced only one tumour in 4 mice after a long latency period (8-12 weeks) (Quintanilla et al., 1991). It seems therefore, that although a strong correlation exists between levels of mutant p21 ras and the tumorigenic potential of a cell, the genetic background of the cell in particular additional genetic alterations, must also play a role.

5.8 Overexpression of normal H-ras in squamous carcinoma cells appears to induce a suppressive effect on tumour development

Overexpression of normal H-ras in the spindle cell lines A5 and D3, was unable to produce a detectable suppressive effect on tumour development. However, the inherent genetic instability, and high levels of endogenous mutant H-ras characteristic of these cells, makes them perhaps intrinsically less amenable to the proposed suppressive effect, if any, of normal H-ras. It may be that the mutant:normal ratio plays an important role at an earlier stage of tumour development.

Preliminary experiments with the squamous cell lines B9 and SN161, reveal slight differences in tumour latency (1-3mm rather than 3-6mm nodules 11 days post-injection) in some of the normal transfectant and infectant clones. However, histological examination does not indicate any significant differences in the differentiated state of the tumours (data not shown), although this may not entirely rule out a suppressive role for normal H-ras, since the tumorigenically suppressed A5 and D3 tumours were still spindle in morphology.

It is thus still too early to draw firm conclusions on the suppressive role of normal H-ras in the squamous cell carcinoma stage of tumour progression. However, if real, it may explain the impetus for the alterations in gene dosage of mutant:normal H-ras, concurrent with the squamous to spindle transition. If normal H-ras is still able to induce a suppressive effect on tumour development in the squamous cell carcinoma, it may thus be necessary to down-regulate expression of the normal gene, as well as increase the copy number of mutant H-ras. Further analysis of the B9 and SN161 normal transfectant and infectant clones is clearly essential.

5.9 Normal ras as a suppressor of tumour development

Tumour suppressor genes are thought to fall into two distinct classes, those which are tumorigenic solely by virtue of their loss or inactivation, and those referred to as "recessively-activated proto-oncogenes". The latter have been descibed as "proto-oncogenes which exert a neoplastic effect when loss of one allele unmasks an activating mutation in its homologue" (Green, 1988). The H-ras proto-oncogene may fall into this class of tumour suppressor gene. The interaction of suppressor genes with proto-oncogenes has been studied by analysing the suppression of cells known to contain an activated proto-oncogene. Studies with the HT1080 human fibrosarcoma and EJ human bladder cell lines which contain an activated N-ras and H-ras respectively, have generated some interesting observations. Suppression of EJ tumorigenicity was seen not to involve any changes in the level of mutant H-ras protein (Geiser et al., 1986). From this it was postulated to occur by a post-translational mechanism, by competition with the ras protein or substrates. In addition, suppression appeared to be dosage dependent, in that diploid fibroblasts could suppress near-diploid HT1080 cells but not near-tetraploid HT1080 variants (Benedict et al., 1984). It was also shown that suppression of HT1080 cells appeared to be mediated by chromosome 1, the chromosome where N-ras itself resides, suggesting that normal N-ras itself was capable of tumour suppression, although the results of Paterson and colleagues do not confirm this (Paterson et al., 1987). In addition to these cell fusion studies are the observations that losses of all three ras alleles occur in several tumour types (Kraus et al., 1984; Diamond et al., 1988; Guerrero et al., 1985). We have some evidence to suggest that in some cases loss of normal H-ras may be occur by transcriptional down-regulatory mechanism. This seems to be the case for carcinoma SN158 which shows a mitotic recombination distal to H-ras thus the normal allele remains intact. However, RNase protection analysis of this particular tumour shows the complete absence of normal transcript. These data together lend some support to the theory of ras as a suppressor of

tumourigenesis. Indeed this proposed suppression by normal ras may account for the necessity for trisomy of chromosome 7. It seems that a single point mutation is not in itself dominant, and duplication in the form of chromosome non-disjunction is necessary to enable tumour development to continue.

The concept of normal H-ras as a tumour suppressor gene, and the fact that it may function in a dose-dependent manner, provoke an interest in understanding the mechanism of such tumour suppression.

5.10 A proposed mechanism for tumour suppression by normal H-ras

The proposed suppressive effect of normal ras on its' oncogenic counterpart most likely involves some form of competition between the proteins, possibly for an effector molecule.

Some interesting observations that may pertain to this tentative mechanism, have been considered by Bourne and Stryer, in a recent Nature News and Views article (Bourne and Stryer, 1992). Studies of heterotrimeric G proteins, in particular the G protein α-subunit, have shown that both GAP and effector activities reside in the same polypeptide. Association of such activities in the same molecule may provide a precedent for regulation of the mammalian p21 ras pathway. The slow turn-off of α-GTP may act to maintain the effector molecule refractory to further stimuli, by prolonging the effector-GDP bound complex. Although still very speculative, such a model may offer a mechanism by which normal p21-ras can sequester the available effector molecules and maintain them in an inactive complex. In this way, it can be imagined how the normal protein could suppress the oncogeneic effect of the mutant product when both proteins are present at approximately equal ratios.

5.11 Conclusions from the *in vitro* approach to investigating the role of H-ras in tumorigenesis

In summary, it seems that neither overexpression of mutant H-ras in squamous cell lines, or overexpression of normal H-ras in spindle cell lines can dramatically alter their respective phenotypes. Clearly another locus is involved in the squamous to spindle transition. This observation correlates with data from cell fusion studies, which infer that the spindle phenotype is recessive, indicating a loss of genetic material. The exact nature of these alterations remain uncertain. Transfection and re-expression of E-cadherin in the spindle cell line carB was unable to alter the morphology of the spindle cells. Experiments reintroducing E-cadherin into A5 and D3 cells are in progress, yet it seems that E-cadherin in itself is not sufficient.

Extensive searches of the mouse genome using the microsatellite approach may identify candidate loci that are responsible for the transition event. The fact that the spindle phenotype is reversible, as shown by fusion studies with epithelial cells, may suggest that the gene or genes of interest may be identified by transfection of a cDNA expression library, followed by selection for epithelial revertants. This may be accomplished by selecting cells on the basis of induced re-expression of E-cadherin, or of the keratin genes. Both these markers have been shown to be re-expressed in the epithelial revertants derived by fusion of a spindle with a squamous cell line (A.Stoler, in press). These experiments are in progress in our laboratory.

In respect to the proposed suppressive effect of normal H-ras, no conclusive evidence for the existence of such a mechanism has been realised from the present studies. Even those cell lines which displayed high level expression of normal H-ras as well as loss of mutant H-ras, for example A5 pHO6N1 2 and 4, showed no dramatic difference in terms of a suppressed phenotype than the likes of A5 Homer 3, which showed a similar reduction in copy number of mutant, but no expression of exogenous normal H-ras.

The evidence to date to support a suppressor role for normal p21 ras has come mainly from the studies indicating loss or under-representation of the normal gene in several tumour types. However, these losses of normal H-ras may be as an indirect consequence of increasing the dosage of the mutant gene, most probably in the form of a non-disjunction event.

The difficulty exists in distinguishing whether the alterations in gene dosage ratio occur as a mechanism for increasing the relative amount of mutant protein, possibly as a driving force for *in vivo* tumour growth, or whether they result in order to overcome some form of suppressive effect instigated by the normal gene. From the present studies, some suggestion of suppression, in respect to tumour size, of some of the squamous transfectant and infectant clones may represent genuine tumour suppression. However, these results await more detailed analysis. Thus we are still unable to fully rule out a role for normal ras in suppressing tumour development, in particular at early stages of tumorigenesis, where the gene dosage ratios may be more critical. The necessity for the trisomy of mouse chromosome 7 in the development of benign papillomas, may indicate that it is at this stage that the mutant:normal gene dosage ratio is critical.

Perhaps the most definitive method of examining the role of normal H-ras in early tumour development is to use the *in vivo* approach outlined previously, and the results of which are discussed in the Section below.

5.12 Gene targeting: the *in vivo* approach to investigating the role of H-ras

5.12.1 Alternative approaches to achieving the same goal

Alternative strategies have been employed to attempt to "knock-out" the H-ras gene in ES cells and in other relevant cell lines. Initial efforts to use direct nuclear microinjection to introduce a targeting construct were unsuccessful due to technical difficulties. In addition, a promoterless targeting construct failed to produce viable ES clones following G418 selection possibly because of the low endogenous levels of H-ras in ES cells.

Further experiments have been carried out, in collaboration with Martin Hooper and Alan Clarke at the Department of Pathology, Edinburgh, using a targeting construct with both positive and negative selectable markers. This vector termed the PNS construct has been electroporated a number of times into ES cells, and the cells selected with geneticin and gancyclovir. The resultant clones have been screened both by Southern blot hybridisation and PCR analysis. In addition, to ensure that the PCR screening technique was accurate, a positive control plasmid was generated by the method outlined in Section 4.3.3.

The end result, after screening over 400 double $G418^R/GANC^R$ resistant colonies, is that so far it has not been possible to isolate a positive, legitimate recombinant.

Several articles from laboratories involved in the targeting field, have reported a linear correlation between the length of homology of the targeting construct and the frequency of homologous recombination (Hasty et al., 1991c; Mansour et al., 1988). This prompted us to increase the length of homology, from the initial 3kb, for the PNS construct to a total of 7kb for the PNS.EXT construct. In addition, an insertion-type vector was created, based on the "hit-and-run" approach. It was reported by Bradley and colleagues that insertion vectors targeted 9-fold more efficiently than replacement vectors possessing the same homologous sequences (Hasty et al., 1991c).

The H-ras "hit and run" vector possesses 9kb of H-ras homology, mostly upstream of the coding exons. Experiments with these latest constructs are currently in progress in Edinburgh, in collaboration with M.Hooper and A. Clarke.

5.12.2 Summary of the gene targeting data

Despite a great deal of effort, in the generation of the different targeting constructs, and mastering of the various techniques, a legitimate H-ras targeted clone has not yet been isolated. Various factors have been reported to effect the frequency of homologous recombination. Amongst the most prominent is the length of homology that the vector has in relation to the gene of interest. Although vectors with as little as 1.4kb of homology have proved successful (Doetschman et al., 1988), in general the greater the length of homology the better the gene targeting frequency. However, reviewing the literature for the frequencies of gene targeting, it becomes immediately obvious that certain genes are more easily targeted than others. For instance disruption of the N-myc gene occurred in 20% of the $G418^R$ colonies (Charron et al., 1990) compared to only 1 in 5000 for N-ras (Cases and Dautry, 1992). The precise reason for such a variation in frequencies is unclear, but may involve a number of factors. The H-ras gene may simply fall into the class of genes which are particularly difficult to target. The fact that other groups have reportedly been trying to inactivate H-ras in ES cells, and as yet no successful results have been published, perhaps supports this theory.

One possible biological explanation for the difficulty encountered in targeting H-ras, is that inactivation of one allele of H-ras would provide a growth disadvantage to the cell, similar perhaps to the haplo-insufficiency seen in the developmental Pax genes (Gruss and Walther, 1992). If this were the case, it would perhaps explain why a correctly targeted ES cell clone has not yet been isolated.

However, in a recent article, the successful inactivation of the N-ras gene in murine ES cells was detailed (Cases and Dautry, 1992). Although perhaps distinct from H-ras, the inactivation of one allele of N-ras did not appear to have a detrimental effect on the *in vitro* growth of the cells. In addition, chimareic mice were established from blastocyst injection of the targeted ES cells, indicating that inactivation of one allele had little effect on the developmental potential of embryonic stem cells. As for H-ras, the story remains unclear. An abstract article from a meeting (Finney and Bishop, 1991), outlines the successful homologous recombination of H-ras in Rat-1 fibroblasts. Although the details are sketchy, and no article on this matter has since been published, hope remains for the successful targeting of the H-ras gene in ES cells. If it is felt, that the gene dosage of the normal H-ras gene is critical for the proper growth and maintenance of the cells, then ES cell lines carrying transfected copies of the normal H-ras gene, can be experimentally generated and tested in the system.

Finally if successful, the targeted mice would be studied in the context of our skin carcinogenesis system. Heterozygous null mice if treated with carcinogens may provide an answer to the proposed suppressor role of normal H-ras. If as previously discussed, trisomy of chromosome 7 is necessary to overcome some suppressive effect of proto-oncogenic ras, then initiation of the skin in heterozygous null mice by point mutation of H-ras would result in a target cell with no normal H-ras. This may lead to a reduction in the latency period for papilloma development or indeed an increase in the papilloma yield. One can only speculate as to the consequences of a reduction or complete loss of H-ras in mouse skin tumorigenesis, or indeed in the overall process of cellular differentiation concurrent with normal embryonic development.

These as with many other aspects of ras biology await further investigation.

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